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# Physiological Impacts of Lampricides on Invasive Sea Lamprey (Petromyzon marinus) and Non-target Fishes

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# Physiological Impacts of Lampricides on Invasive Sea Lamprey (*Petromyzon marinus*) and Non-target Fishes

By

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**ABSTRACT**

Parasitic sea lamprey (*Petromyzon marinus*) ingest large quantities of blood from fishes using their oral disc and rasping tongue, most often killing the host. In the early 1900s, sea lamprey invaded the Laurentian Great Lakes, decimating sport, commercial and culturally significant fisheries. Since the early 1960s, chemical control using the lampricides 3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide have helped to reduce sea lamprey populations by 90%. Lampricides are applied to larval lamprey nursery streams targeting many generations of lamprey at once. However, there is concern about the potential adverse effects of lampricides on other fishes, particularly vulnerable lake sturgeon (*Acipenser fulvescens*) populations. In larval lamprey and rainbow trout (*Oncorhynchus mykiss*), TFM disrupts mitochondrial function, reducing ATP supply and depleting essential energy reserves such as glycogen and phosphocreatine (PCr), leading to death. Yet, little is known about the physiological effects of niclosamide. The goals of this thesis were to better understand how lampricide exposure adversely affected the physiology of the lake sturgeon, and to compare the physiological effects of niclosamide to TFM in larval lamprey, rainbow trout and lake sturgeon. Accordingly, brain, liver and muscle were collected from larval lamprey, trout and sturgeon exposed to TFM or niclosamide, followed by determination of tissue energy reserves (glycogen, glucose), high energy phosphates (ATP, PCr), and acid-base balance. In larval lamprey, brain was most sensitive to niclosamide, resulting in significant reductions in glycogen. In sturgeon and trout, liver was most sensitive to niclosamide and TFM as characterized by large reductions in glycogen concentration, with lesser declines in brain. Niclosamide exposure also caused notable reductions in muscle glycogen stores and intracellular pH in all three species, which could compromise their capacity to perform vigorous activity in the hours following treatment.

In all three species, lampricide-induced metabolic disturbances were corrected within 24 h, suggesting that long-term eco-physiological effects on trout and sturgeon populations were unlikely. However, the demonstrated ability of surviving larval lamprey to completely recover from lampricide treatment could also undermine sea lamprey control efforts by leading to increased numbers of parasitic juvenile sea lamprey that could go on to cause substantial damage to Great Lake's fisheries.

## CO-AUTHORSHIP

Chapters 3, 4 and 6: LC-MS/MS measurements of water niclosamide concentrations were conducted in the laboratory of Dr. Mark Servos, Department of Biology, University of Waterloo, by Dejana Mitrovic, a technician in the laboratory of Dr. Michael Wilkie. All toxicity experiments, experimental exposures, tissue collection and subsequent tissue and data analyses, as well as writeup were completed by Razvan Adrian Ionescu.

Chapter 5: experimental preparation and exposure of juvenile lake sturgeon to TFM, and tissue collection was performed by Scott Hepditch, an MSc student in the laboratory of Dr. Michael Wilkie. All subsequent tissue and data analyses, as well as writeup were completed by Razvan Adrian Ionescu.

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## 1. INTRODUCTION

### 1.1 The sea lamprey invasion and lampricides

The sea lamprey (*Petromyzon marinus*) is a parasitic jawless fish that feeds on the blood of large teleost (bony) fishes (Beamish and Potter, 1975; Farmer *et al.*, 1975; Farmer, 1980). It is native to the North Atlantic Ocean, but in the early 20$^{th}$ century sea lamprey invaded the Laurentian Great Lakes, contributing to the devastation of commercial and recreational, some of which were culturally important to First Nations (GLFC, 2011). The resulting damage to the Great Lakes' ecosystem ultimately led to the economic devastation of communities that relied on the lakes and fishery for their livelihoods. The underlying cause of this devastation was that the elimination of apex predators by parasitic sea lamprey subsequently led to a population explosion of other invasive species such as alewife (*Alosa pseudoharangus*) and rainbow smelt (*Osmerus mordax*; Smith and Tibbles, 1980; GLFC, 2011; Hansen *et al.*, 2016). Responding to this ecological and economic emergency, the Canadian and United States (US) governments signed the Convention on Great Lakes Fisheries in 1954, forming the Great Lakes Fisheries Commission (GLFC), with a mandate to develop a comprehensive program to eliminate/control invasive sea lamprey populations in the Great Lakes basin (GLFC, 2011). As a result, sea lamprey became the subject of intensive research on how to eradicate their populations, and to help restore Great Lakes' fisheries (Christie *et al.*, 2003; Krueger and Marsden, 2007; Siefkes, 2017).

Early measures for sea lamprey control (SLC) included physical and even electrical barriers to prevent adult sea lamprey from spawning (Applegate *et al.*, 1957). Electrical barriers were eventually discontinued because they were dangerous and impractical to operate (Smith *et al.*, 1974; Smith and Tibbles, 1980; see also Miehls *et al.*, 2020 for review). However, existing

dams used for flood control or electricity generation, and specially constructed low-head barrier dams and weirs were highly effective at preventing sea lamprey spawning and continue to be an essential tool for effective sea lamprey control (McLaughlin *et al.*, 2007; Siefkes, 2017). However, barriers alone could not control sea lamprey populations due to the massive number and complexity of rivers and streams that drained into the Great Lakes. For the last sixty years, the other essential method of SLC has been chemical control using the piscicides (lampricides) 3-trifluoromethyl-4-nitrophenol (TFM) and 2′,5-dichloro-4′-nitrosalicylanilide, better known as niclosamide (Bayluscide®), which are applied to rivers and streams infested with larval sea lamprey (ammocoetes; Applegate *et al.*, 1957, 1961; Lawrie, 1970; McDonald and Kolar, 2007; McLaughlin *et al.*, 2007). Although lampricides have been used for nearly sixty years to control sea lamprey populations in the Great Lakes, only recently have we begun to understand the mode of action of TFM and its effects on the physiology of sea lamprey. In addition, we know little about how TFM affects sensitive non-target fish species such as the lake sturgeon (*Acipenser fulvescens*), a species at risk in the Great Lakes. Even less is known about how niclosamide exerts its toxic effects on lamprey and non-target fishes. The overall goal of this thesis is to compare the physiological effects of niclosamide to those of TFM, and to contrast the physiological responses of sea lamprey to TFM and niclosamide to those of two non-target fishes, the rainbow trout (*Oncorhynchus mykiss*) and juvenile lake sturgeon.

## 1.2 Natural history of the sea lamprey

As one of 41 agnathan (jawless) fish species belonging to the order Petromyzontiformes and family Petromyzontidae, sea lamprey are found primarily in waters of the Northern Hemisphere on both coasts of the North Atlantic (Potter and Gill 2003; Renaud, 2011). After

hatching in freshwater streams and rivers, larval sea lamprey drift downstream and burrow into the soft sediment (Figure 1.1; Beamish and Potter, 1975; Sutton and Bowen, 1994). In the tributaries of the Great Lakes, larval sea lamprey are functionally blind, and spend 3-7 years burrowed in the sediments filter-feeding on detritus, biofilm, algae, diatoms and suspended organic matter (Figure 1.1; Beamish and Potter, 1975; Potter, 1980; Holmes and Youson, 1994; Sutton and Bowen, 1994; Manzon *et al.*, 2015). Feeding and respiration take place by drawing water into the oral cavity via an oral hood located anteriorly to the oral cavity. The water currents are generated by a muscular vellum, located anterior to the pharynx, which directs the water across the gills in a unidirectional fashion. Using the elastic recoil of the brachial skeleton, water exits via gill slits (branchiopores), allowing gas exchange to take place. The food particles are trapped in the pharynx by mucus secreted by the endostyle, and then directed to the gut (Rovainen, 1996). Once body mass and body length reach a critical threshold, usually greater than 2.5 g and 120 mm (Holmes and Youson, 1994), and lipid stores are sufficiently elevated, the highly complex, larval sea lamprey enter a multi-staged metamorphosis lasting 3-4 months (Lowe *et al.*, 1973; Youson, 2003; Manzon *et al.*, 2015). Sea lamprey metamorphosis is characterized by changes in body colouration, from light-dark brown to blue-black with a metallic sheen, and the appearance of eyes. Another distinct change is the loss of the oral hood, which is replaced by an oral disc ornamented with numerous teeth and a rasping tongue (Mallatt, 1996; Rovainen, 1996; Renaud *et al.*, 2009). When sea lamprey complete metamorphosis and enter the juvenile parasitic phase, the elaborate dentition of the oral disc and the rasping tongue are used to latch onto fish and feed on their blood (Figure 1.1; Mallatt, 1996; Rovainen, 1996; Renaud *et al.*, 2009). Juvenile parasitic sea lamprey consume considerable volumes of blood from large salmonids and other game fishes, sturgeons, and in marine environments, even sharks

and cetaceans (Beamish and Potter, 1975; Nichols and Hamilton, 2004; Wilkie *et al.*, 2004,; Gallant *et al.*, 2006; Renaud *et al.*, 2009). In the juvenile parasitic stage, sea lamprey ingest 3-10% of their body mass in blood and by the latter part of the parasitic phase this value may rise as high as 30% (Farmer *et al.*, 1975; Farmer, 1980). Fishes attacked by parasitic sea lamprey become functionally anaemic and are prone to secondary infection of the wound caused by lamprey attachment (Swink, 2003; Patrick *et al.*, 2009). These are the likely reasons why so many fish die after being parasitized. At the end of the juvenile parasitic stage, adult sea lamprey enter a second non-trophic period as they begin to migrate upstream to spawn and then die (Figure 1.1; Beamish and Potter, 1975).

Although sea lampreys are anadromous, and native to the north Atlantic Ocean, the ability of juvenile sea lamprey to survive in fresh water allowed them to invade and establish populations in the Laurentian Great Lakes, the Finger Lakes (New York) and Lake Champlain (Vermont and New York; Beamish, 1980; Smith and Tibbles, 1980; Eshenroder, 2009). However, the origin of sea lamprey in these waters remains a subject of debate (Eshenroder, 2009, 2014). Based on microsatellite DNA studies, it has been proposed that sea lamprey populations of the Atlantic and the Great Lakes are genetically different, which suggests that sea lamprey were indigenous to Lake Ontario, and possibly the Finger Lakes and Lake Champlain in New York and Vermont (Bryan *et al.*, 2005; Waldman *et al.*, 2004, 2006; D'Aloia *et al.*, 2015). On the other hand, primarily due to lack of historical documentation of sea lamprey in the region, persuasive arguments have been made that sea lamprey entered Lake Ontario from the Atlantic via the Hudson River in New York, following the construction of the Erie Canal in the early 1800s (Lawrie, 1970; Eshenroder, 2009, 2014). It is generally understood that Niagara Falls acted as a natural barrier to sea lamprey, restricting them to Lake Ontario. With modifications of

the Welland Canal completed in the early 1900s, however, sea lamprey were subsequently able to bypass Niagara Falls, entering Lake Erie circa early 1920s and eventually the Upper Great Lakes (Lawrie, 1970). By the 1950s, uninhibited sea lamprey predation along with unsustainable fishing practices (Pycha and King, 1975; Coble *et al.*, 1990) had nearly eliminated populations of top predators such as lake trout (*Salvelinus namaycush*) and burbot (*Lota lota*) from the upper Great Lakes, while decimating other important commercial, recreational and Indiginous fisheries, such as lake whitefish (*Coregonus clupeaformis*) and walleye (*Sander vitreus*; Smith and Tibbles, 1980).

### 1.3 Brief history of lampricides

Following the formation of the GLFC in 1954, intesive research began in an effort to identify a compound(s) that could be used to selectively control or eradicate sea lamprey (GLFC 2011). Following testing of 4,346 compounds (mostly organic), 15 agents were identified as being more toxic to sea lamprey than to rainbow trout. These compounds were all halogenated mono-nitrophenols (phenols containing one nitro group), with structural activity analyses indicating that compounds derived from dinitrophenols, such as 5-chloro-2-nitrophenol, 3,4,6-trichloro-2-nitrophenol and 3-tri-fluoromethyl-2-nitrophenol, and those derived from 4-nitrophenol including 2,5-dichloro-4-nitrophenol, 3-bromo-4-nitrophenol and TFM, which contained nitro groups in the para position and halogen groups in the meta position, had the greatest selectivity to sea lamprey (Applegate *et al.*, 1957; Applegate *et al.*, 1966). Although there were other potential lampricides identified, TFM was selected for its higher selectivity to sea lamprey and for its appropriate chemical properties, including water solubility (Applegate *et al.*, 1966; Thingvold and Lee, 1981).

The first field trials of TFM took place in early 1960s with the treatment of Lake Superior, followed by other Great Lakes in subsequent years (Schnick, 1972; Smith *et al.,* 1974; Smith and Tibbles, 1980). TFM is a halogenated, phenolic, aromatic compound which is light yellow at room temperature (Figure 1.2A). With a pKa of 6.38 (McConville *et al.*, 2016), TFM is a phenolic weak acid which easily dissociates to form the ionized species TFM-$O^-$, an important aspect to understanding its pharmacokinetics and treatment efficacy in field applications (Hubert, 2003). It has been reported that TFM undergoes relatively fast photodegradation in aquatic systems, with a half life of 3-5 days, when tested in the laboratory under natural water and sunlight conditions, with rates being accelerated at higher pH (Carey *et al.*, 1988; Ellis and Mabury, 2000). Conversely, more recent studies have shown that in small river systems which reach the Great Lakes in just a few kilometers, TFM experiences virtually no photodegradation (McConville *et al.*, 2017a, 2017b).

By the early 1960s the SLC program incorporated niclosamide into lampricide treatment regimes, frequently using it as an adjuvant to increase TFM toxiciy without loss of specificity (see McDonald and Kolar, 2007; Wilkie *et al.*, 2019 for reviews). It is important to note that niclosamide is not restricted to SLC, but is also used as a molluscicicde to treat waters infested with snails which are the intermediate host for the *Schistosoma japonicum* parasite which causes schistosomiasis in humans (Lardans and Dissous, 1998; Joubert *et al.*, 2001; Zhao *et al.*, 2015). In addition, niclosamide is also used to treat human and animal cestode (flatworm) and trematode (fluke) infections (Köhler, 2001; McKellar and Jackson, 2004), and more recently shown promise for treating various human cancers (e.g. Sack *et al.,* 2011; Li *et al.*, 2014; You *et al.*, 2014; Liang *et al.,* 2017).

Niclosamide, like TFM, is a phenolic compound comprised of two substituted aromatic rings linked by an amide functional group (Figure 1.2B). Similar to TFM, niclosamide is a weak phenolic acid with a $pK_a$ of 6.25 (Dawson, 2003), which easily dissociates, at higher pHs into its ionized form (Figure 1.2B). Niclosamide is much more lipophilic than TFM, rendering it more difficult to keep in aqueous solution (Tomlin, 1994). This reason, and the fact that niclosamide has higher toxicity (discussed later), is why it is most often used as an adjuvant (1-2%) to TFM (Dawson 2003).

Niclosamide comes in granular and emulsifiable concentrate or wettable powder form, each having a specific use. The granular form of niclosamide (granular Bayluscide®) is used for sea lamprey population surveys, while Bayluscide® (20% emulsifiable concentrate or 70% wettable powder) is applied to fast flowing or deep lentic waters, usually in conjunction with TFM at 1-2% concentrations of TFM (Dawson, 2003; Barber and Steeves, 2019). The addition of 1% niclosamide reduces the amount of TFM required for lampricide applications by 40% (Boogaard *et al.*, 2003; Gutreuter and Boogaard, 2007), without loss of TFM specificity (Dawson, 2003).

In addition to lampricides, barriers have been highly effective at preventing adult sea lamprey from spawning. Together barriers to sea lamprey migration and lampricides have resulted in 90 % reductions in sea lamprey populations in the Great Lakes from the peak values reached in the 1950s (Siefkes 2017). Another less widely used method of SLC was the sterilization of males using the chemical bisazir, which were then released to compete with fertile males for mates, but has since been discontinued (Siefkes, 2017; Miehls *et al.*, 2020). The sterilized male release technique (SMRT; 1991-2011) program was discontinued due to uncertainties regarding its success, introduced at least in part, by a failure to establish proper

metrics, such as adquate estimation of natural sea lamprey population size in the St. Marys River and the number of males that could be sterilized and released (Bravener and Twohey, 2016). More recently, biological control using lamprey specific pheremones have been developed for use as attractants/repllents to increase trapping success and/or divert lamprey away from spawning habitat (Wagner *et al.*, 2006; McLaughlin *et al.*, 2007; Sorensen and Hoye, 2007; Li *et al.*, 2012; Johnson *et al.*, 2015; Siefkes, 2017). However, this method has not yet been fully implemented, but field trials are underway to determine if lamprey specific pheromones could be a viable method of supplementary SLC (Siefkes 2017).

Despite these advances in our understanding of sea lamprey biology and the development of novel control tactics, lampricides will continue to be used to manage populations of sea lamprey in the Great Lakes for the foreseeable future. Yet, only recently have we begun to understand the mechanism(s) of TFM toxicity in sea lamprey and non-target organisms, and we know even less about niclosamide (Wilkie *et al.*, 2019). Such knowledge is required to further improve lampricide effectiveness and to better understand and mitigate the potential harmful effects that lampricides could have on non-target aquatic vertebrates and invertebrates, from both an individual and population standpoint (Boogaard *et al.*, 2003; McDonald and Kolar, 2007).

## 2. TOXICOLOGY

### 2.1 Lampricides uncouple mitochondrial oxidative phosphorylation

Both TFM and niclosamide have been long regarded as uncouplers of oxidative phosphorylation, primarily because they are phenolic and are weakly acidic (McLaughlin and Dilger, 1980; Hollingworth and Gadelhak, 1998; Skulachev, 1998; Kadenbach, 2003; Moridani *et al.*, 2003; Ozaki *et al.*, 2008; Bireanu *et al.*, 2011; Solaini *et al.*, 2011; Huerta *et al.*, 2020).

The process of oxidative phosphorylation (aerobic respiration) occurs in the mitochondria beginning with the oxidation of substrates (NADH and $FADH_2$) generated by the citric acid cycle (aka. Tricarboxylic Acid Cycle or Kreb‘s Cycle). Substrate oxidation takes place at respiratory complexes of the electron transport chain (ETC) located on the inner mitochondrial membrane, which frees electrons to be transported from one complex to the next (Complex I-IV). The oxidative process takes places when the electrons reach their final destination (Complex IV) where they combine with oxygen and protons to form water. As electrons are passed along the respiratory complexes, $H^+$ are pumped out of the mitochondrial matrix into the intermembrane space by respiratory complexes I, III and IV, which creates an electrochemical gradient across the inner membrane, referred to as the proton motive force. The low permeability of the inner membrane compels $H^+$ ions to return to the matrix through ATP-synthase (aka. Complex V). Passage of $H^+$ from the intermembrane space to the matrix through ATP-synthase energizes the phosphorylation of ADP to ATP, linking or coupling the oxidation of respiratory substrates on the ETC to phosphorylation of ADP to ATP (see Brookes, 2005; Scatena *et al.*, 2007 for review).

Some uncouplers of oxidative phosphorylation are thought to act as protonophores (proton shuttles), increasing the $H^+$ permeability of the inner mitochondrial membrane, allowing $H^+$ to non-specifically diffuse to the matrix. This degrades the proton motive force, subsequently lowering the production of ATP. In rainbow trout and sea lamprey, it has been demonstrated using isolated mitochondria that TFM uncouples oxidative phosphorylation in this manner (Birceanu *et al.*, 2011; Huerta *et al.*, 2020). Similar experiments have not yet been performed on sea lamprey or non-target fishes using niclosamide.

## 2.2 Effects of uncoupling of oxidative phosphorylation on physiology

A consequence of TFM-induced uncoupling of oxidative phosphorylation is a mismatch between ATP supply and demand, leading to decreases in anerobic energy reserves such as glycogen and phosphocreatine, with the brain being particularly vulnerable (Wilkie *et al.,* 2007; Birceanu *et al.,* 2009; Clifford *et al.,* 2012; Henry *et al.,* 2015). Similar effects were reported for TFM-exposed rainbow trout (Birceanu *et al.,* 2014), suggesting that that high energy phosphagen and glycogen stores could be used as biomarkers of lampricide exposure and sensitivity in fishes (Wilkie *et al.*, 2019). High energy phosphagens are transitory sources of ATP in times when energy demands are increased, such as during burst exercise, or when ATP supply is diminished due to external stressors such as $O_2$ starvation. Glycolysis is also used to sustain ATP supply when energy demands increase or ATP supply is reduced, providing a larger source of ATP when PCr reserves are insufficient to meet ATP demand (Hochachka 1991). Exposure to toxic concentrations of TFM was indeed reported to significantly deplete brain glycogen concentrations in sea lamprey (Birceanu *et al.,* 2009; Clifford *et al.,* 2012; Henry *et al.,* 2015) and rainbow trout (Birceanu *et al.*, 2014). The brain is highly reliant on glucose from glycogen, and these results indicate that death from TFM exposure may occur due to depletion of glycogen reserves leading to ATP starvation of the central nervous system (CNS). However, effects on other organs such as the heart, cannot be fully ruled out because it also accumulates TFM (Lech and Statham, 1975; Statham and Lech, 1975).

## 2.3 TFM selectivity, lampricide detoxification and elimination

TFM accumulates in the liver of teleost fishes where it is detoxified by Phase II biotransformation (Figure 1.3; Lech and Costrini, 1972; Kane *et al.,* 1994; Hubert *et al.,* 2005b;

Bussy *et al.,* 2017a; Foubister, 2018), which results in the addition of water soluble functional groups, such as glucuronic acid and sulfate esters. The addition of these functional groups are broadly used to render xenobiotics and endogenous compounds, such as steroids, more water soluble, which enables excretion via biliary and renal routes (Clarke *et al.,* 1991; Kalant and Roschlau, 1998). Lech and Statham (1975) suggested that in rainbow trout, TFM is primarily biotransformed in the liver and kidneys, where TFM-glucuronide accumulates. While it was long thought that glucuronidation was the primary path of TFM biotransformation in the liver *in vivo* (Lech and Costrini, 1972; Lech, 1974; Lech and Statham, 1975; Kane *et al.*, 1994), recent studies suggest that TFM is also conjugated with sulfate in non-target fishes, such as rainbow trout, lake sturgeon and bluegill (Bussy *et al*., 2017a, 2017b). Using liver homogenates of sea lamprey and non-target fishes, as well as in intact sea lamprey exposed to TFM, Bussy *et al.* (2017a, 2017b) recently demonstrated that TFM undergoes Phase I biotransformation, to a reduced form of TFM, 3-trifluoromethyl-4-aminophenol (TFMa; Figure 1.3). Consequentially, the accumulation of TFMa in intact sea lamprey is likely due to their reduced capacity to form TFM conjugates, TFM-glucuronide and TFM-sulfate, which would divert the accumulation of TFM to TFMa and its metabolites. The production of TFMa in non-target fishes is likely less important, physiologically and quantitatively, because of their ability to facilitate glucuronidation and sulfation of TFM (Wilkie *et al.,* 2019). Thus, the selectivity of TFM to sea lamprey may be explained by their comparatively low capacity to use glucuronidation and/or sulfation to biotransform the lampricide. This was demonstrated by using salicylamide to inhibit uridine diphosphate glucuronyltransferase (UDPGT), the enzyme which catalyzes the biotransformation of TFM to TFM-glucuronide (Lech, 1974). In salicylamide-treated rainbow trout there was

dose-dependant glucuronidation inhibition and reduced survival compared to untreated-controls, whereas larval sea lamprey were not affected (Lech, 1974; Lech and Statham, 1975).
In non-target fishes such as salmon (Schultz *et al.*, 1979), TFM is primarily excreted in urine, predominantly as TFM-glucuronide (Hunn and Allen, 1975), while others, such as channel catfish (*Ictalurus punctatus*), rely primarily on their gills for excretion (Allen and Hunn, 1977). Hlina *et al.* (2017) demonstrated, the latter strategy is likely relied upon by larval sea lamprey. Sea lamprey which were injected with $^{14}$C-labelled TFM ($^{14}$C-TFM) cleared over 95% of the load into the water within 24 h, and because sea lamprey's capacity to biotransform TFM via glucuronidation or sulfation is limited, it was concluded that a large proportion of TFM must be cleared at the gills in its hydrophobic (unionized) form.

Similar to TFM, phase I biotransformation has little impact on niclosamide metabolism (Figure 1.4; Van Der Kraak *et al.*, 1994), with the greatest accumulation occurring in the liver of rainbow trout along with 50-150 times higher concentrations in the bile (Statham and Lech, 1975). Mass spectroscopy revealed that the largest proportion of residues was glucuronidated-niclosamide, which would be excretable in urine (Statham and Lech, 1975). Niclosamide detoxification also occurs by sulfation, with significant concentrations of sulfated ester of niclosamide detected in the muscle of rainbow trout and catfish (Hubert *et al.*, 2005) and primarily excreted renally in non-target fishes (Dawson, 2003). However, very little is known about how this process occurs in sea lamprey.

## 3. RESEACH GAPS AND QUESTIONS

Until recently (Wilkie *et al.*, 2007; Birceanu *et al.*, 2009, 2011, 2014; Henry *et al.*, 2015; Huerta *et al.*, 2020), only one study has delved into the mode of action and physiological effects

of TFM in fishes (Christie and Battle 1963), with virtually no studies on niclosamide. The literature on the effects of niclosamide in fishes is mostly limited to toxicity and metabolism studies in combination with TFM (e.g. Statham and Lech, 1975; Schreier *et al.,* 2000; Dawson 2003; Boogaard *et al.,* 2003). While niclosamide is ever present in the SLC program, whether used alone or in combinations with TFM, little is known about how the deleterious effects of niclosamide (alone) are imparted on sea lamprey and what adverse effects are experienced by non-target fishes that may occupy the same habitats during treatment. Because of the frequent use of niclosamide in the SLC program it is important to understand how this chemical affects sea lamprey, to help to delineate the pathway of niclosamide toxicity and to better understand its interactions with TFM. A better understanding of TFM-niclosamide interactions could lead to more effective use of lampricides to control sea lamprey. This is an important consideration for several reasons: it addresses growing concerns about pesticides entering aquatic environments, could result in improved protection of non-target aquatic species, and greater reliance on TFM-niclosamide mixtures would be more cost effective because of the relative scarcity and high cost of TFM compared to niclosamide (Christie and Goddard, 2003).

A better knowledge of TFM and niclosamide toxicity and their interactions could also yield knowledge that can be used to prevent or mitigate possible adverse effects of lampricides in non-target fishes. Of particular concern are juvenile lake sturgeon, which are known to be highly sensitive to lampricides (Boogaard 2003; O'Connor et al. 2017). Lakes sturgeon are a species at risk throughout the Great Lakes basin and the remainder of their native range which reaches as far as the North Saskatchewan River in Edmonton, Alberta to the west, St. Roche de Aulnaires on the St. Lawrence River to the east, Seal River, a western tributary of Hudson Bay to the north, and the Mississippi River and its main southward tributaries to southern Arkansas, in the south

(Ferguson and Duckworth, 1997). Recovery efforts are underway, however, to re-establish lake sturgeon populations but these efforts are hindered by their slow sexual maturation, 12-15 years for males and 18-27 years for females (Scott and Crossman, 1973; Bruch, 1999), and non-iteroparous reproduction, spawning every 4-9 years for females and 1-3 years for males (Roussow, 1957; Fortin *et al.*, 1996). After hatching lake sturgeon larvae spend the first year of their life in streams and rivers which may also be inhabited with larval sea lamprey, where they may be subjected to lampricide exposure. They are most vulnerable to lampricide as young-of-the-year (YOY) juveniles, particularly when they are less than <100 mm in length (Boogaard *et al.*, 2003; McDonald and Kolar 2007), which is related to higher uptake rates of TFM in the YOY lake sturgeon compared to 1+ animals (Hepditch *et al.*, 2019). It is, therefore, of utmost importance to explore the physiological effects of TFM and niclosamide in lake sturgeon in order to better understand how lamprey control measures can be improved to better protect this species from the adverse effects of lampricides, while maintaining the effectiveness of the SLC program. The overarching goal of this dissertation was to characterize underlying mechanisms of toxicity, the physiological effects, and the resilience of larval sea lamprey, and two non-target fish species, larval lake sturgeon and rainbow trout, to TFM and niclosamide. Rainbow trout was used because, in addition to its physiology being widely studied and understood, it has also proved to be a very useful model organisms for learning about the non-target effects of TFM (Birceanu *et al.*, 2014). Thus, it is the logical choice as model organism comparing the physiological effects of niclosamide exposure to those of TFM. Accordingly, toxicology and biochemical techniques were used to determine changes in energy stores (e.g ATP, PCr, glycogen) and metabolites (e.g. pyruvate, lactate) in different tissues (e.g. muscle, brain, liver) of these fish following exposure to TFM or niclosamide.

## 4. RESEARCH OBJECTIVES

The specific objectives of this thesis were to: (i) compare the physiological effects of niclosamide to TFM in larval sea lamprey, rainbow trout and lake sturgeon; (ii) determine if/how the responses of larval sea lamprey to niclosamide differ from those in rainbow trout and lake sturgeon exposed to niclosamide (iii) characterize how quickly these fishes recovered from sub-lethal niclosamide exposure. To address these objectives sea lamprey, rainbow trout and lake sturgeon were exposed to either TFM and/or niclosamide for 9 h. The 9 h exposure time was chosen because normally this corresponds to the length of time that lampricides are applied to waterways during a given treatment. To quantify the corresponding physiological disturbances experienced by the fishes, muscle, brain, and liver were collected and frozen for later measurements of internal energy stores (e.g. ATP, PCr, glycogen) metabolites (e.g. pyruvate, lactate) and muscle pHi. Niclosamide-exposed sea lamprey (Chapter 2) and rainbow trout (Chapter 3) responses were compared to responses reported in literature for TFM, whereas the respective responses to TFM and niclosamide addressed in Chapters 4 and 5. Sub-sets of each species were also transferred to clean well water following niclosamide exposure, followed by tissue collection at 24 h, to determine if lampricide-induced physiological disturbances were corrected.

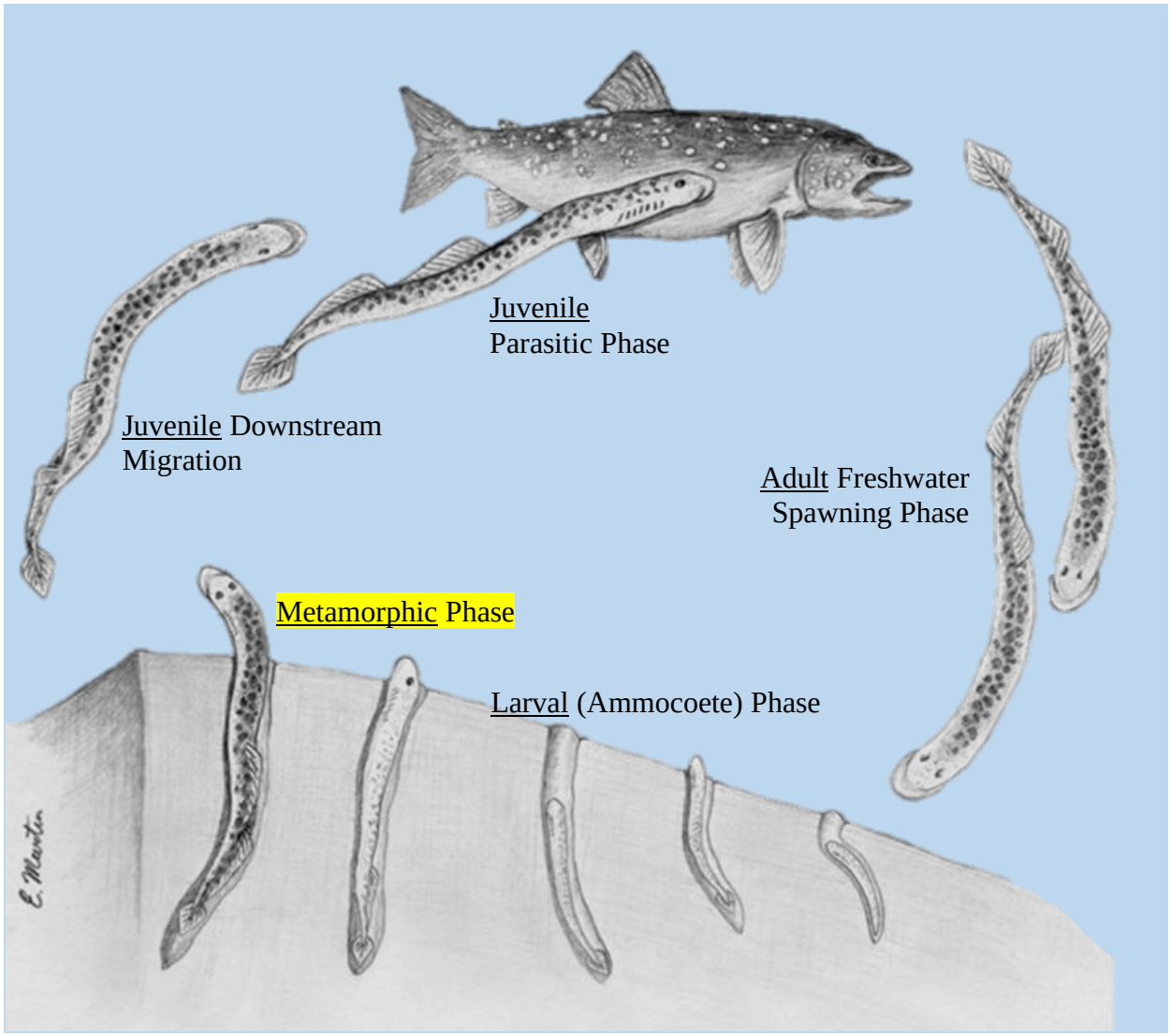


**Figure 1.1 Sea lamprey life cycle**

**Figure 1.1 Sea lamprey life cycle.** After hatching, larval sea lamprey (ammocoetes) burrow into the substrate of rivers and streams draining into the Atlantic Ocean or Laurentian Great Lakes and live as filter feeders. After 3-7 years, the larvae undergo a non-trophic, multi-staged metamorphosis lasting 3-4 months that is characterized by morphological and physiological changes that include the development of an oral disk complimented with teeth, a rasping tongue and the appearance of eyes. Following metamorphosis, juvenile sea lamprey migrate downstream to open waters such as the Atlantic Ocean, or to the Great Lakes in the case of landlocked, invasive populations of sea lamprey. During the juvenile parasitic phase, sea lamprey prey on a variety of fishes including lake trout and white fish in the Great Lakes. The parasitic stage lasts 12-20 months, after which adult sea lamprey stop feeding and migrate upstream, spawn and then die. Figure adapted from Wilkie (2009).

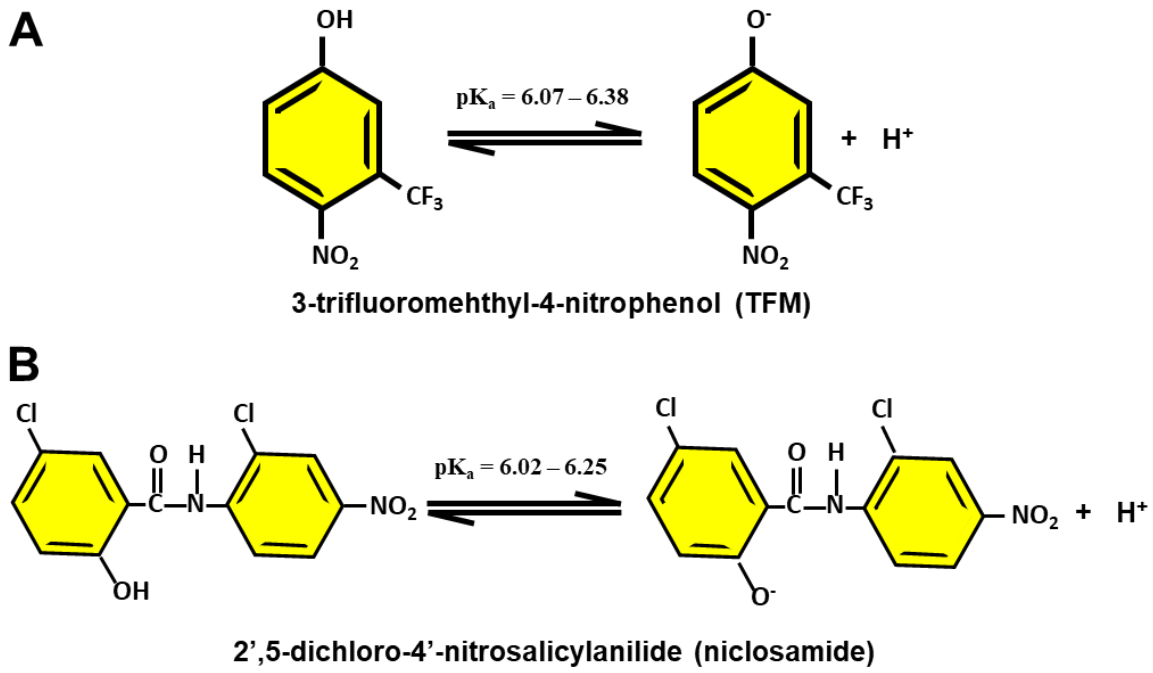


**Figure 1.2 Chemical structures of TFM and niclosamide**

**Figure 1.2 Chemical structures of TFM and niclosamide.** Both (A) TFM and (B) niclosamide are weak acids with pKa of 6.07 – 6.38 and 6.02 - 6.25, respectively. At low pH both chemicals are predominantly in the un-ionized (phenolic), more diffusible form and at high pH they are predominantly in the ionized (phenolate), less diffusible form.

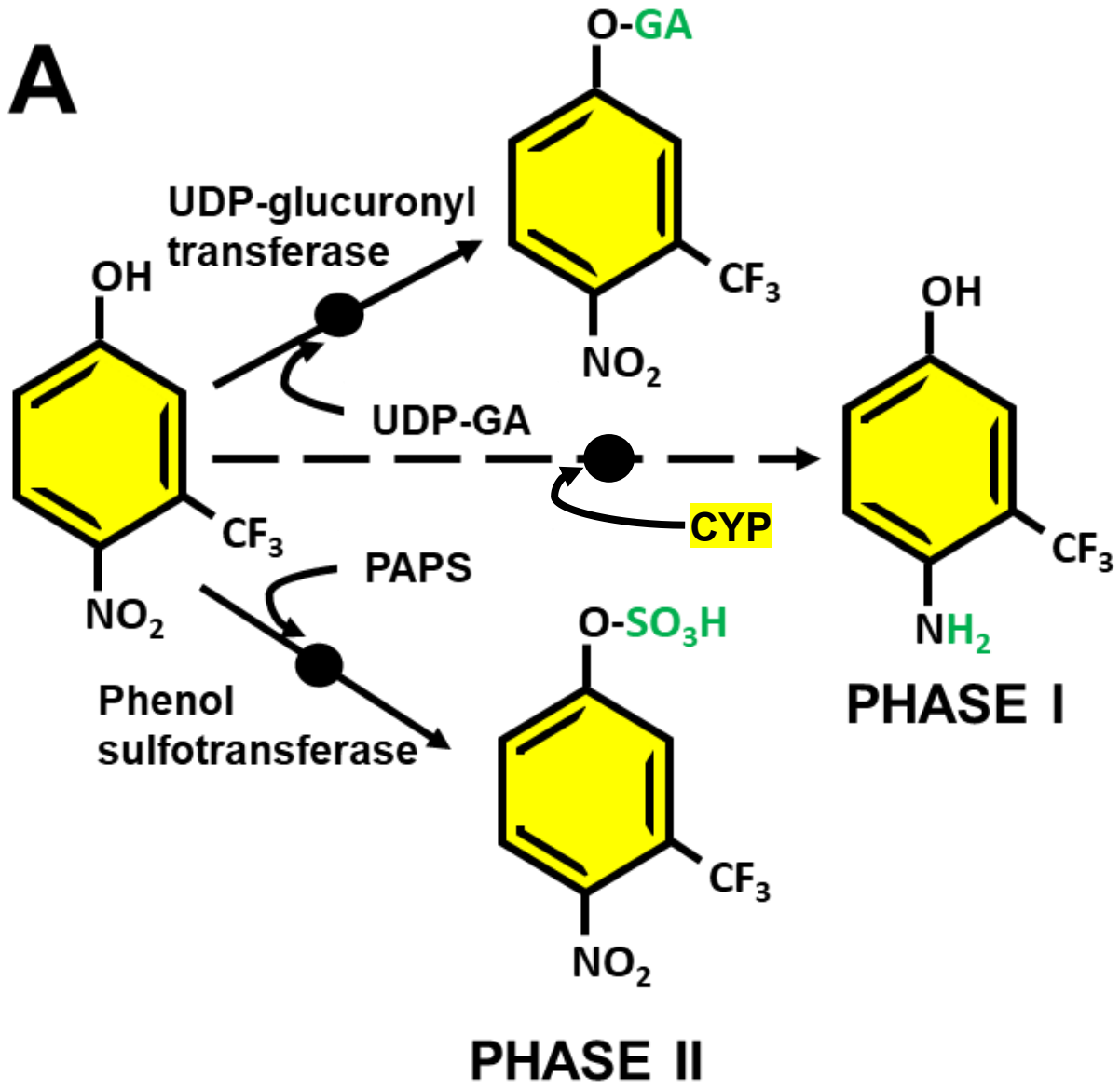


**Figure 1.3 Mechanisms of TFM detoxification**

**Figure 1.3 Mechanisms of TFM detoxification.** TFM is primarily detoxified in non-target fishes using Phase II biotransformation, with Phase I pathways possibly playing a minor role. In Phase II biotransformation, TFM can be conjugated to form TFM-glucuronide, in an enzyme mediated reaction catalyzed by UDP-glucuronyltransferase (UDPGT), leading to the attachment of a glucuronic acid functional group (GA) from UDP-glucuronic acid (UDPGA) to the hydroxyl group of TFM. Sulfation takes place via a sulfotransferase, which catalyzes the addition of a sulfate functional group to the hydroxyl functional site of TFM by conversion of inorganic sulfate to the substrate 3'-phosphoadenosine-5'-phosphosulphate (PAPS).

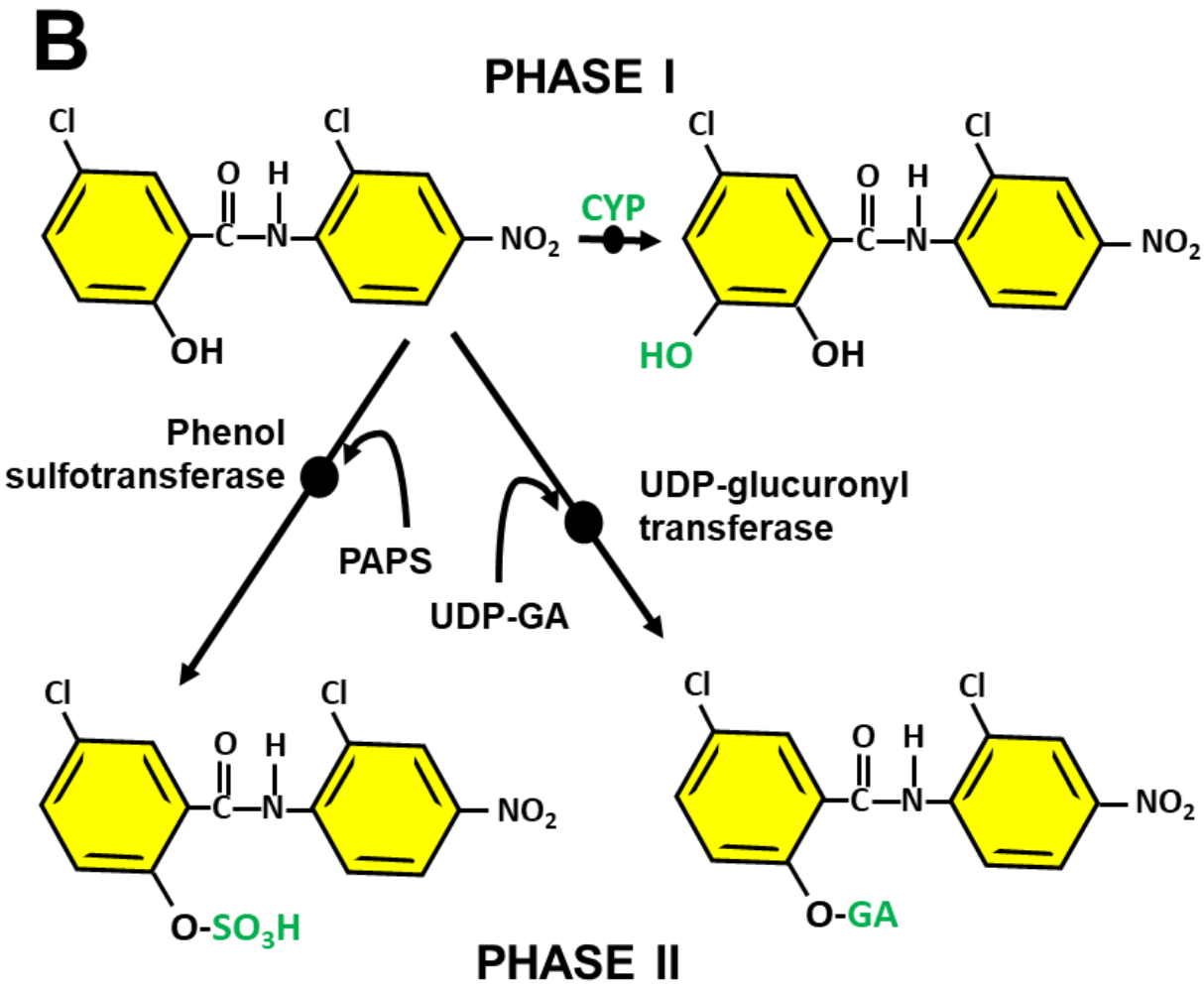


**Figure 1.4 Mechanisms of niclosamide detoxification**

**Figure 1.4 Mechanisms of niclosamide detoxification.** Niclosamide is primarily detoxified in non-target fishes using Phase II biotransformation, with Phase I pathways possibly having a minor role via cytochrome P450 enzymes (CYP). For glucuronidation, UDP-glucuronyl transferase (UDPGT) catalyzes the transfer of a glucuronic acid functional group (GA) from UDP-glucuronic acid (UDPGA) to the hydroxyl group of chlorophenol ring of niclosamide, resulting in the glucuronide conjugate. For sulfation, sulfotransferase catalyzes the addition of sulfate functional group to the hydroxyl functional site of TFM, by conversion of inorganic sulfate to the substrate 3'-phosphoadenosine-5'-phosphosulphate (PAPS).

# CHAPTER 2:

# Experimental and Analytical Methods

This thesis comprises experiments on three different fish species, sea lamprey (*Petromyzon marinus*), rainbow trout (*Oncorhynchus marinus*) and lake sturgeon (*Acipenser fulvescens*), to determine the toxicity and physiological effects of two pesticides (lampricides), 3-trifluoromethyl-4-nitrophenol (TFM) and 2',5-dichloro-4'-nitrosalicylanalide (niclosamide; aka Bayluscide®, Bayer 73®). Because many of the processes and analyses (e.g. metabolite extraction, enzyme linked assays, intracellular pH determination) are similar throughout the thesis, this chapter is intended as a global materials and methods description. Where differences occur, they will be described in detail. The reader is encouraged to refer back to this chapter for reference when reading the following chapters.

## 1. ANIMAL HUSBANDRY

### 1.1 Larval sea lamprey

Larval sea lamprey (total length 124 ± 5.7 mm; mass 2.3 ± 0.1 g; N = 150) were captured from tributaries draining into Lake Huron or Lake Michigan by United States Fish and Wildlife biologists using pulsed D.C. electrofishing and temporarily held in the aquatic facilities at the United States Geological Survey's Hammond Bay Biological Station (HBBS), Millersburg, Michigan. Groups of larvae (N = 250) were then shipped by overnight courier, in plastics bags filled with ice cold, oxygen saturated water, that were contained in hard-sided 70 L coolers, to Wilfrid Laurier University, Waterloo, Ontario, Canada, and then housed in 110 L polypropylene tanks lined on the bottom with ~10 cm of fine sand and continuously receiving Wilfrid Laurier University well water at a replacement rate of ~1 L $min^{-1}$, after passing it through an aerated head tank placed ~2.5 m above the water surface, before entering the tank via a 1.25 cm (I.D.) hose. Water quality was monitored daily for temperature (15° ± 0.4°C), dissolved $O_2$ (DO; 99.2 ± 0.4

%) and pH (8.12 ± 0.04) using a DO meter (Pro2030, YSI Integrated Systems & Services, St Petersburg, FL, USA) and pH meter (Oakton pHTestr 20, Thermo Fisher, Waltham, MA, USA). Titratable alkalinity averaged 268 ± 3 mg $L^{-1}$ as $CaCO_3$ measured using a commercial kit (AL-AP MG/L (2444301), HACH Limited, London, Ontario, Canada). Stocking density was 4.6 g $L^{-1}$ and the fish were fed once a week with a slurry of Baker's Select Fresh Yeast (1.0 g $fish^{-1}$; Fleishmann's, Mississauga, ON) mixed with well water. Sea lamprey were allowed to acclimate to the laboratory for at least two weeks prior to start of experiments. All experiments followed Canadian Council of Animal Care (CCAC) guidelines and were approved by the WLU Animal Care Committee.

1.2 Rainbow trout

Rainbow trout (total length 163 ± 1.45 mm; mass 45 ± 1.08 g; N = 100) were acquired in August 2018 from Rainbow Springs Hatchery, Thamesford, Ontario, Canada and housed, the same day, in a 400 L polypropylene tank continuously receiving Wilfrid Laurier University well water at a replacement rate of ~1 L $min^{-1}$, after passing it through a degassing column (30 cm X 15 cm diameter) filled with biological beads and suspended ~ 0.75 m above the water surface. Water quality was monitored daily for temperature (15 ± 0.5$^{o}$C), dissolved $O_2$ (DO; 98.6 ± 0.5 %) and pH (8.22 ± 0.05) using a DO meter (Pro2030, YSI Integrated Systems & Services, St Petersburg, FL, USA) and pH meter (Oakton pHTestr 20, Eutech Instruments; Thermo Fisher, Waltham, MA, USA). Titratable alkalinity averaged 238 ± 5 mg $L^{-1}$ $CaCO_3$ as measured using a commercial kit (AL-AP MG/L (2444301), HACH Limited, London, Ontario, Canada). Water hardness, and the concentrations of $Na^+$ and $Cl^-$ averaged ~350 mg $L^{-1}$ as $CaCO_3$, ~1.1 mmol $L^{-1}$ and 0.0 mmol $L^{-1}$, respectively. The fish were fed 3 times a week with size #1 floating fish

pellets (~2% total body mass; EWOS, Cargill Incorporated, Minneapolis, MN, USA). Fish were allowed to acclimate to the laboratory for at least two weeks prior to start of experiments. All experiments followed Canadian Council of Animal Care (CCAC) guidelines and were approved by the WLU Animal Care Committee.

1.3 Lake sturgeon (TFM exposure)

Young-of-the-year (YOY; total length 118 ± 1.87 mm; weight 4.36 ± 0.20 g; N = 300) lake sturgeon were graciously provided by Dr. Gary Anderson, Department of Biological Sciences, University of Manitoba. They were the progeny of 4 female and 5 male lake sturgeon, caught on the Winnipeg River (50º17'52"N, 95º32'51"W), from which eggs and sperm were collected by members of the Anderson lab in April 2015 fertilized, and then hatched in mid-late May 2015 (Bjornson and Anderson, 2018). The YOY lake sturgeon were subsequently shipped to Wilfrid Laurier University on December 5, 2015, where they were housed in a multi tank G-HAB aquatic system (Pentair Aquatic Eco-Systems, Apopka, FL, USA) equipped with a 5-phase filtration (large particulate, biological, small particulate, carbon and UV) system and kept on recirculation with partial water replacement of 120L per day. Water was a 50:50 mix of City of Waterloo dechlorinated water and Wilfrid Laurier University reverse osmosis (RO) water to achieve a conductivity of 700-900 µS (alkalinity =150-200 mg $L^{-1}$ $CaCO_3$) and pH of 8.0 ± 0.1. Stocking densities were approximately 12 g biomass $L^{-1}$. The fish were fed twice daily on frozen blood worms (~2% total body mass; Brine Shrimp Direct, Ogden, UT, USA). Water parameters [pH, dissolved $O_2$ (DO), conductivity] were checked daily while chlorine and total ammonia were checked weekly. Experiments proceeded in the first week of April 2016 and followed

Canadian Council of Animal Care guidelines and were approved by the WLU Animal Care Committee.

1.4 Lake sturgeon (niclosamide exposure)

Lake sturgeon fertilized eggs, in the eyed stage, were provided courtesy of Joe Hunter, Sustainable Sturgeon Culture, Emo, Ontario, Canada, and reared at the University of Guelph Alma Aquaculture Research Station, Alma, Ontario, Canada in May 2018. Young-of-the-year (YOY; total length 128.8 ± 5.7 mm; mass 9.1 ± 3.5 g; N = 200) lake sturgeon were subsequently transported to Wilfrid Laurier University on March 15, 2019, where they were housed in a circular tank (water volume ~ 400 L) continuously receiving aerated Wilfrid Laurier University well water (temperature = 14.5 ± 0.5$^{o}$C; alkalinity = 280 ± 5 mg $L^{-1}$ $CaCO_3$; pH = 7.93 ± 0.1; DO = 95 ± 2%) at a rate of 1 L $min^{-1}$ replacement. Stocking density was approximately 4 g biomass $L^{-1}$. The fish were fed daily (2% body weight) with a 2:1 slurry of frozen blood worms (Brine Shrimp Direct, Ogden, UT, USA) mixed with size 0 commercial fish pellets (EWOS, Cargill incorporated, Minneapolis, MN, USA). Water quality parameters [temperature, pH, dissolved $O_2$ (DO) and alkalinity] were checked daily while chlorine and total ammonia were checked weekly. Experiments were completed following at least 2 weeks acclimation to holding environment and followed Canadian Council of Animal Care guidelines and were approved by the WLU Animal Care Committee.

## 2 NICLOSAMIDE TOXICITY TESTS

### 2.1 Larval sea lamprey

Prior to niclosamide toxicity tests, food was withheld for at least 48 h to limit ammonia buildup in the static experimental tanks, after which the sea lamprey were transferred to glass aquaria (N = 4 per aquaria) containing 8 L of aerated Wilfrid Laurier University well water, of the same chemistry described above, and allowed to acclimate overnight. The day of experiments, the water was changed (approximately 75 %) to eliminate the possibility of ammonia build-up confounding results. All experiments were conducted using field formulation niclosamide (Bayluscide® emulsifiable concentrate, containing 16.9% active ingredient; Coating Place Inc., Verona, WI, USA) provided courtesy of the Sea Lamprey Control Centre, Sault St. Marie, Ontario, Canada. Larval sea lamprey (N = 12 each; N = 4 in triplicate at each concentration) were exposed to various concentrations (0.005, 0.01, 0.025, 0.05, 0.1 or 0.25 mg $L^{-1}$) of niclosamide, dissolved in 50% methanol, for up to 24 h, while control fish (N = 12) were held for 24 h in the absence of niclosamide. Fish were monitored for mortalities at 1, 2, 3, 6, 9, 12 and 24 h of exposure, and dead fish were removed, weighed, measured for length, and the time of death recorded. Immobility was used as criteria for determination of death, which was confirmed by removing the fish and pinching the tail by hand. The niclosamide 9-h $LC_{50}$ and 95% confidence levels for sea lamprey were calculated using the log-probit method based on Litchfield Jr and Wilcoxon (1949) using online software (https://jvadams.shinyapps.io/LW1949demo/).

2.2 Lake sturgeon

Prior to toxicity tests, lake sturgeon were fasted for 24 h to limit ammonia buildup in the static experimental tanks, and then transferred to aquaria (n = 3 per aquaria) containing 8 L of aerated Wilfrid Laurier University well water, of the same chemistry described above, and allowed to acclimate overnight. The day of experiments, the water was changed (approximately 75 %) to eliminate the possibility of ammonia build-up confounding results. All experiments were conducted using field formulation of niclosamide (Bayluscide® emulsifiable concentrate, containing 16.9% active ingredient; Coating Place Inc., Verona, WI, USA) provided courtesy of the Sea Lamprey Control Centre, Sault St. Marie, Ontario, Canada. Lake sturgeon (N = 12 each) were exposed to various concentrations (0.05, 0.075, 0.10, 0.11, 0.13, 0.17, 0.25, 0.50 or 1.00 mg $L^{-1}$) of niclosamide, diluted with 50% methanol, for up to 12 h, while control fish (N = 12) were held for 12 h in the absence of niclosamide. Fish were monitored for mortalities at 1, 2, 3, 6, 9 and 12 h of exposure, and dead fish were removed, weighed, measured for length and time of death recorded. The niclosamide 9-h $LC_{50}$ and 95% confidence levels for lake sturgeon were calculated using the log-probit method based on Litchfieldand Wilcoxon (1949) using software available online at (https://jvadams.shinyapps.io/LW1949demo/).

## 3. EXPERIMENTAL PROCEDURES

3.1 Larval sea lamprey

All experiments were conducted using the same stock of field formulation niclosamide (Bayluscide® emulsifiable concentrate, containing 16.9% active ingredient; Coating Place Inc., Verona, WI, USA), provided courtesy of the Sea Lamprey Control Centre, Sault St. Marie, Ontario, Canada, dissolved in 50 % methanol. Experimental glass aquaria (N = 24; 10 L

maximum volume) were filled with 8 L of Wilfrid Laurier University well water (for water chemistry see Table 2.1) and placed in a flow-through water bath to control temperature. Twelve hours prior to experiments, larval sea lamprey (n = 3 each) were transferred to experimental aquaria, and food was withheld to limit ammonia accumulation in the static systems. Control fish (not exposed to niclosamide) were sampled at 0 h (n=6) and at 9 h (n=6) to rule out possible temporal effects on energy stores and metabolite concentrations that could have confounded data interpretation. At the start of experiments, sufficient niclosamide was added to each of the treatment aquaria to achieve the nominal concentration of 0.11 mg $L^{-1}$, the 9-h $LC_{50}$ determined from the toxicity experiments described above. The fish in each of the aquaria receiving niclosamide were exposed to the lampricide for 1, 3, 6 or 9 h at which time they were euthanized with an overdose of 1.0 g $L^{-1}$ tricaine methanesulfonate (TMS; Syndel Laboratories Canada, Nanaimo, BC, Canada) buffered with 2.0 g $L^{-1}$ $NaHCO_3$, followed by blood and tissue (brain, liver, kidney and carcass) collection for later analysis of tissue energy stores and metabolites. A sub-set of surviving sea lamprey exposed to niclosamide for 9 h were subsequently transferred to new aquaria containing clean (no niclosamide) well water for 24 h, to assess post-niclosamide recovery, after which they were euthanized, followed by blood and tissue collection. Immediately following euthanasia, each fish was patted dry with paper towel, weighed and measured for total length. Blood was collected by caudal transection into 500 µL polypropylene centrifuge tubes that had been pre-rinsed with $Na^+$-heparin (Sigma-Aldridge, Oakville, Ontario, Canada; 15 mg $Na^+$-heparin in 25 mL Courtland's Saline) and temporarily set aside on ice. Liver, kidney and brain were then excised, snap frozen in liquid nitrogen, stored at -80°C and saved for later analyses. The carcass (whole body minus the viscera and head) was then freeze clamped in liquid $N_2$ using pre-cooled aluminum tongs and stored at -80°C for later analyses of

tissue energy stores, metabolites and intracellular pH (Wang *et al.*, 1994). The blood that had been set aside was then separated into 2 aliquots to be used for ion content or lactate analyses. The whole blood designated for lactate analysis was acidified with 2 volumes of 8% perchloric acid (PCA) containing 1 mmol $L^{-1}$ ethylenediaminetetraacetic acid (EDTA, Sigma-Aldridge Canada, Oakville, Ontario, Canada), centrifuged (Centrifuge 5415D; Eppindorf Canada Ltd., Mississauga, Ontario, Canada) at 10,000 *g* for 5 minutes and frozen in liquid $N_2$. The second blood aliquot designated for ion analyses was centrifuged, as above, and the plasma transferred to a new heparin-coated microcentrifuge tubes, which were immediately snap frozen in liquid $N_2$ and stored at -80$^{o}$C.

3.2 Rainbow trout

Prior to experiments, the rainbow trout were fasted for 24 h, then transferred one at a time to one of 24 experimental glass aquaria containing 8 L of WLU well-water (for water chemistry see Table 2.1), under static conditions, and allowed to acclimate overnight. The aquaria were positioned in a flow-through water bath, which maintained a constant temperature of 15$^{o}$C.

All experiments were conducted using same field formulation of niclosamide as described above for sea lamprey experiments. The morning of experiments, approximately 75 % of the water was replaced with a fresh batch of well water at the appropriate temperature to ensure that experimental results were not confounded by build-ups of waste ammonia in the water, which was never detectable. After approximately 30 minutes, each aquaria (except controls or shams) were dosed with 10 mL of 120 mg $L^{-1}$ niclosamide dissolved in 50 % methanol, to achieve a nominal niclosamide concentration of 0.15 mg $L^{-1}$, the pre-determined 9-h $LC_{50}$ of niclosamide to rainbow trout in Wilfrid Laurier University well water (Darren Foubister,

unpublished data). Water samples were taken at the start (0 h; immediately after adding niclosamide) and conclusion (9 h) of experiments for measurements of niclosamide concentrations (described below). All aquaria were well aerated, achieving dissolved oxygen levels of 98% ±1. A sub-set of fish exposed to the same volume of 50 % methanol, minus the niclosamide, served as shams to ensure that any physiological changes observed in niclosamide-exposed fish were not a result of solvent exposure. The rainbow trout were exposed to niclosamide for 1, 3, 6 or 9 h (n = 12 per sample period), or 9 h followed by 24 h of depuration in niclosamide-free water (n = 12). Control fish were sampled at the beginning of the experiment or after 9 h to ensure that temporal changes in energy stores did not obscure findings (n = 8 each). Shams were sampled after 9 h (n = 7). At each designated sample period, fish were euthanized one at a time by adding directly to each container a liquid slurry (10 mL) of tricaine methanesulfonate buffered with $NaHCO_3$ to achieve a final concentration of 1.0 g $L^{-1}$ and 2.0 g $L^{-1}$, respectively. After euthanization, each fish was patted dry with paper towel, weighed and measured for total length. Blood was collected by post-anal transection of the caudal peduncle, into a heparinized 1.5 mL microcentrifuge tube and then centrifuged at 10, 000 *g* for 4 minutes. The resultant plasma supernatant was transferred to a new heparinized tube and flash frozen in liquid nitrogen and stored at -80$^{o}$C for later ion analyses. The liver and brain were then excised, snap frozen in liquid nitrogen and stored at -80$^{o}$C for later analysis. A filet of white muscle (1-2 g) was then collected from the lateral trunk of the fish, immediately freeze clamped using pre-chilled aluminum tongs in liquid nitrogen, and stored at -80$^{o}$C for later analyses (Wang *et al.*, 1994a).

3.3 Lake sturgeon (TFM exposure)

Lake sturgeon (N = 32) were transferred from holding tanks to a 37L glass aquarium and acclimated for four weeks in reconstituted water set to moderate alkalinity (150 mg $l^{-1}$ $CaCO_3$), using methods described by American Public Health Association (APHA, 1999) and American Society of Testing and Materials (APHA, 2007). Briefly, RO water was reconstituted with addition of adequate amounts of $CaSO_4 \bullet H_2O$ (BioShop Canada Inc. Burlington, Ontario, Canada), KCl (VWR International LLC, West Chester, PA, USA) and $MgSO_4$ (BioShop Canada Inc.) to control water hardness (~100 mg $l^{-1}$ $CaCO_3$) and $NaHCO_3$ (BioShop Canada Inc.) to establish desired alkalinity. The aquarium was fitted with activated carbon and biological filtration systems and received 50% water changes every other day. Water composition was confirmed by use of flame atomic absorption spectroscopy (AAS; PinAAcle 900T, Perkin Elmer, Waltham, MA, USA) for $Na^+$ and $Ca^{2+}$, a colorimetric assay for $Cl^-$ (Zall *et al.*, 1956), a pH meter (YSI Integrated Systems & Services, St Petersburg, FL, USA), and a commercial titratable alkalinity kit (HACH, London, Ontario, Canada). During acclimation, the fish were fed daily on frozen blood worms (2% of body weight).

All experiments were conducted using field formulation of TFM (Clariant SFC GMBH WERK, Griesheim, Germany; 35% active ingredient dissolved in isopropanol), provided courtesy of the Sea Lamprey Control Centre, Fisheries and Oceans Canada (DFO), Sault Ste. Marie, ON. Experimental aquaria (N = 4; 20 L maximum volume) were filled with 15 L reconstituted water of moderate alkalinity (150 mg $L^{-1}$ $CaCO_3$; pH = 8.36 ± 0.011). Seventy-two hours prior to experiments, lake sturgeon (n = 8 each) were transferred to experimental aquaria, and food was withheld to limit ammonia accumulation in the static systems. One aquarium was designated as a control tank (no TFM) for the duration of the experiment. At the start of

experiments, sufficient TFM was added to each of the treatment aquaria to achieve at nominal TFM concentration of 4.7 mg $L^{-1}$, which was equivalent to the sea lamprey minimum lethal concentration (MLC) of 4.7 mg $L^{-1}$, which was determined from tables published by Bills *et al.* (2003), based on the measured water pH and alkalinity of the test water. The fish in each of the 3 aquaria receiving TFM were exposed to the lampricide for 3, 6 or 9 h after which they were euthanized with an overdose of 1.0 g $L^{-1}$ tricaine methanesulfonate, buffered with 2.0 g $L^{-1}$ $NaHCO_3$. Of the 8 lake sturgeon in the control tank, 4 were euthanized at the start of the experiments and 4 at the conclusion, to determine if there was temporal variation in physiological parameters to be measured. Immediately after euthanizing the fish, each was patted dry with paper towel, weighed and measured for total length. The liver and brain were then excised, snap frozen in liquid nitrogen and stored at -80°C and saved for later analyses. The carcass was freeze clamped using pre-chilled aluminum tongs in liquid nitrogen and stored at -80$^{o}$C for later analyses (Wang *et al.*, 1994).

Rainbow trout (*Oncorhynchus mykiss*; Rainbow Springs, Thamesford, ON, Canada; N = 4; total length 157.7± 0.7 mm; mass = 11.55 ± 0.9 g), also acclimated to the same medium alkalinity water, were sampled under control conditions (no TFM), and were subsequently used as positive controls for assays conducted on lake sturgeon tissues.

### 3.4 Lake sturgeon (niclosamide exposure)

All experiments were conducted using the same stock of field formulation of niclosamide, dissolved in 50 % methanol, described above. Experimental aquaria (N = 24; 10 L maximum volume) were filled with 8 L of Wilfrid Laurier University well water (for water chemistry see Table 2.1) and placed in a flow-through water bath to control temperature. Twelve

hours prior to experiments, the sturgeon (n = 3 each) were transferred to experimental aquaria, and food was withheld to limit ammonia accumulation in the static systems. For each of the two experimental series, control fish (not exposed to niclosamide) were sampled at 0 h (N=6) and at 9 h (N=6) to rule out possible temporal effects on energy stores and metabolite concentrations that could have confounded data interpretation. At the start of experiments, sufficient niclosamide was added to each of the treatment aquaria to achieve the nominal concentration of 0.11 mg $L^{-1}$, the 9-h $LC_{50}$ determined from the toxicity experiments described above. The fish in each of the aquaria receiving niclosamide were exposed to the lampricide for 1, 3, 6 or 9 h after which they were euthanized with an overdose of 1.0 g $L^{-1}$ tricaine methanesulfonate buffered with 2.0 g $L^{-1}$ $NaHCO_3$, followed by collection of blood and tissue (brain, liver, carcass) for later analysis of tissue energy stores and metabolites. A sub-set of surviving lake sturgeon exposed to niclosamide for 9 h were subsequently transferred to new aquaria containing clean (no niclosamide) well water for 24 h, to assess post-niclosamide recovery, after which they were euthanized, followed by blood and tissue collection.

Immediately following euthanasia, each fish was patted dry with paper towel, weighed and measured for total length. Blood was then collected by caudal transection, into heparin- (600 mg $L^{-1}$ $Na^+$-heparin in Courtland's Saline) coated microcentrifuge tubes, and the liver and brain were then excised, snap frozen in liquid nitrogen and stored at -80°C and saved for later analyses. The carcass (whole body minus the viscera and head) was freeze clamped using pre-chilled aluminum tongs in liquid nitrogen and stored at -80°C for later analyses of tissue energy stores, metabolites and intracellular pH (Wang *et al.*, 1994). The blood was separated into 2 aliquots to be used for ion content or lactate analyses. The whole blood designated for lactate analysis was acidified with 2 volumes of 8% PCA containing 1 mmol $L^{-1}$ EDTA, centrifuged at

10, 000 *g* for 5 minutes and then frozen in liquid $N_2$. The second blood aliquot designated for ion analyses was centrifuged, as above, and the plasma was transferred to new heparin-coated microcentrifuge tubes, which were immediately snap frozen in liquid $N_2$ and stored at -80ºC.

## 4. QUANTIFICATION OF LAMPRICIDES IN WATER

### 4.1 Niclosamide

Water samples were collected from all experimental tanks containing niclosamide at 0 h (immediately after addition of niclosamide), followed by 9 h (conclusion of exposure). Water samples were collected from the middle of the tanks in 20 mL glass scintillation vials and stored at -20°C until analysis. Prior to analysis samples were completely thawed at room temperature and then vortexed for 20 s. From each sample, 5 mL of water was transferred to a new clean glass test tube. Quality control samples (matrix spikes) were prepared with 5 mL of (deionized) Milli-Q water in a glass test tube spiked with 100 µg $L^{-1}$ niclosamide-(2-chloro-4-nitrophenyl-$^{13}C$) hydrate (NIC-$C^{13}$) as the internal sample chemical (200 µg $L^{-1}$) and samples were vortexed again for an additional 20 s. Samples were filtered through 0.45 µm glass fiber filters (Pall Corporation, Michigan, USA) using a vacuum filtration apparatus (15 mL, Sigma Aldrich) and 1 ml of the eluent was transferred into a 2 mL amber glass vial ready for analysis.

For LC-MS/MS analyses, samples were measured for the niclosamide using an Agilent 1260 HPLC with 6460 Triple Quad and Agilent Jetstream ESI source in negative ionization mode. An Agilent Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm) was used to chromatographically separate the analyte. Samples were injected at 10 µL sample volume, 35°C constant temperature, flow rate of 0.8 mL $min^{-1}$ and with the gradient flow. Mobile phase A was Milli-Q water and B was acetonitrile. The gradient was 0 min: 80, 20; 1 min: 80, 20; 10 min: 0,

100; 12 min: 0, 100; 12.1 min: 80, 20 (numbers expressed in percentiles of mobile phase A, B). The instrument source parameters were temperature of 400°C, the gas temperature of 230°C, gas flow 12 L $min^{-1}$, nebulizer at 275.8 kPa, and a capillary voltage of 2500V. The calibration curve ranged from 0 µg $L^{-1}$ to 500 µg $L^{-1}$ of each standard. Niclosamide concentrations were calculated and adjusted for background noise using a niclosamide (0 – 500 mg $L^{-1}$) standard curve linear equation ($y = 1.0001x – 0.0008$; $R^2 = 0.9998$).

4.1 TFM

Water samples from each tank were collected at the beginning of the experiment, and every 3 h thereafter, followed by the immediate measurement of TFM concentration using a plate spectrophotometer (Absorbance = 395 nm; Epoch2 Microplate Reader, BioTek Winooski, VT, USA) and precision standards (provided courtesy of USGS, Hammond Bay, MI, USA) to verify that TFM concentrations remained constant.

## 5. TISSUE PREPERATIONS FOR ANALYSES

Procedures for tissue metabolite extraction followed those outlined in Bergmeyer (1983), and modified as described in Wilkie *et al*. (1997, 2001). Briefly, using an insulated mortar and pestle, sea lamprey or lake sturgeon carcasses were ground to a fine powder under liquid $N_2$. and samples acidified for 10 minutes with 4 volumes of 8% PCA containing 1 mmol $L^{-1}$ EDTA, then centrifuged at 4°C and 10,000 *g* for 5 min. For rainbow trout, frozen pieces of white muscle were macerated using a hammer, transferred to pre-tared 2.0 mL microcentrifuge tube and weighed, followed by the addition of 4 volumes of 8% PCA containing 1 mmol $L^{-1}$ EDTA. After adding 2 stainless steel beads (2.3 mm diameter; BioSpec Products, Bartlesville, OK, USA) and capping

the tubes, muscle tissues were homogenized using a bead homogenizer (Precellys 24, Bertin Technologies, Montigny-le-Bretonneux, France) at 6,000 RPM, for 2 runs of 5 seconds, with a 15 s pause between runs, then centrifuged at 4°C and 10,000 *g* for 5 min.

An aliquot (50 µL) of the resulting supernatant was neutralized (~pH = 7) using 3 mol $L^{-1}$ $K_2CO_3$ (VWR International LLC, Mississauga, ON, Canada), and set aside for glucose and glycogen analyses. The remaining supernatant was neutralized (~pH = 7) in a half volume of 2 mol $L^{-1}$ KOH (EDM Millipore Canada Ltd, Etobicoke, ON, Canada) containing 0.4 mol $L^{-1}$ imidazole and 0.4 mol $L^{-1}$ KCl (Sigma-Aldridge, Oakville, Ontario, Canada) and saved for ATP, ADP, phosphocreatine (PCr), creatine, pyruvate and lactate analyses. Immediately after preparation, all the homogenized samples were flash frozen in liquid nitrogen and stored at -80°C until analyzed. Procedures for analyzing brain, liver and kidney tissue were similar to carcass but due to the smaller amounts of tissue available, 3-7 mg lamprey brain and, < 50 mg sturgeon and trout brain, or 100-150 mg liver and kidney, tissue was added to microcentrifuge tubes and homogenized on ice using a hand-held, motorized, plastic pestle homogenizer (Gerresheimer Kimble Kontes LLC, Dusseldorf, Germany) in 7 volumes (lamprey brain) or 4 volumes (sturgeon and trout brain, liver and kidney) of 8% PCA: 1 mmol $L^{-1}$ EDTA mixture. The homogenates were neutralized and then flash frozen in liquid nitrogen and stored at -80°C as described above. The whole blood was acidified with 8% PCA, centrifuged for 5 min at 4°C and 10,000 *g*, and the supernatant was subsequently used in lactate assays.

## 6. ANALYTICAL TECHNIQUES

Tissue (carcass, brain, kidney and liver) glucose and glycogen were determined on the neutralized extracts based on methods described by Bergmeyer (1983). First, the tissue extracts

were mixed with one-part 2 mol $L^{-1}$ acetate buffer (Sigma-Aldridge Canada, Oakville, Ontario, Canada), followed by the addition of 40 units (U) of amyloglucosidase (Sigma-Aldridge Canada) and allowed to incubate, with occasional mixing, for 2 h at 37ºC. The glycogen digestion was terminated by addition of 70% PCA and neutralized using 3 mol $L^{-1}$ $K_2CO_3$. Prior to glycogen digestion a sub-sample of extract was saved for determination of free glucose, expressed as µmol $g^{-1}$ wet weight, which subsequently was subtracted from total tissue glucose, yielding the glycogen concentration expressed as µmol glucosyl units $g^{-1}$ wet weight. Glucose concentration was determined spectrophotometrically at 340 nm using a microwell plate spectrophotometer (Epoch 2; BioTek, Winooski, VT, USA). The neutralized carcass extracts obtained from the second aliquot were analyzed spectrophotometrically at 340 nm using micro-modification of enzyme-linked assays outlined in Bergmeyer (1983) for glucose (hexokinase; HK), ATP (HK, and glucose-6-phosphatase; G6PDH), PCr (creatine kinase; CK), ADP (pyruvate kinase; PK, and lactate dehydrogenase; LDH), creatine (CK, PK and LDH), pyruvate (LDH) and lactate (LDH). Assays on brain and kidney were restricted to ATP, PCr, glucose, glycogen and lactate due to the limited amounts of tissue that were available, while liver assays were restricted to glucose and glycogen. Energy stores, except glycogen, were expressed as µmol $g^{-1}$ wet weight. Plasma lactate (lake sturgeon, niclosamide experiments and sea lamprey) was analyzed in the same manner as described above for other tissues.

## 7. INTRACELLULAR pH DETERMINATION

Carcass/muscle intracellular pH (pHi) measurements followed methods described by Pörtner (1990). Briefly, carcass/muscle (trunk minus internal viscera and head) was ground to a fine powder under liquid nitrogen using an insulated mortar and pestle. Ground tissue (~100 mg)

was combined with 400 µL ice-cold metabolic inhibitor cocktail containing 150 mmol $L^{-1}$ KF and 6 mmol $L^{-1}$ nitrilotriacetic acid sodium salt ($Na_2NTA$) to create a slurry. The samples were then vortexed for 10 seconds and pulsed in a centrifuge for ~10 seconds at 4°C. The resultant supernatant was used to measure pH at 15°C (acclimation temperature of the experimental fish) using a micro pH probe (Biotrade, Hamilton Bonaduz AG, Bonaduz, Switzerland) and meter (ION85 Analyzer, Radiometer, Copenhagen, Denmark). The pH electrode was calibrated using clinical standards (pH 7.0 and pH 10.0; VWR International LLC, Mississauga, Ontario, Canada) prior to measurement of samples and regularly checked for drift during the measurement process. All pH readings of samples and pH standards were allowed to stabilize for 3 minutes before final readings were recorded.

**8. PLASMA ION DETERMINATION**

Plasma $Na^+$ concentrations was determined on 10 µL aliquots diluted in 9990 µL (1:1000) 2% nitric acid. The $Na^+$ ion concentrations in each sample were determined using a 1000 mg $L^{-1}$ sodium atomic spectroscopy standard (Pure Lot #: 18-154NAX1; Perkin Elmer Corporation, Waltham, MA, USA) and flame atomic absorption spectroscopy (AAS; PinAAcle 900T, Perkin Elmer, Waltham, MA, USA). Plasma $Cl^-$ concentrations were determined on 20 µL of undiluted sample using a chloride analyzer (Chloride Analyser 926, Cole Parmer, Vernon Hills, IL, USA) standardized with a 100 mmol $L^{-1}$ chloride meter standard (Sherwood Scientific Ltd, Cambridge, UK).

## 9. STATISTICAL ANALYSES

All statistical data analyses were performed using Prism® 8.3.1 (GraphPad Software Inc, La Jolla, CA, USA). Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. In instances where the data were not normally distributed, standard deviations were significantly different from one another or were not transformable, data were analysed using non-parametric ANOVA (Kruskal-Wallis test), followed by Dunn's multiple comparison test. All data were expressed as mean ±SEM, with the level of significance set to $P \leq 0.05$.

**Table 2.1 Experimental Water Chemistry.**

Summary of water types (WW: well water; RDI: reconstituted deionized water), water chemistry (temperature, titratable alkalinity, pH and DO: dissolved oxygen) used for exposing fish to lampricides in the different experiments (SL: sea lamprey; RT: rainbow trout; LS: lake sturgeon; Nic: niclosamide exposure; TFM: TMF exposure). Results are presented as mean readings ± SEM.

| Experiment | Water Type | Temperature (ºC) | Alkalinity (mg $L^{-1}$ CaCO3) | pH | DO (%) |
|---|---|---|---|---|---|
| SL (Nic) | WW | 15.0 ± 0.4 | 268 | 8.12 ± 0.04 | 99.2 ± 0.4 |
| RT (Nic) | WW | 15.0 ± 0.5 | 238 | 8.22 ± 0.05 | 98.6 ± 0.5 |
| LS (TFM) | RDI | 13.8 ± 0.0 | 150 | 8.34 ± 0.0 | 93.0 ± 0.3 |
| LS (Nic) | WW | 14.5 ± 05 | 280 | 7.93 ± 0.1 | 95.2 ± 0.6 |

# CHAPTER 3:

# Niclosamide (2',5-dichloro-4'-nitrosalicylanilide): The Other Lampricide, and its Effects on Larval Sea Lamprey (*Petromyzon marinus*)

## 1. INTRODUCTION

The lampricide, TFM has been in use since the late 1950s as part of the integrated pest management program to control populations of parasitic sea lamprey in the Laurentian Great Lakes of North America (Lawrie, 1970; Olson and Marking, 1973; Smith and Tibbles, 1980; Hubert, 2003; McDonald and Kolar, 2007b). However, a second lampricide, 2',5-dichloro-4'-nitrosalicylanilide, better known as niclosamide (aka. Bayluscide®; Bayer 73®), has been used alongside TFM since the 1960s (Howell *et al.*, 1964). Niclosamide is often used in combination with TFM, most often as a TFM: 1% niclosamide mixture, which can reduce the amount of TFM needed by 40%, while maintaining its selectivity, and effectiveness (Boogaard *et al.*, 2003; Dawson, 2003; Gutreuter and Boogaard, 2007). The granular form of niclosamide, granular Bayluscide™, is also used for population surveys, and in lentic waters or large, deep river systems, due to its propensity to sink to the bottom where it can act directly on larvae in their burrows (Dawson 2003; Wilkie et al. 2019).

Niclosamide is not restricted to lamprey control, it is also commonly used as a molluscicide, applied to waters infested with snails, which are the primary hosts of the parasite, *Schistosoma japonicum*, which causes schistosomiasis in humans (Lardans and Dissous, 1998; Joubert *et al.*, 2001; Zhao *et al.*, 2015). It can also be used as a drug in humans and animals for the treatment of trematode (fluke) and cestode (tapeworm) intestinal parasite infections (Köhler, 2001; McKellar and Jackson, 2004), and it is being investigated for its potential to treat certain cancers (Sack *et al.*, 2011; Li *et al.*, 2014; Liu *et al.*, 2014; You *et al.*, 2014; Alasadi *et al.*, 2018).

Niclosamide is much more toxic and less specific than TFM (Dawson, 2003; Wilkie *et al.*, 2019). Acute toxicity studies have demonstrated that non-target fishes, such as American eel

(*Anguilla rostrata*), rainbow trout (*Oncorhynchus mykiss*) and white sucker (*Catostomus commersonii*), have sensitivity ratios (non-target 12 h LC50/lamprey MLC) to TFM that are 3-5 times higher than the concentration required to kill larval sea lamprey using TFM (Applegate and King Jr., 1962; Bills *et al.*, 2003; Dawson, 2003) (Wilkie *et al.*, 2019). The specificity of TFM to sea lamprey is related to the higher capacity of non-target fishes to detoxify TFM via glucuronidation and/or sulfation (Olson and Marking, 1973; Lech and Statham, 1975; Kane *et al.*, 1994; Bussy *et al.*, 2017b, 2017a). However, there is much less variation in the toxicity of niclosamide between sea lamprey and non-target fishes, making it less specific (Dawson, 2003; Wilkie *et al.*, 2019).

Like TFM, it is hypothesized that niclosamide imparts its toxicity in sea lamprey by uncoupling mitochondrial oxidative phosphorylation (Wilkie *et al.*, 2019), leading to a mismatch between ATP demand and ATP supply (Birceanu *et al.*, 2009, 2011). However, this has not been directly shown in sea lampreys. Niclosamide is a halogenated phenol, belonging to a class of compounds called salicylanilides which have also been shown to uncouple oxidative phosphorylation in different biological systems, a property which explains its known or potential effectiveness in the treatment of not only intestinal parasites and cancer, but diabetes and even viral, bacterial and microbial infections (Köhler, 2001; McKellar and Jackson, 2004; Wu *et al.*, 2004; Sack *et al.*, 2011; Li *et al.*, 2014; Liu *et al.*, 2014; You *et al.*, 2014; Tao *et al.*, 2016; Alasadi *et al.*, 2018; Chen *et al.*, 2018; Gwisai *et al.*, 2018). Thus, it is likely that niclosamide imparts similar physiological effects as TFM, albeit with higher potency. For this reason, it is important to assess the effects of niclosamide alone in sea lamprey in order to determine if niclosamide has a similar mechanism of toxicity as TFM in these fish, and to be able to later compare and contrast these effects with the effects of niclosamide on non-target fishes, such as

rainbow trout (*Oncorhynchus mykiss*), as well as species as risk such as the lake sturgeon (*Acipenser fulvescens*).

The goal of this study was to learn more about the physiological effects and mechanism of niclosamide toxicity in larval sea lamprey. This life stage was chosen because lampricide treatments in the field target the larval lamprey phase, in order to eliminate multiple generations at once. It was predicted that physiological responses to niclosamide in sea lamprey would lead to a multi-system depletion of energy stores, similar to those reported for TFM in sea lamprey (Wilkie *et al.*, 2007; Birceanu *et al.*, 2009; Clifford *et al.*, 2012). To test this hypothesis, acute toxicity tests for niclosamide were first conducted on larval sea lamprey to determine the 9 h $LC_{50}$ of the chemical. Using this information, larval sea lamprey were exposure to their specific niclosamide 9 h $LC_{50}$, followed by tissue collection at different intervals of exposure (1, 3, 6, 9 h, 24 h recovery). Energy stores and metabolite concentrations were then quantified in various tissues (ATP, phosphocreatine, glucose, glycogen and lactate in brain and carcass; glucose and glycogen in liver; creatine, pyruvate and ADP in carcass; lactate and, $Na^+$ and $Cl^-$ ions in blood) to assess if lampricide-induced energy declines were taking place accompanied by increased reliance on anaerobic processes. Blood ion concentrations in the blood were quantified as an indicator of possible niclosamide-induced gill epithelia damage (e.g. ionocytes). To test the hypothesis that niclosamide resulted in greater reliance on anaerobic glycolysis for ATP production resulting in metabolic acidosis, intracellular pH (pHi) in the carcass was also measured.

## 2. RESULTS

### 2.1 Niclosamide toxicity

Niclosamide-exposed sea lamprey (N = 12 each concentration) experienced death in a dose- and time-dependent manner. For control lamprey (no niclosamide exposure), and those exposed to nominal niclosamide concentrations of 0.005, 0.01 mg and 0.025 mg $L^{-1}$ niclosamide, no deaths occurred over 24 h. At 0.05 mg $L^{-1}$ exposure 2 deaths were recorded at 24 h, with 4 deaths at concentration of 0.1 mg $L^{-1}$ after 9 h, 3 deaths after 12 h exposure, with the remaining 5 dying by 24 h. With exposure to 0.25 mg $L^{-1}$ niclosamide all 12 fish died by 3 h of exposure. The corresponding 9-h $LC_{50}$ and 12-h $LC_{50}$ were 0.111 mg $L^{-1}$ (CL = 0.091- 0.135) and 0.095 (0.078-0.115) mg $L^{-1}$, respectively (Figure 3.1).

### 2.2 Measured water niclosamide concentrations

Nominal niclosamide concentration was 0.111 mg $L^{-1}$, however, LC-MS/MS analysis revealed that measured concentrations were 0.088 ± 0.008 mg $L^{-1}$ at start (0 h) and 0.087 ± 0.005 mg $L^{-1}$ at end (9 h), thus, there was no significant degradation of the lampricide over time.

### 2.3 Effects of time on energy stores and metabolites in control sea lamprey

There were no significant differences in the concentrations of ATP, PCr, glycogen, lactate and other metabolites measured in the brain, kidney, liver, carcass or blood of control fish (not exposed to niclosamide) sampled at the beginning of the experiment (0 h) and those sampled after 9 h (Supplemental Table 3.3S). Therefore, data from the two control groups were combined for each set of analyses used to quantify the effects of niclosamide exposure on larval sea lamprey.

2.4 Niclosamide effects on energy stores and metabolites in sea lamprey brain

The ATP concentrations in the brain of sea lamprey held under control conditions averaged 1.4 ± 0.1 μmol $g^{-1}$ ww, and PCr averaged 7.0 ± 0.3 μmol $g^{-1}$ ww (Figure 3.2). Exposure of sea lamprey to the 9-h $LC_{50}$ of niclosamide, nominal concentrations of 0.11 mg $L^{-1}$, was characterized by significantly reduced brain ATP throughout exposure, by 20 – 25%, before returning to pre-exposure levels after 24 h recovery (Figure 3.2A). Brain PCr concentrations trended downward following niclosamide exposure, with values significantly reduced by approximately 33% after 6 and 9 h, before returning to pre-exposure levels after 24 h of recovery (Figure 3.2B).

Brain glucose, glycogen and lactate concentrations in non-exposed control lamprey averaged 4.2 ± 0.1 μmol $g^{-1}$ ww, 91.0 ± 2.4 μmol glucosyl units $g^{-1}$ ww and 2.5 ± 0.2 μmol $g^{-1}$ ww, respectively (Figure 3.3). Although brain glucose concentrations were reduced by approximately 25% throughout niclosamide exposure, these reductions were not statistically significant, and returned to pre-exposure concentrations within 24 h of recovery (Figure 3.3A). Notably, niclosamide led to rapid and sustained decreases in brain glycogen levels, which were significantly reduced by approximately 20-25 % after 3 h, and by 40-50% between 6 and 9 h of niclosamide exposure. This was followed by a complete restoration of brain glycogen concentrations to pre-exposure levels after 24 h recovery (Figure 3.3B). In the presence of niclosamide, there were corresponding increases in brain lactate concentrations, which increased by 4- to 5-fold between 3 h and 9 h of exposure, before returning to pre-niclosamide levels after 24 h recovery (Figure 3.3C).

2.5 Effects of niclosamide on energy stores in sea lamprey liver

Liver glucose concentrations in sea lamprey held under control conditions averaged 1.9 ± 0.1 μmol $g^{-1}$ ww and glycogen concentrations averaged 9.1 ± 0.5 μmol glucosyl units $g^{-1}$ ww (Figure 3.4). Liver glucose remained unchanged in the presence of niclosamide (Figure 3.4A). However, glycogen concentration decreased in a stepwise manner through the entire exposure period, with concentrations approximately 70 % lower than controls after 9-h. Glycogen concentrations were restored to pre-exposure levels after the 24 h recovery period (Figure 3.4B).

2.6 Effects of niclosamide on energy stores and metabolites in sea lamprey kidney

The ATP concentrations in the kidney of sea lamprey held under control conditions averaged 1.2 ± 0.1 μmol $g^{-1}$ ww, and PCr averaged 0.5 ± 0.1 μmol $g^{-1}$ ww (Figure 3.5). Exposure of sea lamprey to niclosamide was characterized by a significant 60 % reduction of ATP, which was sustained through 3-9 h of exposure, before returning to pre-exposure levels after 24 h recovery (Figure 3.5A). Kidney PCr concentrations were unaffected by niclosamide exposure (Figure 2.5B). Kidney glucose, glycogen and lactate concentrations in un-exposed control lamprey averaged 1.2 ± 0.1 μmol $g^{-1}$ ww, 25.7 ± 0.9 μmol glucosyl units $g^{-1}$ ww and 1.9 ± 0.2 μmol $g^{-1}$ ww, respectively (Figure 3.6). Kidney glucose concentration was not affected by niclosamide exposure (Figure 3.6A), but glycogen underwent an immediate and sustained decrease of approximately 80 % after 9 h (Figure 3.6B). This was accompanied by 2- to 2.5-fold increases in lactate compared to controls. Both kidney glycogen and lactate concentrations were near control concentrations following 24 h recovery (Figure 3.6C).

2.7 Effects of niclosamide on energy stores and metabolites in sea lamprey carcass

ATP and ADP concentrations in carcass of sea lamprey controls averaged 2.6 ± 0.2 μmol $g^{-1}$ ww and 1.1 ± 0.1 μmol $g^{-1}$ ww (Figure 3.7). In the presence of niclosamide, carcass ATP concentrations were not significantly altered (Figure 3.7A), with no changes observed in ADP concentrations (Figure 3.7B). Although there were no significant changes in the ATP/ADP ratios compared to controls (0.4 ± 0.1), a significant change did occur between lamprey exposed to niclosamide for 3 h (0.3 ± 0.0) and 9 h (1.4 ± 0.4; Table 3.1)

Lamprey carcass PCr and creatine concentrations for controls averaged 32.0 ± 1.6 μmol $g^{-1}$ ww and 5.9 ± 0.3 μmol $g^{-1}$ ww, respectively (Figure 3.7). Carcass PCr concentrations underwent an immediate and sustained reduction of greater than 50 % for the first 6 h of niclosamide exposure, approaching 80% after 9 h (Figure 3.7C). After 24 h recovery, carcass PCr concentrations returned to within 30 % of pre-exposure levels and were no longer statistically significant from control values ($P > 0.99$; Figure 3.7C). Creatine concentration in the carcass was increased by approximately 21 and 24% after 6 and 9 h of exposure and returned to pre-exposure levels after 24 h recovery (Figure 3.7D).

Glucose and glycogen in carcass of sea lamprey controls averaged 1.1 ± 0.1 μmol $g^{-1}$ ww and 19.3 ± 0.6 μmol glucosyl units $g^{-1}$ ww (Figure 3.8A, B). No changes were observed for carcass glucose concentration during exposure to niclosamide (Figure 3.8A), but glycogen was significantly decreased by approximately more than 20% after 3 h, and almost 35% after 9 h, before returning to pre-exposure levels after 24 h recovery (Figure 3.8B). Carcass pyruvate and lactate concentrations in sea lamprey averaged 0.2 ± 0.0 μmol $g^{-1}$ ww and 3.5 ± 0.3 μmol $g^{-1}$ ww, respectively (Figure 3.8). In the presence of niclosamide, carcass pyruvate concentrations significantly increased by approximately 3-fold in the first 1- 3 h of exposure and remained

significantly elevated through 9 h (Figure 3.8C). Pyruvate concentration returned to pre-exposure levels after 24 h recovery (Figure 3.8C). Consistent with the increase in pyruvate, carcass lactate concentration steadily increased during the niclosamide exposure period, peaking at a concentration that was approximately 3-fold higher than controls, before returning to baseline concentrations after 24 h recovery (Figure 3.8D). Similar increases were observed in the plasma, in which plasma lactate was 5- to 7-fold greater than observed in control lamprey, before returning to pre-exposure concentrations after 24 h recovery (Figure 3.8E).

2.8 Effects of niclosamide on sea lamprey carcass intracellular pH (pHi)

The carcass intracellular pH (pHi) of control sea lamprey averaged 7.22 ± 0.02 for controls. The presence of niclosamide resulted in a significant acidosis in the carcass, characterized by decreases in pHi to values of pH 6.96 and pH 6.68 after 6 h and 9 h, respectively. The acid-base disturbance was rapidly corrected, however, returning to pre-exposure levels (pH = 7.21 ± 0.02) after 24 h of recovery (Figure 3.8F).

2.9 Effects of niclosamide on plasma ions in sea lamprey

Concentrations of $Na^+$ and $Cl^-$ in blood plasma of sea lamprey controls averaged 95.6 ± 1.0 mmol $L^{-1}$ and 92.0 ± 0.4 mmol $L^{-1}$, respectively, but no significant changes were observed in the presence of niclosamide (Table 3.2).

## 3. DISCUSSION

### 3.1 Effects of niclosamide on energy stores and metabolites

The brain of lampreys have unusually high glycogen concentrations that are integral to maintaining glucose homeostasis in the central nervous system (CNS; Rovainen 1970; Murat *et al.*, 1979; 1993; Foster *et al.*, 1993a; Clifford *et al.*, 2012; Weil *et al.*, 2018). First reported by Rovainen (1970) and later confirmed by Rovainen *et al.* (1971), it is the surrounding meninges that contain the majority (~90%) of the brain's glycogen reserve in lamprey. This siutation is unlike most vertebrates, in which the liver is the main organ of glucose homeostasis (Polakof *et al.*, 2012). Indeed, the lamprey liver is characterized by much lower concentrations of glycogen and appears to play a relatively minor role in maintaining glucose homeostasis (O'Boyle and Beamish, 1977). As in all chordates, glucose is an essential energy source for the CNS, and homeostatic disturbances such as hypoglycemia can severely impact its function (Hochachka *et al.*, 1993). The glycogen stores in the meninges of sea lamprey provides the brain with vast glucose reserves which can be used to maintain the demands of the CNS (Rovainen *et al.*, 1969, 1971; Rovainen, 1970). In addition, substantially high activity of the enzyme glucose-6-phosphatase in the meninges, provides the catalyst for the conversion of glycogenolysis-derived glucose-6-phosphate into glucose in sea lamprey and river lamprey brain (Rovainen *et al.*, 1971; Murat *et al.*, 1979). This arrangement may even contribute to the unusually high hypoxia tolerance of lampreys (Potter *et al.*, 1970). However, the present results also demonstrate that brain glycogen concentrations are very sensitive to disturbances caused by niclosamide exposure that interfere with ATP production and supply, and likely contribute to death. While brain glucose levels were not significantly reduced, exposure to the 9-h $LC_{50}$ of niclosamide caused an

immediate and sustained depletion in brain glycogen in sea lamprey, declining by more than 50% by 9 h exposure. These results are similar to those reported in sea lamprey exposed to TFM, in which exposure to the 12-h $LC_{50}$ or $LC_{99.9}$ of TFM induced comparable reductions in brain glycogen (Clifford et al. 2012; Henry et al. 2014). As previously demonstrated, TFM interferes with mitochondrial ATP production in sea lamprey (Birceanu *et al.*, 2011), which leads to increased reliance on glycolysis and high energy phosphagens such as phosphocreatine (PCr) and phosphoarginine for ATP production in lampreys and molluscs, respectively (Viant *et al.*, 2001; Wilkie *et al.*, 2007; Birceanu *et al.*, 2009). As a known uncoupler of oxidative phosphorylation (e.g. Sack *et al.*, 2011; Li *et al.*, 2014b; Alasadi *et al.*, 2018), niclosamide was expected to behave similarly to TFM, but niclosamide is much more potent leading to marked reductions in glycogen at concentrations that were roughly 1/10 of the TFM levels known to interfere with glycogen homeostasis during exposure in water of similar chemistry (Birceanu et al. 2009). The greater potency of niclosamide was further demonstrated by significantly decreased brain ATP and PCr levels that resulted from niclosamide exposure, which was not observed with TFM (Clifford *et al.*, 2012; Henry *et al.*, 2015).

The 5- to 6-fold increases in sea lamprey brain lactate concentrations that accompanied glycogen depletion in the presence of niclosamide strongly suggests that ATP supply was being maintained, at least in part, by anaerobic glycolysis. Glycolysis results in the generation of 2 molecules of lactate per glucosyl unit of glycogen/glucose (Hochachka *et al.*, 1993). However, the stoichiometry of brain lactate accumulation to glycogen depletion was well below this 2:1 ratio, suggesting that the lactate was either exported from the brain or catabolized (Clifford *et al.*, 2012; Henry *et al.*, 2015). In retrospect, export of lactate from the brain via monocarboxylate transporters (MCT; cf. Clifford *et al.*, 2012) seems unlikely because it would result in the loss of

an energy-rich C-skeleton. The most likely scenario is that the lactate was oxidized *in situ* by the brain and/or used as a substrate for gluconeogenesis, as demonstrated in adult sea lamprey (Foster *et al.*, 1993). Although, glucose remains the primary substrate for neuronal oxidative ATP production, the lactate generated within the astrocytes plays an important supplementary role in this process in other vertebrates (Hochachka and Somero, 2002; Yip *et al.*, 2017). The lamprey brain, like other vertebrate brains, contains astrocytes which provide metabolic support to neurons of the brain, maintain neuronal ion homeostasis, as well as guiding neurodevelopment (Rovainen, 1979; Cinelli *et al.*, 2017; Weil *et al.*, 2018). The astrocytes also play an important role in glucose uptake from the blood, which is then shuttled to neurons, or undergoes glycolysis resulting in the generation of lactate which is subsequently exported to the neurons by the astrocytes via astrocytic and neuronal MCTs. Within the neuron, this lactate is subsequently oxidized to pyruvate within the cell cytosol before entering the citric acid cycle within the mitochondria, which ultimately fuels ATP production via oxidative phosphorylation (see Hochachka and Somero, 2002; Yip *et al.*, 2017 for reviews).

Normally, lactate oxidation would augment ATP production by driving the TCA cycle, with the subsequent generation of the reducing equivalents required to drive the mitochondrial respiratory chain (aka. electron transport chain) that generates the proton motive force needed to phosphorylate ADP to ATP via ATP synthase. While niclosamide and TFM would be expected to reduce ATP production by degrading the proton motive force, the upstream respiratory chain (oxidative processes) would still be intact, resulting in continued consumption of reducing equivalents and oxygen by the mitochondria. Indeed, this is demonstrated by the increased rates of respiration (state IV) observed after exposing mitochondria isolated from adult sea lamprey and trout to physiologically relevant concentrations of TFM (Birceanu et al. 2011). By keeping

the respiratory pathways upstream of ATP synthase intact, lactate oxidation within neurons and/or gluconeogenesis within astrocytes would be expected to continue, but only until ATP dependent processes, ion homeostasis in particular, began to degrade during the terminal period of exposure.

Another possible route of lactate catabolism, more likely to take place in the astrocytes, is gluconeogenesis, provided ATP is still being generated at sufficient rates to energize the process, which would be questionable in the face of reduced rates of oxidative phosphorylation. Some ATP could arise from anaerobic glycolysis, and the TCA cycle could also potentially generate 2 ATP from GTP, generated through the conversion succinyl-coA to succinate in the citric acid cycle (Boyer, 2006). Because gluco(glyco)neogenesis requires ATP it is unclear how much lactate would be consumed by this process as opposed to oxidation to pyruvate, but it would be relatively easy to determine using $^{14}$C-lactate, $^{14}$C-glucose and other radio-tracers (e.g. Foster *et al.*, 1993).

Niclosamide exposure induced no significant changes in liver or kidney glucose but the observed depletion of glycogen was similar to observations made in the liver of larval sea lamprey exposed to the respective 12-h $LC_{50}$ and $LC_{99.9}$ of TFM for 3 h and 6 h (Birceanu *et al.*, 2009; Clifford *et al.*, 2012). Although the weight of evidence suggests that the lamprey liver plays a relatively minor role in glucose homeostasis (O'Boyle and Beamish, 1977; Larsen, 1978), the responses to both niclosamide and TFM suggests that liver glycogen stores may be mobilized when ATP supply is compromised. Similarly, the kidney may also play an underappreciated role in glucose homeostasis, with 3-fold higher concentrations of glycogen than the liver. The presence of significant glucose-6-phosphatase activity (Rovainen *et al.*, 1971; Murat *et al.*, 1979), which converts glucose-6-phosphate arising from glycogen catabolism to

glucose, in the liver and kidney also indicates that together these tissues could serve as a significant glucose reservoir in larval lampreys under extreme environmental conditions. Nevertheless, the role of the liver in maintaining glucose homeostasis in larval sea lamprey still pales in comparison to the liver of teleost fishes, which stores 5- to 10-fold higher concentrations of glycogen (Plisetskaya and Kuz'mina, 1971; O'Boyle and Beamish, 1977; Larsen, 1978; Larsen *et al.*, 2001; Barcellos *et al.*, 2010).

The significant amounts of glycogen stored in the kidney were not surprising since most vertebrates, including sea lamprey, rely on the kidneys to help maintain circulating glucose levels (Shanghavi and Weber, 1999). This is reflected in the fact that niclosamide-exposed lamprey experienced a significant decline in kidney glycogen while glucose remained at steady state levels. These observations differ from those observed in larval, juvenile, and adult sea lamprey exposed to 12-h TFM $LC_{50}$, in which there were no changes in kidney glycogen concentrations (Henry *et al.*, 2015). This observation could be related to the relatively short duration of TFM exposure (3 h), as well as the greater potency of niclosamide. Some of the observed glycogen depletion may have also been due to *in situ* catabolism to fuel renal processes.

The significant depletion in kidney ATP and elevation in lactate also implies that anaerobic glycolysis was, at least in part, supplying the energy needs of this organ. Notably, phosphocreatine concentrations in the kidneys were unaffected by niclosamide exposure, similar to findings for TFM exposed lamprey (Henry *et al.*, 2015). The low control PCr levels (< 0.5 μmol $g^{-1}$ ww) reported here, however, may be reflective of a relatively low anaerobic capacity in this organ compared to other tissues in the lamprey, such as muscle (> 20-fold higher; Wilkie *et al.*, 2001; Henry *et al.*, 2015) and brain (Birceanu *et al.*, 2009; Clifford *et al.*, 2012; Henry *et al.*,

2015). While there was no evidence of altered ion or osmotic balance during niclosamide exposure, the present findings do raise the possibility that renal failure could potentially contribute to death in non-surviving lamprey.

### 3.2 Similarities between niclosamide exposure and exercise

In sea lamprey exposed to niclosamide, carcass (trunk minus viscera) glycogen concentrations were significantly depleted in a stepwise manner, over time, while glucose concentrations remained unaffected. Muscle glycogen in fishes is generally used to fuel burst activity (Milligan and McDonald, 1988; Wang *et al.*, 1994a; Kieffer, 2000; Wilkie *et al.*, 2001). It plays little to no role in glucose homeostasis in teleosts because of a lack of glucose-6-phosphatase, which catalyzes the conversion of glucose-6-phosphate arising from glycogen breakdown into glucose (Panserat *et al.*, 2000). The selective advantage of this strategy in fish such as trout, is that it allows them to retain high amounts of glycogen in the muscle, which acts as an "energy spring" that can fuel sudden, rapid bursts of exercise, leading to glycogen depletion and lactate accumulation, followed by gradual restoration of glycogen reserves over several hours, using lactate as glyconeogenic substrate (Omlin and Weber, 2013). The situation is likely similar in the muscle of both larval and adult sea lamprey, which also experience rapid depletion of muscle glycogen accompanied by corresponding increases in lactate, and gradual restoration of glycogen and lactate elimination during recovery (Boutilier *et al.*, 1993; Wilkie *et al.*, 2001). Sea lamprey likely require rapid bursts of activity during each of these life stages, such as during burrowing as larvae or during the upstream spawning migration and the act of spawning by adults (Beamish and Potter, 1975).

In the presence of niclosamide, it is likely that basal metabolic demands of the muscle could not be met, even at rest, forcing them to rely on glycolysis to make up for any ATP shortfall. The observed decrease in glycogen, and the virtual depletion of PCr in the carcass, during niclosamide exposure lend further support to this interpretation (Figure 3.7 and 3.8). Further, the muscle (carcass) of sea lamprey is composed of a mix (mosaic; Boutilier *et al.*, 1993) of red (oxidative) and white (glycolytic) muscle fibres (Peters and Mackay, 1961; Meyer, 1979), which would make it even more imperative to ensure that an adequate supply of ATP was provided to meet the basal demands of the muscle, even at rest. The near depletion of PCr, along with the reductions in glycogen and corresponding increases in pyruvate and lactate, caused by niclosamide mimicked the responses of the larval lamprey to exhaustive exercise. But rather than increasing ATP demand, it was compromised ATP supply that explained the response.

The mobilization of PCr was likely triggered by elevations in the ATP/ADP ratio (Table 3.1), resulting in the CPK reaction shifting toward production of PCr, which buffers ATP reserves (Moyes and West, 1995). In the present study, ADP was unaltered but creatine was elevated above controls at 6 and 9 h of niclosamide exposure. Although calculations of ATP/ADP ratios resulted in high variability, significant elevations were observed between 3 and 9 h exposure (Table 3.1); however, creatine charge ([PCr]/[total creatine]) experienced immediate and sustained decreases (Table 3.1). From these results, it is clear that PCr, along with glycogen, were buffering ATP supply, which prevented any significant reduction in ATP in niclosamide-exposed lamprey.

Niclosamide also resulted in a significant metabolic acidosis in the carcass, as reflected by the marked decrease in pHi by more than 0.5 pH units. Similar declines in white muscle pHi are usually associated with vigorous exercise and increased reliance on glycolytic ATP

generation, due to increased rates of ATP hydrolysis (Hochachka *et al.*, 1993). An acidosis of this magnitude would also promote increased PCr dephosphorylation (Schulte *et al.*, 1992), as was previously observed in exhaustively exercised larval sea lamprey that experienced marked reductions in muscle glycogen and PCr, with corresponding elevations in pyruvate and lactate, as well as high post-exercise acid excretion (Wilkie *et al.*, 2001). Even though most of the lactate is retained in muscle, some leakage likely occurred from the muscle and possibly other organs, in the presence of niclosamide. It is not clear if lactate production in red blood cells (RBCs) would increase but may be worth investigating in the future.

While exposure to niclosamide resulted in significant metabolic disturbances in larval sea lamprey, surviving fish which were transferred to clean water experienced a relatively speedy (within 24 h) and total recovery of metabolic status. These results resemble findings for adult and larval sea lamprey, which readily (0.5 – 4 h) re-established energy stores, lactate and acid-base balance following exhaustive exercise (Boutilier *et al.*, 1993; Wilkie *et al.*, 2001). It is important to note that the full recovery points to the resilience of larval lamprey to niclosamide exposure, with an absence of any residual effects (e.g. 'niclosamide hangover') which could jeopardize their survival. Such resiliance to lampricide exposure suggests that inadequate doses of TFM and/or niclosamide due to dilution arising from side-channels, tributaries or upwellings, or even precipitation, may exlain why some lamprey survive lampricide treatments. Careful vigilance would therefore be required to ensure that there are no surviving animals following treatment, to minimize the risk of residual sea lamprey. The intent of the sea lamprey control program is to target larval sea lamprey the year before they begin metamorphsosis (Siefkes 2017). Residual sea lamprey therefore undermine this key objective, because it ultimately leads to increased numbers of juvenile sea lamprey and greater parasitism of the Great Lake's fishes.

3.3 Effects of niclosamide on ion balance

Fishes living in freshwater environments, face osmo- and ion-regulatory challenges which they overcome by a diversity of strategies. Diffusive ion loss can be limited by decreasing epithelial ion permeability (Chasiotis *et al.*, 2012) and/or increasing ability to uptake ions from the environment (Evans *et al.*, 2005). For compensation of diffusive ion loss, fish use a variety of specialized gill (or skin) cells (ionocytes) which have specific transporters or groups of transporters which facilitate transepithelial active transport of $Na^+$, $Cl^-$ and $Ca^{2+}$ (see Evans *et al.*, 2005 for review). Ionocytes also regulate pH balance by excreting acidic ($H^+$) or basic (e.g. $HCO_3^-$) equivalents (Goss and Wood, 1990a,b). There are a number of fundamental transporters responsible for $Na^+$ uptake and $H^+$ excretion, such as $Na^+/H^+$ exchanger (NHE), $Na^+/K^+$-ATPase (NKA), $H^+$-ATPase (HA) and $Na^+$ channel, for $Cl^-$ uptake and base secretion, such as $Na^+/Cl^-$ cotransporter (NCC) and anion exchanger (AE), and for $Ca^{2+}$ uptake such as the epithelial calcium channel (ECaC) and plasma membrane $Ca^{2+}$-ATPase (PMCA), among others, which are relatively well conserved across fish species (see Dymowska *et al.*, 2012 for review). Because active transport is energetically expensive, ionocytes (aka. mitochondrion rich cells (MRC)) possess a large number of mitochondria for ATP production, and must posses a specific group of apical and basolateral transporters and channels which provide directional passage of ions or acid-base equivalents (Dymowska *et al.*, 2012). Conversely, the distribution of MRCs, their morphology, functional sub-types and strategies for ion and acid-base homeostasis are comparatively species-specific.

There are numerous similarities with ionocytes found in lamprey gills. Ionocytes are generally rich in mitochondria, which are required for the generation of ATP needed to power

active ion transport. Ionocytes such as intercalated mitochondrion-rich cells (IMRCs) and ammocoete mitochondrion-rich cells (AMRCs) in larval sea lamprey gill epithelia, play a role in the active uptake and secretion of monovalent ions; the ultrastructural and functional characteristic of these cells in sea lamprey have been thoroughly studied (Bartels and Potter, 2004; Reis-Santos *et al.*, 2008; Zydlewski and Wilkie, 2012; Sunga *et al.*, 2020). Briefly, since the IMRCs in the sea lamprey gill epithelia share ultrastructural characteristics with other members of this cell group conserved in certain ion transporting epithelia of various vertebrates (Brown and Breton, 1996), they likely perform the same basic function as those cells.

In larval sea lamprey approximately 60% of the gill lamellar surface is occupied by AMRCs, with mitochondria occupying about one-third of the cell volume (Mallatt *et al.*, 1995). The high density of mitochondria in AMRCs and positive histochemical reaction for carbonic anhydrase (CA; Conley and Mallatt, 1988), has lead to the widely accepted view that in larval sea lamprey these cells are responsible for taking up $Na^+$ via $Na^+/H^+$ exchanger (NHE) and/or $Cl^-$ uptake via $Cl^-/HCO_3^-$ exchanger (Youson and Freeman, 1976; Mallatt and Ridgway, 1984). In sea lamprey, there may also be an indirect coupling of apical extrusion of $H^+$ via V-ATPase and $Na^+$ uptake. In this case, the V-type $H^+$-ATPase (V-ATPase) contributes to the generation of the electrochemical gradient, as it does in teleosts (Dymowska *et al.*, 2012; Edwards and Marshall, 2012), which is necessary to drive uptake of $Na^+$ via an apical $Na^+$ channel (subunit of ENaC; Ferreira-Martins *et al.*, 2016; Sunga *et al.*, 2020). Similarly, V-ATPase has been proposed to play a role in promoting ammonia excretion at the gill, via the Rhesus glycoprotein metabolon, whereby extrusion of $H^+$ by V-ATPase and/or NHE, leads to ammonia trapping to form $NH_4^+$, from diffusion across apical Rhcg glycoproteins (Wright and Wood, 2009). Recently, a study investigated the distribution of V-ATPase to the lamellae of larval sea lamprey, and reported that

distribution decreased following metamorphosing, concluding that V-ATPase may have a more important role in acid-base regulation and $Na^+$ uptake in FW (Sunga *et al.*, 2020).

Many histological studies have reported TFM-induced damage and/or alterations to larval sea lamprey gills (Christie and Battle, 1963; Mallatt *et al.*, 1994), specifically MRCs (Mallatt *et al.*, 1994) and MR platelet cells (Bartels and Potter, 2004). Similar damage caused by niclosamide cannot be dismissed but given the lack of change in plasma ion $Na^+$ and $Cl^-$ concentrations, it suggests that such damage did not significantly contributed to niclosamide toxicity in larval sea lamprey. The lack of change in blood plasma ions also suggests that niclosamide does not interfere with gill mediated ionoregulation. Similary, no changes were obeserved in blood plasma $Na^+$ and $Cl^-$ of TFM-exposed larval sea lamprey (Birceanu *et al.*, 2009). While it is still a possibility that niclosamide-mediated oxidative phosphorylation deficiency resulted in decreased rates of $Na^+$ and/or $Cl^-$ uptake, the exposure time was likely too short to produce measurable changes in plasma $Na^+$ and $Cl^-$ levels, which often only develop after several days, following disturbances such as exposure to acid or alkaline pH (e.g. Wood *et al.*, 1988; Wilkie and Wood, 1991). Future experiments utilizing radiotracers such as $^{22}Na^+$ and $^{36}Cl^-$ could prove very useful in answering this question.

## 3.4 Summary and conclusions

This study presents evidence that niclosamide interferes with ATP production in larval sea lamprey, resulting in increased reliance on PCr and glycolysis to maintain basal ATP requirements. Prolonged reliance on glycolysis in all likelihood leads to glucose starvation of the nervous system, ultimately leading to death. Similar to findings in TFM-exposed sea lamprey and rainbow trout (Birceanu *et al.*, 2009, 2011, 2014), the present results support the hypothesis

that niclosamide also interferees with mitochondrial oxidative phosphorylation. Additional studies using isolated mitochondria are required to test this hypothesis, however. The present study also demonstrates that niclosamide-exposed larval sea lamprey readily recover (within 24 h) from adverse effects on metabolism, similar to TFM-exposed larval lamprey (Clifford *et al.*, 2012), which could potentially result in residual lampreys that survive lampricide treatment. It is imperative to continue using lampricides to control sea lamprey populations, but care must be taken to ensure that residual survival is minimized while ensuring that non-target organisms including fishes and other aquatic vertebrates (Boogaard *et al.*, 2003) are protected from any adverse effects of treatment. In the present study, the physiological effects of niclosamide were independently evaluated, which provided us with insight into its mechanism of action in lampreys. However, it should be kept in mind that its is usually used in combination with TFM in TFM/1% niclosamide mixtures, which increases TFM efficacy and reduces the amount of lampricide, with minimal decreases in the selectivity of TFM. While the present study did not precisely mimic field protocols, these experiments allowed for a direct comparison between the physiological effects imparted by TFM and niclosamide, providing insight into how these two lampricides might interact. Based on the similar affects on ATP production, and the corresponding depletion of metabolic energy stores that result, these findings suggest that niclosamide enhances the effects of TFM by interfering with similar metabolic pathways. However, future physiological studies using TFM/1% niclosamide mixtures are needed to learn more about the nature of TFM-niclosamide interactions in sea lamprey, as well as non-target fishes.

**Table 3.2 ADP/ATP ratios and creatine charge in sea lamprey exposed to niclosamide.**

Changes in ATP/ADP ratios ([ADP]/[ATP]) and creatine charge ([PCr]/[total creatine]) in the carcass of sea lamprey (*Petromyzon marinus*) held under control conditions (no niclosamide), during exposure to niclosamide at a nominal concentration of 0.11 mg L-1 (9 h LC50) for up to 9 h, or following a 24 h depuration period in clean (no niclosamide) water. Data are expressed as mean ± S.E.M.

| Treatment | [ADP]/[ATP] ± SEM (N) | Creatine Charge ± SEM (N) |
|---|---|---|
| Control | 0.4 ± 0.1 (10) | 0.8 ± 0.0 (10) |
| 1 h | 0.9 ± 0.3 (9) | 0.6 ± 0.0 (9) |
| 3 h | 0.3 ± 0.0 (10) | 0.6 ± 0.1 (10) |
| 6 h | 0.4 ± 0.1 (9) | 0.7 ± 0.0 (9) |
| 9 h | 1.4 ± 0.4 (7) | 0.1 ± 0.0 (7) |
| 24 h Rec. | 0.5 ± 0.1 (8) | 0.8 ± 0.1 (8) |

**Table 3.3 Effects of niclosamide exposure on sea lamprey ion balance.**

The concentrations of $Na^+$ and $Cl^-$ in the blood plasma of sea lamprey (*Petromyzon marinus*) held under control conditions (no niclosamide), during exposure to niclosamide at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for up to 9 h, or following a 24 h depuration period in clean (no niclosamide) water. Data are expressed as mean ± S.E.M.

| Treatment | $Na^+$ mmol $L^{-1}$ ± SEM (N) | $Cl^-$ mmol $L^{-1}$ ± SEM (N) |
|---|---|---|
| Control | 95.6 ± 1.0 (8) | 92.0 ± 0.4 (8) |
| 1 h | 93.3 ± 1.6 (7) | 90.7 ± 0.7 (7) |
| 3 h | 96.2 ± 0.1(6) | 91.2 ± 1.0 (6) |
| 6 h | 97.3 ± 1.9 (5) | 93.1 ± 1.5 (5) |
| 9 h | 95.9 ± 1.7 (4) | 93.1 ± 1.0 (4) |
| 24 h Rec. | 96.3 ± 0.1 (6) | 92.0 ± 0.8 (6) |

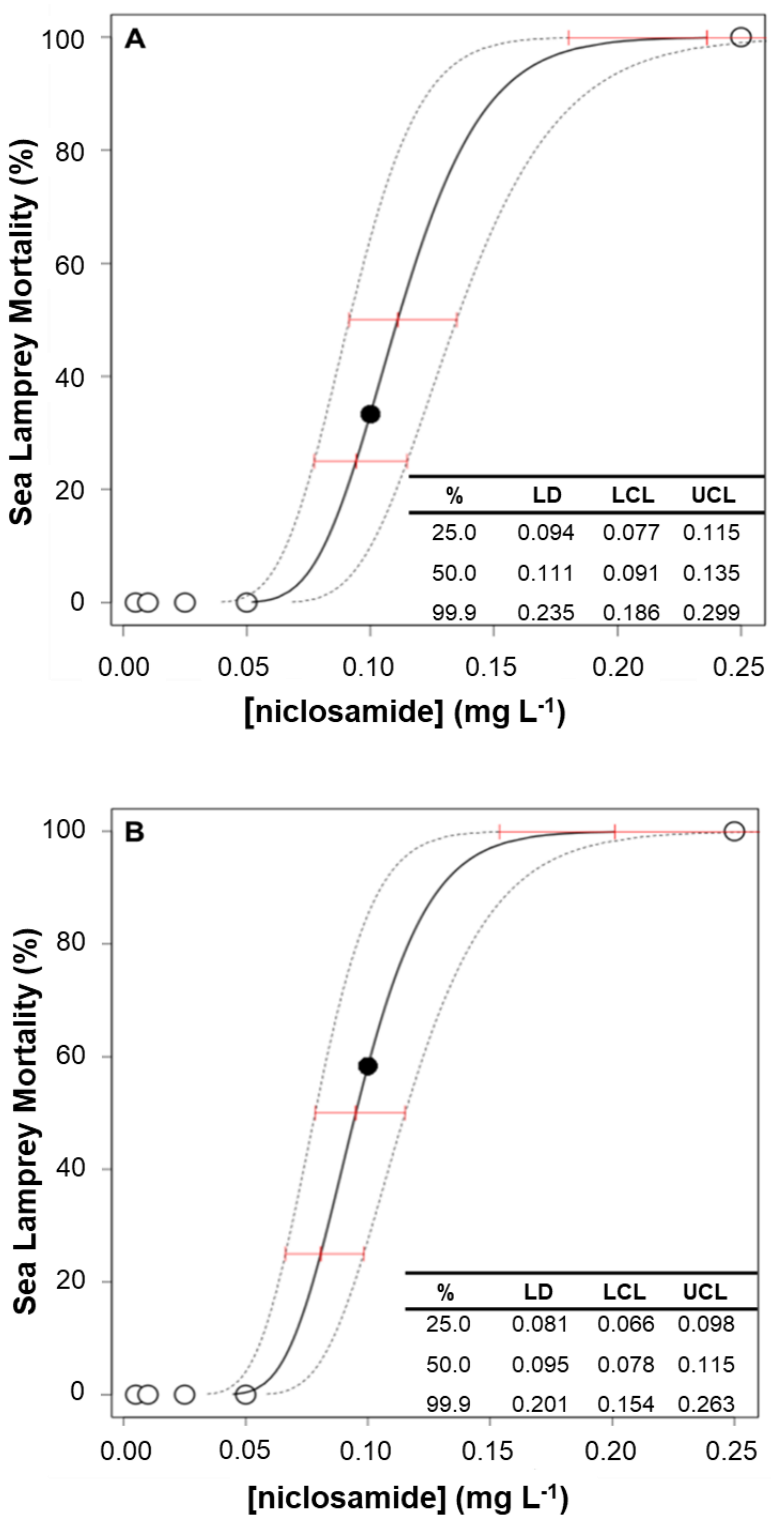


**Figure 3.1 Sea lamprey toxicity curve for niclosamide**

**Figure 3.1 Sea lamprey toxicity curve for niclosamide.** Plot of observed data with the Litchfield Wilcoxon fitted model on arithmetic scale (solid black curve; dashed curves 95% LCL and UCL) for sea lamprey (*Petromyzon marinus*) niclosamide 9 h (A) and 12 h (B) toxicity tests. At each of the specified percentages affected, the predicted lethal doses and their 95% confidence levels are depicted in red. Observations with 0% or 100% affected sea lamprey are shown as white circles. Inset: Table lists specific lethal concentration percentages (%), predicted lethal dose (ED), 95% lower confidence level (LCL) and 95% upper confidence level (UCL).

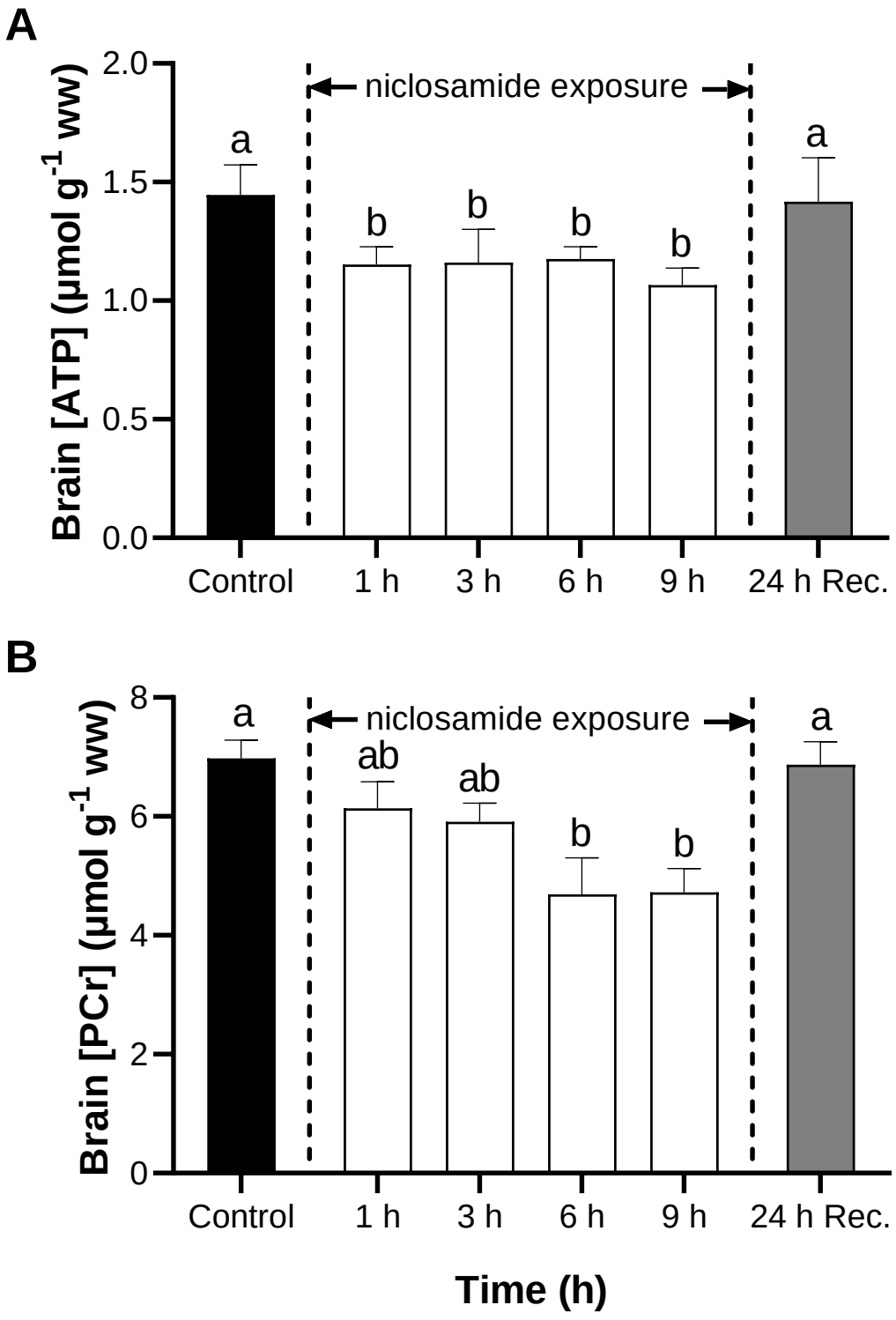


**Figure 3.2 Brain ATP and phosphocreatine reserves in sea lamprey exposed to niclosamide**

**Figure 3.2 Brain ATP and phosphocreatine reserves in sea lamprey exposed to niclosamide.** Changes in the concentrations of (A) ATP and (B) PCr in the brain of larval sea lamprey (*Petromyzon marinus*) during niclosamide exposure (open bars) at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 7), 3 h (n = 6), 6 h (n = 5) and 9 h (n = 4), and following exposure to niclosamide (24 h recovery; n = 4; grey bars) or held under control conditions (no niclosamide; n = 9; black bars). Note in some instances (e.g. 6 h and 9 h exposure) brain excision from some individual fish was not possible, however, other tissues were collected. Data are expressed as the mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

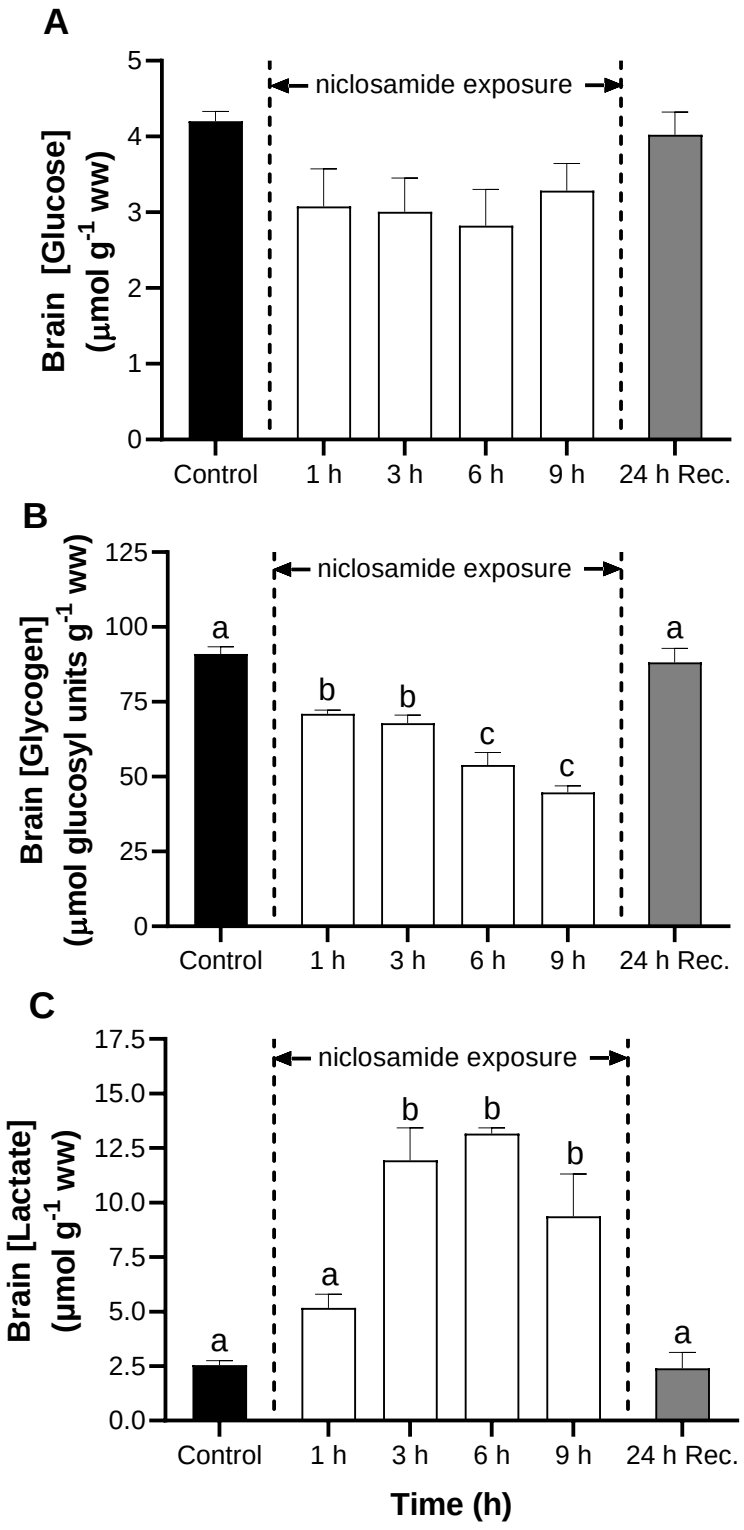


**Figure 3.3 Brain energy reserves and metabolites in sea lamprey exposed to niclosamide**

**Figure 3.3 Brain energy reserves and metabolites in sea lamprey exposed to niclosamide.** Changes in the concentrations of (A) glucose, (B) glycogen and lactate (C) in the brain of larval sea lamprey (*Petromyzon marinus*) during niclosamide exposure (open bars) at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 7), 3 h (n = 6), 6 h (n = 5) and 9 h (n = 4), and following exposure to niclosamide (24 h recovery; n = 4; grey bars) or held under control conditions (no niclosamide; n = 9; black bars). Note in some instances (e.g. 6 h and 9 h exposure) brain excision from some individual fish was not possible, however, other tissues were collected. Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

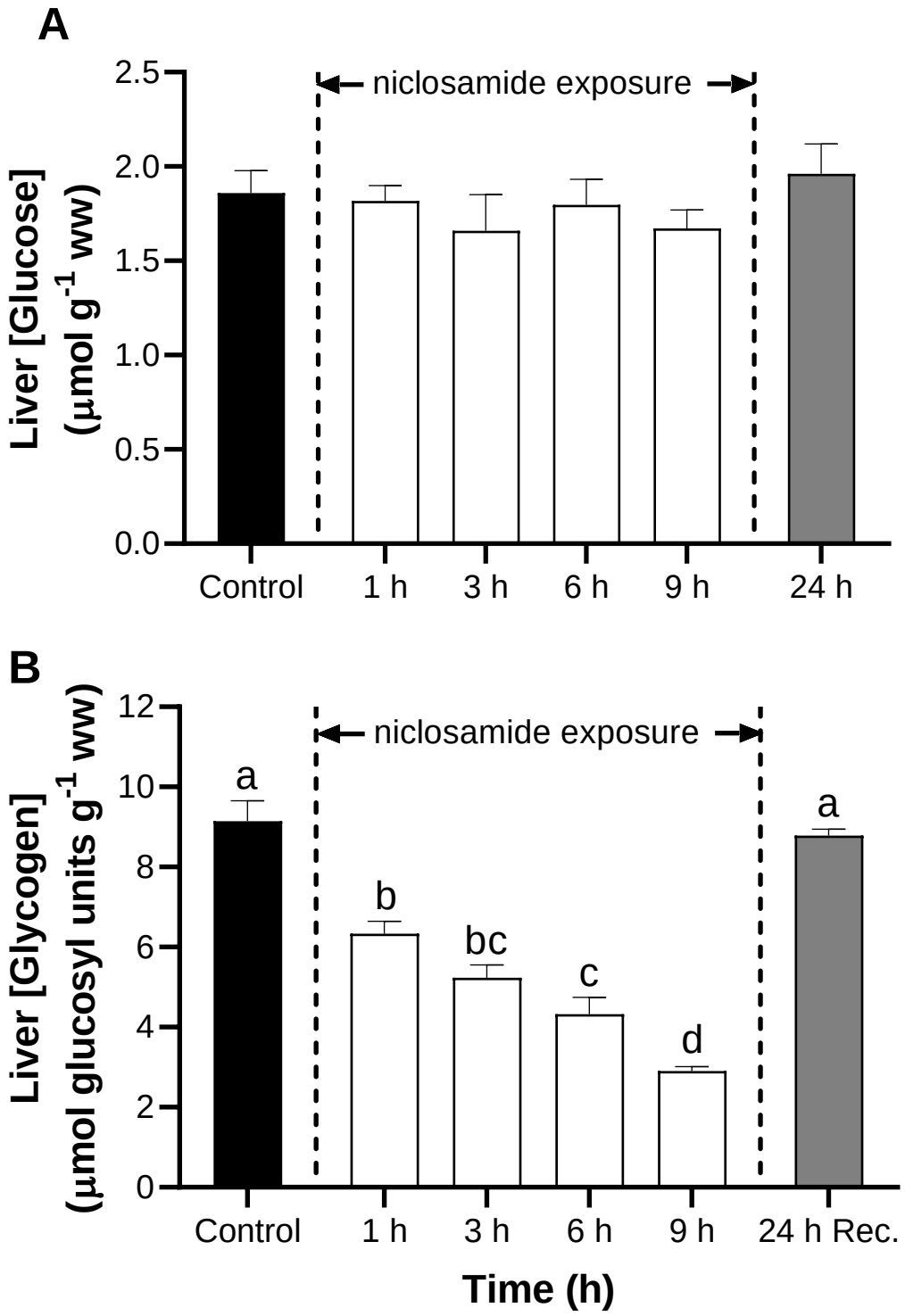


**Figure 3.4 Liver energy reserves in sea lamprey exposed to niclosamide**

**Figure 3.4 Liver energy reserves in sea lamprey exposed to niclosamide.** Changes in the concentrations of (A) glucose and (B) glycogen in the liver of larval sea lamprey (*Petromyzon marinus*) during niclosamide exposure (open bars) at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 11), 3 h (n = 7), 6 h (n = 7) and 9 h (n = 10), and following exposure to niclosamide (24 h recovery; n = 6; grey bars) or held under control conditions (no niclosamide; n = 10; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

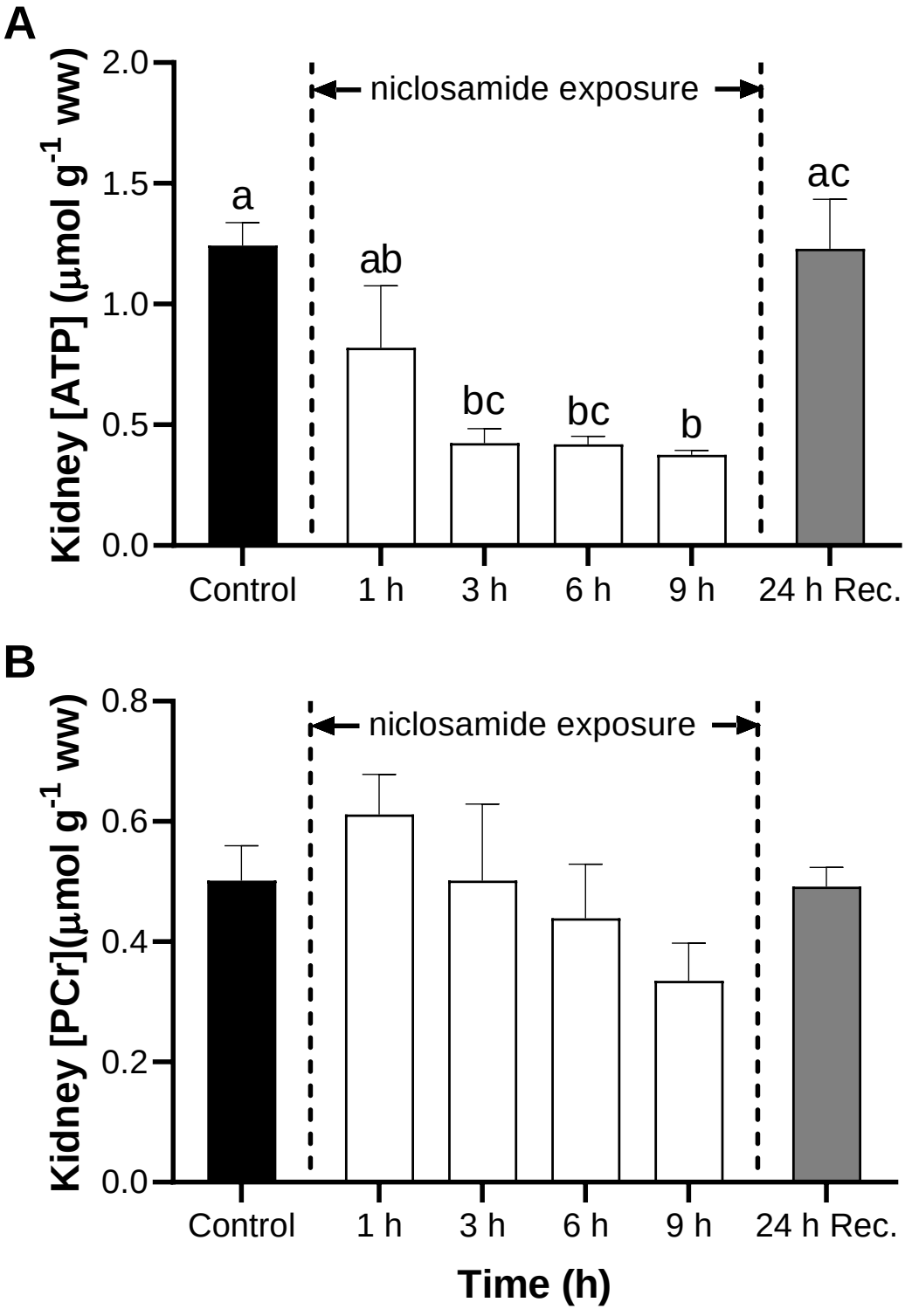


**Figure 3.5 Kidney energy molecules in sea lamprey exposed to niclosamide**

**Figure 3.5 Kidney energy molecules in sea lamprey exposed to niclosamide.** Changes in the concentrations of (A) ATP and (B) PCr in the kidney of larval sea lamprey (*Petromyzon marinus*) during niclosamide exposure (open bars) at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 10), 3 h (n = 6), 6 h (n = 8) and 9 h (n = 6), and following exposure to niclosamide (24 h recovery; n = 6; grey bars) or held under control conditions (no niclosamide; n = 10; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

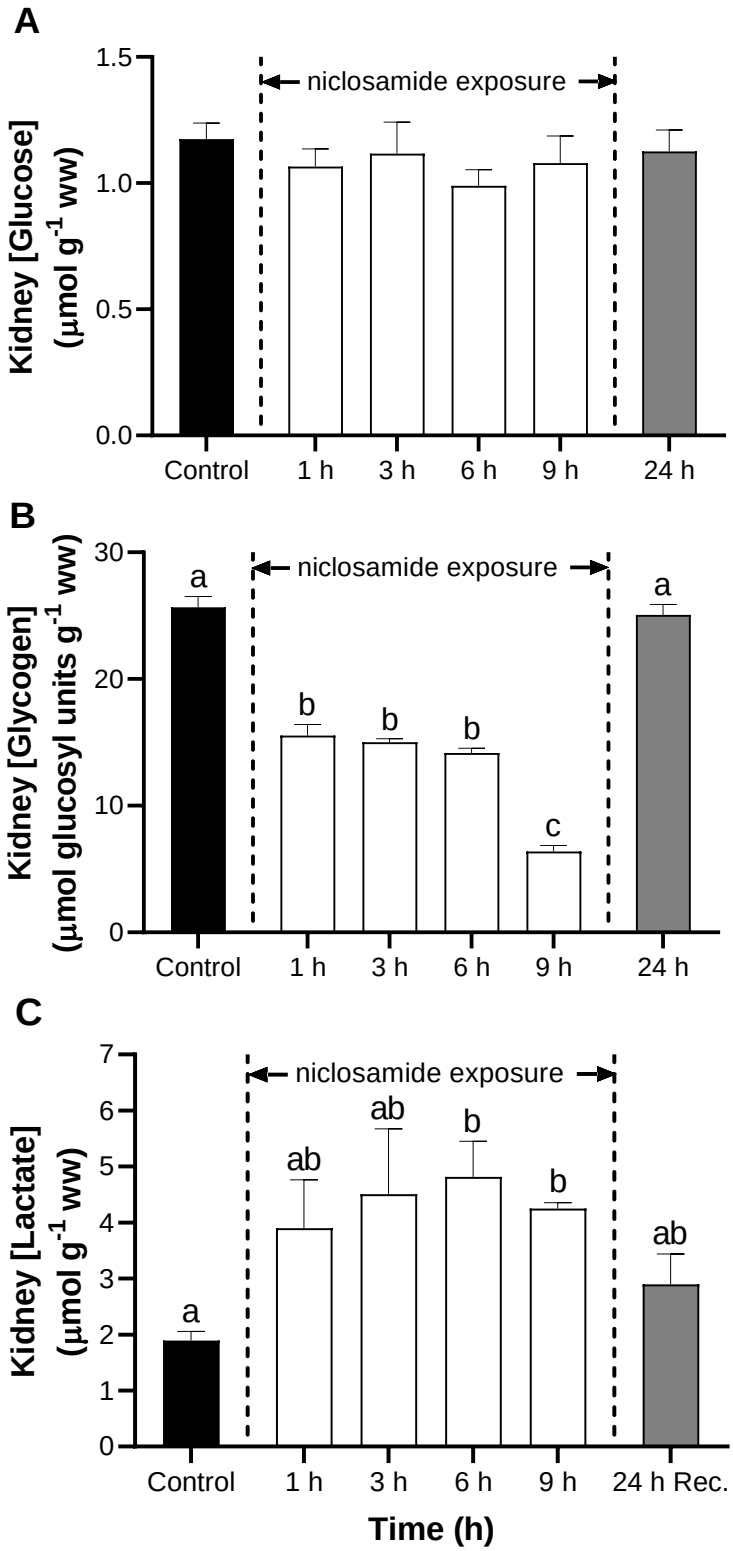


**Figure 3.6 Kidney energy molecules and metabolites in sea lamprey exposed to niclosamide**

**Figure 3.6 Kidney energy molecules and metabolites in sea lamprey exposed to niclosamide.** Changes in the concentrations of (A) glucose, (B) glycogen and lactate (C) in the kidney of larval sea lamprey (*Petromyzon marinus*) during niclosamide exposure (open bars) at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 10), 3 h (n = 6), 6 h (n = 8) and 9 h (n = 6), and following exposure to niclosamide (24 h recovery; n = 4; grey bars) or held under control conditions (no niclosamide; n = 10; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

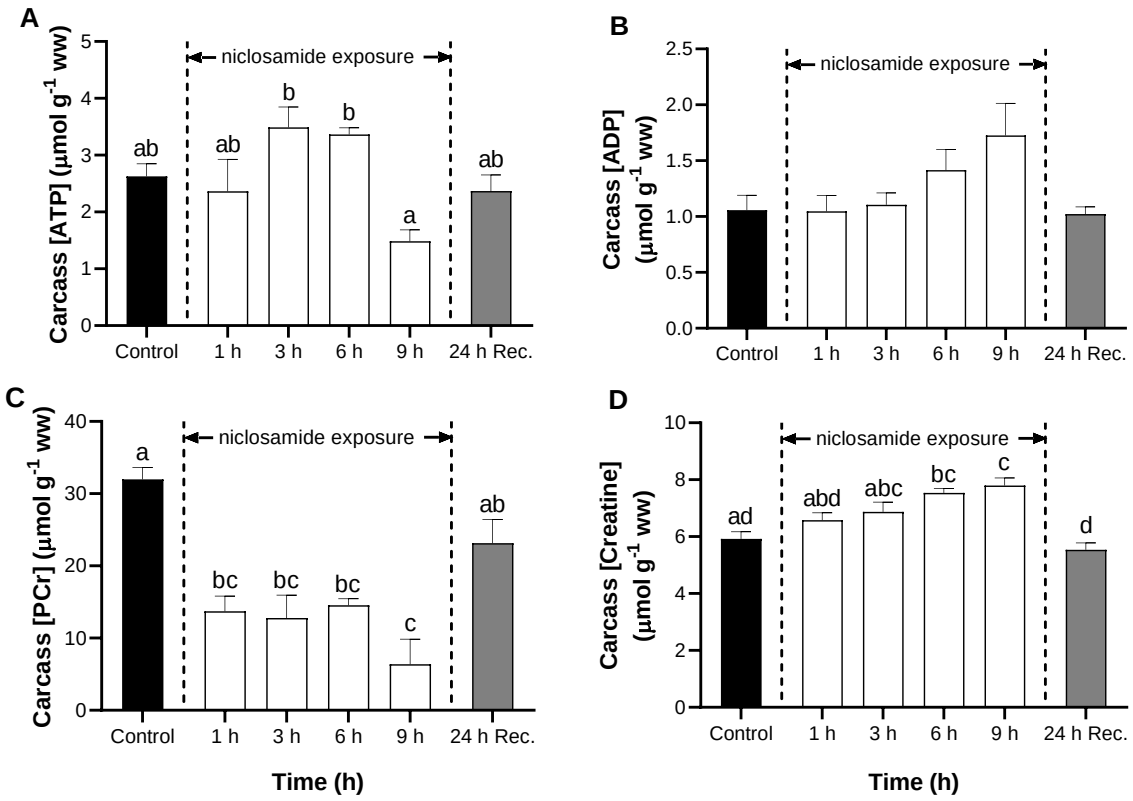


**Figure 3.7 Carcass energy molecules and metabolites in sea lamprey exposed to niclosamide**

**Figure 3.7 Carcass energy molecules and metabolites in sea lamprey exposed to niclosamide.** Changes in the concentrations of (A) ATP, (B) ADP, (C) PCr and (D) creatine in the carcass of larval sea lamprey (*Petromyzon marinus*) during niclosamide exposure (open bars) at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 9), 3 h (n = 10), 6 h (n = 9) and 9 h (n = 7), and following exposure to niclosamide (24 h recovery; n = 8; grey bars) or held under control conditions (no niclosamide; n = 10; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

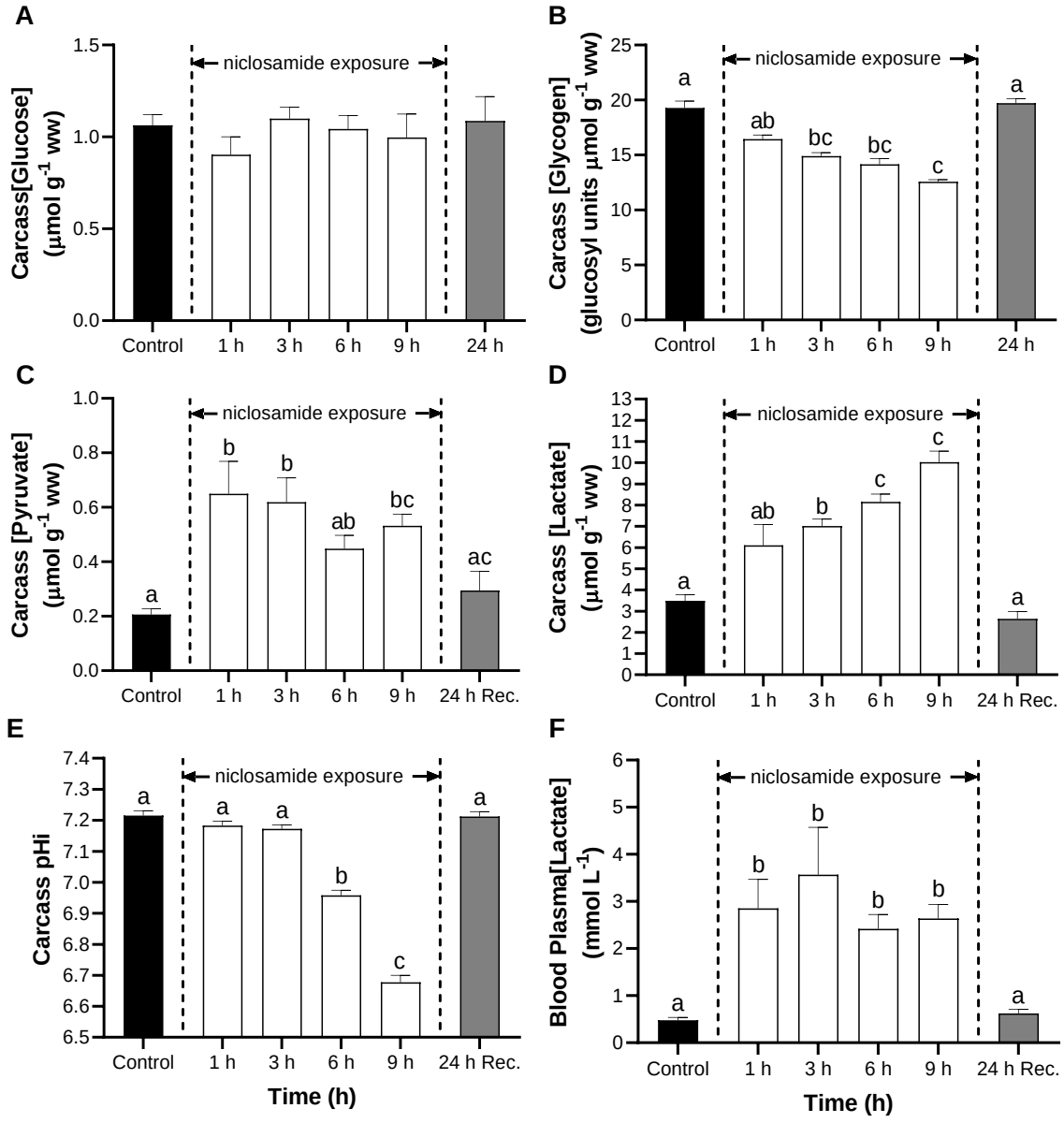


**Figure 3.8 Carcass energy molecules and metabolites in sea lamprey exposed to niclosamide**

**Figure 3.8 Carcass energy molecules and metabolites in sea lamprey exposed to niclosamide.** Changes in the concentrations of (A) glucose, (B) glycogen (C), pyruvate (D) lactate, and (E) pHi in the carcass and (F) lactate in plasma of larval sea lamprey (*Petromyzon marinus*) during niclosamide exposure (open bars) at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (carcass n = 9; plasma n = 7), 3 h (carcass n = 10; plasma n = 6), 6 h (carcass n = 9; plasma n = 5) and 9 h (carcass n = 7; plasma n = 4), and following exposure to niclosamide (24 h recovery; carcass n = 8; plasma n = 6; grey bars) or held under control conditions (no niclosamide; carcass n = 10; plasma n = 8; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

**SUPPLIMENTAL MATERIAL**

**Table 3.4S Summary of control analyses in all experimental aspects in sea lamprey.**

Comparison of control (no niclosamide) measuring concentrations of energy stores and metabolites in the brain, kidney, liver and muscle of sea lamprey (*Petromyzon marinus*) sampled at 0 h (start) and 9 h (end). Muscle intracellular pH (pHi) control measurements were similarly compared as were blood plasma lactate and ions. Data are expressed as mean ± S.E.M. No significant differences were observed.

| Brain Assays | Control Start [µmol $g^{-1}$ ww ± SEM (n)] | Control Finish [µmol $g^{-1}$ ww ± SEM (n)] |
|---|---|---|
| ATP | 1.5 ± 0.2 (5) | 1.4 ± 0.1 (4) |
| PCr | 7.0 ± 0.5 (5) | 6.9 ± 0.4 (4) |
| Glucose | 4.3 ± 0.1 (5) | 4.1 ± 0. 3 (4) |
| Glycogen | 90.4 ± 2.3 (5) | 91.7 ± 4.9 (4) |
| Lactate | 2.6 ± 0.2 (5) | 2.3 ± 0.4 (4) |

| Kidney Assays | Control Start [µmol $g^{-1}$ ww ± SEM (n)] | Control Finish [µmol $g^{-1}$ ww ± SEM (n)] |
|---|---|---|
| ATP | 1.3 ± 0.1 (6) | 1.2 ± 0.1 (4) |
| PCr | 0.5 ± 0.1 (6) | 0.5 ± 0.1 (4) |
| Glucose | 1.1 ± 0.0 (4) | 1.2 ± 0.1 (4) |
| Glycogen | 25.0 ± 0.7 (4) | 26.3 ± 1.6 (4) |
| Lactate | 2.0 ± 0.2 (6) | 1.8 ± 0.2 (4) |

| Liver Assays | Control Start [µmol $g^{-1}$ ww ± SEM (n)] | Control Finish [µmol $g^{-1}$ ww ± SEM (n)] |
|---|---|---|
| Glucose | 1.9 ± 0.2 (6) | 1.7 ± 0.2 (4) |
| Glycogen | 8.5 ± 0.6 (6) | 10.1 ± 0.7 (4) |

| Muscle Assays | Control Start [µmol $g^{-1}$ ww ± SEM (n)] | Control Finish [µmol $g^{-1}$ ww ± SEM (n)] |
|---|---|---|
| ATP | 2.7 ± 0.4 (6) | 2.5 ± 0.1 (4) |
| PCr | 31.8 ± 2.5 (6) | 32.2 ± 2.1 (4) |
| ADP | 1.0 ± 0.2 (6) | 1.1 ± 0.2 (4) |
| Creatine | 0.2 ± 0.0 (6) | 0.2 ± 0.0 (4) |
| Glucose | 1.1 ± 0.1 (6) | 1.1 ± 0.1 (4) |
| Glycogen | 18.7 ± 0.8 (6) | 20.1 ± 0.9 (4) |
| Pyruvate | 1.0 ± 0.2 (6) | 1.1 ± 0.2 (4) |
| Lactate | 3.7 ± 0.3 (6) | 3.2 ± 0.6 (4) |

| Muscle Assays | Control Start [µmol $g^{-1}$ ww ± SEM (n)] | Control Finish [µmol $g^{-1}$ ww ± SEM (n)] |
|---|---|---|
| pHi | 7.45 ± 0.0 (8) | 7.40 ± 0.0 (8) |

| Plasma Assays | Control Start [mmol $L^{-1}$ ± SEM (n)] | Control Finish [mmol $L^{-1}$ ± SEM (n)] |
|---|---|---|
| Lactate | 0.4 ± 0.0 (4) | 0.4 ± 0.1 (4) |

| Plasma Ions | Control Start [mmol $L^{-1}$ ± SEM (n)] | Control Finish [mmol $L^{-1}$ ± SEM (n)] |
|---|---|---|
| $Na^+$ | 95.8 ± 1.9 (4) | 95.4 ± 1.1 (4) |
| $Cl^-$ | 91.8 ± 0.5 (4) | 92.3 ± 0.7 (4) |

# CHAPTER 4:

# High Physiological Resilience of Rainbow Trout (*Oncorhynchus mykiss*) to Sub-Lethal Exposure to the Pesticide 2′, 5-dichloro-4′-nitrosalicylanilide (Niclosamide).

## 1. INTRODUCTION

The invasion of the Great Lakes by the sea lamprey (*Petromyzon marinus*), along with overfishing, devastated culturally significant, commercial and recreational fisheries in the mid-20th century (GLFC, 2011). In response, the governments of the United States and Canada formed the Great Lakes Fisheries Commission (GLFC) in 1954, which was charged with creating a sea lamprey research and control program to eliminate sea lamprey from the Great Lakes basin (GLFC, 2011; Siefkes, 2017). After testing over 4,000 chemicals in the 1950s, scientists identified 3-trifluoromethyl-4-nitrophenol (TFM) as a potential lampricide, which specifically targeted larval sea lamprey (ammocoetes; Applegate *et al.*, 1957, 1966). In the early 1960s, 2',5-dichloro-4'-nitrosalicylanalide (niclosamide; aka. Bayluscide® or Bayer 73®) was added to the lamprey control program (Howell *et al.*, 1964). Niclosamide is often co-applied with TFM, at 1-2% concentrations (Dawson, 2003), which reduces the amount of TFM needed by more than 40% while maintaining its efficacy and selectivity to sea lampreys (Boogaard *et al.*, 2003; Gutreuter and Boogaard, 2007). In some cases, a granular formulation of niclosamide (granular Bayluscide®) is used for population surveys or treatment of deep lentic waters or in rivers with very high discharge where the application of TFM is not practical or effective (Dawson, 2003; Wilkie *et al.*, 2019).

Niclosamide is also used as a molluscicide to treat waters infested with snails that served as vectors for schistosomes, which are parasitic flukes that cause the debilitating disease schistosomiasis in tropical countries, particularly in Africa (Lardans and Dissous, 1998; Joubert *et al.*, 2001; Zhao *et al.*, 2015; WHO, 2017). Niclosamide also has other existing and potential therapeutic uses in humans and animals including cancer therapy, where niclosamide causes mitochondrial uncoupling of oxidative phosphorylation of tumor cells (Alasadi *et al.*, 2018), as

well as inducing cell cycle arrest, growth inhibition and apoptosis (Li *et al.*, 2014). In type 2 diabetes (T2D) mice, oral administration of the ethanolamine salt of niclosamide (NEN) also acted as a mitochondrial uncoupler of oxidative phosphorylation, resulting in reduced diabetic response (Tao *et al.*, 2016).

While it is now understood that TFM exerts its toxicity in rainbow trout and sea lamprey by uncoupling mitochondrial oxidative phosphorylation, thereby inducing an energy shortfall (Birceanu *et al.*, 2011, 2014), gaps remain in our understanding of how niclosamide affects non-target species following lampricide applications, not to mention following its use as the recommended molluscicide to prevent the transmission of schistosomiasis by snails (WHO, 2017). Because niclosamide is much more potent than TFM, with a 9 h $LC_{50}$ that is $1/10^{th}$ that of TFM (Boogaard *et al.*, 2003; Newton *et al.*, 2017; Wilkie *et al.*, 2019), its effects on mitochondrial oxidative phosphorylation and anaerobic energy reserves would be expected to be more severe than those of TFM, but these potential effects have not yet been examined.

In the present study, I hypothesized that rainbow trout exposure to niclosamide will result in effects similar to those seen in TFM-exposed rainbow trout, but at much lower concentrations. Specifically, I predicted that niclosamide exposure will lead to reductions in ATP and PCr, leading to increased dependence on glycolysis, as well as cause disturbances to blood ion homeostasis due to the impairment of active (ATP-dependent) ion transport at the gill. Furthermore, I hypothesized that prolonged dependence on glycolysis due to niclosamide exposure will result in metabolic acidosis in rainbow trout. Accordingly, I exposed rainbow trout to respective 9 h $LC_{50}$ of niclosamide, and measured tissue (brain, liver, muscle) ATP, ADP, PCR, creatine, pyruvate, lactate, glucose and glycogen over 9 h exposure, to facilitate direct comparisons to previously studies addressing the physiological effects of TFM on sea lamprey

and rainbow trout. To test for blood ion and acid-base disturbances in niclosamide-exposed fish, I measured blood plasma $Na^+$ and $Cl^-$ concentration and white muscle intracellular pH (pHi) over the 9 h exposure period.

## 2. RESULTS

### 2.1 Measured water niclosamide concentrations

LC-MS/MS analysis of water samples taken from experimental tanks at start (0 h) and end (9 h) of experiments revealed that measured niclosamide concentrations were slightly higher than nominal concentrations (0.15 mg $L^{-1}$), averaging 0.18 mg $L^{-1}$ ± 0.01 at the start of experiments and dropped slightly below at the end of experiments, averaging 0.13 mg $L^{-1}$ ± 0.0 (Table 4.1).

### 2.2 Effects of sham exposure compared to controls

There were no differences in the respective concentrations of glucose, glycogen, pyruvate, ATP, ADP, phosphocreatine measured in muscle, brain or liver of control (not exposed to TFM) and sham treated fish (Table 4.1S). Nor were any differences observed between control samples collected at the start of experiments compared to those sampled after 9 h (Table 4.1S). Accordingly, all control data were pooled for comparison to niclosamide-treated fish.

### 2.3 Effects of niclosamide on energy stores and metabolites in rainbow trout brain

The concentration of ATP in the brain of rainbow trout held under control conditions averaged 0.6 ± 0.0 µmol $g^{-1}$ ww, and PCr averaged 4.2 ± 0.5 µmol $g^{-1}$ ww (Figure 4.1). Following exposure to niclosamide, the concentration of ATP in the brain underwent an

immediate and sustained reduction of approximately 50 % compared to the control measurements, returning to pre-exposure levels after 24 h recovery (Figure 4.1A). Brain PCr concentrations were unchanged in the presence of niclosamide (Figure 4.1B).

Brain glucose, glycogen and lactate concentrations in rainbow trout controls averaged 0.9 ± 0.0 μmol $g^{-1}$ ww, 5.6 ± 0.2 μmol glucosyl units $g^{-1}$ ww and 8.1 ± 0.4 μmol $g^{-1}$ ww, respectively (Figure 4.2). Brain glucose concentrations were unchanged in the presence of niclosamide (Figure 4.2A), but glycogen concentrations were significantly reduced by approximately 60% at 3, 6 and 9 h relative to controls, with levels returning to pre-exposure concentrations after 24 h of recovery (Figure 4.2B). Brain lactate concentrations in rainbow trout were significantly elevated in the presence of niclosamide, by approximately 20% in relation to controls, returning to pre-exposure levels by 24 h recovery (Figure 4.2C).

2.4 Effects of niclosamide on energy stores in rainbow trout liver

Liver glucose concentrations in rainbow trout held under control conditions averaged 16.6 ± 1.2 μmol $g^{-1}$ ww and glycogen concentrations averaged 43.1 ± 2.2 μmol glucosyl units $g^{-1}$ ww (Figure 4.3). Liver glucose remained unchanged in the presence of niclosamide relative to controls (Figure 4.3A) but glycogen concentrations were significantly reduced by approximately 25% and 40% at 6 and 9 h, respectively, compared to controls, returning to pre-exposure levels after 24 h recovery (Figure 4.3B).

2.5 Effects of niclosamide on energy stores, metabolites and pHi in rainbow trout muscle

White muscle ATP and ADP concentrations in rainbow trout controls averaged 7.3 ± 0.3 μmol $g^{-1}$ ww and 1.0 ± 0.0 μmol $g^{-1}$ ww, respectively (Figure 4.4). In the presence of niclosamide,

muscle ATP concentrations were significantly reduced by approximately 38% and 75% at 6 and 9 h, respectively, relative to controls, returning to control concentrations after 24 h recovery (Figure 4.4A). White muscle concentrations of ADP remained unchanged in the presence of niclosamide (Figure 4.4B).

White muscle PCr and creatine concentrations in rainbow trout controls average 27.6 ± 0.5 μmol $g^{-1}$ ww and 21.0 ± 1.3 μmol $g^{-1}$ ww, respectively (Figure 4.4). Muscle PCr concentrations were significantly reduced in the presence of niclosamide by approximately 45% at 9 h, relative to controls, returning to pre-experimental levels after 24 h recovery (Figure 4.4C) and creatine was significantly reduced approximately 35% at 3, 6 and 9 h compared to controls, returning to pre-exposure concentrations after 24 h recovery (Figure 4.4 D).

Concentrations of glucose and glycogen in white muscle of rainbow trout controls averaged 0.9 ± 0.0 μmol $g^{-1}$ ww and 13.6 ± 0.2 μmol glucosyl units $g^{-1}$ ww (Figure 4.5). No changes were observed in muscle glucose concentration in the presence of niclosamide (Figure 4.5A), but glycogen concentrations were significantly depleted by approximately 40% and 70% at 6 and 9 h, respectively, compared to controls, returning to pre-experimental levels after 24 h of recovery (Figure 4.5B).

Pyruvate and lactate concentrations in white muscle of rainbow trout controls averaged 0.3 ± 0.0 μmol $g^{-1}$ ww and 2.2 ± 0.1 μmol $g^{-1}$ ww, respectively (Figure 4.5). In the presence of niclosamide muscle pyruvate concentrations were significantly increased by approximately 45% relative to controls and returned to pre-experimental levels after 24 h recovery (Figure 4.5C). White muscle lactate levels were significantly impacted by niclosamide, with approximately 5-fold (1 h), 6.5-fold (3 and 6 h) and 9-fold (9 h) increases relative to controls. However, lactate also returned to pre-experimental concentrations after 24 h recovery (Figure 4.5D).

The intracellular pH (pHi) of control rainbow trout white muscle averaged 7.44 ± 0.02 for controls. The presence of niclosamide resulted in acidosis in the muscle, characterized by significant pHi decreases (0.11, 0.15 and 0.19 pH units) to 7.33 ± 0.01, 7.29 ± 0.01 and 7.25 ± 0.01 at 3, 6 and 9h, respectively, relative to controls, returning to pre-experimental levels (pH = 7.42 ± 0.02) after 24 h of recovery (Figure 4.6).

2.6 Effects of niclosamide on blood plasma ions in rainbow trout

Concentrations of $Na^+$ and $Cl^-$ in blood plasma of rainbow trout controls averaged 143.6 ± 2.2 mmol $L^{-1}$ and 116.8 ± 1.0 mmol $L^{-1}$, respectively (Table 4.1).

## 3. DISCUSSION

3.1 Effects of niclosamide on energy stores and metabolites in the brain

Exposure of rainbow trout to sub-lethal concentrations of niclosamide resulted in a significant decrease of ATP and glycogen in the brain, consistent with niclosamide's known uncoupling effects on mitochondrial oxidative phosphorylation (Park *et al.*, 2011; Jurgeit *et al.*, 2012; Alasadi *et al.*, 2018). These observations are similar to those of rainbow trout exposed to TFM, which is known to uncouple mitochondrial oxidative phosphorylation, thus reducing ATP production (Birceanu *et al.*, 2011, 2014). Typically, when ATP supply fails to meet demand due to increased energy requirements in vertebrates, such as during vigorous exercise in muscle or due to hypoxia or anoxia, ATP is formed from high energy phosphagens such as PCr in which the creatine phosphokinase (CK) mediated dephosphorylation of PCr transfers a phosphate group to ADP to sustain ATP levels (Hochachka *et al.*, 1993; McLeish and Kenyon, 2005; Wallimann *et al.*, 2011). Curiously, this was not observed in the trout brain, as PCr

concentrations were sustained throughout the niclosamide exposure period despite the sustained reduction in ATP. Nor were significant reductions in PCr observed in the brain of trout exposed to TFM (Birceanu *et al.*, 2014), which again suggests that its role in buffering ATP concentrations was minimal in this tissue. This was unlike the situation previously reported in larval sea lamprey exposed to niclosamide (Chapter 2) or TFM (Birceanu *et al.*, 2009; Clifford *et al.*, 2012), in which slight declines in brain ATP levels were accompanied by persistent reductions in PCr.

Brain PCr concentrations typically undergo precipitous declines in response to drops in ATP supply caused by drops in $O_2$ due to restricted blood supply in mammals or environmental hypoxia or anoxia in many fishes species (e.g. Van Den Thillart *et al.*, 1989; Van Waarde *et al.*, 1990; Wallimann *et al.*, 2011). One possible explanation for the apparent lack of PCr mobilization during niclosamide exposure in the trout brain with niclosamide and TFM exposure could be its extensive reliance on glucose oxidation and oxidative phosphorylation for ATP production (Soengas and Aldegunde, 2002), along with a corresponding low anaerobic capacity characterized by relatively low glycogen stores and PCr compared to more hypoxia/anoxia tolerant fishes including larval sea lamprey, bullhead (*Ictalurus nebulosus*), common carp (*Cyprinus carpio*) and crucian carp (*Carassius auratus*) (DiAngelo and Heath, 1987; van Raaij *et al.*, 1994; Lardon *et al.*, 2013). Compared to the responses of common carp and bullhead to hypoxia, the ATP yield from brain PCr and glycogen mobilization in rainbow trout is much less, which likely explains their much lower tolerance to oxygen starvation (DiAngelo and Heath, 1987; van Raaij *et al.*, 1994).

To better understand why there was no change in PCr in the face of lower ATP production, further examination of the creatine kinase (CK)/PCr system could be informative.

The CK/PCr system is multifunctional, acting not only as a short-term buffer of ATP, but it also links sites of ATP production within the mitochondria to sites of ATP utilization in the cytosol (e.g. ATPase pumps), and it also acts as a metabolic regulator (see Wallimann *et al.*, 2011 for review). These actions are mediated by different isoforms of CK, that have distinctive sub-cellular and tissue specific distribution patterns. Isoforms include mitochondrial CK (mCK) which is coupled to ATP export from the mitochondria via adenine nucleotide transporters, which has different properties compared to cytosolic CK, which buffers $H^+$ arising from ATP hydrolysis. Another cytosolic isoform of CK mainly functions to buffer ATP stores, the better-known role of the CK/PCr system. This complexity therefore raises the possibility that the results observed here could also be due to differences in the relative complement and properties of CK isoforms present in the different sub-cellular regions of the brain of the trout, compared to other species and/or tissues such as the muscle which has a much higher relative anaerobic capacity due to its reliance on PCr and endogenous glycogen stores to sustain high rates of ATP production for burst exercise. Future studies looking at the relative complements and properties of CK isozymes present in the different subcellular compartments of muscle and brain, not to mention other tissues, are needed to resolve these issues, however (Wallimann *et al.*, 2011).

Wilkie *et al.* (2007) first suggested that death from TFM was likely to occur when the glucose supply to the brain was sufficiently depleted and no longer able to meet the demands of the nervous system. Notably, the reduction in brain ATP and glycogen observed in rainbow trout were much more pronounced with niclosamide exposure than those observed by Birceanu *et al*., (2014) for TFM, which likely reflects the greater potency of the former. The oxidation of glucose, provided via plasma from mobilized glycogen stores in the liver, as well as the brain itself, is primarily responsible for sustaining the ATP supply in the brain (Soengas and

Aldegunde, 2002; Polakof *et al.*, 2007; 2012). In fact, the rainbow trout brain, uses more glucose per unit mass than any other organ in the body (Washburn *et al.*, 1992), but it also has relatively low glycogen reserves (< 5- 6 µmol glucosyl units $g^{-1}$ ww; e.g. Figure 4.2; DiAngelo and Heath, 1987). In contrast, the brain of sea lampreys have glycogen concentrations that typically exceed 100 µmol glucosyl units $g^{-1}$ ww (Rovainen *et al.*, 1969; Foster *et al.*, 1993; Clifford *et al.*, 2012).

In larval sea lamprey, such high brain glycogen concentrations could be an adaptation that enables them to tolerate hypoxic conditions, which can occur in their burrow-dwelling habitat (Potter *et al.*, 1970). Indeed, in anoxia tolerant fishes, such as the goldfish (*Carassius auratus*) and crucian carp, brain glycogen may be as high as 200 µmol glucosyl units $g^{-1}$ ww, and a key adaptation that enables them to survive in $O_2$ starved waters beneath the ice and snow covered shallow lakes and ponds (Nilsson, 2001; Nilsson and Lutz, 2004; Vornanen and Paajanen, 2006). Under such conditions the high brain glycogen, along with massive liver glycogen stores, of crucian carp and goldfish serves as an important anaerobic energy reserve at times when oxygen supply to the brain is lowered or eliminated, allowing reliance on glycolysis to maintain ATP production when supply is limited due to limited or absent aerobic metabolism (Rovainen *et al.*, 1969; Vornanen and Paajanen, 2006). Less hypoxia-tolerant fish species, such as rainbow trout, generally have low basal concentrations of brain glycogen, therefore they mainly rely on liver glycogen stores to meet glucose demands of nervous tissues, achieved by increases in glycogenolysis in response to glucagon release in the face of depleting glucose supply (Soengas and Aldegunde 2002; Polakof *et al.*, 2012). Thus, the very pronounced (50 %) drops in liver glycogen that were observed during niclosamide exposure in the present study likely reflect the need to sustain the glucose supply to the brain, where it was subsequently metabolized by anaerobic glycolysis.

When oxidative ATP production is not possible due to lack of oxygen resulting from ischemia, environmental anoxia/hypoxia or compounds that directly interfere with mitochondrial ATP production, such as niclosamide, it becomes detrimental to brain function (Soengas and Aldegunde, 2002). A further obstacle is that anaerobic glycolysis and the subsequent hydrolysis of ATP generates metabolic acid ($H^+$; Hochachka and Mommsen, 1983), which may further disturb the central nervous system (CNS) function. There was insufficient brain tissue to measure how niclosamide affected intracellular pH in the brain. There was a significant metabolic acidosis in the muscle, however, which has a substantial, 3-fold higher non-bicarbonate buffer capacity than the brain (Milligan and Wood 1986), suggesting that there was likely a much larger drop in brain pHi. The presence of severe acidosis in the brain, combined with ATP supply limitations, could have further compounded any niclosamide-induced physiological disturbances to CNS function.

At first glance, observed decreases in brain glycogen with niclosamide exposure appear to be disproportionately larger than total lactate accumulation, in which the stoichiometry would result in a lactate appearance to glycogen consumption ratio of 2:1. As a preferred substrate for neurons (Hochachka *et al.*, 2002; Soengas and Aldegunde, 2002; Barros *et al.*, 2020), however, the lactate was likely oxidized in the mitochondria. Although, niclosamide impairs ATP production by uncoupling oxidative phosphorylation, there is no *a priori* reason why mitochondrial pyruvate or lactate oxidation would be reduced because the components of the respiratory chain (electron transport chain) would remain intact and likely unaffected. Although glucose is the dominant fuel for neurons, its partial metabolism in astrocytes gives rise to lactate, which is subsequently exported to the neurons via a “lactate-shuttle” where it is subsequently oxidized (Hochachka and Somero, 2002). Thus, even in the presence of niclosamide, lactate

oxidation would continue, or be enhanced in the face of increased rates of glycolysis, thereby generating reducing equivalents that would continue to feed the electron transport chain (ETC) leading to continued or increased consumption of oxygen, which is the final electron acceptor in the terminal step (protein complex IV) of the ETC (Hochachka and Somero, 2002). Indeed, the State IV rates of respiration of trout isolated liver mitochondria were enhanced by 2- to 3-fold in the presence of TFM and 2,4-dinitrophenol, which are also well-established uncouplers of oxidative phosphorylation (Birceanu *et al.*, 2011).

A less likely fate for lactate was that it was simply transported out of the brain using monocarboxylic acid (MCT) transporters. The MCT family of transporters facilitates the movement of monocarboxylic acids including lactate, pyruvate and ketone bodies across the plasma membrane of different tissues, including muscle and nervous tissue (see Halestrap 2013 for review). Lactate transport is thought to take place via Lac-$H^+$ co-transport mediated by several different MCT isoforms including MCT1 and MCT2, which generally promote lactate uptake, including by neurons in the mammalian CNS. On the other hand, MCT4 is known to promote lactate export from glial cells to neurons via the so-called astrocyte-neuron lactate shuttle. MCT1 and MCT4 are also known to promote the export of lactate by red and white mammalian skeletal muscle, respectively, and by cardiac muscle (MCT1; Halestrap 2013). Little attention has been given to fish MCTs, but it has been demonstrated that a lack of MCT4 gene expression likely accounts for the retention of lactate in trout white skeletal muscle (Omlin and Weber 2013), which is subsequently used to fuel glycogen re-synthesis following exercise (see Wood 1991; Kieffer 2000 for reviews). The same study also was notable in that only MCT1a and MCT2, which account for neuronal lactate uptake, were expressed in brain, but not MCT4 which is involved in lactate export (Omlin and Weber, 2013).

3.2 Niclosamide has similar effects on muscle metabolic status as exhaustive exercise

Many authors have conducted exhaustive exercise experiments on rainbow trout, investigating metabolic stress and recovery (see Milligan, 1996; Kieffer, 2000 for reviews). While the results from these experiments can vary, the overall trend is that rainbow trout readily recover their metabolic status, often in less than 12 h, following exercise. The values reported in the present study for ATP, PCr, glycogen and lactate in muscle of rainbow trout controls (at rest) are similar to, and fall within the reported ranges (4-6, 20-42, 7-16 and 0.5-11µmol $g^{-1}$ ww, respectively) in resting trout (Milligan and Wood, 1986a; Tang and Boutilier, 1991; Ferguson *et al.*, 1993; Kieffer *et al.*, 1994; Wang *et al.*, 1994). Moreover, the reductions in in energy stores in the muscle of niclosamide exposed rainbow trout and their restoration of ATP, PCr, glycogen and lactate during recovery from exposure, closely resemble those observed in exhaustively exercised trout (Milligan and Wood, 1986a; Tang and Boutilier, 1991; Ferguson *et al.*, 1993; Kieffer *et al.*, 1994; Wang *et al.*, 1994; Wang *et al.*, 1994). Interestingly, although metabolic acidosis was observed in white muscle of niclosamide exposed rainbow trout, there was only a decrease in pHi of approximately 0.2 units, which is approximately one third that observed in rainbow trout following exhaustive exercise experiments (Wood *et al.*, 1983; Milligan and Wood, 1986; Wang *et al.*, 1994). These are important observations because it provides further support for the hypothesis that niclosamide imparts its toxicity in rainbow trout via disruption of oxidative ATP production.

3.3 Effects of niclosamide on blood plasma ions

Neither plasma $Na^+$ and $Cl^-$ concentrations were altered by niclosamide exposure (Table 1), similar to results observed for TFM in rainbow trout by Birceanu *et al.* (2014), and observed

for TFM in sea lamprey (Birceanu *et al.,* 2009; Henry *et al.,* 2015). Thus, it is unlikely that any gill damage resulted from niclosamide exposure. In contrast, transmission electron microscopy (TEM) ultrastructural analysis of the gills of larval sea lamprey exposed to lethal concentrations (9 h $LC_{100}$) of TFM and niclosamide (Bayer 73®) revealed damage to almost exclusively to ion-uptake cells on the gill lamellae of lamprey (Mallatt et al 1994). Damage included cell rounding, enlargement of mitochondria and widening of intracellular spaces in presumptive ion-uptake cells, which are now recognized as mitochondrion-rich cells or ionocytes (Wilson and Laurent 2000; Evans et al. 2004; Dymowska *et al.*, 2012). Compared to TFM, niclosamide exposure also resulted in a greater incidence of necrosis to ion-uptake cells (Mallatt *et al.,* 1994). However, rainbow trout gills were unaltered by lethal concentrations of either lampricide (Mallatt *et al.*, 1994), which at least partially explains the lack of ionic disturbance observed in the present study.

The present results also lend no support to the hypothesis that ATP shortfalls due to niclosamide exposure would substantially impair active transport mediated ion uptake by ionocytes in the gill. Such cells are rich in mitochondria, which are required to generate the ATP needed to power active ion transport. For instance, in trout, $Na^+$ balance is thought to be dependent on freshwater ionocytes in which protons are pumped across the apical membrane of cells via ATP-dependent, V-ATPase proteins, which help generate an electrical gradient that promotes $Na^+$ uptake via apical $Na^+$ channels (Edwards and Marshall, 2012). To date, however, there is no evidence of an epithelial $Na^+$ channel (ENaC) in the gills of teleosts, leading to the alternate hypothesis that apical $Na^+$ uptake takes place via $Na^+/H^+$ (NHE) exchange (see Dymowska *et al.*, 2012 for review). However, it has been demonstrated that acid-sensing ion channels (ASICs) are expressed in the gills of rainbow trout and adult zebrafish (*Danio rerio*)

and likely play a role in $Na^+$ uptake (Dymowska *et al.*, 2014, 2015). Regardless of events occurring at the apical membrane, in either case, branchial $Na^+$ uptake would depend upon low intracellular $Na^+$ mediated by basolateral $Na^+/K^+$-ATPase. In fact, immunohistochemical analysis has shown that trout ASIC localizes to $Na^+/K^+$-ATPase-rich cells in the gill (Dymowska *et al.*, 2014). Yet the present study suggests that niclosamide has little effect on $Na^+$ transport, despite its known effects on mitochondrial ATP production.

Less is known about $Cl^-$ uptake, which is thought to occur, via an apical $Cl^-/HCO_3^-$ +exchanger. However, other ions such as $Ca^{2+}$ are known to be mediated by secondary active transport via an epithelial $Ca^{2+}$ channel, down an electrochemical gradient maintained by a basolateral $Ca^{2+}$-$Na^+$ exchange (Dymowska *et al.*, 2012). While it remains possible that impaired oxidative phosphorylation resulted in decreased rates of $Na^+$ and/or $Cl^-$ uptake, the time of exposure was in all likelihood too brief to elicit measurable changes in $Na^+$ and $Cl^-$ balance, which often take days to develop following insults such as exposure to acid or alkaline pH (e.g. (Wood *et al.*, 1988; Wilkie and Wood, 1991). Future experiments, using radio-tracers (e.g. $^{22}Na^+$; $^{36}Cl^-$; $^{45}Ca^{2+}$) would shed more light on this question.

### 3.4 TFM and niclosamide: a challenging balance between life and death

In the present study the reported rainbow trout niclosamide 9 h $LC_{50}$ was nominally 0.15 mg $L^{-1}$ which induced adverse physiological effects in rainbow trout despite the sub-lethal concentrations used (present study), leading to disturbances in energy metabolism. However, as demonstrated in this study, rainbow trout are physiologically highly resilient, recovering relatively quickly (within 24 h) following withdrawal from niclosamide exposure. This resilience can be attributed to the fact that rainbow trout begin eliminating niclosamide (mostly conjugated

forms) almost immediately after exposure (Statham and Lech, 1975; Allen *et al.*, 1976; Dawson *et al.*, 1996), and have been reported to withstand very long exposures (up to 35 days) at low concentrations (0.005 mg $L^{-1}$; Hubert *et al.*, 1996).

Sea lamprey control personnel are continually presented with the challenge of ensuring the protection of non-target fishes while administering lampricide treatments to waterways. The fast sinking, granular form of niclosamide (Bayluscide®) is used in lentic areas, in population surveys, and in very large fast-flowing waters of large rivers, such as the St. Mary's River which drains Lake Superior, to control populations of larval sea lamprey (Jones *et al.*, 2015). Under such conditions, it is much less likely that non-target fishes such as rainbow trout will be exposed to high concentrations of niclosamide, such as those used in this study, for very long periods because they can either escape the niclosamide by moving up in the water column to evade the lampricide, or it will quickly dissipate.

For typical lampricide applications, it is usually more preferable to use the combination of niclosamide (1-2 %) with TFM under such conditions because the amount of TFM is significantly reduced, decreasing the amount of pesticide released into the aquatic ecosystem, as well as decreasing the costs (Gutreuter and Boogaard, 2007). It has been reported that niclosamide use in the Great Lakes has risen over the past 15 years (Wilkie *et al.*, 2019). While even brief exposures of sub-lethal concentrations of niclosamide lead to physiological disturbances in energy homeostasis, rainbow trout fully recover following exposure, devoid of residual effects. Whether or not this is the case in other non-target fishes remains to be determined, but such knowledge could have important implications for predicting how other populations of fish respond to niclosamide. Therefore, it is more important than ever to develop a

better understanding of niclosamide effects, alone and combined with TFM, not only in rainbow trout, but in as many non-target fishes as possible, especially species at risk native to the basin. Although TFM and niclosamide are typically applied in mixtures, it is unclear if TFM and niclosamide interact in a strictly additive, less than additive (antagonistic) or greater than additive fashion (synergistic). Given the need to develop additional methods of sea lamprey control to minimize the risk of lampricide resistance evolving (Dunlop et al. 2019; Christie et al. 2020), a better understanding of the underlying mechanisms of niclosamide-TFM interactions could inform the development of new adjuvants for lampricide treatment including greener, more environmentally benign lampricides. One approach to learn more about niclosamide-TFM interactions may be to standardize the toxicity of each lampricide using the concentration addition model by equating the respective 9-h $LC_{50}$ to a toxic unit (Newman and Unger, 2003; Playle, 2004), and then measure the response of the fish following exposure to the same toxic unit value. In this way the toxic potency of various combinations of the two chemicals can be determined in rainbow trout. By performing similar experiments on sea lamprey, it would be possible to calculate the best mixtures to achieve MLC of target organism while minimizing risk to rainbow trout.

## 3.5 Summary and Conclusions

Niclosamide-induced depletion of brain ATP and glycogen suggests that rainbow trout became increasingly reliant on glycolysis, further supported by elevated lactate in the brain and mobilization of liver glycogen. Energy depletion and increased dependence on anaerobic processes was also evident in muscle where ATP, PCr and glycogen were severely depleted with a concomitant increase in pyruvate and lactate, resulting in metabolic acidosis. These results are

similar to TFM-exposed rainbow trout (Birceanu *et al.*, 2009, 2014), suggesting that niclosamide toxicity has a similar mode of action, specifically disruption of ATP production. Indeed, the most pronounced effect of niclosamide in rainbow trout is on ATP synthesis; however, the fish readily recover from exposure. From the disturbances observed in the muscle, it may be inferred that in the interim period following niclosamide exposure, exercise (swimming) performance could be impaired. Impaired swimming performance could affect predator evasion, foraging or upstream migration, depending on the time of year. Moreover, the rapid restoration of homeostasis in the brain suggest that CNS mediated activities including behaviour or autonomic regulation would only be temporarily altered followed exposure. The resilience of rainbow trout facing relatively short (9 h) exposure of sub-lethal niclosamide concentrations, suggests that the short-term homeostatic disturbances arising from sub-lethal concentrations are only temporary and unlikely to have negative prolonged effects on salmonid fishes that are inadvertently exposed to niclosamide.

**Table 4.1 Measured water niclosamide concentrations.**

The nominal concentration of niclosamide (0.15 mg $L^{-1}$) was used to expose rainbow trout (*Oncorhynchus mykiss*). Water niclosamide concentrations were measured using LC-MS/MS, in samples taken from experimental tanks at the start (0 h) and end (9 h) of experiments.

| Treatment | Measured Water [niclosamide](mg $L^{-1}$ ± SEM (n) |
|---|---|
| 0 h | 0.18 ± 0.07 (22) |
| 9 h | 0.13 ± 0.0 (22) |

**Table 4.2 Effects of niclosamide on rainbow trout blood plasma ions.**

Changes in the concentrations of $Na^+$ and $Cl^-$ in the blood plasma of rainbow trout (*Oncorhynchus mykiss*) during and following exposure to niclosamide (24 h recovery) at a nominal concentration of 0.15 mg L-1 (9 h LC50) for up to 9 h or held under control conditions (no niclosamide). Data are expressed as mean ± S.E.M. No significant differences were observed.

| Treatment | $Na^+$ mmol $L^{-1}$ ± SEM (n) | $Cl^-$ mmol $L^{-1}$ (± SEM; n) |
|---|---|---|
| Control | 143.7 ± 3.4 (22) | 116.9 ± 1.3 (23) |
| 1 h | 143.0 ± 2.3 (11) | 112.2 ± 4.3 (12) |
| 3 h | 141.5 ± 2.9 (11) | 115.4 ± 2.6 (11) |
| 6 h | 144.3 ± 3.3 (12) | 114.2 ± 1.7 (11) |
| 9 h | 147.0 ± 4.0 (9) | 119.6 ± 3.1 (10) |
| 24 h Rec. | 143.3 ± 3.1 (11) | 116.8 ± 2.8 (12) |

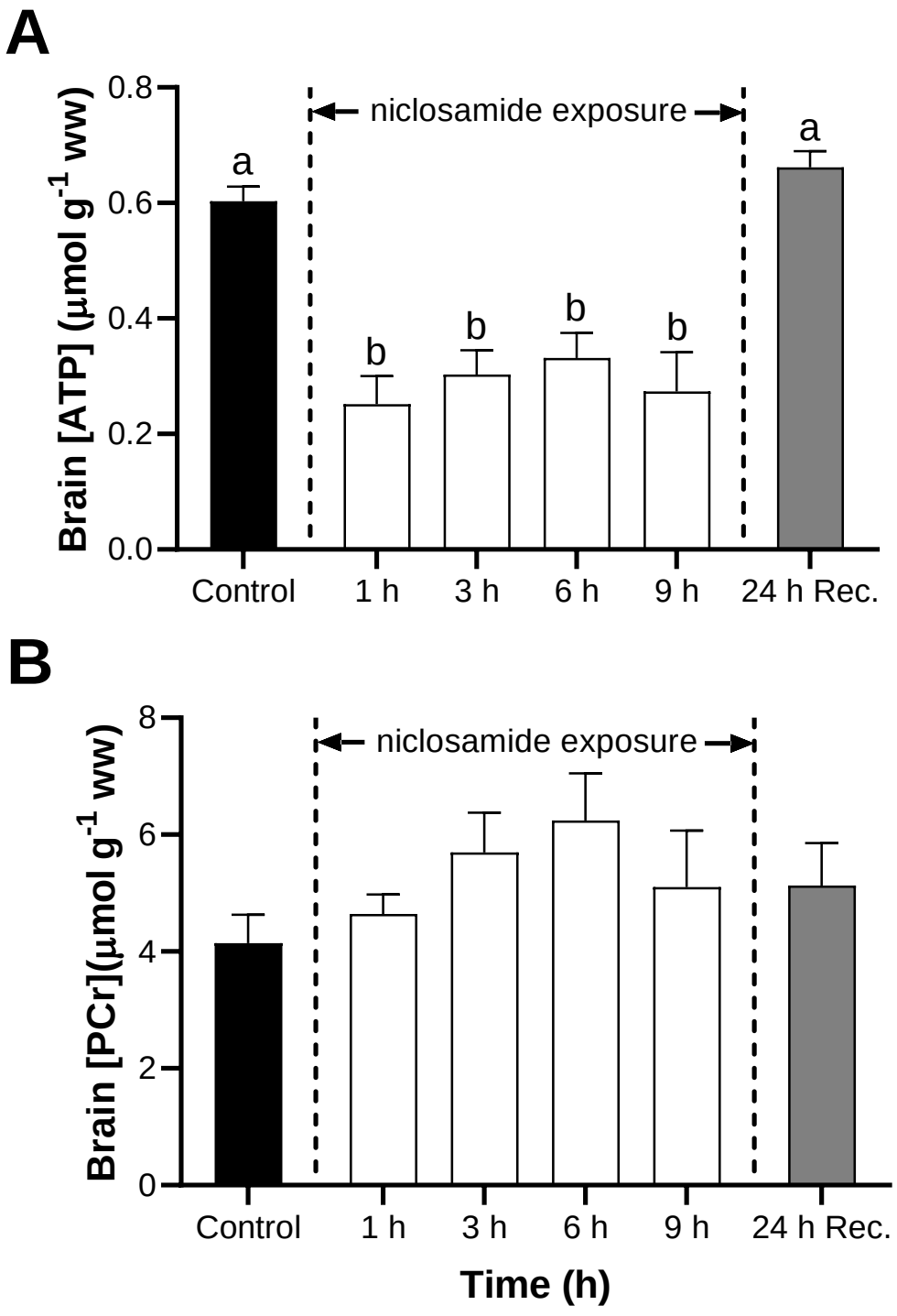


**Figure 4.1 Energy reserves in brain of rainbow trout**

**Figure 4.1. Energy reserves in brain of rainbow trout.** Changes in the concentrations of (A) ATP and (B) PCr in the brain of rainbow trout (*Oncorhynchus mykiss*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 12; grey bars) at a nominal concentration of 0.15 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 13), 3 h (n = 11), 6 h (n = 11) and 9 h (n = 10), or held under control conditions (no niclosamide; n = 22; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

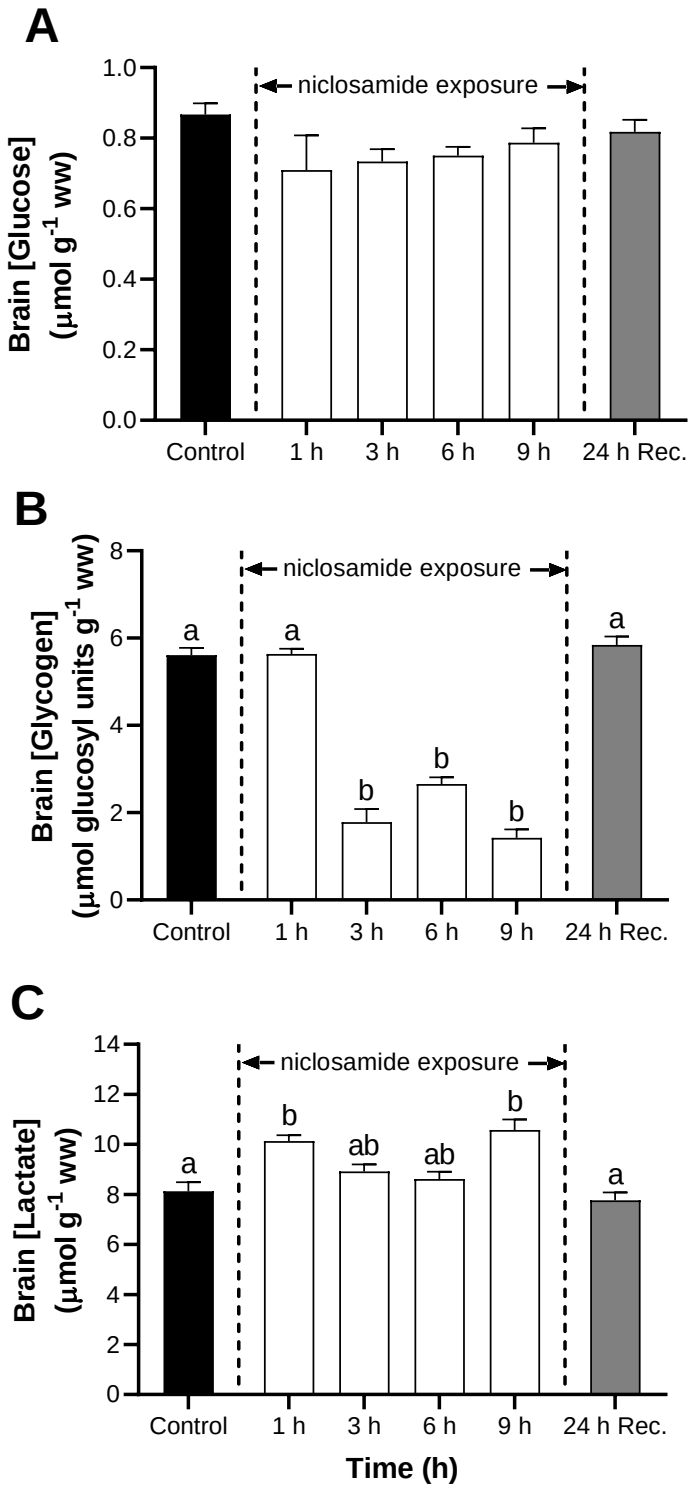


**Figure 4.2 Energy stores and metabolites in brain of rainbow trout**

**Figure 4.2 Energy stores and metabolites in brain of rainbow trout.** Changes in brain concentrations of (A) glucose, (B) glycogen and (C) lactate in the brain of rainbow trout (*Oncorhynchus mykiss*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 12; grey bars) at a nominal concentration of 0.15 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 13), 3 h (n = 11), 6 h (n = 11) and 9 h (n = 10), or held under control conditions (no niclosamide; n = 22; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

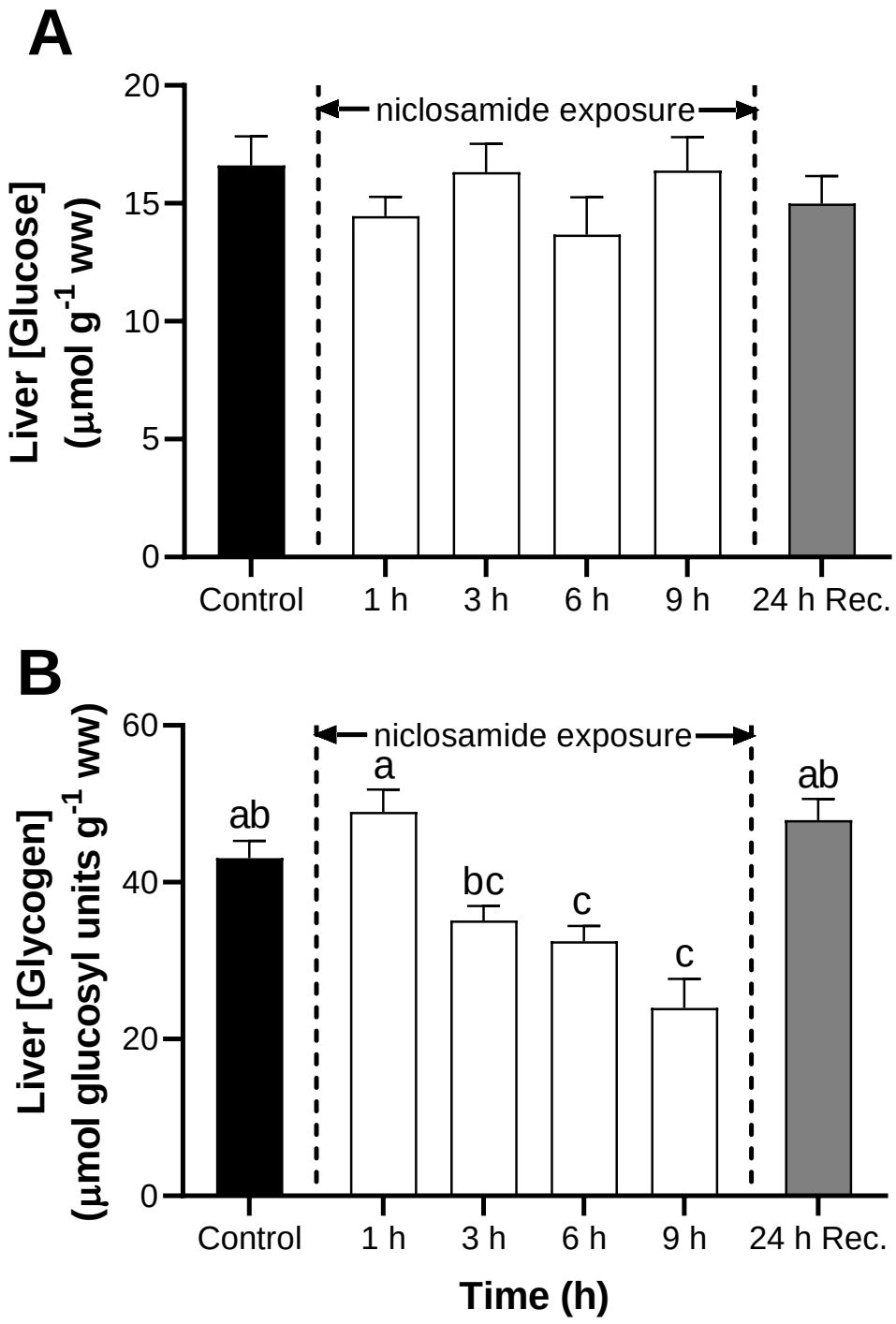


**Figure 4.3 Energy stores in liver of rainbow trout**

**Figure 4.3 Energy stores in liver of rainbow trout.** Changes in liver concentrations of (A) glucose and (B) glycogen in the liver of rainbow trout (*Oncorhynchus mykiss*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 12; grey bars) at a nominal concentration of 0.15 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 12), 3 h (n = 11), 6 h (n = 12) and 9 h (n = 10), or held under control conditions (no niclosamide; n = 23; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

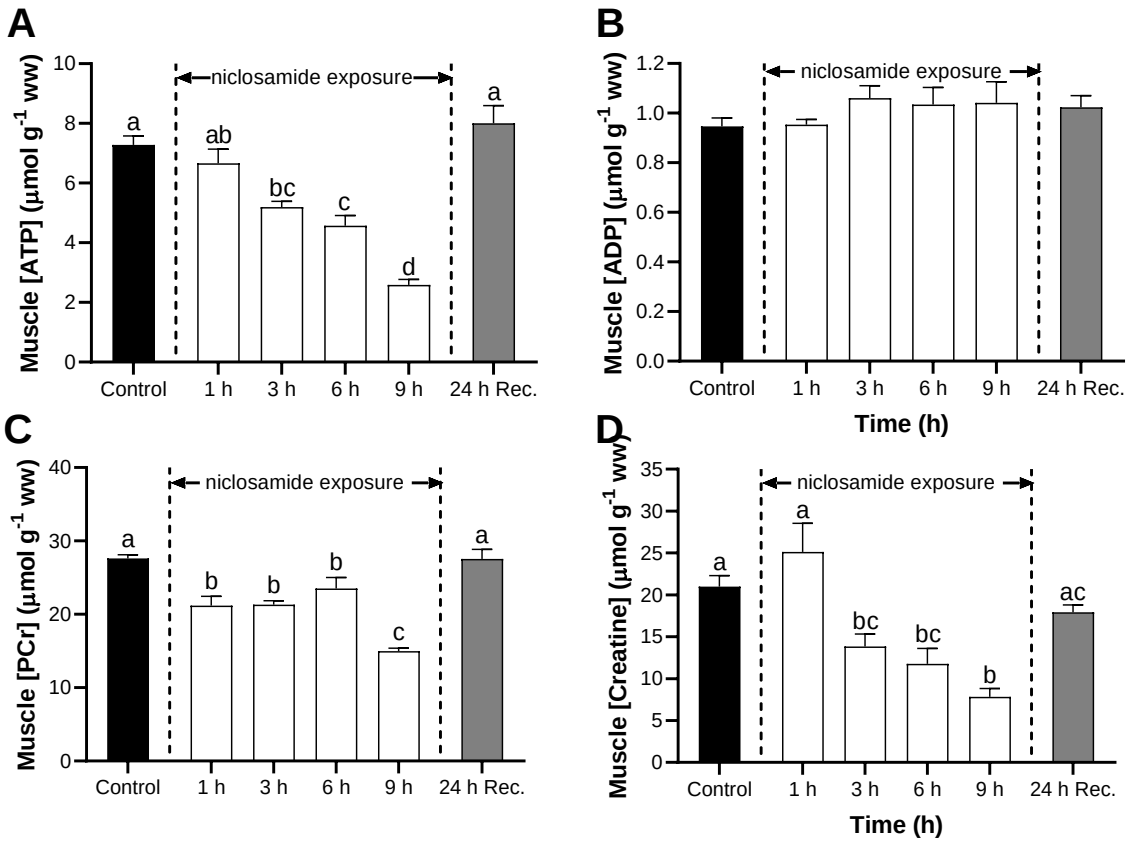


**Figure 4.4 Energy stores in muscle of rainbow trout**

**Figure 4.4 Energy stores in muscle of rainbow trout.** Changes in muscle concentrations of (A) ATP, (B) ADP, (C) PCr and (D) creatine in the muscle of rainbow trout (*Oncorhynchus mykiss*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 12; grey bars) at a nominal concentration of 0.15 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 12), 3 h (n = 11), 6 h (n = 10) and 9 h (n = 9), or held under control conditions (no niclosamide; n = 23; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

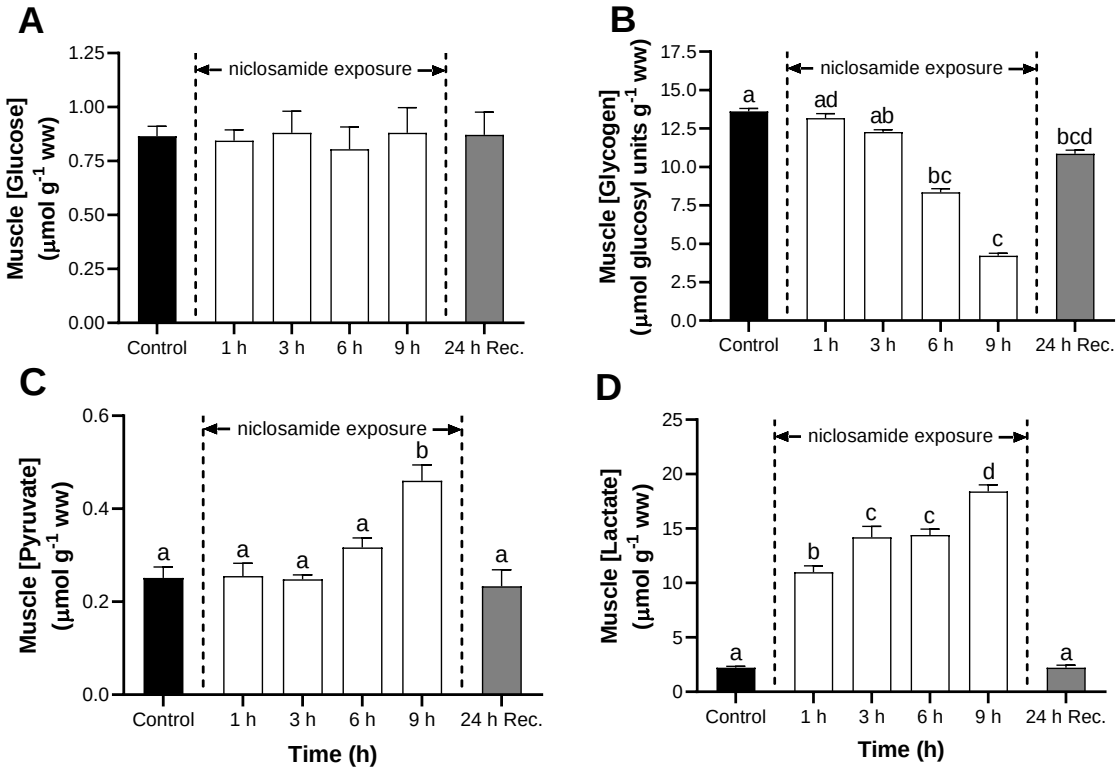


**Figure 4.5 Energy stores and metabolites in muscle of rainbow trout**

**Figure 4.5 Energy stores and metabolites in muscle of rainbow trout.** Changes in muscle concentrations of (A) glucose, (B) glycogen (C) pyruvate and (D) lactate in the muscle of rainbow trout (*Oncorhynchus mykiss*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 12; grey bars) at a nominal concentration of 0.15 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 12), 3 h (n = 11), 6 h (n = 11) and 9 h (n = 9), or held under control conditions (no niclosamide; n = 23; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

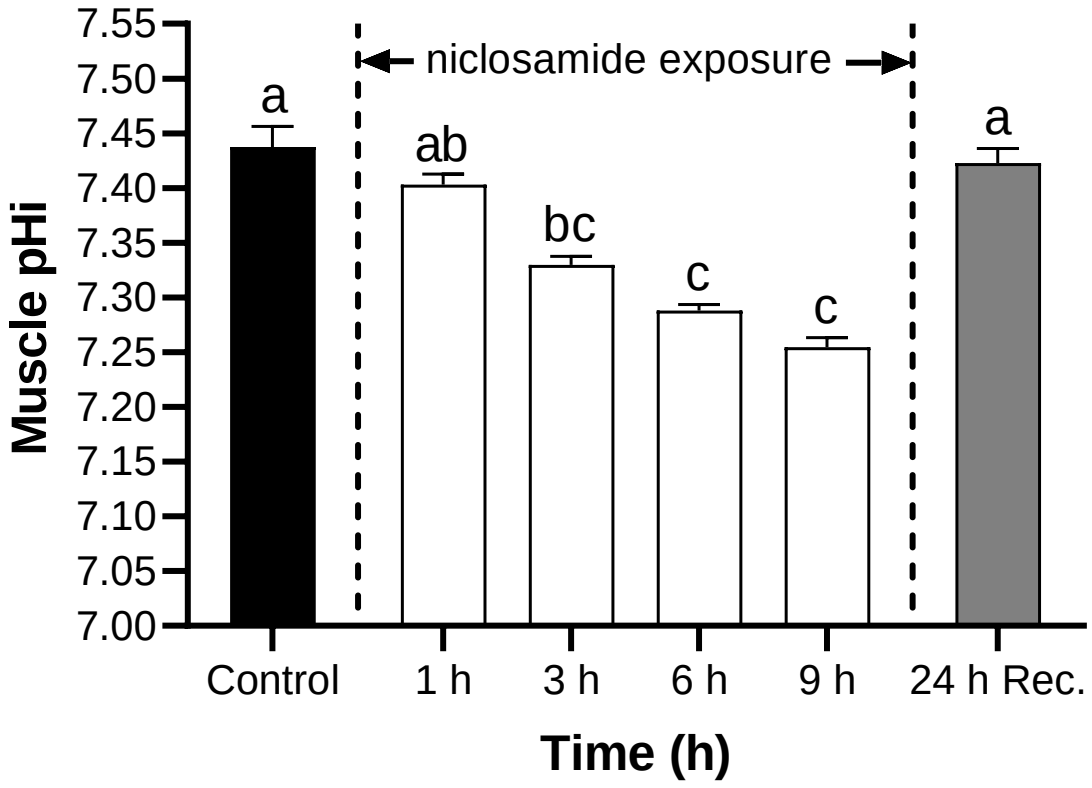


**Figure 4.6 Intracellular pH in muscle of rainbow trout**

**Figure 4.6 Intracellular pH in muscle of rainbow trout.** Changes in pHi in the muscle of rainbow trout (*Oncorhynchus mykiss*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 12; grey bars) at a nominal concentration of 0.15 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 12), 3 h (n = 11), 6 h (n = 11) and 9 h (n = 9), or held under control conditions (no niclosamide; n = 23; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

**SUPPLIMENTAL MATERIAL**

**Table 4.3S Summary of Controls.**

Comparison of controls (no niclosamide) measuring concentrations of energy stores and metabolites in the brain, liver and muscle of rainbow trout (*Oncorhynchus mykiss*) sampled at 0 h (start), sham (50% methanol: no niclosamide) and 9 h (finish). Muscle intracellular pH (pHi) control measurements were similarly compared as were blood plasma ions. Data are expressed as mean ± S.E.M. No significant differences were observed.

| Brain Assays | Control Start [µmol $g^{-1}$ ww ± SEM (n)] | Control Sham [µmol $g^{-1}$ ww ± SEM (n)] | Control Finish [µmol $g^{-1}$ ww ± SEM (n)] |
|---|---|---|---|
| ATP | 0.6 ± 0.1 (8) | 0.6± 0.0 (6) | 0.6 ± 0.0 (8) |
| PCr | 4.2 ± 0.6 (8) | 4.2 ±1.4 (6) | 4.0 ± 0.7 (8) |
| Glucose | 0.9 ± 0.1 (8) | 0.9 ± 0.0 (6) | 0.9 ± 0.0 (8) |
| Glycogen | 5.5 ± 0.3 (8) | 5.6 ± 0.2 (6) | 5.7 ± 0.4 (8) |
| Lactate | 8.2 ±0.8 (8) | 7.5 ± 0.3 (6) | 8.6 ± 0.6 (8) |

| Liver Assays | Control Start [µmol $g^{-1}$ ww ± SEM (n)] | Control Sham [µmol $g^{-1}$ ww ± SEM (n)] | Control Finish [µmol $g^{-1}$ ww ± SEM (n)] |
|---|---|---|---|
| Glucose | 16.8 ± 2.3 (8) | 15.2 ± 1.6 (7) | 17.6 ± 2.5 (8) |
| Glycogen | 43.2 ± 4.3 (8) | 42.4 ± 4.6 (7) | 43.5 ± 3.0 (8) |

| Muscle Assays | Control Start [µmol $g^{-1}$ ww ± SEM (n)] | Control Sham [µmol $g^{-1}$ ww ± SEM (n)] | Control Finish [µmol $g^{-1}$ ww ± SEM (n)] |
|---|---|---|---|
| ATP | 7.2 ± 0.6 (8) | 7.3 ± 0.6 (7) | 7.3 ± 0.4 (8) |
| PCr | 27.8 ± 0.8 (8) | 27.5 ± 1.4 (7) | 26.8 ± 0.4 (8) |
| ADP | 1.0 ± 0.1 (8) | 1.0 ± 0.1 (7) | 0.9 ± 0.1 (8) |

| | | | |
|---|---|---|---|
| Creatine | 21.0 ± 1.8 (8) | 20.6 ± 3.2 (7) | 21.4 ± 2.0 (8) |
| Glucose | 0.9 ± 0.0 (8) | 0.8 ± 0.0 (7) | 0.9 ± 0.0 (8) |
| Glycogen | 13.3 ± 0.6 (8) | 13.8 ± 1 (7) | 13.8 ± 0.1 (8) |
| Pyruvate | 0.2 ± 0.0 (8) | 0.2 ± 0.0 (7) | 0.3 ± 0.1 (8) |
| Lactate | 2.1 ± 0.2 (8) | 2.3 ± 0.4 | 2.3 ± 0.2 (8) |

| Muscle Assays | Control Start [pH ± SEM (n)] | Control Sham [pH ± SEM (n)] | Control Finish [pH ± SEM (n)] |
|---|---|---|---|
| pHi | 7.48 ± 0.01 (8) | 7.47 ± 0.06 (7) | 7.40 ± 0.02 (8) |

| Plasma Ions | Control Start [mmol $L^{-1}$ ± SEM (n)] | Control Sham [mmol $L^{-1}$ ± SEM (n)] | Control Finish [mmol $L^{-1}$ ± SEM (n)] |
|---|---|---|---|
| $Na^+$ | 141.8 ± 3.7 (8) | 139.1 ± 3.6 (7) | 150.4 ± 3.5 (7) |
| $Cl^-$ | 115.4 ± 2.4 (8) | 116.6 ± 2.6 (7) | 118.7 ± 3.8 (7) |

# CHAPTER 5:

# Ecologically Relevant Concentration of The Lampricide 3-trifluoromethyl-4-nitrophenol (TFM) Reduces Energy Stores and Intracellular Muscle pH in Juvenile Lake Sturgeon (*Acipenser fulvescens*)

## 1. INTRODUCTION

Since the early 1960s, the lampricide, 3-trifluoromethyl-4-nitrophenol (TFM) has been used in an integrated pest management program to control populations of invasive sea lamprey (*Petromyzon marinus*) in the Great Lakes. Based on population surveys, TFM is applied to nursery streams infested with larval sea lamprey every 3-4 years, eradicating several generations at once (Smith and Tibbles, 1980; Bills *et al.*, 2003; Boogaard *et al.*, 2003; McDonald and Kolar, 2007b). TFM has proven to be a very effective tool in controlling sea lamprey populations, mainly because of the lampricide's specificity to ammocoetes (Applegate and King, 1962; Lech and Costrini, 1972; Lech and Statham, 1975) most cases, non-target fishes can tolerate 3-5 times higher TFM concentrations than what is required to kill sea lamprey (Applegate and King, 1962; Bills *et al.*, 2003; Boogaard *et al.*, 2003; Wilkie *et al*., 2019). The higher tolerance to TFM in non-target fishes is attributed to a greater capacity to detoxify TFM to TFM-glucuronide, using the enzyme uridine diphosphate glucuronyltransferase (UDPGT; Olson and Marking, 1973; Lech, 1974; Lech and Statham, 1975; Kane *et al.*, 1993, 1994) , rendering the lampricide more water soluble and easier to excrete via urine or feces (Clarke *et al.*, 1991).

The amounts of TFM added to the water are based on the 9-h $LC_{99.9}$ of the lampricide to larval sea lampreys, also referred to as the minimum lethal concentration (MLC). Typically, TFM is applied at 1.2-1.5 times the MLC to ensure complete eradication of larval sea lamprey in a stream (Bills *et al.*, 2003; Christie *et al.*, 2003; O'Connor *et al.*, 2017). Because, the toxicity of TFM is inversely proportional to water pH and alkalinity, these chemical parameters are closely monitored and the application rates of TFM adjusted accordingly to ensure that concentrations are kept at levels sufficient to kill the lamprey, but to also ensure that excess TFM is not applied (Barber and Steeves, 2019). Excess TFM use is not only wasteful, it can also pose a risk to non-

target organisms. Despite every effort to monitor TFM concentration and water pH during treatments, occasional non-target fish mortalities do occur, unfortunately (McDonald and Kolar, 2007). It is therefore important to define physiological effects of TFM toxicity in non-target fishes to better predict how vulnerable different fish populations to the lampricide.

Various studied focusing on the toxicity of TFM in non-target fishes, have demonstrated that there is broad inter-species variability in the sensitivity to this lampricide (Applegate and King, 1962; Marking and Olson, 1975; Bills and Leif, 1976; Johnson *et al.*, 1999), with juvenile lake sturgeon (*Acipenser fulvescens*) one of the most sensitive non-target species (Boogaard *et al.*, 2003). Their delayed sexual maturity (~25 years for females; ~15 years for males) and a long spawning cycle (4-5 years for females; 1-2 years for males; Scheidegger, 2012), along with vulnerability to sea lamprey parasitism, particularly in the early juvenile stages (Patrick *et al.*, 2009), and its threatened status in the Great Lakes (OMNRF, 2019), has raised concern about this species vulnerability to lampricides. There is limited knowledge on the effects of TFM in lake sturgeon (Boogaard *et al.*, 2003; Middaugh *et al.*, 2014; Sakamoto *et al.*, 2016; Bussy *et al.*, 2017a, 2017b; O'Connor *et al.*, 2017; Hepditch *et al.*, 2019). However, it is clear that lake sturgeon, especially in the first year of life, are much more susceptible to TFM, than other non-target fishes (Boogaard et al. 2003; McDonald and Kolar 2007; O'Connor et al. 2017). There are no studies which delve into the mechanism of TFM toxicity in these fish, but such knowledge could be very helpful in developing strategies of lampricide application to help with ongoing preservation efforts of lake sturgeon in the Great Lakes.

The goal of this study was to learn more about the physiological effects and mechanism of TFM toxicity in lake sturgeon. I predicted that physiological responses to TFM in lake sturgeon would lead to a multi-system depletion of energy stores, similar to those reported for

sea lamprey (Wilkie *et al.*, 2007; Birceanu *et al.*, 2009; Clifford *et al.*, 2012)and for rainbow trout (Birceanu *et al.*, 2011, 2014). To test my hypothesis, juvenile lake sturgeon were exposed to previously published sea lamprey minimum lethal concentrations (MLC) of TFM (Bills *et al.*, 2003) for up to 9 h and I measured glycogen, lactate, ATP and phosphocreatine in the carcass, brain and liver. In addition, I hypothesized that as lake sturgeon increasingly relied on glycolysis to compensate for shortfalls in ATP supply, that it would result in metabolic acidosis in the muscle. To determine the presence of an acidosis I measured intracellular pH (pHi) in the carcass at the various time points of TFM exposure.

## 2. RESULTS

### 2.1 Experimental water parameters

The water chemistry (pH, DO, alkalinity) and temperature measurements for acclimation and experimental tanks ([TFM] exposed fish) are summarized in supplemental data (Table 5.1S). Briefly, alkalinity averaged 148 ± 2 mg $L^{-1}$ as $CaCO_3$ in the acclimation tank, which was virtually identical to that of the experimental tanks, which averaged 150 ± 1 mg $L^{-1}$ as $CaCO_3$. Water pH averaged 8.43 ± 0.02 and 8.36 ± 0.01 in the acclimation and experimental tanks, respectively. Temperature was also the same, averaging 13.6-13.9 ± 0.1°C, and dissolved $O_2$ was always greater than 90 % saturation. Measured water TFM concentrations averaged 4.6 ± 0.0 mg $L^{-1}$, compared to nominal concentration of 4.7 mg $L^{-1}$. No significant differences in measured TFM concentrations were observed between tanks, and no TFM degradation occurred over the duration of the experiments (Table 5.1S). For the purpose of clarity, discussion will continue to refer to the nominal concentration of TFM.

2.2 Positive controls

Rainbow trout were held under control conditions (no TFM) for a period of 9 h under identical water conditions (alkalinity = 150 mg $L^{-1}$ $CaCO_3$) used for lake sturgeon experiments. Positive control analyses on rainbow trout muscle were conducted in conjunction with lake sturgeon analyses for the same assays (Table 5.2S).

2.3 Effects of TFM on energy stores and metabolites in lake sturgeon brain

There were no significant differences in ATP, PCr, glycogen or lactate measurements observed in the brain of control lake sturgeon, not exposed to TFM, between the start (0 h) and end (9 h) of the experiment. Accordingly, all control measurements were pooled and depicted as a single control measurement.

The concentration of ATP in the brain of control lake sturgeon, not exposed to TFM, averaged 0.29 ± 0.03 µmol $g^{-1}$ ww, and phosphocreatine averaged 0.48 ± 0.04 µmol $g^{-1}$ ww (Figure 5.1). Following exposure to TFM, the concentration of ATP in the brain underwent an immediate and sustained reduction of approximately 60 % compared to the control measurements (Figure 5.1A). Similarly, brain PCr was reduced by approximately 50% at 6 and 9 h, compared to controls (Figure 5.1B).

Brain glucose and glycogen concentrations were relatively low in lake sturgeon, averaging 0.81 ± 0.13 µmol $g^{-1}$ ww and 0.13 µmol $g^{-1}$ ww, respectively (Figure 5.2). Brain glucose concentrations were unchanged in the presence of TFM (Figure 5.2A) but glycogen was significantly depleted by 50% at 6 and 9 h relative to the control fish (Figure 5.2B). Lactate levels in TFM exposed sturgeon brain significantly increased from 4.6 ± 0.5 µmol $g^{-1}$ ww in the

controls to 7.9 ± 0.4 μmol $g^{-1}$ ww after 6 h, peaking at 9.3 ± 0.5 μmol $g^{-1}$ ww after 9 h, an increase of approximately 45-50% (Figure 5.2C).

2.4 Effects of TFM on energy stores in lake sturgeon liver

As for liver, control data collected at the start of (0 h) and the end (9 h), were not significantly different (data not shown). Thus, in the absence of any temporal variation in liver glucose or glycogen stores, all control measurements were pooled. Liver glucose and glycogen concentrations were markedly depleted in lake sturgeon exposed to TFM. In control animals, liver glucose concentrations averaged 2.6 ± 0.5 μmol $g^{-1}$ ww, while liver glycogen concentrations were much higher averaging 58.3 ± 5.4 μmol glucosyl units $g^{-1}$ ww (Figure 5.3). In lake sturgeon exposed to TFM, liver glucose concentrations were approximately 50 % lower than controls at 3 h and 6 h, but not quite significantly different at 9 h ($P = 0.11$; Figure 5.3A). Glycogen concentrations underwent a significant and rapid, sustained depletion of 50% in the same TFM exposed fish, which was significant at all time points (Figure 5.3B).

2.5 Effects of TFM on energy stores, metabolites and pHi in lake sturgeon carcass

The control data collected at the start (0 h) and end of the experiment (9 h) were also pooled in carcass, as described above. The carcass represented the whole body of the sturgeon, minus the brain, liver and other viscera. In the controls, sturgeon carcass ATP and PCr concentrations averaged 1.5 ± 0.1 μmol $g^{-1}$ ww and 5.5 ± 0.3, respectively (Figure 5.4). However, in the group exposed to TFM, there was a marked reduction in ATP concentrations of approximately 56% by 9 h of exposure compared to the control fish (Figure 5.4A). Surprisingly,

there were no notable changes in PCr (Figure 5.4B), ADP (Figure 5.4C) and creatine (Figure 5.4D) in the animals exposed to TFM.

While glucose concentrations in the carcass of TFM-exposed lake sturgeon did not significantly differ from controls, glycogen concentrations were significantly affected by TFM exposure declining by approximately 50-80 % compared to the control values of 1.20 ± 0.08 µmol glucosyl units $g^{-1}$ ww (Figure 5.5B). Concomitantly, there was a dramatic 5-fold and 3.5-fold increase in carcass pyruvate (Figure 5.5C) and lactate levels (Figure 5.5D) at 9 h of TFM exposure, respectively, over controls, which was measured at 0.25 ± 0.04 µmol $g^{-1}$ ww and 1.38 ± 0.22 µmol $g^{-1}$ ww, respectively (Figures 5.5C and 5.5D).

The intracellular pH (pHi) of carcass averaged 7.16 ± 0.01 in lake sturgeon not exposed to TFM. However, exposure to TFM resulted a mild acidosis, characterized by a significant decrease in pHi to 7.09 ± 0.01 by 9 h of TFM exposure (Figure 5.6).

## 3. DISCUSSION

### 3.1 TFM interferes with ATP production by lake sturgeon

Exposure of juvenile lake sturgeon to TFM, at concentrations that could be encountered during a typical field application of the lampricide, resulted in significant decreases in brain ATP, PCr and glycogen, with corresponding increases in lactate. These observations are consistent with the known mode of action of TFM, which targets the mitochondria resulting in a decrease in oxidative phosphorylation and subsequent reductions in ATP production (Birceanu et al. 2011), and correspondingly greater reliance on anaerobic metabolic processes such as the dephosphorylation of phosphocreatine to buffer ATP supplies, and greater reliance on glycolysis (Wilkie et al. 2007; Clifford et al. 2012; Henry et al. 2014). As noted in previous studies both the

supply of PCr and glycogen, the primary substrate of glycolysis, are finite, and when these reserves are lowered, and ATP supply can no longer match ATP demands, the fish are unable to maintain homeostasis, leading to death (Wilkie et al. 2019). Indeed, Wilkie et al. (2007) originally suggested that death likely resulted when the brain was deprived of sufficient glucose to meet the demands of the nervous system.

The brain is one of the most metabolically active organs in vertebrates (Sokoloff, 1989; Magistretti, 1999), typically comprising 0.1-1% of the body weight in non-mammalian vertebrates, but accounting for 2.7-3.4% of total body energy expenditure in ectotherms (Hylland *et al.*, 1997; Purdon and Rapoport, 1998). This is because neural processes are metabolically expensive (Laughlin *et al.*, 1998), with approximately 50-60% of the brain's ATP being dedicated to electrical activity (Hylland *et al.*, 1997; Purdon and Rapoport, 1998). Thus, the brain is highly sensitive to physiological disturbances or compounds that interfere with oxidative ATP production, including TFM.

The supply of ATP to the brain is sustained by the oxidation of glucose, supplied via the plasma from the catabolism of endogenous glycogen stores in the liver, as well as in the brain itself (Soengas and Aldegunde, 2002; Polakof *et al.*, 2007, 2012). In fact, the brain consumes more glucose per unit mass than other organ in rainbow trout (Washburn *et al.*, 1992). However, glycogen stores are relatively low in the brain of the lake sturgeon, especially in relation to larval sea lamprey in which brain glycogen can exceed 100 μmol glucosyl units $g^{-1}$ ww (Rovainen, 1970; Foster *et al.*, 1993; Clifford *et al.*, 2012) and in fishes that are hypoxia or anoxia tolerant such as the crucian carp (*Carassius carassius*), in which concentrations of glycogen have been reported to be as high as 200 μmol glucosyl units $g^{-1}$ ww (Vornanen and Paajanen, 2006). High brain glycogen in these fishes likely serves as an important anaerobic energy reserve when $O_2$

supply to the brain is limited, allowing the fish to rely on glycolysis to sustain ATP production when supply is limited due to impaired or absent aerobic metabolism (Rovainen, 1970; Vornanen and Paajanen, 2006). This may explain why the goldfish *(Carassius auratus*), an anoxia-tolerant cousin of crucian carp, is highly tolerant to the other, more potent lampricide, niclosamide (Wilkie *et al.*, 2019). Other less hypoxia-tolerant fishes including the lake sturgeon primarily rely on liver glycogen reserves to meet the glucose demands of the nervous system, with rates of glycogenolysis increasing in response to glucagon release as glucose supply diminishes (Polakof *et al.*, 2012).

An inability to oxidize glucose due to a lack of oxygen (ischemia, environmental anoxia or hypoxia) or compounds that directly interfere with mitochondrial ATP production such as TFM (Niblett and Ballantyne, 1976; Birceanu *et al.*, 2011) are clearly detrimental to brain function (Soengas and Aldegunde, 2002; Polakof *et al.*, 2012). Another complication is that anaerobic glycolysis also results in the generation of lactate and metabolic acid ($H^+$), which can further compromise neural function. In lake sturgeon, exposure to TFM impaired ATP production in the brain and liver, but also the carcass of the animals. It was not possible to collect sufficient amounts of tissue to measure brain intracellular pH, but measurements in the muscle demonstrated that there was a significant metabolic acidosis. The evidence suggests that this was due to increased rates of glycolysis because in addition to reductions in glycogen, there were significant increases in lactate as well as pyruvate, the end-product of glycolysis, seen in the carcass of the lake sturgeon. Whether or not similar acid-base disturbances extend to the neural tissue, which would compound any reductions in anaerobic energy reserves, remains an open question, but should be investigated in future studies.

Unlike in larval sea lamprey, in which it was reported that TFM caused reductions of brain glycogen of more than 50 µmol/g ww (Clifford *et al.*, 2012; Henry *et al.*, 2015), the absolute reductions in lake sturgeon were less than 1/10 of this value because of its very low basal concentrations of glycogen in the brain. Some fishes, including lampreys, which may experience frequent periods of hypoglycemia due to factors such as non-trophic life stages and/or limited food supply (e.g. during winter), tend to depend on endogenous brain glycogen as a proximate energy source, rather than exogenous carbohydrates; however, these fishes generally have very high levels of brain glycogen (Plisetskaya, 1985; Schmidt and Wegener, 1988; Foster et al., 1993; Henry et al., 2015). The relatively low concentrations of brain glycogen observed in the present study suggests that it is a relatively unimportant source for maintaining glucose levels in the brain of lake sturgeon. Rather, the liver, where glycogen concentrations were approximately 60-fold higher than in the brain, is likely the major glucose pool for the central nervous system, as it is in many other aquatic and terrestrial vertebrates (Soengas and Aldegunde, 2002). In such cases glucose levels in the brain are sustained by glycogenolysis in the liver (Soengas and Aldegunde, 2002; Polakof *et al.*, 2012). The fact that lactate concentrations in the brain were 10-fold higher strongly suggests that there was increased anaerobic metabolism of circulatory glucose, rather than brain glycogen, following TFM exposure.

As previously demonstrated, oxidative ATP production in the mitochondria is impeded by TFM, triggering rapid depletion of energy stores, not only in the brain, but in other vital, metabolically active tissues such as the liver (Birceanu *et al.*, 2009, 2014). Indeed, glycogen levels in the liver of lake sturgeon were severely depleted and remained depleted for the duration of the TFM exposure period. Thus, severe glucose and glycogen depletion observed in the liver

of TFM-exposed lake sturgeon is reflective of this organ mobilizing these energy stores in response to lower brain glucose levels. In all likelihood, death would likely follow once the supply of liver glycogen was exhausted, when brain glucose supply could no longer be sustained.

### 3.2 TFM may impact ecological and physiological performance in lake sturgeon

It is evident that TFM induces metabolic disturbances in juvenile lake sturgeon which result in depletion of neural and hepatic energy stores (present study), presumably via uncoupled mitochondrial oxidative phosphorylation, as observed in sea lamprey and TFM-resistant rainbow trout (Birceanu *et al.*, 2011). However, in conjunction with impairement of neural and hepatic metabolic processes, TFM could have other serious effects on physiology that impact lake sturgeon survival. Sakamoto *et al.* (2016) explored the effects of TFM on olfaction and behaviour in young-of-the-year (YOY) lake sturgeon. Using electro-olfactography (EOG), they showed that TFM interfered with olfactory sensory neurons (OSNs) which are attuned to food cues, and the ciliated sensory neurons which are specific to migration and alarm cues. In the presence of ecologically relevant concentrations of TFM, lake sturgeon EOG responses to odour cues, alarm cues and food cues were severely diminished by 52%, 64% and 80%, respectively, relative to controls (Sakamoto *et al.*, 2016). When given a choice between food cues and hatchery water, control fish spent 66% of their time in the food cue end of the aquarium while TFM exposed fish showed no preference, and TFM exposed fish consumed 36% less food than control fish (Sakamoto *et al.*, 2016). Additionally, lake sturgeon were able to detect the lampricide in water regardless of lighting (day vs. night), as demonstrated by 120% greater amplitude of EOGs in TFM exposed fish compared to water blank (Sakamoto *et al.*, 2016). This group also performed activity analyses of lake sturgeon, reporting that, compared to control fish,

TFM expose fish were 79% more active but had 19% slower acceleration and 16% slower velocity, (Sakamoto *et al.*, 2016).

By integrating the results from the present study with those offered by Sakamoto *et al.* (2016), it might be speculated that the diminished energy stores (ATP, PCr and glycogen) observed in the brain of TFM exposed lake sturgeon, could have contributed to the processing impairment of electric signals from the OSNs, which could be manifest as significantly altered EOGs. Indeed, the mechanisms of olfactory impairment could be at the level of the olfactory neurons themselves, where ATP is needed to repolarize neurons following action potentials. Presumably, TFMs inhibition of oxidative phosphorylation would extend to olfactory neurons which are also enriched with mitochondria (Zielinski *et al.*, 1996; Daghfous *et al.*, 2012; Fluegge *et al.*, 2012). It seems probable that the decreases of energy stores observed in the brain of lake sturgeon would extend to the rest of the neural system, thus, directly affecting olfaction at the sensory neurons (Mirza *et al.*, 2009; Green *et al.*, 2010; Tierney *et al.*, 2010; Dew *et al.*, 2014). The increased activity observed in TFM exposed lake sturgeon by Sakamoto *et al.* (2016), would likely increase the fish's vulnerability to predation while diminishing muscle energy stores (carcass ATP and glycogen; present study). Increased vulnerability to predation would further be exacerbated by diminished swimming performance (velocity, acceleration; Sakamoto *et al.*, 2016). Interestingly, lake sturgeon carcass PCr concentrations were maintained at only slightly diminished (non-significant) concentrations while ATP depletion continued throughout TFM exposure. This is unlike the results observed in the brain, where ATP and PCr were significantly reduced with TFM. Since PCr was not severely affected by TFM, it is not surprising that, as components of the creatine kinase equilibrium, ADP and creatine were also unaffected in the carcass. However, the observed TFM-induced decreases in muscle glycogen suggests that

lake sturgeon were heavily relying on this energy store to supplement ATP demands in this tissue, which implies that with continued lampricide exposure they would experience increased predation vulnerability and decreased swimming performance, as mentioned above.

### 3.3 Implications for lake sturgeon population enhancement and sea lamprey control

Parasitism of culturally-significant, commercial and recreational fishes still has the potential to cause serious harm to the Great Lakes fishery, which is annually worth more than 7 billion dollars (Krantzberg and De Boer, 2008; GLFC, 2011). The use of lampricides such as TFM remains an integral component of the sea lamprey control program (Siefkes, 2017; Wilkie *et al.*, 2019). However, the risks of adverse effects on non-target fishes also need to be considered, especially those that are species at risk and/or of cultural importance. Efforts have been underway in the Great Lakes to restore lake sturgeon populations, particularly by the U.S. Fish and Wildlife Service (LRBOI-USFWS, 2017). However, there is concern by government agencies and First Nations that TFM could potentially undermine these efforts due to lampricide applications, particularly TFM which has been identified as a source of age-0 lake sturgeon mortality (Johnson *et al.*, 1999; Boogaard *et al.*, 2003; O'Connor *et al.*, 2017). In fact, in some larval sea lamprey-infested streams containing sensitive populations of lake sturgeon, fisheries personnel have attempted to remove as many of the age-0 lake sturgeon prior to the application of TFM, returning them to the stream once the lampricide has dissipated and is no longer at toxic concentrations (LRBOI-USFWS, 2017). Unfortunately, the effectiveness of this measure is unproven, and may prove cost restrictive. Thus, other alternatives to protect lake sturgeon from TFM toxicity need to be considered. Reducing the concentrations of TFM applied to streams

containing YOY lake sturgeon has also been tried, preserving lake sturgeon but again leading to increased numbers of parasitic lamprey and damage to fisheries (Dobiesz *et al.*, 2018).

Not treating streams containing lake sturgeon is not likely a feasible approach because it would lead to increased numbers of parasitic sea lamprey in receiving waters (lakes) of affected rivers. In fact, lake sturgeon themselves could be more vulnerable to sea lamprey parasitism if TFM treatments ceased on affected rivers as recently demonstrated using a generalized Great Lakes sturgeon population model which related changes in lake sturgeon populations to sea lamprey-associated mortality (Dobiesz *et al.*, 2018). This model assumes that while age-0 lake sturgeon are found to be the most susceptible to TFM toxicity (Johnson *et al.*, 1999; Boogaard *et al.*, 2003; O'Connor *et al.*, 2017), a second age-related factor exists, involving the sea lamprey control program: sea lamprey predation on sub-adult (ages 7-24) lake sturgeon (Dobiesz *et al.*, 2018). Dobiesz *et al.* (2018) determined that there would be a 5.7% population increase in adult lake sturgeon in the absence of TFM use, if sea lamprey predation were not changed. However, the study convincingly pointed out that eliminating TFM use would lead to an increase in sea lamprey populations ultimately leading to as much as 37% reduction in adult lake sturgeon. Thus, it was concluded that the benefits of protecting age-0 lake sturgeon by eliminating TFM would be greatly outweighed by the increased mortality suffered by adult lake sturgeon due to sea lamprey parasitism (Dobiesz *et al.*, 2018). Thus, eliminating TFM use in lake sturgeon nursery streams is not an option. Rather, it would be more beneficial to develop sea lamprey control approaches that reduce or eliminate TFM toxicity to age-0 lake sturgeon, while maintaining toxicity to larval sea lamprey, to promote the restoration of the lake sturgeon populations (Dobiesz *et al.*, 2018).

Based on the present study, lake sturgeon experience metabolic perturbations to TFM that include depleted liver glycogen stores, likely leading to impaired glucose delivery to the brain. Thus, measures that reduce the magnitude of such disturbances are needed to preserve lake sturgeon populations, while ensuring that larval lamprey eradication is not compromised. Recently, Hepditch et al. (2019) demonstrated that the greater susceptibility of lake sturgeon to TFM toxicity in their early juvenile stages (< 100 mm in length) resulted from substantially higher mass-specific rates of TFM uptake compared to larger 1+ animals (Hepditch *et al.*, 2019). Thus, consideration might be given to delaying TFM treatment until the autumn when the fish are likely to reach sizes larger than 100 mm in length, when rates of TFM uptake would be much lower and their capacity to detoxify the lampricide might be expected to be higher. Another approach may be exposing the fish to lower TFM concentrations over longer periods (Long and Low TFM treatment). The lower concentration of TFM in the water would mean that sturgeon would take up an overall lower quantity of the lampricide, allowing them to 'keep up' with detoxification and elimination of the toxin. In this way, the toxicity load to juvenile lake sturgeon is reduced, presumably along with physiological perturbations. At the same time, the longer exposure of sea lamprey to TFM, albeit at a lower concentration, would likely keep residual survival to a minimum due to this animal's limited capacity to detoxify the lampricide. Furthermore, a combination of long and low plus time limitations could be employed. Even if some larger lake sturgeon remained in the stream, this measure would likely reduce the magnitude of the non-lethal non-target effects, such as impaired olfaction and swim performance; however, the reversibility and magnitude of olfaction impairment by TFM requires further research.

Overall, our understanding on the effects of TFM on lake sturgeon has improved over the past few years but more research is still necessary especially as it pertains to recovery rates from TFM exposure, as well as, long term and chronic effects, especially in juveniles. In addition, more studies are necessary to assess the reversibility and magnitude of olfaction impairment and possible impairment of swim performance on lake sturgeon. While the current study shows that TFM at ecologically significant concentrations can have serious consequences in juvenile lake sturgeon, it is likely that these effects would be greatly reduced in larger fish (age 1+ or older).

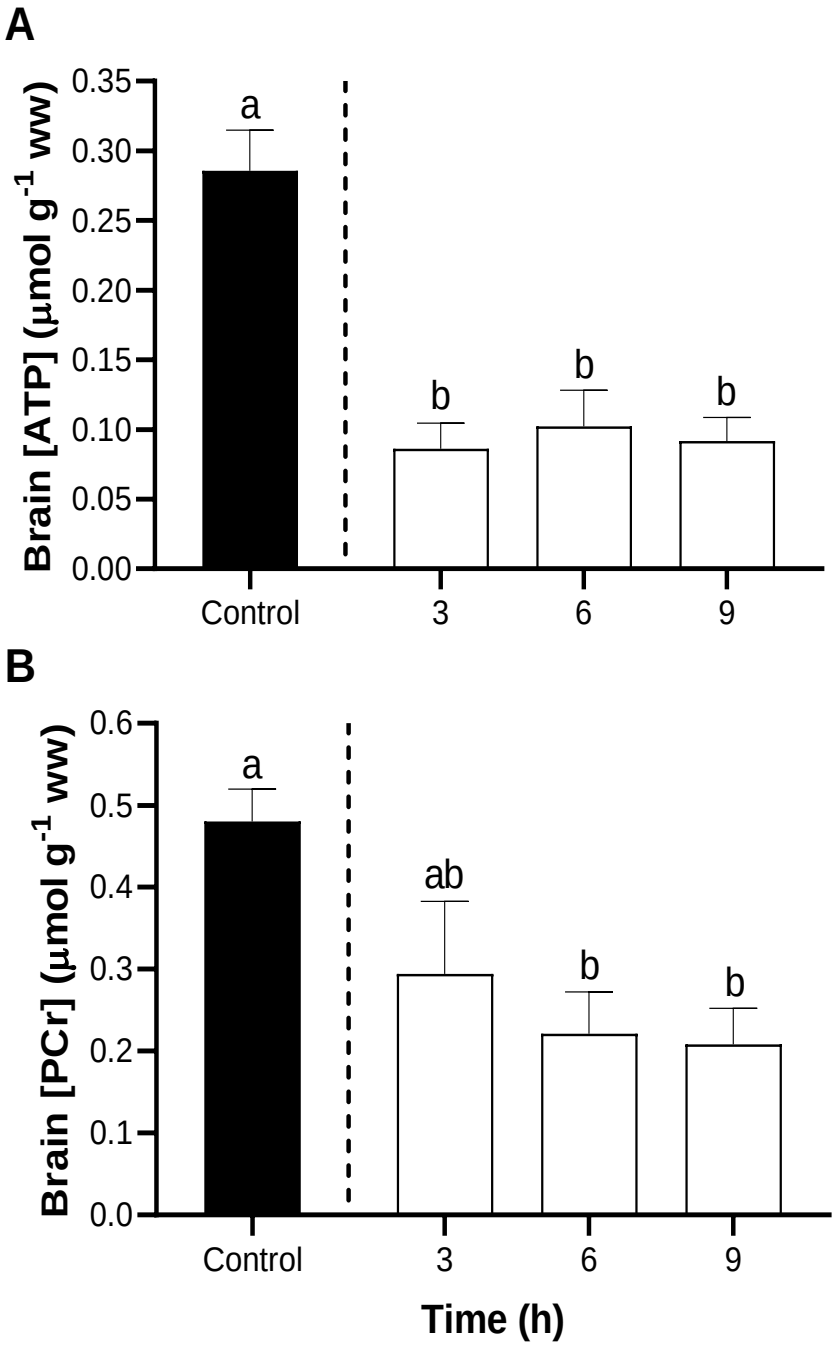


**Figure 5.1 Energy stores in brain of TFM-exposed lake sturgeon**

**Figure 5.1 Energy stores in brain of TFM-exposed lake sturgeon.** Changes in brain concentrations of (A) adenosine triphosphate (ATP) and (B) phosphocreatine (PCr) in control (non-exposed) lake sturgeon, and following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at measured concentrations of 4.6 mg ± 0.1 $L^{-1}$, for 3, 6 and 9 h. Data are expressed as mean ± S.E.M (n = 8). Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

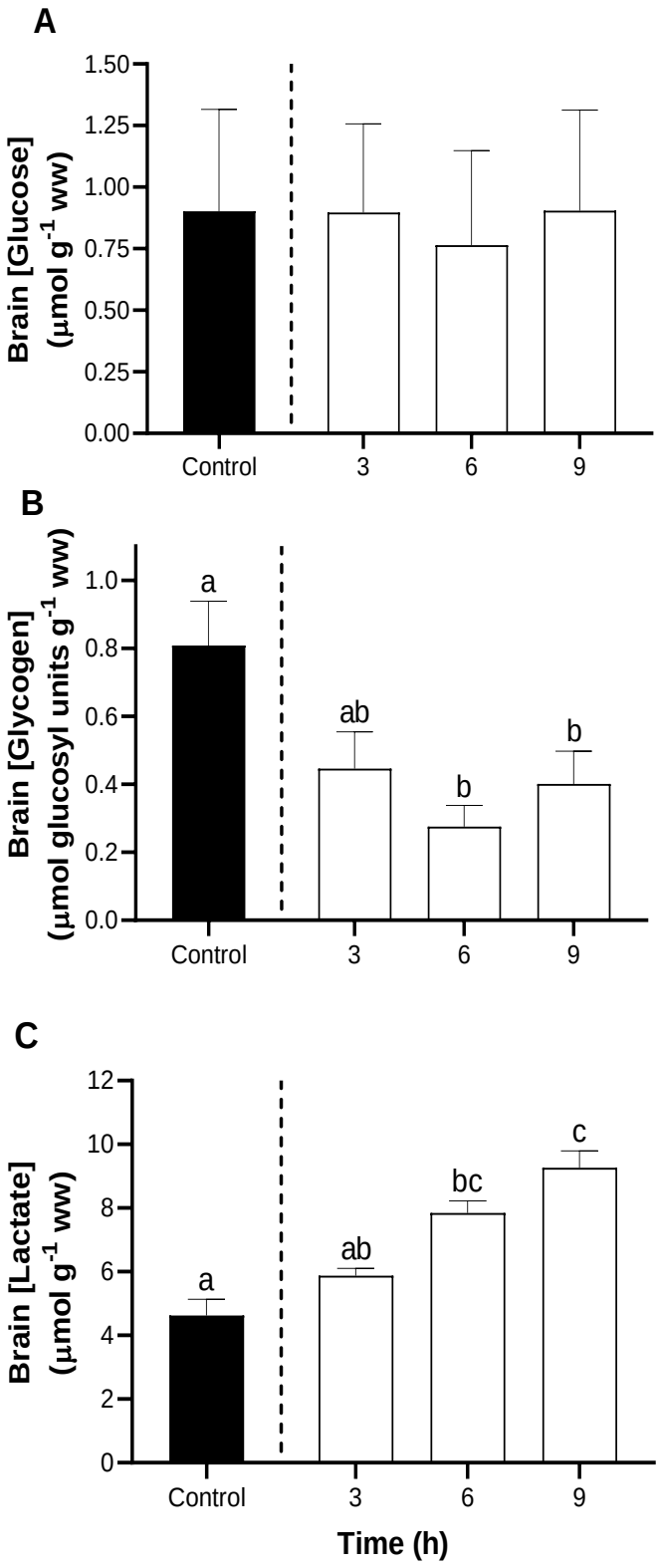


**Figure 5.2 Energy stores and metabolites in brain of TFM-exposed lake sturgeon.**

**Figure 5.2 Energy stores and metabolites in the brain of TFM-exposed of lake sturgeon.** Changes in the concentrations of (A) glucose (B) glycogen and (C) lactate in the brains of control (non-exposed) lake sturgeon, and following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at measured concentrations of 4.6 mg ± 0.1 $L^{-1}$, for 3, 6 and 9 h. Data are expressed as mean ± 1 S.E.M (n = 7-8). Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

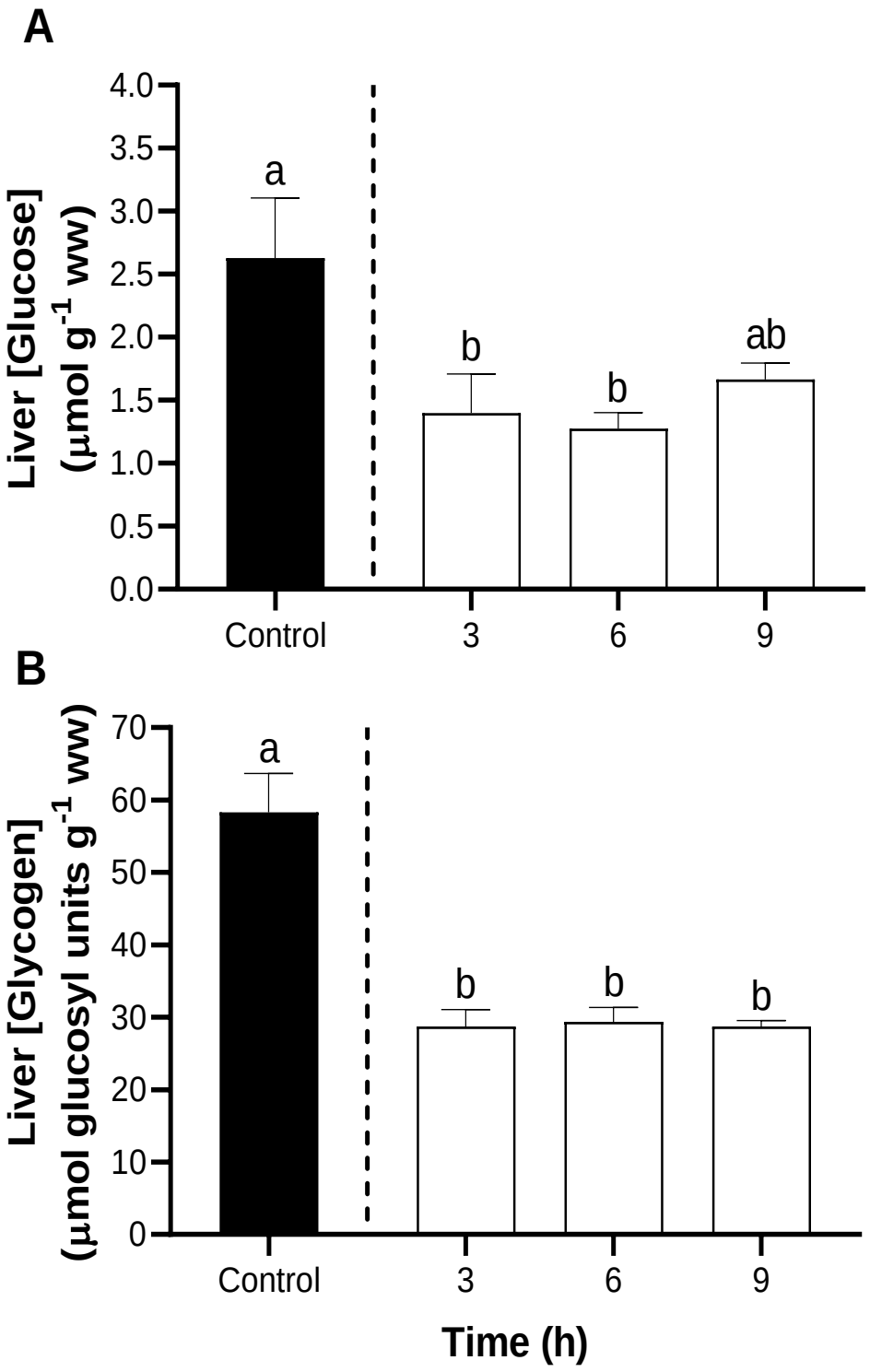


**Figure 5.3 Energy stores in liver of TFM-exposed lake sturgeon**

**Figure 5.3 Energy stores in liver of TFM-exposed lake sturgeon.** Changes in the liver concentrations of (A) glucose and (B) glycogen in control (non-exposed) lake sturgeon, and following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at measured concentrations of 4.6 ± 0.1 mg $L^{-1}$, for 3, 6 and 9 h. Data are expressed as mean ± S.E.M (n = 8). Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

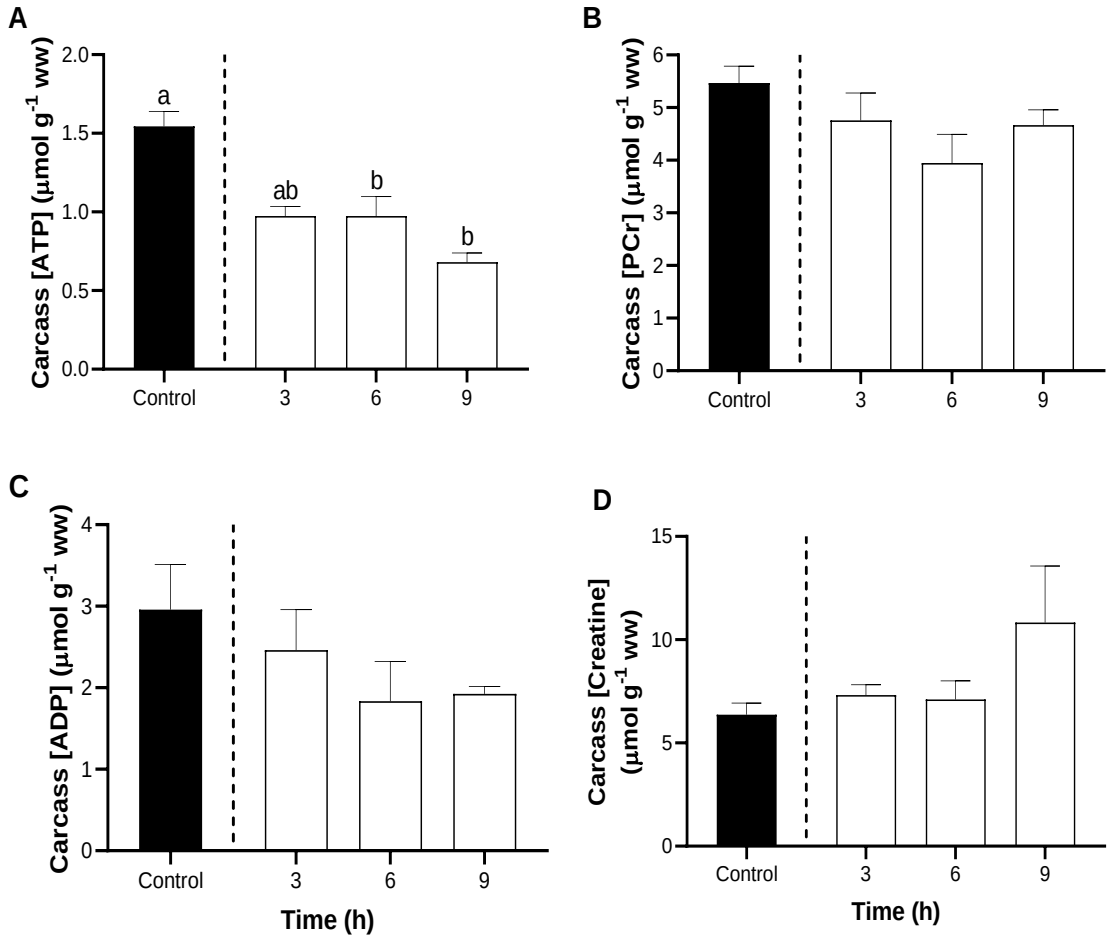


**Figure 5.4 Energy reserves and metabolites in TFM-exposed carcass or lake sturgeon**

**Figure 5.4 Energy reserves and metabolites in TFM-exposed carcass or lake sturgeon.** Changes in carcass concentrations of (A) adenosine triphosphate (ATP), (B) Phosphocreatine (PCr), (C) adenosine diphosphate (ADP) and (D) creatine in control (non-exposed) lake sturgeon, and following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at measured concentrations of $4.6 \pm 0.1$ mg $L^{-1}$, for 3, 6 and 9 h. Data are expressed as mean ± S.E.M (n = 8). Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

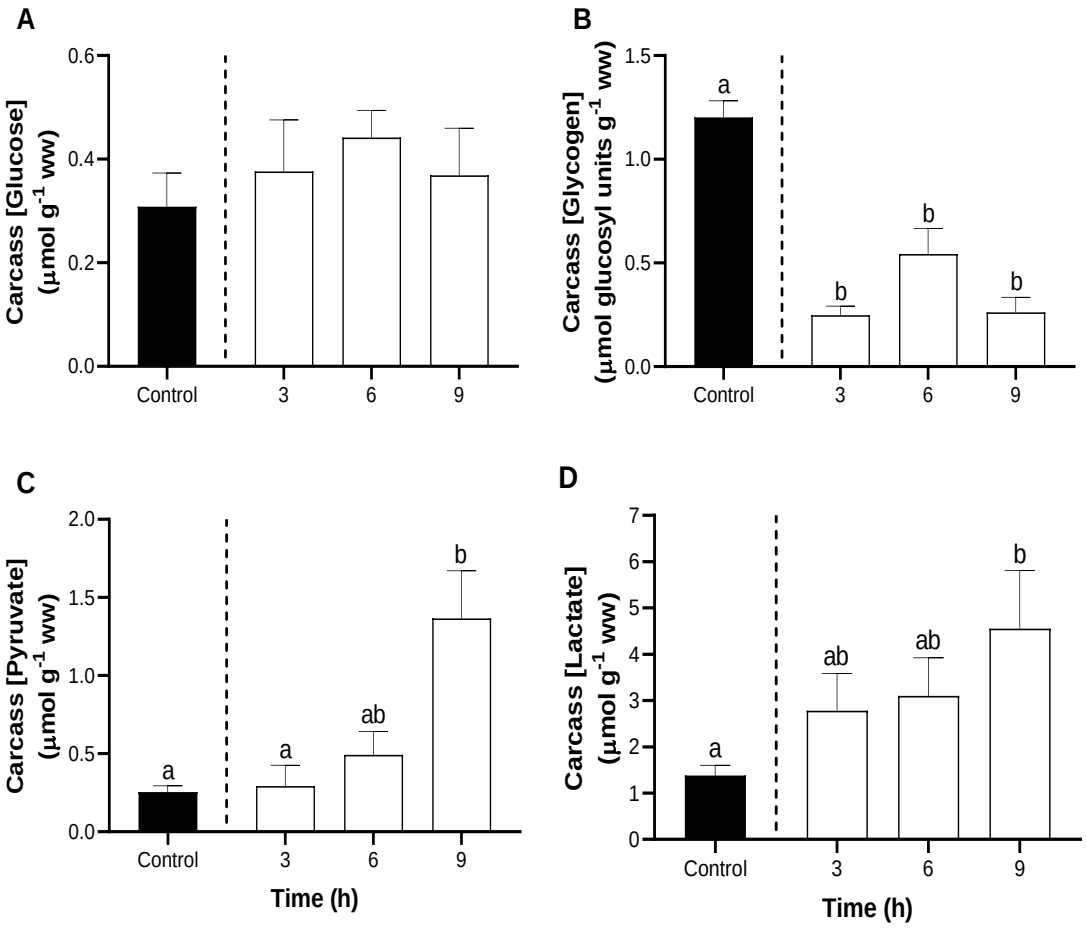


**Figure 5.5 Energy stores and metabolites in TFM-exposed carcass of lake sturgeon**

**Figure 5.5 Energy stores and metabolites in TFM-exposed carcass of lake sturgeon.** Changes in carcass concentrations of (A) glucose, (B) glycogen, (C) pyruvate and (D) lactate in control (non-exposed) lake sturgeon, and following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at measured concentrations of 4.6 ± 0.1 mg $L^{-1}$, for 3, 6 and 9 h. Data are expressed as mean ± S.E.M (n = 8). Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

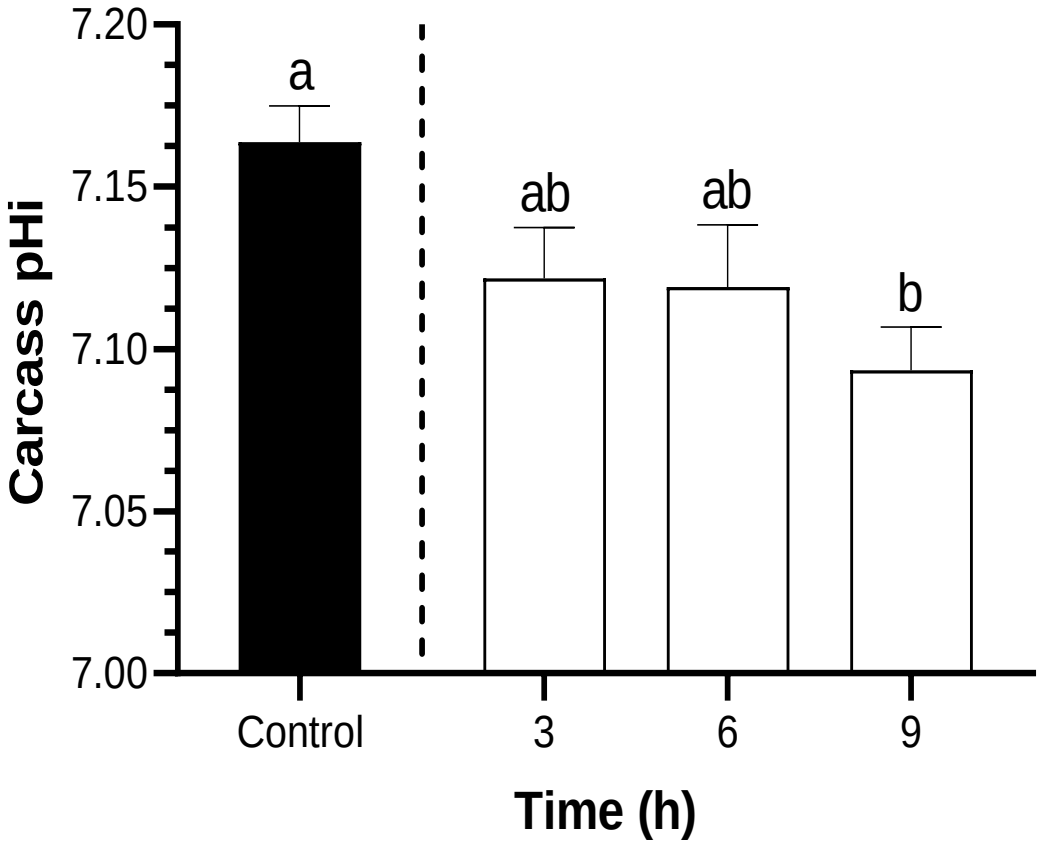


**Figure 5.6 Intracellular pH in carcass of TFM-exposed lake sturgeon**

**Figure 5.6 Intracellular pH in carcass of TFM-exposed lake sturgeon.** Changes in carcass pHi in in control (non-exposed) lake sturgeon and following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at measured concentrations of 4.6 ± 0.1 mg $L^{-1}$, for 3, 6 and 9 h. Data are expressed as mean ± S.E.M (n = 8). Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

**SUPLIMENTAL DATA**

**Table 5.1S List of water chemistry.**

Water parameters (alkalinity, pH, dissolved $O_2$ (DO), temperature). Measurements were conducted daily, during acclimation and water parameters plus [TFM] were measured throughout the experiments (N = 4), expressed as mean ± SEM.

| Aquaria | Alkalinity ($CaCO_3$ $L^{-1}$) | pH | DO (%) | Temperature (ºC) | [TFM] (mg $L^{-1}$) |
|---|---|---|---|---|---|
| **Acclimation** | 148 ± 2 | 8.43 ± 0.02 | 93.7 ± 0.3 | 13.6 ± 0.1 | N/A |
| **Experimental** | 150 ± 1 | 8.36 ± 0.01 | 93.0 ± 0.3 | 13.9 ± 0.1 | 4.6 ± 0.0 |

**Table 5.2S Positive control assay results using rainbow trout muscle.**

Animals were held under control conditions (no TFM) for a period of 9 h under medium alkalinity (150 mg $L^{-1}$ $CaCO_3$) water conditions identical to that used for lake sturgeon experiments. Positive control analyses on rainbow trout muscle were conducted in conjunction with lake sturgeon analyses for the same assays expressed as mean µmol $g^{-1}$ ww (±SEM), except where otherwise indicated.

| Assay | Rainbow Trout µmol $g^{-1}$ ww (±SEM) | (n) | Lake Sturgeon µmol $g^{-1}$ ww (±SEM) | (n) |
|---|---|---|---|---|
| ATP | 6.8 ± 0.6 | 6 | 1.5 ± 0.1 | 8 |
| PCr | 20.4 ± 1.3 | 6 | 5.5 ± 0.3 | 8 |
| ADP | 2.7 ± 0.1 | 4 | 3.0 ± 0.6 | 8 |
| Free Creatine | 23.5 ± 5.6 | 4 | 6.4 ± 0.6 | 8 |
| Pyruvate | 0.4 ± 0.0 | 4 | 0.3 ± 0.0 | 8 |
| Lactate | 1.4 ± 0.2 | 4 | 1.4 ± 0.2 | 8 |
| Glucose | 0.8 ± 0.1 | 4 | 0.3 ± 0.1 | 8 |
| Glycogen | 1.2 ± 0.3[1] | 4 | 1.2 ± 0.1[1] | 8 |
| pHi | 7.14 ± 0.04[2] | 6 | 7.16 ± 0.01[2] | 8 |

[1]Expressed as mean µmol glucosyl units $g^{-1}$ ww (±SEM).

[2]Expressed as mean pH units (±SEM).

# CHAPTER 6:

# The Effects of Exposure to the Lampricide, Niclosamide, on Energy Metabolism and Intracellular Muscle pH in the Lake Sturgeon (*Acipenser fulvescens*)

## 1. INTRODUCTION

Although once widespread in lakes and rivers of the central US, Great Lakes and the Hudson Bay drainages of Canada, lake sturgeon (*Acipenser fulvescens*) populations were decimated in the late 19$^{th}$ to early 20$^{th}$ centuries due to overfishing and habitat degradation (Harkness and Dymond, 1961; Scott and Crossman, 1973; Becker, 1983). Efforts to restore lake sturgeon populations in the Great Lakes has been hindered by the time it takes for the fish to reach sexual maturity (~25 years for females: ~15 years for males) and long spawning cycle (4-5 years for females; 1-2 years for males; Scheidegger, 2012). It has also been suggested that sea lamprey (*Petromyzon marinus*) parasitism, especially in the early juvenile stages, has contributed greatly to the delayed recovery of this species at risk (SAR) in the Great Lakes (Patrick *et al.*, 2009; Dobiesz *et al.*, 2018).

Lake sturgeon, however, have also been shown to be at least as susceptible to TFM as sea lamprey during their early juvenile stages (< 100 mm) as well as to to TFM/1% niclosamide mixtures (Boogaard *et al.*, 2003; O'Connor *et al.*, 2017). To determine how TFM/niclosamide mixtures affect lake sturgeon, a necessary first step is to determine how they respond to niclosamide alone. While there is increasing understanding regarding toxicity and physiological effects of TFM in lake sturgeon (Chapter 5; Boogaard *et al.*, 2003; Middaugh *et al.*, 2014; Sakamoto *et al.*, 2016; Bussy *et al.*, 2017a, 2017b; O'Connor *et al.*, 2017; Hepditch *et al.*, 2019), there is no data on how niclosamide (alone) adversely affects this species, especially early juveniles, in order to delineate, compare and contrast the mode(s) of toxicity of the two lampricides. Such information could prove very helpful in developing lampricide application strategies to effectively control sea lamprey without compromising sturgeon conservation efforts in the Great Lakes.

The goal of this study was to acquire a better understanding of the physiological effects and mechanism of niclosamide toxicity in lake sturgeon. I predicted that physiological responses to niclosamide in lake sturgeon would lead to a depletion of energy stores, especially in the brain, similar to those reported for TFM in this species (Chapter 5). To test this hypothesis, the 9-h $LC_{50}$ of niclosamide to juvenile lake sturgeon was determined, after which they were exposed to this concentration for 9 h, during which time tissue (brain, muscle, liver) and blood samples were taken at different time intervals. The resiliency of lake sturgeon to sub-lethal niclosamide exposure was then assessed by collecting additional samples following a 24 h post-niclosamide exposure recovery period. Brain, muscle and liver were subsequently analyzed for glucose, glycogen concentration and lactate accumulation, along with ATP and phosphocreatine, and related metabolites. Intracellular pH (pHi) was also measured in the muscle to quantify the extent of any disturbances to acid-base balance during and following niclosamide exposure. As with TFM, I hypothesized that during niclosamide exposure, lake sturgeon would increase their reliance on glycolysis to compensate for shortfalls in ATP supply, and altered acid-base balance.

## 2. RESULTS

### 2.1 Niclosamide toxicity

Lake sturgeon (N = 12 each concentration) exposed to niclosamide experienced death in a dose and time-dependent manner. For controls (no niclosamide exposure), 0.05 and 0.075 mg $L^{-1}$ niclosamide no deaths occurred for full exposure time (24 h). At 0.1 mg $L^{-1}$ exposure 1 death was recorded after 9 h. At niclosamide concentration of 0.11 mg $L^{-1}$, 4 lake sturgeon died after 6 h and 1 after 9 h exposure. With exposure to 0.13 mg $L^{-1}$ niclosamide 6 fish died after 3 h and 6 after 6 h. At 0.17 mg $L^{-1}$ of niclosamide 10 fish after 3 h and 2 after 6 h exposure. At niclosamide

concentration of 0.25 mg $L^{-1}$, 1 fish died after 1 h and 11 after 3 h. With exposure to 0.50 or 1.00 mg $L^{-1}$ niclosamide, all fish died after 1 h exposure. While predicted toxicity and respective 95% confidence levels for 9, 12 and 24 h differ slightly, the toxicity curve for 9 h exposure and 95% confidence levels (Figure 6.1) is the same for 12 h and 24 h exposure due to the fact that all partial deaths occurred by 9 h.

2.2 Measured water niclosamide concentrations

Nominal concentration of niclosamide for experiments examining physiological responses of the sturgeon to the lampricide was 0.111 mg $L^{-1}$. LC-MS/MS analysis revealed that measured concentrations were 0.110 ± 0.003 mg $L^{-1}$ at start (0 h) and 0.111 ± 0.002 mg $L^{-1}$ at end (9 h). There was no significant difference between nominal and measured niclosamide concentrations nor was there degradation of the lampricide over time.

2.3 Effects of time on energy stores and metabolites in control lake sturgeon

There were no significant differences in the concentrations of ATP, PCr, glycogen, lactate and other metabolites measured in the brain, liver, carcass or blood of control fish (not exposed to niclosamide) sampled at the beginning of the experiment (time 0 hours) and those sampled after 9 h (Supplemental Table 6.2S). Therefore, data from the two control groups were combined for each set of analyses used to quantify the effects of niclosamide exposure on the lake sturgeon.

2.4 Niclosamide effects on energy stores and metabolites in lake sturgeon brain

The ATP concentrations in the brain of lake sturgeon held under control conditions averaged 0.4 ± 0.0 μmol $g^{-1}$ ww, and PCr averaged 0.5 ± 0.0 μmol $g^{-1}$ ww (Figure 6.2). Exposure of lake sturgeon to the measured 9-h $LC_{50}$ of niclosamide, 0.1 mg $L^{-1}$, was characterized by a downward trend in brain ATP through the exposure period, which was significantly reduced, by approximately 36%, after 9 h, before returning to pre-exposure levels after 24 h recovery (Figure 6.2A). Brain PCr concentrations were not significantly affected by niclosamide exposure (Figure 6.2B).

Brain glucose, glycogen and lactate concentrations in lake sturgeon controls averaged 1.1 ± 0.1 μmol $g^{-1}$ ww, 1.6 ± 0.1 μmol glucosyl units $g^{-1}$ ww and 6.3 ± 0.4 μmol $g^{-1}$ ww, respectively (Figure 6.3). Brain glucose concentrations experienced an immediate and sustained reduction of approximately 50-60 % in the presence of niclosamide, returning to pre-exposure levels within 24 h of recovery (Figure 6.3A). Brain glycogen levels remained unchanged throughout the experiment (Figure 6.3B). Brain lactate concentrations in lake sturgeon underwent immediate and sustained elevation in the presence of niclosamide, by at least 35% in relation to controls, returning to pre-experimental levels by 24 h recovery (Figure 6.3C).

2.5 Effects of niclosamide on energy stores in lake sturgeon liver

Liver glucose concentrations in lake sturgeon held under control conditions averaged 3.5 ± 0.4 μmol $g^{-1}$ ww and glycogen concentrations averaged 52.0 ± 1.2 μmol glucosyl units $g^{-1}$ ww (Figure 6.4). Liver glucose remained unchanged in the presence of niclosamide relative to controls (Figure 6.4A) but glycogen concentrations were significantly reduced by approximately

30% and 75% at 6 and 9 h, respectively, compared to controls, returning to pre-exposure levels after 24 h recovery (Figure 6.4B).

2.6 Effects of niclosamide on energy stores and metabolites in lake sturgeon carcass

ATP and PCr concentrations in the carcass of lake sturgeon controls averaged 1.0 ± 0.1 μmol $g^{-1}$ ww and 5.7 ± 0.3 μmol $g^{-1}$ ww, respectively (Figure 6.5). In the presence of niclosamide, carcass ATP concentrations were significantly reduced by approximately 60% at 6 h, relative to controls, recovering to near control concentrations of 0.7 ± 0.1 μmol $g^{-1}$ ww at 6 and 9 h and returning to pre-experimental levels after 24 h recovery (Figure 6.5A). Carcass PCr concentrations experienced immediate and sustained reduction of approximately 60% in the presence of niclosamide relative to controls, returning to pre-exposure levels after 24 h recovery (Figure 6.5B).

Carcass ADP and creatine concentrations in lake sturgeon controls average 3.2 ± 0.2 μmol $g^{-1}$ ww and 5.4 ± 0.4 μmol $g^{-1}$ ww, respectively, remaining unchanged in the presence of niclosamide (Figures 6.5C, 6.5D).

Concentrations of glucose and glycogen in carcass of lake sturgeon controls averaged 0.6 ± 0.1 μmol $g^{-1}$ ww and 2.1 ± 0.3 μmol glucosyl units $g^{-1}$ ww (Figure 6.6). No changes were observed carcass glucose concentration during exposure to niclosamide (Figure 6.6A). Nor were glycogen concentrations altered compared to controls (Figure 6.6B). However, a significant difference was present between carcass glycogen concentrations at 3h (1.3 ± 0.2 μmol glucosyl units $g^{-1}$ ww) and 9 h (2.3 ± 0.2 μmol glucosyl units $g^{-1}$ ww). Carcass glycogen levels returned to control concentrations after 24 h recovery (Figure 6.6B).

Pyruvate and lactate concentrations in carcass of lake sturgeon controls averaged 0.2 ± 0.0 µmol $g^{-1}$ ww and 0.6 ± 0.1 µmol $g^{-1}$ ww, respectively (Figure 6.6). In the presence of niclosamide carcass pyruvate concentrations experienced an immediate and sustained elevation of approximately 50% relative to controls and returned to pre-exposure levels after 24 h recovery (Figure 6.6C). In the presence of niclosamide, carcass lactate levels were significantly increased (2-fold at 1, 6 and 9h; 3-fold at 3 h), relative to controls, returning to pre-experimental concentrations after 24 h recovery (Figure 6.6D).

2.7 Effects of niclosamide on blood plasma lactate and ions in lake sturgeon

Blood plasma lactate concentrations in lake sturgeon controls averaged 0.5 ± 0.1 µmol and experienced an immediate significant >2-fold which was sustained for the duration of lampricide exposure. Lactate concentrations returned to pre-experimental levels after 24 h recovery (Figure 6.6E).

Concentrations of $Na^+$ and $Cl^-$ in blood plasma of lake sturgeon controls averaged 125.5 ± 1.0 mmol $L^{-1}$ and 115.1 ± 1.0 mmol $L^{-1}$, respectively (Table 6.1). No significant changes were observed in lake sturgeon blood plasma $Na^+$ or $Cl^-$ ions in the presence of niclosamide.

2.8 Effects of niclosamide on lake sturgeon carcass intracellular pH (pHi)

The carcass intracellular pH (pHi) of control lake sturgeon averaged 7.12 ± 0.002 for controls. The presence of niclosamide resulted in acidosis in the muscle, characterized by significant pHi decreases (0.02, 0.04 and 0.15 pH units) to 7.09 ± 0.0, 7.08 ± 0.0 and 7.02 ± 0.01 at 3, 6 and 9h, respectively, relative to controls, returning to pre-experimental levels (pH = 7.11 ± 0.0) after 24 h of recovery (Figure 6.6F).

## 3. DISCUSSION

### 3.1 Niclosamide interferes with ATP production by lake sturgeon

Young-of-the-year lake sturgeon exposed to niclosamide at the species-specific 9 h $LC_{50}$ (0.11 mg $L^{-1}$) experienced significant decreases in brain ATP and glucose, with corresponding increases in lactate levels. As with TFM (Birceanu *et al.*, 2011), niclosamide targets the mitochondria, uncoupling oxidative phosphorylation, leading to a decrease in ATP production (e.g. Li *et al.*, 2014; Tao *et al.*, 2016; Alasadi *et al.*, 2018). In sea lamprey this subsequently results in greater reliance on anaerobic metabolic processes, such as dephosphorylation of phosphocreatine (PCr) to maintain the supply of ATP, and a greater reliance on glycolysis which leads to marked glycogen depletion in the brain (Wilkie *et al.*, 2007; Clifford *et al.*, 2012; Henry *et al.*, 2015). The situation is similar in lake sturgeon exposed to sea lamprey MLC of TFM (Chapter 5), but there are some notable differences in the lake sturgeon's responses to TFM and to niclosamide. One difference is the lack of PCr mobilization in the brain in response to niclosamide exposure. Exposure of rainbow trout and larval sea lamprey to TFM results in a depletion of brain PCr (Birceanu *et al.*, 2009, 2014; Clifford *et al.*, 2012), a common response when ATP demand is increased, or supply is compromised (Hochachka *et al.*, 1993). PCr is normally utilized as a temporary buffer to maintain ATP supply when demands increase, such as during bursts of muscular activity, in response to decreases in ADP/ATP ratios (Moyes and West, 1995; McLeish and Kenyon, 2005).

Another difference was the absence of changes in brain glycogen with niclosamide exposure, a defining feature of TFM exposure in sea lamprey and rainbow trout (Birceanu *et al.*, 2009, 2014; Clifford *et al.*, 2012). The simplest explanation for these observations is that lake

sturgeon brain has very low anaerobic capacity, characterized by very low concentrations of phosphocreatine, less than 1 μmol $g^{-1}$ ww, which are about 1/5 to 1/10 the concentrations measured in the brain of sea lamprey and rainbow trout (Birceanu *et al.*, 2009, 2014; Clifford *et al.*, 2012). Similarly, brain glycogen concentrations are less than 1 μmol $g^{-1}$ ww, about 1/10 concentrations made in rainbow trout and 2-orders of magnitude lower than concentrations measured in sea lamprey brain, which are amongst the highest measured in any ectothermic vertebrate (Rovainen, 1970; Foster and Moon, 1989; Clifford *et al.*, 2012). These findings are consistent with earlier work that suggested sturgeon have a relatively low glycolytic capacity compared to teleosts, as suggested by relatively low activities of hexokinase, phosphofructokinase, and lactate in brain, red muscle and liver (Singer *et al.*, 1990). Thus, unlike larval sea lamprey, and to a lesser extent, rainbow trout, brain glycogen and PCr likely provide little, if any, ATP to the central nervous system (CNS) of lake sturgeon when oxidative ATP production is compromised.

Glucose is the preferred substrate for oxidation in the brain of vertebrates (Polakof *et al.*, 2012). In most teleost fishes glucose arises from hepatic glycogen stores, and is transported across the blood brain barrier from the circulatory system, and then used for ATP production via oxidative phosphorylation within the brain (Soengas and Aldegunde, 2002). However, in lampreys, benthic and several hypoxia/anoxia tolerant teleosts, the proximate glucose source is high concentrations of brain glycogen (Soengas and Aldegunde, 2002). The low concentration and absence of any change in brain glycogen reserves with niclosamide exposure suggests that sturgeon nervous system primarily relies on exogenous glucose to meet their ATP demands. The stepwise decreases in hepatic glycogen reserves that took place during the niclosamide exposure period also suggests that hepatic glycogenolysis was needed to provide glucose.

Glucose concentrations in the brain were also reduced by approximately 50 %, however, suggesting glucose demand or supply to the brain was curtailed. Due to limited volume of plasma, glucose concentrations were not measured, but given the fact that liver concentrations of glucose were unaffected by niclosamide exposure, it seems unlikely that the glucose supply to the brain was limited. Rather, these findings suggest that glucose demands in the brain increased during niclosamide exposure, resulting in lower steady state concentrations of the fuel in this organ. One possibility is that ATP production by the sturgeon CNS is thought to rely on other aerobic substrates for ATP production, such as non-esterified fatty acids or lactate, in addition to glucose (Singer *et al.*, 1990). Thus, increased reliance on anaerobic glycolysis by the brain could explain the sustained reduction in glucose concentration that was observed. This, plus decreased oxidation of lactate, could explain the disproportionate increase in the concentration of this metabolite. However, death would likely result once the hepatic glycogen stores were depleted, as the brain would be starved of its primary energy reserve, glucose (Wilkie *et al.*, 2007). Another complication of niclosamide exposure would be metabolic acidosis in the brain, which would arise from increased reliance on anaerobic glycolysis to produce ATP (Hochachka 1993). It was not possible to collect sufficient amounts of tissue to measure brain intracellular pH (pHi), but measurements in the carcass suggests that there was a significant metabolic acidosis as demonstrated by significant decreases in pHi of 0.1 pH units, after 9 h exposure. However, the closely related white sturgeon (*Acipenser transmontanus*), and many other fishes exhibit preferential pH regulation, in which pH disturbances arising from exogenously-induced acid-base insults such as hypercarbia, result in extracellular acid-base disturbances (blood pH) but intracellular pH disturbances are relatively minor or completely absent (Baker *et al.*, 2009; Shartau *et al.*, 2019). For instance, white sturgeon subjected to hypercarbia ($P_{CO2}$ = 1.5 kPa)

experienced 0.2 reductions in extracellular pH after 6 h, which was accompanied by compensatory increase in brain, liver and heart pHi (Baker *et al.*, 2009). It is difficult to ascertain if disturbances in muscle pHi extended to the brain during niclosamide exposure, but it could be a fruitful future research direction. In fish, mechanisms compensating for hypercapnia in the brain are proposed to affect olfaction and lateralization (Heuer *et al.*, 2016) by the disruption of the gamma-aminobutyric acid$_A$ ($GABA_A$) neurotransmitter receptor, resulting in reduction in avoidance of chemical alarm cues (Heuer *et al.*, 2016). Such olfaction impairment has been demonstrated in YOY lake sturgeon exposed to TFM (Sakamoto *et al.*, 2016). Thus, if niclosamide-induced acidosis were to extend to the neural system of lake sturgeon, such chemical alarm cue disturbances would diminish the ability of the fish to avoid the lampricide, leading to prolonged exposure.

While niclosamide imparts some adverse physiological effects even at sub-lethal concentrations, lake sturgeon readily recovered within 24 h following exposure. Of particular interest is the recovery of glycogen, the primary substrate of glycolysis. Studies have shown that glycogen recovery following exhaustive exercise in fish may take up to 12 h but recovery is variable and species dependant (Kieffer, 2000). In mammals it has been long believed that glycogen regeneration is described by the Cori cycle in which muscle produced lactate is transported to the liver via the blood, where it is converted to glucose, then transported back to the muscle via blood where it is used in glycogen synthesis (Newsholme and Leech, 1983). In skeletal muscle of fish, however, the Cori cycle is of little physiological consequence in re-synthesis of glycogen (Gleeson, 1991; Fournier and Guderly, 1992; West *et al.*, 1994). Instead, muscle glycogen re-synthesis appears to occur *in situ* using lactate as the primary substrate. One line of evidence shows that following exhaustive exercise, there was 80-85% of total lactate

retention in the muscle with clearing being coincidental with glycogen replacement (Wood *et al.*, 1983; Milligan and Wood, 1986; Pagnotta and Milligan, 1991). Additionally, the rate of observed lactate clearance in the muscle cannot be accounted for by *in vivo* lactate turnover rates (Cornish and Moon, 1985; Weber *et al.*, 1986; Milligan and McDonald, 1988). It is likely that glycogen levels were restored in this manner, during recovery from niclosamide exposure. This would account for restoration of lactate levels to pre-exposure levels as the substrate is being used to replenish glycogen. As the fish continues to clear the body of niclosamide during recovery, stress on energy metabolism would be diminished, allowing the recuperation of ATP and PCr reserves. With lower dependence on anaerobic glycolysis, the generation of lactate and protons would also be decreased, thus, carcass pHi would also be expected to return to pre-exposure (baseline) levels.

### 3.2 Effects of niclosamide on blood plasma ions

Uncoupling of mitochondrial oxidative phosphorylation by niclosamide might also be expected to impair gill-mediated iono- and osmoregulation, which require ATP for primary and secondary active transport to take-up ions from fresh water via mitochondria rich cells (aka. MRCs or ionocytes; see Evans *et al.*, 2005 for review). Previously, Mallatt *et al.* (1994) demonstrated that exposure to lethal concentrations (9 h $LC_{100}$) of TFM and niclosamide (Bayer 73®) altered the ultrastructure of gills in larval sea lamprey and rainbow trout. Using transmission electron microscopy (TEM), they observed damage to presumed ion-uptake cells (now known as mitochondria rich cells or ionocytes; Evans *et al.*, 2005) such as cell rounding, enlargement of mitochondria and intracellular space widening. While both lampricides caused similar damage, niclosamide exposure resulted in higher incidence of necrosis in the ionocytes

(Mallatt *et al.,* 1994). The authors suggested that the damage observed in the gills of lampricide exposed sea lamprey may have comprised ion and osmoregulation, and contributed to their high sensitivity to TFM (Mallatt *et al.,* 1994). Indeed, ion and osmoregulation in freshwater depends upon ATP dependent ion pumps including the $Na^+/K^+$-ATPase, V-ATPases, and $Ca^{2+}$-ATPases characteristic of ionocytes (Evans *et al.*, 2005). In the present study, however, plasma $Na^+$ and $Cl^-$ concentrations in lake sturgeon were unaffected by niclosamide exposure (Table 6.1), suggesting that acute disturbances to gill-mediated ion regulation do not contribute to niclosamide-induced physiological disturbances or mortality. These findings are similar to those observed in rainbow trout and sea lamprey exposed to TFM (Birceanu *et al.*, 2009; Birceanu *et al.*, 2014; Henry *et al.,* 2015), as well as in lake sturgeon (Sorensen, 2015). Nor did TFM exposure (12 h $LC_{50}$) have any marked effects on rates of $Na^+$ uptake, whole body ion ($Na^+$, $K^+$) concentrations (Birceanu *et al.*, 2009, 2012), or on plasma ion ($Na^+$, $K^+$) concentrations in trout or sea lamprey (Birceanu *et al.*, 2009, 2012; Henry *et al.,* 2015).

### 3.3 Impacts of niclosamide on the ecophysiology of lake sturgeon

It is possible that in conjunction with impairement of neural and hepatic metabolic processes, niclosamide could have other serious physiological effects, impacting lake sturgeon survival. For example, it has been recently demonstrated that fishes experience olfaction impaiment when exposed to certain environmental toxicants such as metals and pesticides (Mirza *et al.*, 2009; Green *et al.*, 2010; Tierney *et al.*, 2010; Dew *et al.*, 2014). Impaired olfaction can lead to failure in perception of chemosensory cues and response, in turn resulting in maladaptive behaviour (Weis and Weis, 1995; Norris *et al.*, 1999; Tierney *et al.*, 2010; Sovová *et al.*, 2014). Using electro-olfactography (EOG), Sakamoto *et al.* (2016) showed that in YOY lake sturgeon

TFM interfered with olfactory sensory neurons (OSNs) and ciliated sensory neurons, adapted to recognizing food cues, and migration and alarm cues, respectively. Additionally, activity analyses of lake sturgeon revealed that TFM-expose fish were 79% more active but had 19% slower acceleration and 16% slower velocity, compared to controls (Sakamoto *et al.*, 2016). Because TFM and niclosamide are both halogenated phenolic compounds which are thought to impart their toxicity in a similar fashion, it is quite possible that niclosamide could impair olfaction and behaviour in lake sturgeon similar to TFM. It might be speculated that the diminished brain energy stores (ATP and glucose) in niclosamide-exposed lake sturgeon would extend to the mitochondria-enriched olfactory neurons (Zielinski *et al.*, 1996; Daghfous *et al.*, 2012; Fluegge *et al.*, 2012), directly affecting olfaction and the sensory neurons (Mirza *et al.*, 2009; Green *et al.*, 2010; Tierney *et al.*, 2010; Dew *et al.*, 2014) and contributing to the processing impairment of electric signals from the OSNs. Since repolarization of neurons following action potentials requires ATP, it would be worthwhile to investigate if niclosamide exposure could manifest as significantly altered EOGs, similar to observations in TFM exposed lake sturgeon (Sakamoto *et al.*, 2016).

The increased activity observed in TFM exposed lake sturgeon by Sakamoto *et al.* (2016), would likely increase the fish's vulnerability to predation due to diminishing muscle energy stores (ATP and glycogen; Chapter 5). It would be expected that lake sturgeon would also experience predation vulnerability in the presence of niclosamide due diminishing muscle energy stores (carcass ATP, PCr and glycogen), albeit the fact that niclosamide-exposed fish experienced higher variability in the concentration of these molecules, compared to TFM-exposed fish. Increased vulnerability to predation would further be exacerbated by diminished swimming performance (velocity, acceleration; Sakamoto *et al.*, 2016). In the current study,

niclosamide-exposed fish experienced severe depletion in carcass PCr and variable ATP decline. It may be implied that niclosamide induced increased activity in the presence of the lampricide, thus, necessitating the buffering of ATP by PCr. Other components of the creatine kinase equilibrium, ADP and creatine were unaffected in the carcass.

### 3.4 Implications for lake sturgeon population enhancement and sea lamprey control

The potential of lamprey parasitism to cause serious harm to Great Lakes fisheries, worth in excess of 7 billion dollars annually, is still present (Krantzberg and De Boer, 2008; GLFC, 2011). While use of lampricides such as TFM and niclosamide remains an integral component of the sea lamprey control program (Siefkes, 2017; Wilkie *et al.*, 2019), the risks of adverse effects on non-target fishes also need to be considered and mitigated where necessary. For instance, the potential harm that lampricide application could have on culturally important species at risk (SAR) such as the lake sturgeon, needs to be weighed against the potential harm of sea lamprey parasitism to Great Lakes fisheries. Indeed, recent analysis suggests that ineffective or reduced use of lampricides could lead to greater sea lamprey parasitism and mortality of lake sturgeon populations (Dobiesz *et al.*, 2018). Efforts have been underway in the Great Lakes to restore lake sturgeon populations, particularly by the U.S. Fish and Wildlife Service (LRBOI-USFWS, 2017). On the other hand, there is concern by government agencies and First Nations that lampricide applications could undermine these efforts, particularly TFM which has been identified as a source of age-0 lake sturgeon mortality (Johnson *et al.*, 1999; Boogaard *et al.*, 2003; O'Connor *et al.*, 2017). Understanding how niclosamide and TFM affects the physiology of lake sturgeon, is therefore, an important first step in improving our understanding of the potential impacts that lampricide application could have on this SAR.

Attempts to reduce lampricide (TFM) concentrations (from 1.3-1.5 x MLC to 1 x MLC) applied to streams containing YOY lake sturgeon were implemented by the US Fish and Wildlife Service in the early 2000s, but such efforts led to increased numbers of parasitic lamprey and damage to fisheries, therefore, were abandoned (Dobiesz *et al.*, 2018). Not treating streams containing lake sturgeon is not likely feasible because the increase in numbers of parasitic sea lamprey in receiving waters (lakes) of affected rivers would render lake sturgeon themselves more vulnerable to sea lamprey parasitism. This was recently demonstrated using a generalized Great Lakes sturgeon population model, relating lake sturgeon populations changes to sea lamprey-associated mortality (Dobiesz *et al.*, 2018). Assuming that while age-0 lake sturgeon are found to be the most susceptible to TFM toxicity (Johnson *et al.*, 1999; Boogaard *et al.*, 2003; O'Connor *et al.*, 2017), sea lamprey parasitism on sub-adult (ages 7-24 years) lake sturgeon can result in 32% mortality (Dobiesz *et al.*, 2018). Dobiesz *et al.* (2018) determined that the benefits of reducing sea lamprey predation far outweighed the marginal increase (5.7%) in adult lake sturgeon in the absence of TFM use.

The current study demonstrates that sub-lethal concentrations of niclosamide imparts physiological disturbances in juvenile lake sturgeon, characterized by reduced ATP and glycogen reserves. At first glance, one might conclude that such disturbances could potentially compromise lake sturgeon survival. But this seems unlikely, given the good news that these fish readily recover from exposure to niclosamide within 24 h. This opens the door to other approaches that could be used to apply lampricides, that minimize lampricide accumulation and minimize physiological disturbances when treating streams where lake sturgeon and larval sea lamprey populations overlap. One possible beneficial approach would be to use lower concentrations of lampricides for longer time blocks (long and low approach) in rivers known to

contain juvenile lake sturgeon, reducing the amount of TFM or TFM/niclosamide (1-2 %) entering the water, presumably decreasing the physiological effects of lampricides and increasing survival. Given that TFM uptake is inversely related to body size (Hepditch *et al.*, 2019), delaying treatments until later in the summer or early autumn might also be considered when the sturgeon are larger, and accumulate less lampricide. It would also be beneficial to learn more about how niclosamide and/or TFM effects olfaction and behaviour, to determine if lampricide avoidance, food acquisition, swimming performance and predation are affected, as has been done in only a few studies (Middaugh *et al.*, 2014; Sakamoto *et al.*, 2016). Such knowledge would inform fisheries managers and policy makers of the most feasible way to protect lake sturgeon while maintaining the integrity of the sea lamprey control program.

**Table 6.3 Effects of niclosamide exposure on lake sturgeon ion balance.**

The concentrations of $Na^+$ and $Cl^-$ in the blood plasma of rainbow trout (*Oncorhynchus mykiss*) held under control conditions (no niclosamide), during exposure to niclosamide at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for up to 9 h, or following a 24 h depuration period in clean (no niclosamide) water. Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

| Treatment | $Na^+$ mmol $L^{-1}$ ± SEM (n) | $Cl^-$ mmol $L^{-1}$ ± SEM (n) |
|---|---|---|
| Control | 125.5 ± 1.0 (9) | 115.1 ± 1.0 (9) |
| 1 h | 124.4 ± 2.1 (12) | 113.2 ± 1.3 (12) |
| 3 h | 126.6 ± 4.0 (7) | 116.3 ± 1.8 (7) |
| 6 h | 122.3 ± 2.8 (13) | 113.8 ± 1.2 (13) |
| 9 h | 126.9 ± 2.9 (13) | 114.9 ± 1.2 (13) |
| 24 h Rec. | 123.2 ± 2.9 (6) | 114.3 ± 1.5 (6) |

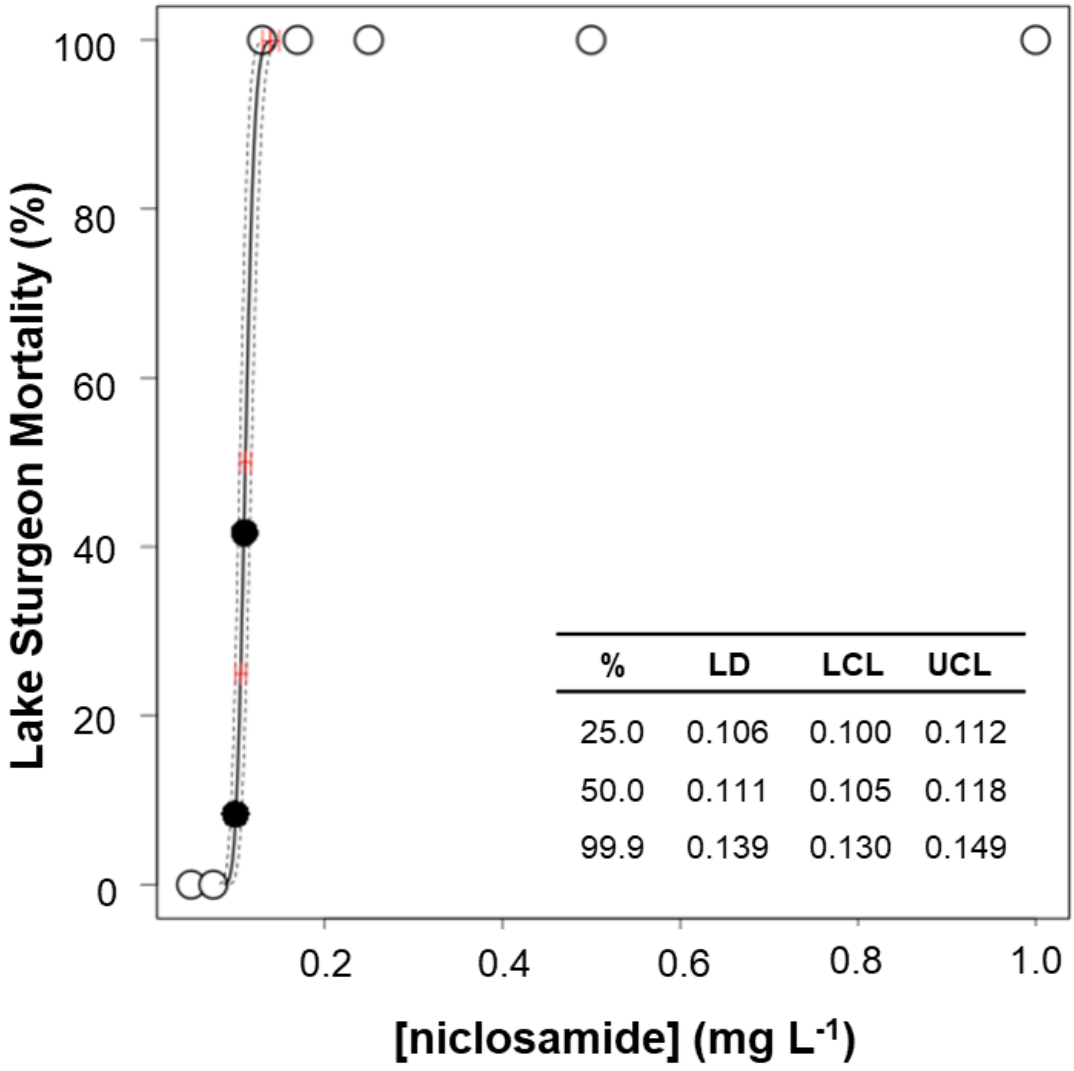


**Figure 6.1 Niclosamide toxicity curve in lake sturgeon**

**Figure 6.1 Niclosamide toxicity curve in lake sturgeon.** Niclosamide toxicity results were calculated using nominal concentrations. Plot of observed data with the Litchfield Wilcoxon fitted model on arithmetic scale (solid black curve; dashed curves 95% LCL and UCL) for lake sturgeon (*Acipenser fulvescens*) niclosamide toxicity experiments. At each of the specified percentages affected, the predicted effective doses and their 95% confidence levels are depicted in red. Observations with 0% or 100% affected lake sturgeon are shown as white circles. Inset table lists specific lethal concentration percentages (%), predicted lethal dose (LD), 95% lower confidence level (LCL) and 95% upper confidence level (UCL).

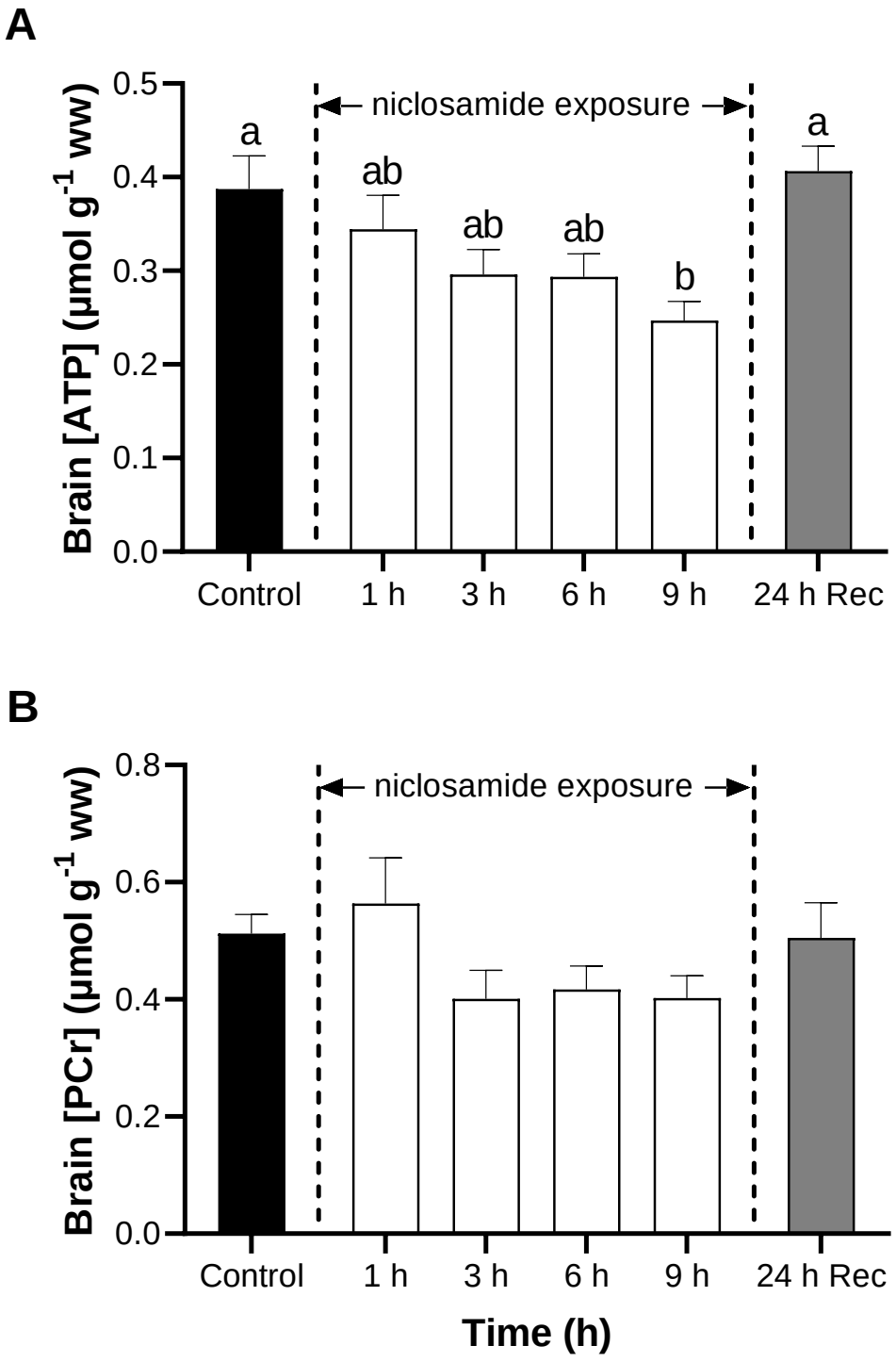


**Figure 6.2 Energy stores in brain or niclosamide-exposed lake sturgeon**

**Figure 6.2 Energy stores in brain or niclosamide-exposed lake sturgeon.** Changes in the concentrations of (A) ATP and (B) PCr in the brain of lake sturgeon (*Acipenser fulvescens*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 11; grey bars) at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 10), 3 h (n = 12), 6 h (n = 12) and 9 h (n = 12), or held under control conditions (no niclosamide; n = 12; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

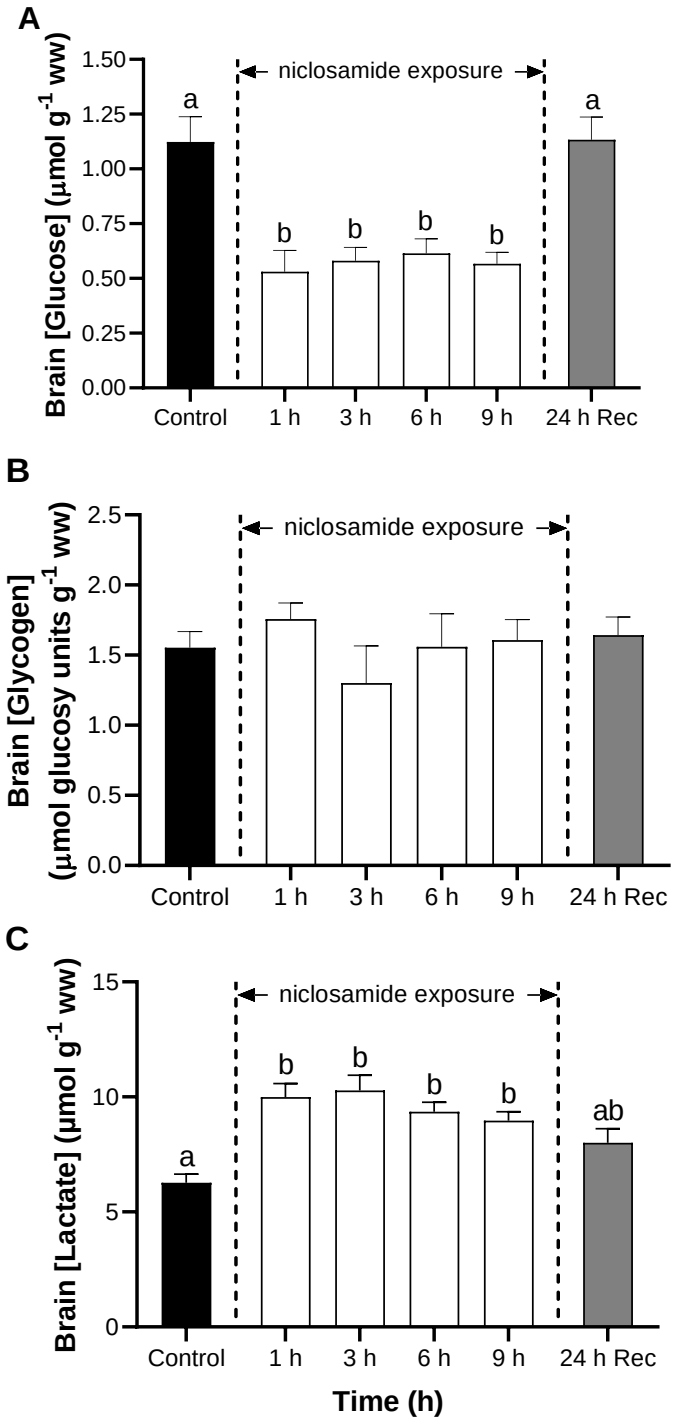


**Figure 6.3 Energy stores and metabolites in brain on niclosamide-exposed lake sturgeon**

**Figure 6.3 Energy stores and metabolites in brain on niclosamide-exposed lake sturgeon.** Changes in the concentrations of (A) glucose, (B) glycogen and (C) lactate in the brain of lake sturgeon (*Acipenser fulvescens*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 11; grey bars) at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 10), 3 h (n = 12), 6 h (n = 13) and 9 h (n = 13), or held under control conditions (no niclosamide; n = 12; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

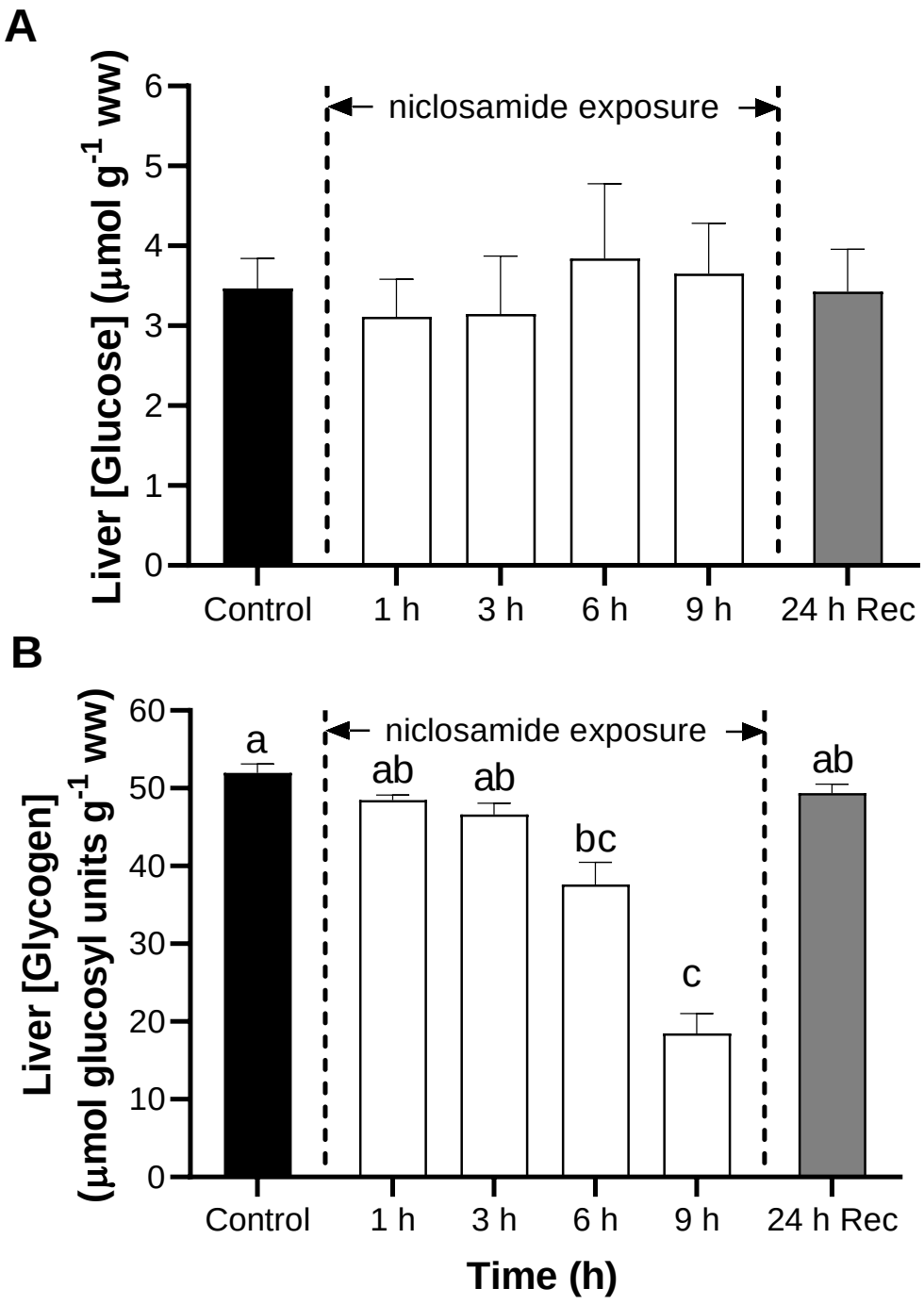


**Figure 6.4 Energy stores in liver of niclosamide-exposed lake sturgeon**

**Figure 6.4 Energy stores in liver of niclosamide-exposed lake sturgeon.** Changes in the concentrations of (A) glucose and (B) glycogen in the liver of lake sturgeon (*Acipenser fulvescens*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 12; grey bars) at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 10), 3 h (n = 10), 6 h (n = 9) and 9 h (n = 11), or held under control conditions (no niclosamide; n = 11; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

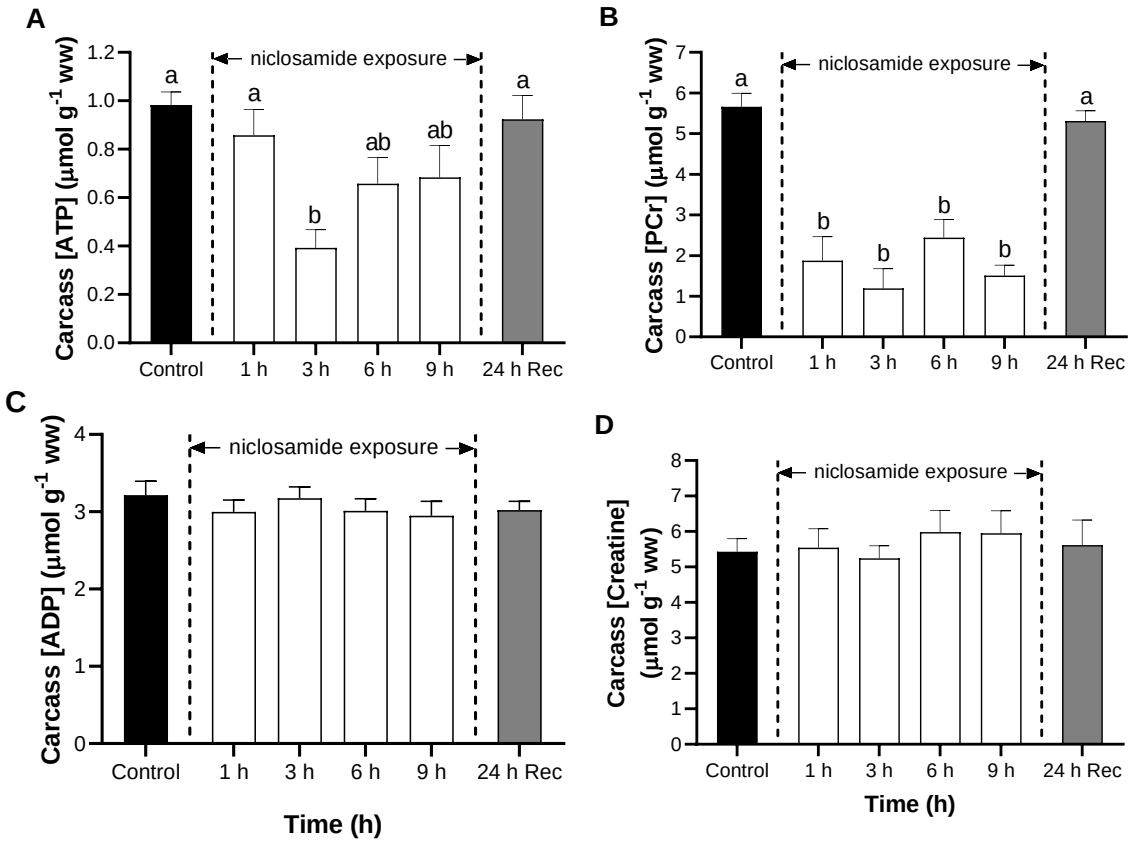


**Figure 6.5 Energy stores in carcass of niclosamide-exposed lake sturgeon**

**Figure 6.5 Energy stores in carcass of niclosamide-exposed lake sturgeon.** Changes in the concentrations of (A) ATP, (B) PCr, (C) ADP and (D) creatine in the muscle of lake sturgeon (*Acipenser fulvescens*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 12; grey bars) at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (n = 12), 3 h (n = 12), 6 h (n = 13) and 9 h (n = 14), or held under control conditions (no niclosamide; n = 12; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

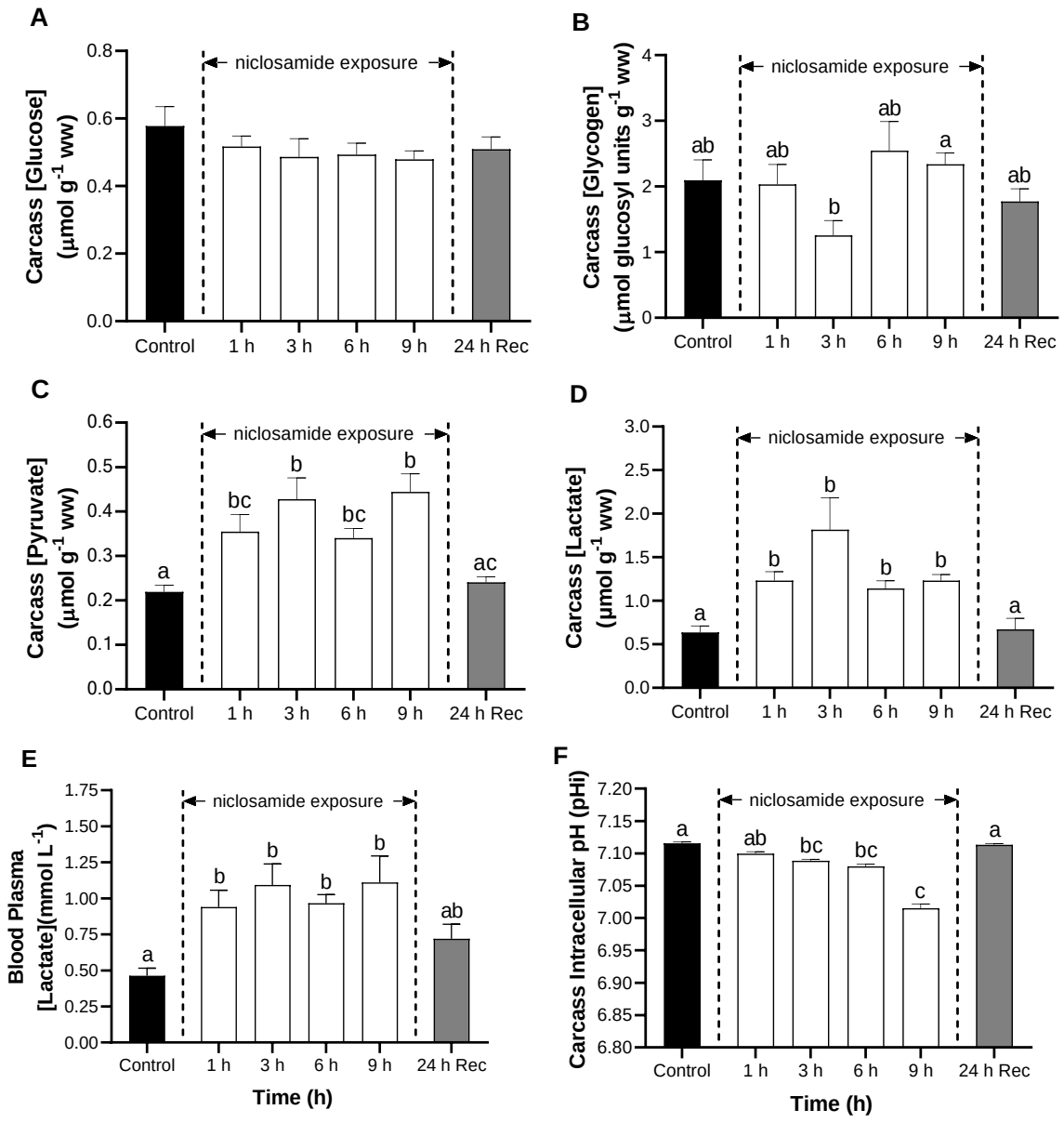


**Figure 6.6 Energy stores and metabolites in carcass of niclosamide-exposed lake sturgeon**

**Figure 6.6 Energy stores and metabolites in carcass of niclosamide-exposed lake sturgeon.** Changes in the concentrations of (A) glucose, (B) glycogen, (C) pyruvate, (D) lactate in the carcass, (E) lactate in blood plasma, and (F) pHi in the carcass of lake sturgeon (*Acipenser fulvescens*) during (open bars) and following exposure to niclosamide (carcass 24 h recovery; n = 12; plasma 24 h recovery; n = 6; grey bars) at a nominal concentration of 0.11 mg $L^{-1}$ (9 h $LC_{50}$) for 1 h (carcass n = 12; plasma n = 12), 3 h (carcass n = 12; plasma n = 7), 6 h (carcass n = 13; plasma n = 13) and 9 h (carcass n = 14; plasma n = 13), or held under control conditions (no niclosamide; carcass n = 12; plasma n = 9; black bars). Data are expressed as mean ± S.E.M. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$).

**SUPPLEMENTAL MATERIAL**

**Table 6.4S Summary of controls.**

Comparison of control (no niclosamide) measuring concentrations of energy stores and metabolites in the brain, liver and muscle of lake sturgeon (*Acipenser fulvescens*) sampled at 0 h (start), and 9 h (finish). Muscle intracellular pH (pHi) control measurements were similarly compared as were blood plasma ions. Data are expressed as mean ± S.E.M. No significant differences were observed.

| Brain Assays | Control Start [μmol $L^{-1}$ ww ± SEM (n)] | Control Finish [μmol $L^{-1}$ ww ± SEM (n)] |
|---|---|---|
| ATP | 0.4 ± 0.0 (6) | 0.4 ± 0.0 (6) |
| PCr | 0.5 ± 0.0 (6) | 0.5 ± 0.0 (6) |
| Glucose | 1.2 ± 0.2 (6) | 1.1 ± 0.2 (6) |
| Glycogen | 1.6 ± 0.2 (6) | 1.5 ± 0.2 (6) |
| Lactate | 5.9 ± 0.8 (6) | 6.7 ± 0.1 (6) |

| Liver Assays | Control Start [μmol $L^{-1}$ ww ± SEM (n)] | Control Finish [μmol $L^{-1}$ ww ± SEM (n)] |
|---|---|---|
| Glucose | 3.5 ± 0.3 (6) | 3.4 ± 0.8 (5) |
| Glycogen | 53.0 ± 2.0 (6) | 50.7 ± 0.6 (5) |

| Muscle Assays | Control Start [μmol $L^{-1}$ ww ± SEM (n)] | Control Finish [μmol $L^{-1}$ ww ± SEM (n)] |
|---|---|---|
| ATP | 1.1 ± 0.1 (7) | 0.9 ± 0.1 (6) |
| PCr | 5.4 ± 0.4 (7) | 5.9 ± 0.6 (6) |
| ADP | 3.2 ± 0.2 (7) | 3.3 ± 0.3 (6) |

| | | |
|---|---|---|
| Creatine | 5.6 ± 0.3 (7) | 5.2 ± 0.8 (6) |
| Glucose | 0.6 ± 0.1 (7) | 0.6 ± 0.1 (6) |
| Glycogen | 2.2 ± 0.5 (7) | 2.0 ± 0.3 (6) |
| Pyruvate | 0.2 ± 0.0 (7) | 0.2 ± 0.0 (6) |
| Lactate | 0.5 ± 0.1 (7) | 0.8 ± 0.1 (6) |

| Muscle Assays | Control Start [pH ± SEM (n)] | Control Finish [pH ± SEM (n)] |
|---|---|---|
| pHi | 7.12 ± 0.0 (7) | 7.11 ± 0.0 (6) |

| Plasma Ions | Control Start [mmol $L^{-1}$ ± SEM (n)] | Control Finish [mmol $L^{-1}$ ± SEM (n)] |
|---|---|---|
| $Na^+$ | 125.7 ± 0.8 (8) | 125.3 ± 2.3 (7) |
| $Cl^-$ | 114.2 ± 1.4 (8) | 116.3 ± 1.3 (7) |

# Chapter 7:

# Synthesis of Research: Models of TFM and Niclosamide Toxicity in Three Fishes and the Implications for Sea Lamprey Control

## 1. SYNOPSIS

Treatments of rivers and streams in the Great Lakes with lampricides are intended to eliminate larval sea lamprey (*Petromyzon marinus*) with little to no acute or chronic effects on non-target fishes or other aquatic organisms. The studies presented in this dissertation, explored the physiological effects of lampricides on sea lamprey and two non-target species, rainbow trout (*Oncorhynchus mykiss*) and lake sturgeon (*Acipenser fulvescens*). A common feature of exposure to the lampricides TFM or niclosamide was diminished production of ATP, leading to a greater reliance on phosphocreatine (PCr) and/or glycolysis to maintain basal ATP needs, accompanied by significant elevations in pyruvate (carcass/muscle, hereafter referred to as muscle) and lactate concentrations, as well as metabolic acidosis. These results lend support to the hypothesis that both TFM and niclosamide act as uncouplers of oxidative phosphorylation in sea lamprey, rainbow trout and lake sturgeon. However, there are subtle differences in the type and magnitude of physiological responses experienced, and strategies employed by each of the fishes to deal with the insult (Figures 7.1 – 7.4).

## 2. PHYSIOLOCIAL EFFECTS OF LAMPRICIDES

### 2.1 Niclosamide and TFM have similar effects on sea lamprey physiology

Ammocoetes responded in a similar manner to niclosamide as to TFM. However, niclosamide was much more potent with corresponding physiological disturbances elicited at much lower concentrations than TFM. One explanation may be that niclosamide elicits disturbances other than uncoupling oxidative phosphorylation such as, damage to DNA (Abreu *et al.*, 2002) and mitochondrial fragmentation, and promotion of apoptotic and autophagic cell death (Park *et al.*, 2011). Niclosamide-exposed larval sea lamprey experienced significant

decreases in brain ATP and phosphocreatine (PCr) concentrations (Figure 7.1). Whereas, in water of identical chemistry, the concentrations of TFM required to elicit similar responses, over a similar time course, were 40-to 50-fold higher (Birceanu et al. 2009; Clifford et al. 2012).

As with TFM, niclosamide exposure results in marked depletion of brain glycogen stores. Unlike higher vertebrates, the lamprey brain contains exceptionally high levels of glycogen (often > 100 μmol $g^{-1}$ ww) reserves, which were reduced by ~50%, with little effect on glucose (Figure 7.1). This was likely because the glycogen reservoir in the brain, at least in surviving lamprey, is sufficiently large to prevent severe depletions of glucose, as evidenced by high glucose-6-phosphatase activity (Rovainen *et al.*, 1971; Murat *et al.*, 1979) needed to convert glycolysis-derived glucose-6-phosphate to glucose (Rovainen *et al.*, 1969, 1971; Rovainen, 1970). The glycogen depletion and concomitant 5- to 6-fold increase in brain lactate concentration in the presence of niclosamide suggests that ATP supply was being maintained, at least in part, by anaerobic glycolysis and to a lesser degree by PCr buffering (Figure 7.1).

The marked depletion in liver glycogen suggests that this organ may play a modest role in sea lamprey glucose homeostasis by mobilizing glycogen, in response to niclosamide-induced comprimised ATP supply. Previous fingdings suggested that sea lamprey liver has little to no involvement in glucose homeostasis (O'Boyle and Beamish, 1977; Larsen, 1978). Thus, it is more probable that glycogen reductions in niclosamide-exposed larval sea lamprey liver may be a result of this organ having gone anaerobic, relying primarily on glycolytic ATP production. Regardless, ammocoetes are significantly less dependant on this organ for glucose homeostasis than their teleost counterparts (Plisetskaya and Kuz'mina, 1971; O'Boyle and Beamish, 1977; Larsen, 1978; Larsen *et al.*, 2001; Barcellos *et al.*, 2010).

The kidney may also play a role in glucose homeostasis (circulating glucose levels), as it does in other vertebrates (Shanghavi and Weber, 1999); this is reflected by the significant depletion in kidney glycogen while glucose remained unaffected. Given that kidney glycogen concentrations are 3-fold higher than the liver, together, may provide an additional glucose pool in ammocoetes. Depleted ATP and glycogen, and increases in lactate concentrations, suggests that anaerobic glycolysis was augmenting this organ's energy needs (Figure 7.1). The already low basal PCr concentrations (<0.5 μmol $g^{-1}$ ww) were unaffected by niclosamide, similar to TFM-exposed lamprey (Henry *et al.*, 2015), suggesting a relatively low anaerobic capacity in the kidney.

As with TFM, muscle glycogen was significantly depleted following niclosamide exposure (Figure 7.1; Birceanu et al. 2009). In fishes, muscle glycogen is normally used for vigorous exercise (Milligan and McDonald, 1988; Wang *et al.*, 1994a; Kieffer, 2000; Wilkie *et al.*, 2001) and plays little to no role in glucose homeostasis (Figure 7.1; Panserat *et al.*, 2000). As in other teleosts such as rainbow trout, larval lamprey may use these glycogen stores in the muscle to fuel sudden, rapid bursts of exercise, such as burrowing (Boutilier *et al.*, 1993; Wilkie *et al.*, 2001), leading to decreases in glycogen and increases in lactate, followed by gradual restoration of glycogen using lactate as glyconeogenic substrate (Omlin and Weber, 2013). The near depletion of PCr and glycogen reduction, with corresponding increases in pyruvate, lactate, and presence of metabolic acidosis caused by niclosamide, mimicked the responses of the larval lamprey to exhaustive exercise and suggest an increased reliance on glycolysis (Figure 7.1). Rather than increased ATP demand, however, it was compromised ATP supply that led to the response. Due to PCr and glycogen buffering, muscle ATP remained unchanged with niclosamide exposure.

2.2 Rainbow trout physiological responses to niclosamide differ from sea lamprey

As observed in sea lamprey, niclosamide exposure of rainbow trout resulted in a marked depletion of brain ATP and glycogen, as the fish became increasingly reliant on glycolysis, and further supported by elevated lactate in the brain and mobilization of liver glycogen. Unlike in lampreys, teleosts primarily rely on the liver to maintain glucose homeostasis and supply to the brain (Plisetskaya and Kuz'mina, 1971; O'Boyle and Beamish, 1977; Larsen, 1978; Larsen *et al.*, 2001; Barcellos *et al.*, 2010; Polakof et al. 2012). This was revealed by the near depletion in liver glycogen, but maintenance of steady state brain glucose concentrations (Figure 7.2). Energy depletion and increased dependence on glycolysis was also evident in muscle where ATP decreases required increased reliance on anaerobic processes for ATP buffering to meet basal energy needs, leading to severely depleted PCr and glycogen concentrations with a concomitant increase in pyruvate and lactate (Figure 7.2). A corresponding metabolic acidosis was observed in the muscle of trout (~ 0.2 units lower pHi), but the magnitude was far less than observed in the larval sea lamprey. The much less pronounced acidosis in the trout was likely due to their much higher non-bicarbonate buffer capacity in this compartment compared to lamprey (Tufts and Boutilier, 1989; Boutilier *et al.*, 1993; Shartau *et al.*, 2019).

These physiological effects of niclosamide are similar to those observed in TFM-exposed rainbow trout (Birceanu *et al.*, 2009, 2014), with the responses seen in the white muscle mimicking those observed following exhaustive exercise (Wood 1993; Moyes and West 1994; Kieffer 2000), specifically, the decrease in pHi, and muscle glycogen and PCr. Like exhaustive exercise, however, the disturbance is rapidly corrected within 24 h. This suggests that exercise performance may be impeded in the interim period following niclosamide exposure, affecting predator evasion, foraging and upstream migration until glycogen reserves, adenylates, and pHi

are restored. However, it also suggests that any adverse effects of sub-lethal niclosamide, not to mention TFM, are rapidly corrected and unlikely to negatively affect these non-target fishes in the longer-term.

2.3 Lampricides lead to depletion of liver glycogen stores in lake sturgeon

As observed in sea lamprey and rainbow trout, niclosamide was much more potent than TFM, showing several response similarities, but produced by less than 1/40 the concentration. Both lampricides led to decreases in lake sturgeon brain energy stores necessitating the use of anaerobic processes to meet ATP needs (Figures 7.3 and 7.4). While the brain relied on both glycogen (glycolysis)-derived ATP and PCr buffered ATP supply in TFM-exposed sturgeon (Figure 7.3), in the presence of niclosamide glucose (glycolysis) reserves were used to meet basal ATP needs of this organ (Figure 7.4; however, the prolonged glycolysis induced by both lampricides resulted in elevated lactate concentrations (Figure 7.3 and 7.4). The differential responses to TFM and niclosamide in regard to PCr, glucose and glycogen may be a reflection of low reserves in this organ, especially glycogen, which were 10- and 100-fold greater in rainbow trout and lamprey. Overall, the brain of lake sturgeon appeared to have a very low anaerobic capacity based on not only the low glycogen stores, but very low concentrations of PCr (< 1 μmol $g^{-1}$ ww), as well as ATP.

Instead of endogenous energy reserves in the brain, lake sturgeon appeared to almost exclusively rely on the liver to maintain glucose homeostasis (Figure 7.3 and 7.4), as reflected by the large reduction in liver glucose (TFM only) and glycogen stores observed in that organ. In line with teleost metabolism, lake sturgeon liver glycogen mobilization was markedly higher than in sea lamprey liver, in fact, it was even higher than that of rainbow trout. The higher liver

glycogen mobilization in niclosamide-exposed lake sturgeon, compared to TFM, may also explain the different brain responses to this lampricide.

Despite having the same 9 h niclosamide $LC_{50}$ (0.11 mg $L^{-1}$) as sea lamprey, physiological responses in lake sturgeon were less pronounced. Lake sturgeon are also one of the most sensitive non-target fish to TFM and TFM/1% niclosamide (Boogaard *et al.*, 2003) especially in the juvenile stages (< 100 mm in length). So why then, do lake sturgeon appear to handle niclosamide toxicity better than sea lamprey? Lake sturgeon have a capacity to detoxify TFM via conjugation to a variety of less toxic end products (Bussy *et al.*, 2017a, 2017b), an ability which is very limited in sea lamprey. Perhaps this ability extends to niclosamide. In fact, it appears that the high sensitivity of juvenile lake sturgeon (<100 mm) to TFM (Boogaard *et al.*, 2003) is related to higher uptake rate of the lampricide at this life stage (Hepditch *et al.*, 2019), and not in the capacity to detoxify. As lake sturgeon reach larger sizes uptake rates are reduced as is the sensitivity to TFM (Boogaard *et al.*, 2003). Similar to experiments by Hepditch *et al.* (2019), future investigation into uptake rates are necessary to determine if the same stands true for niclosamide. Nonetheless, the rapid recovery of lake sturgeon from a relatively brief exposure (9 h) to sub-lethal TFM and niclosamide concentrations, and no evidence of continued physiological disruption, is encouraging. This is important in terms of species conservation and integrity of the sea lamprey control program.

## 3. PROPOSED MODEL FOR TFM AND NICLOSAMIDE TOXICITY

In fishes, TFM is primarily taken up in the un-ionized (TFM-OH) form, entering the gills, diffusing down the corresponding gradient (Figure 7.3). Although the bulk of total TFM (total TFM = TFM-OH + $TFM\text{-}O^-$) is present in water in the ionized form, the more acidic gill

microenvironment shifts speciation toward the unionized form, likely augmenting the inwardly diffusion of TFM-OH down the gradient, leading to greater rates of TFM uptake (Figure 7.3). Acidification of the gill microenvironment can occur in a variety of ways, such as excretion of $H^+$ via V-ATPase and/or $Na^+/H^+$ exchanger, arising from carbonic anhydrase (CA) activity in the cytosol, which catalyzes $CO_2$ hydration to $HCO_3^- + H^+$ (see Evans *et al.*, 2005 for review). The hydration of $CO_2$ may also occur in the microenvironment, possibly via external CA (Wilkie *et al.*, 2019). Although uptake of TFM-$O^-$ is thought to be less important quantitatively, studies have shown that sea lamprey take up appreciable amounts of TFM-$O^-$ from alkaline water containing virtually no TFM-OH (Hlina *et al.*, 2017). The mechanism for ionized TFM uptake is unidentified, but one candidate may include the organic anion transporters (OAT) and organic anion transporter polypeptides (OATP; Figure 7.3), which are used in drug transport and present in various mammalian tissues (see Kovacsics *et al.*, 2017; Huo and Liu, 2018 for reviews), as well as in some fish gills (Armitage *et al.*, 2017), including sea lamprey (Cai *et al.*, 2013). Once TFM enters the blood, it is transported to tissues where its toxic effects are exerted by uncoupling oxidative phosphorylation, thereby decreasing ATP production in the mitochondria, which leads to a variety of physiological disruptions, as described above and depicted in Figure 7.3. Niclosamide is expected to be taken up and behave in a similar manner (Figures 7.1, 7.2 and 7.4).

Uncouplers of oxidative phosphorylation, typically lower transmembrane potential ($\Delta\Psi m$), by acting as protonophores (proton shuttles) or increasing permeability of the inner mitochondrial membrane to protons, effectively 'disconnecting' pumping of protons from ATP-synthase. The resultant reduction in $H^+$ flow through ATP-synthase leads to decreased ATP generation. The volume of research critically focusing on the uncoupling potential of TFM and

niclosamide is surprisingly small, especially compared to research involving other uncouplers (e.g. 2,4 dinitrophenol; Wallace and Starkov, 2000). A more recent study, first demonstrated that TFM uncoupled oxidative phosphorylation in fish, using isolated liver mitochondria from adult sea lamprey and rainbow trout (Birceanu *et al.*, 2011). In addition, this group showed that TFM led to mitochondrial membrane depolarization, indicative of electrochemical gradient breakdown between the intermembrane space and mitochondrial matix. Based on this observation, they concluded that decreased passage of $H^+$ through ATP-synthase would account for decreased ATP production in the presence of this lampricide (Birceanu *et al.*, 2011). Others, tested the effects of Phase I TFM reductive metabolites, such as amino-TFM (TFMa), on cardiac mitochondrial oxygen consumption, and found that complex I and II of the electron transport chain were heavily affected in a dose dependant manner (Huerta *et al.*, 2020). Thus, the toxic effects of TFM are not only exerted on complex V but also Complexes I and II.

The uncoupling potential of salicylanilides such as niclosamide have been shown in helminth parasites (Weinbach and Garbus, 1969; Wilson *et al.*, 1971; Van Den Bossche, 1985), like TFM, acting as protonophores and disrupting the proton motive force, thereby reducing ATP production (Wilson *et al.*, 1971; Kaplay *et al.*, 1970; Jurgeit *et al.*, 2012). The protonated (neutral) form of niclosamide passes from the acidic intermemebrane space, through the inner mitochondrial membrane, to the more alkaline matrix where it releases its ionizable $H^+$, which is followed by diffusion down its electrochemical gradient, back to the intermembrane space, where it traps a new $H^+$ (Terada, 1990). This process occurs in human cancer cell cultures (HeLa cells), where mitochondrial membrane depolarization occurs, leading to apoptosis and autophagy (Park *et al.*, 2011).

The studies presented in Chapters 2, 3 and 5 of this dissertation are the first to report physiological effects of niclosamide in fishes, but the data offered is not exhaustive, especially given that this chemical's potential may not be restricted to uncoupling of oxidative phosphorylation (Khanim *et al.*, 2011; Park *et al.*, 2011). It has been shown that niclosamide inhibits growth of some cancer cells (anti-neoplastic effect; Khanim *et al.*, 2011; Park *et al.*, 2011), while some salisylanilides disrupt pH regulation and inhibit glycolytic enzymes in helminths, thereby impeding their ability to use glycolysis for ATP production (Köhler, 2001). If this is the case in fishes, it may be a contributing factor to the higher potency of niclosamide compared to TFM. These findings suggest that, beyond similarities in their mode of action, there are many unresolved questions in regard to differential pharmokinetics and/or pharmacodynamics of TFM and niclosamide.

## 4. IMPLICATIONS FOR SEA LAMPREY CONTROL

The value of fisheries in the Great Lakes is estimated to exceed seven billion US dollars, but unabated invasive species, such as sea lamprey, impact the economy resulting in large revenue losses every year (Krantzberg and De Boer, 2008; GLFC, 2011). An implementation of an integrative pest management program which primarily relies on chemical control, and barriers and traps to block adult sea lamprey during their spawning migration, has been highly successful in curbing sea lamprey populations, contributing to the recovery of recreational, commercial and culturally significant fisheries of the Great Lakes (GLFC 2011; Siefkes 2017). This thesis has generated a better understanding of modes of action and adverse physiological effects of TFM and niclosamide in sea lamprey and non-target fishes (Figures 7.1-7.4).

The better understanding of how non-target species respond to lampricide exposure could be applied in order to minimize risk of adverse effects. The present findings suggest that the decreased glucose supply to the brain and the depletion of liver glycogen reserves, that are a major consequence of lampricide exposure, are readily reversed following lampricide exposure (within 24 h). With this knowledge, it may be possible to identify times of the year and life stages, where lampricide application is potentially less harmful. For instance, young-of-the-year lake sturgeon are most vulnerable to TFM and niclosamide due to their smaller sizes and higher rates of TFM uptake (Hepditch *et al.*, 2019). Hence, delaying treatments until later in the summer or early autumn when the sturgeon are larger, and better able to correct lampricide-induced disruptions to their energy metabolism, could be adopted as a mitigation measure. It might also be possible to alter methods of TFM-niclosamide application to protect non-target species, without sacrificing lampricide effectiveness. For instance, more prolonged treatment periods at lower concentrations, might still effectively kill larval lamprey without concentrations of TFM and/or niclosamide reaching lethal levels in non-target species. Knowing that non-target fishes such as rainbow trout and lake sturgeon can detoxify TFM more effectively than larval lamprey means that lampricides would be less likely to reach toxic concentrations than in sea lamprey over longer exposure periods. As the present thesis demonstrates, any physiological disturbances resulting from TFM and niclosamide in trout or lake sturgeon would be expected to be lower and completely reversible because less lampricide would accumulate than in sea lamprey.

Combining modifications in lampricide application procedures, with other emerging sea lamprey control strategies, such as using pheromones, genetic-based technologies and ‘next

generation lampricides' (Wilkie *et al.*, 2019), could also be promising approaches to ensure continued success of the sea lamprey control program, and such research must continue. While the effects of TFM and niclosamide on aquatic ecosystems are relatively minimal when used appropriately, some non-target mortalities may occur, and these incidences are the focus of much publicity. Such events and the general aversion to chemical pest control, has led to increasing public and regulatory concerns regarding the use and effects of lampricides on aquatic biota. Therefore, it is of cardinal importance to acquire further knowledge on the biology and sensitivity of non-target species to lampricides, as the current thesis has. Equipped with such knowledge the GFLC, sea lamprey control agents, regulatory agencies, policy makers and the public will have more information that can be used to better predict, identify and mitigate potential adverse effects on non-target organisms arising from lampricide treatment in tributaries draining into the Great Lakes and other waters infested with sea lamprey. Finally, further advancements in sea lamprey control measures, including lampricides, could lead to new approaches for controlling other invasive species (vertebrates, invertebrates, plants and microorganisms) which are straining aquatic ecosystems globally.

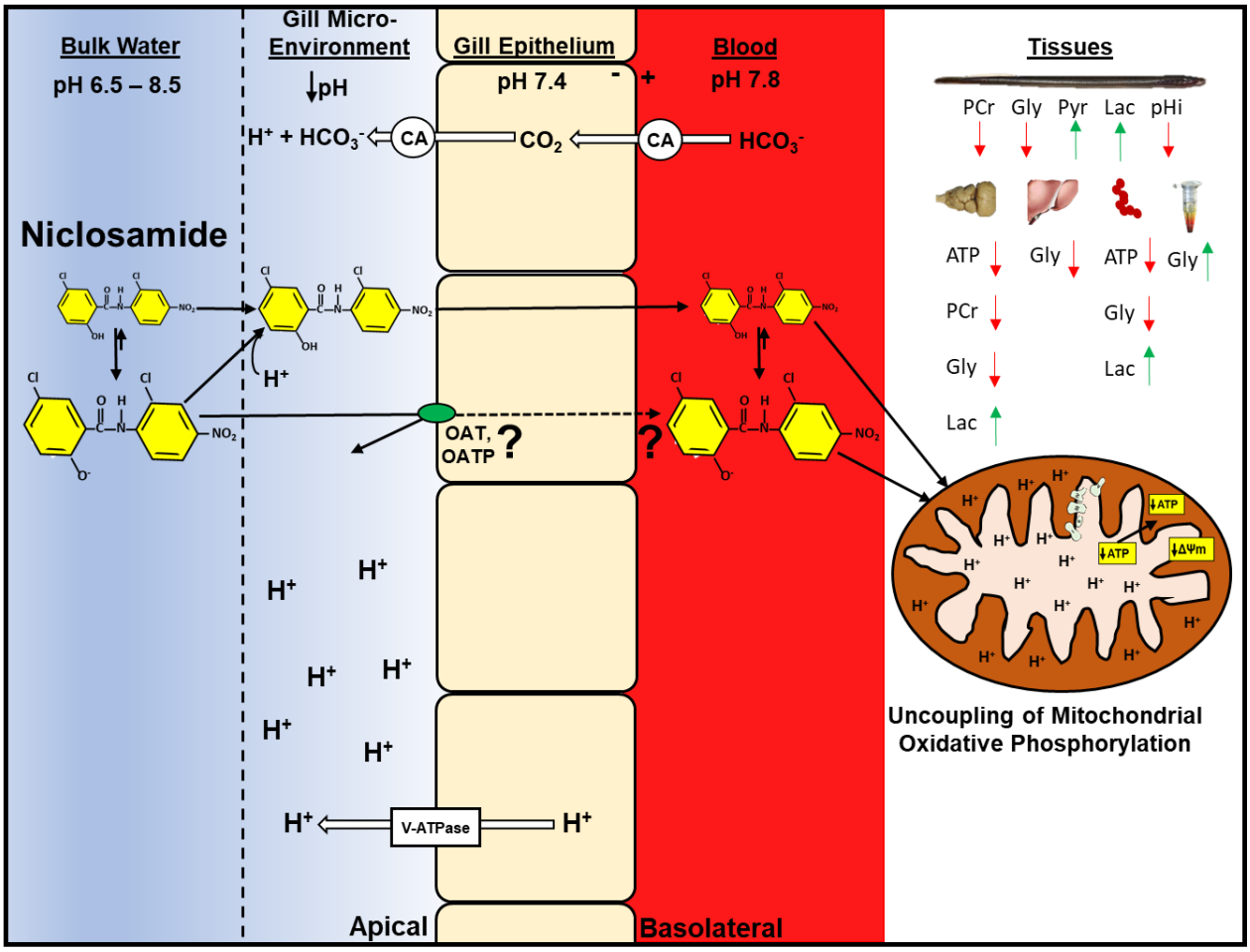


**Figure 7.1 Proposed model for uptake and toxicity of niclosamide in larval sea lamprey**

**Figure 7.1 Proposed model for uptake and toxicity of niclosamide in larval sea lamprey.** Ionized niclosamide (lipophilic; NIC-$O^-$) from bulk water enters the more acidic gill microenvironment of larval sea lamprey (*Petromyzon marinus*) where the equilibrium shifts toward formation of unionized (lipophilic; NIC-OH) species. This increases diffusion rate of NIC-OH through the gill epithelium, entering the blood to be transported to different tissues where niclosamide uncouples mitochondrial oxidative phosphorylation leading to physiological disturbances in muscle, brain, liver, kidney and plasma. NIC-$O^-$ may also enter the gills, facilitated by possible transporters such as organic anion transporters (OAT) and organic anion transporter polypeptides (OATP). Sea lamprey use high concentrations of endogenous brain glycogen to maintain glucose homeostasis of the central nervous system (CNS).

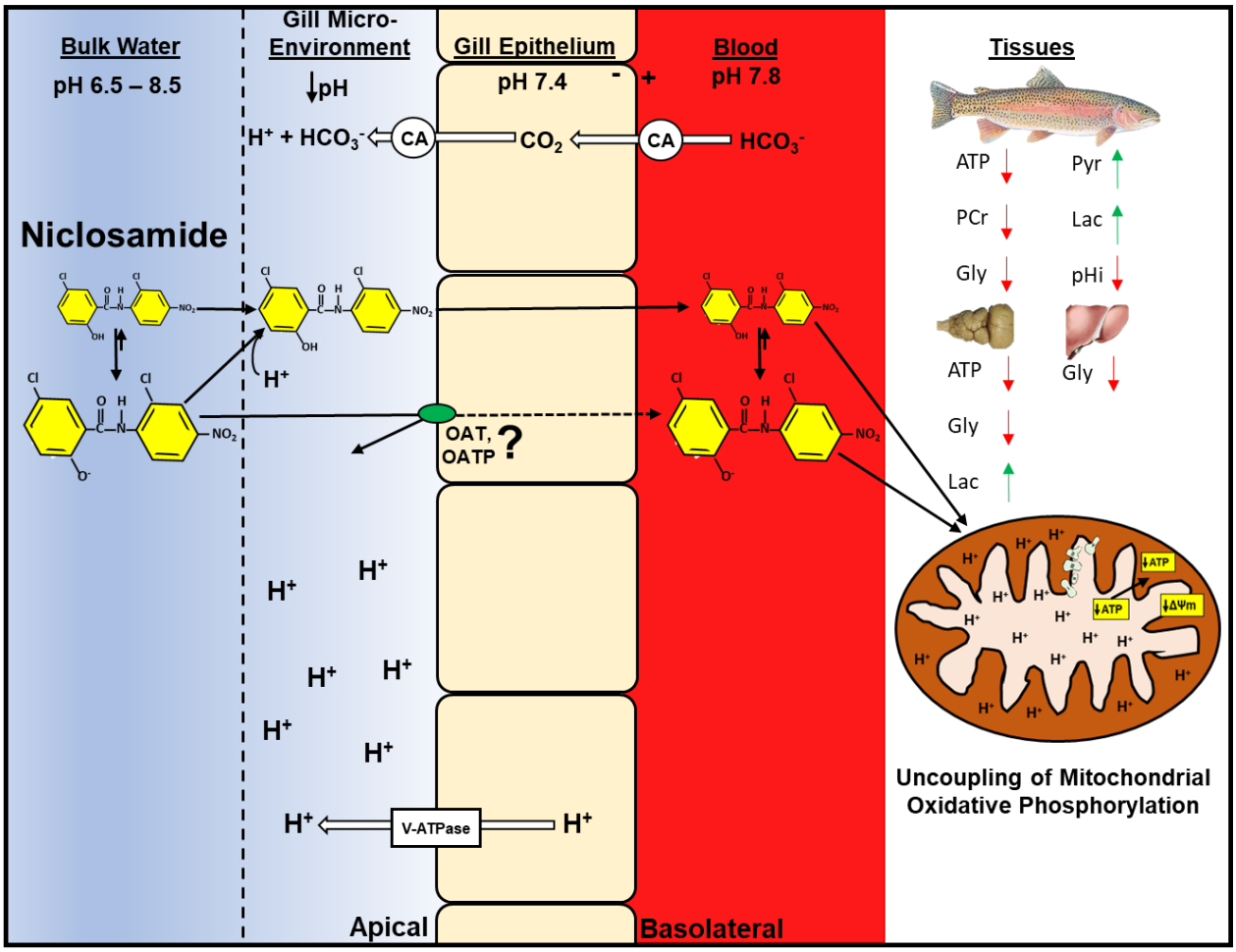


**Figure 7.2 Proposed model for uptake and toxicity of niclosamide in rainbow trout**

**Figure 7.2 Proposed model for uptake and toxicity of niclosamide in rainbow trout.** Ionized niclosamide (lipophilic; NIC-$O^-$) from bulk water enters the more acidic gill microenvironment of rainbow trout (*Oncorhynchus mykiss*) where the equilibrium shifts toward formation of unionized (lipophilic; NIC-OH) species. This increases diffusion rate of NIC-OH through the gill epithelium, entering the blood to be transported to different tissues where niclosamide uncouples mitochondrial oxidative phosphorylation leading to physiological disturbances in muscle, brain and liver. NIC-$O^-$ may also enter the gills, facilitated by possible transporters such as organic anion transporters (OAT) and organic anion transporter polypeptides (OATP). Rainbow trout was most profoundly affected in the muscle by niclosamide, followed by the liver which plays an important role in glucose homeostasis.

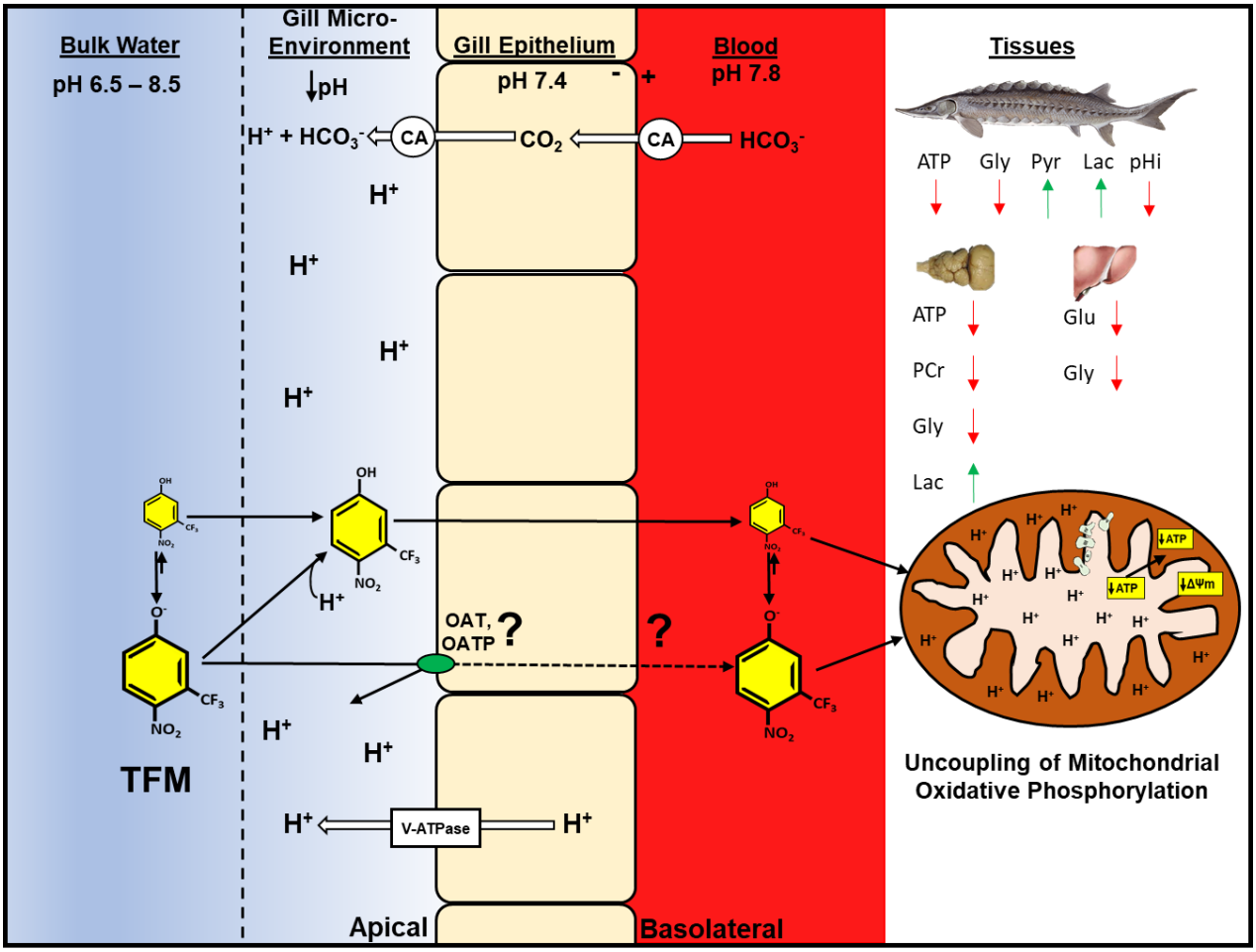


**Figure 7.3 Proposed model for uptake and toxicity of TFM in lake sturgeon**

**Figure 7.3 Proposed model for uptake and toxicity of TFM in lake sturgeon.** Ionized TMF (lipophilic; TFM-$O^-$) from bulk water enters the more acidic gill microenvironment of lake sturgeon (*Acipenser fulvescens*) where the equilibrium shifts toward formation of unionized (lipophilic; TFM-OH) species. This increases diffusion rate of TFM-OH through the gill epithelium, entering the blood to be transported to different tissues where TFM uncouples mitochondrial oxidative phosphorylation leading to physiological disturbances in muscle, brain and liver. TFM-$O^-$ may also enter the gills, facilitated by possible transporters such as organic anion transporters (OAT) and organic anion transporter polypeptides (OATP). Lake sturgeon was most affected by TFM in the liver and brain, with the liver playing an important role in glucose homeostasis.

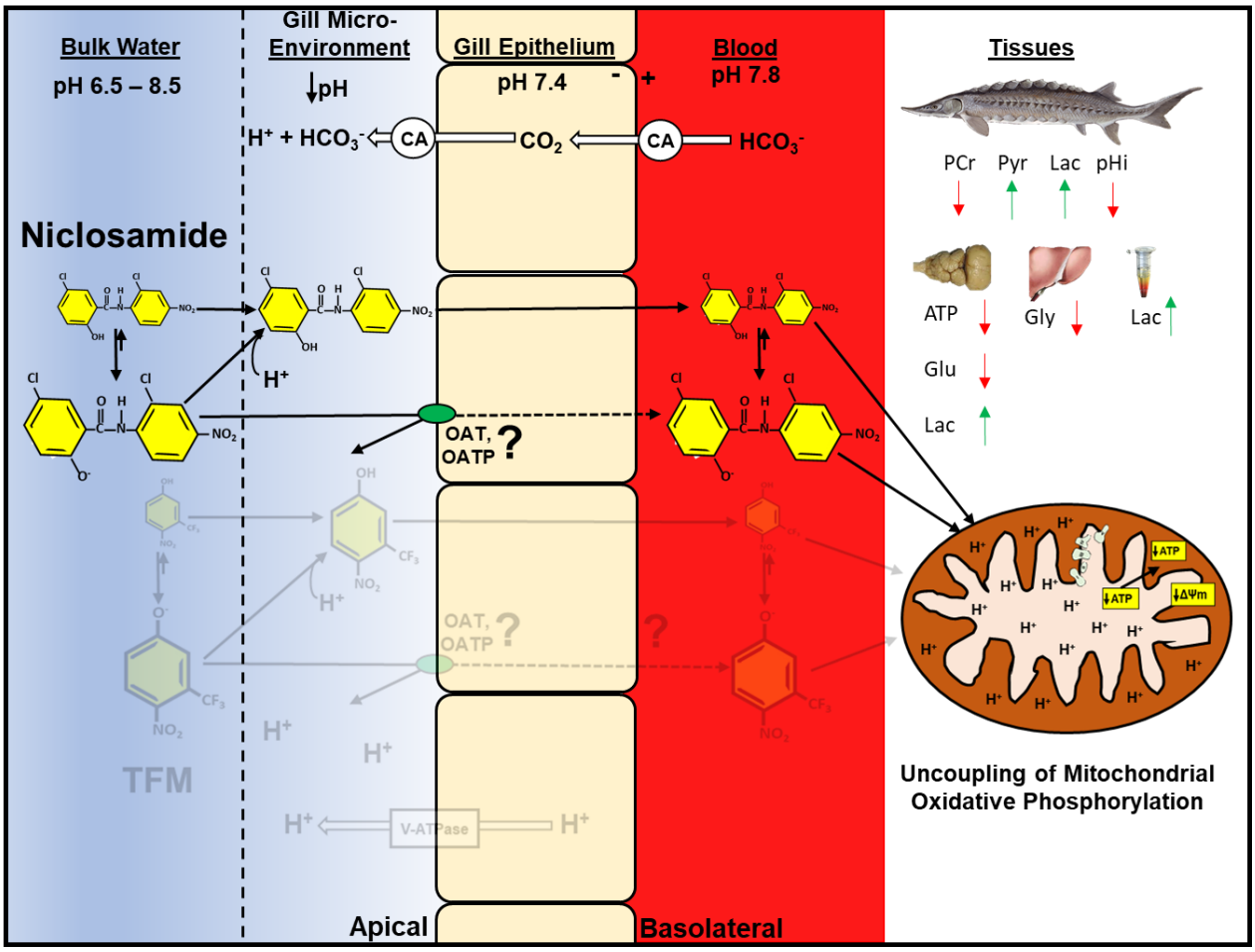


**Figure 7.4 Proposed model for uptake and toxicity of niclosamide in lake sturgeon**

**Figure 7.4 Proposed model for uptake and toxicity of niclosamide in lake sturgeon.** Ionized niclosamide (lipophilic; NIC-$O^-$) from bulk water enters the more acidic gill microenvironment of lake sturgeon (*Acipenser fulvescens*) where the equilibrium shifts toward formation of unionized (lipophilic; NIC-OH) species. This increases diffusion rate of NIC-OH through the gill epithelium, entering the blood to be transported to different tissues where niclosamide uncouples mitochondrial oxidative phosphorylation leading to physiological disturbances in muscle, brain, liver and plasma. NIC-$O^-$ may also enter the gills, facilitated by possible transporters such as organic anion transporters (OAT) and organic anion transporter polypeptides (OATP). Lake sturgeon were most affected by niclosamide in the liver and brain, with the liver playing an important role in glucose homeostasis.

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