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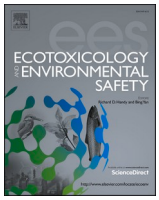

Disturbances to energy metabolism in juvenile lake sturgeon (*Acipenser fulvescens*) following exposure to niclosamide

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ABSTRACT

Since the 1960s, invasive sea lamprey (*Petromyzon marinus*) populations in the Laurentian Great Lakes have been controlled by applying two chemicals, 3-trifluoromethyl-4-nitrophenol (TFM) and 2',5-dichloro-4'-nitrosalicylanilide (niclosamide, aka. Bayluscide®), to streams infested with larval sea lamprey. These "lampricide" applications primarily rely on TFM, and are often combined with 1–2% niclosamide, which increases treatment effectiveness. Niclosamide is also used alone to treat lentic habitats and in rivers with high discharge. However, little is known about niclosamide's possible adverse physiological effects on non-target organisms. Of particular concern is the lake sturgeon (*Acipenser fulvescens*), which is threatened throughout the Great Lakes basin where its habitat often overlaps with larval lamprey. Because niclosamide is believed to impair ATP production by uncoupling oxidative phosphorylation, we determined how it altered metabolic processes and acid-base balance in young-of-the-year (YOY) lake sturgeon exposed to their 9-h LC_{50} of niclosamide (0.11 mg L^{-1}) for 9 h. Exposure to niclosamide led to decreased brain ATP and glucose reserves, and increased lactate, with no effect on brain glycogen. In contrast, substantial (60%) reductions in glycogen were observed in liver, suggesting that hepatic glycogen reserves were mobilized to meet the brain's glucose requirements when ATP supply was impaired during niclosamide exposure. Disturbances in carcass included reduced phosphocreatine (65–70%), 2- and 4-fold increases in pyruvate and lactate, and a slight metabolic acidosis, characterized by a 0.1 unit decrease in intracellular pH (pHi). Each of these disturbances were corrected within 24 h following depuration in clean (niclosamide-free) water. We conclude that if lake sturgeon survive exposure to niclosamide, they are able to rapidly replenish their energy stores (glycogen, ATP, phosphocreatine) and correct any corresponding metabolic disturbances within 24 h.

1. Introduction

Once widespread in and around the Laurentian Great Lakes of North America, lake sturgeon (*Acipenser fulvescens*) populations were decimated in the 19th and early 20th centuries due to overfishing, pollution, and habitat destruction and loss (Becker, 1983; Golder and Associates Ltd, 2011; Harkness and Dymond, 1961; Pratt et al., in press). Efforts to restore lake sturgeon populations in the Great Lakes have been hindered by a continued lack of suitable habitat, barriers to their migration, the length of time it takes for the fish to reach sexual maturity (15–25 years) and long intervals between spawning (up to 5 years; Scheidegger, 2012). As a result, lake sturgeon populations in the Great Lakes-St. Lawrence region are listed as threatened in Canada and the United States (COSEWIC, 2017; Hayes and Caroffino, 2012), with populations considered endangered in the Province of Ontario, Canada (COSSARO, 2017).

Declines in lake sturgeon populations coincided with the invasion of the Great Lakes by parasitic sea lamprey (*Petromyzon marinus*), beginning in late 1800s following modifications to the Welland Canal, which devastated commercial and culturally significant fisheries including lake trout (*Salvelinus namaycush*; Eshenroder, 2014; Lawrie, 1970; Smith and Tibbles, 1980). In response, the Great Lakes Fishery Commission implemented a sea lamprey control program incorporating barriers and traps to prevent adult lamprey from spawning, and chemical control using the piscicides (aka. lampricides) 3-trifluoromethyl-4 nitrophenol (TFM) and niclosamide, which were applied to rivers and streams infested with larval sea lamprey (Siefkes, 2017; Wilkie et al., 2019) Although TFM mainly targets larval lamprey, due to their relatively low

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capacity to detoxify this lampricide (Lech and Statham, 1975; Kane et al., 1994; Bussy et al., 2017a, 2017b), non-target fishes such as the lake sturgeon, have also been shown to be susceptible to TFM in their early juvenile stages (< 100 mm) as well as to TFM/1% niclosamide mixtures (Boogaard et al., 2003; O'Connor et al., 2017).

The toxicity of TFM and niclosamide is related to their impairment of mitochondrial function by uncoupling oxidative phosphorylation (Alasadi et al., 2018; Birceanu et al., 2009; Huerta et al., 2020). With TFM, this results in decreased ATP production and a greater need to rely on anaerobic glycolysis in the brain and muscle to make up for ATP shortfalls, leading to metabolic disturbances that include the depletion of glycogen reserves, reduced concentrations of phosphocreatine, and lactate accumulation in sea lamprey, rainbow trout (*Oncorhynchus mykiss*), and lake sturgeon (Birceanu et al., 2014, 2011, 2009; Clifford et al., 2012; Ionescu et al., 2021). However, less is known about how niclosamide affects the tissue energy stores of non-target fishes such as lake sturgeon, or how resilient they are to episodic, sub-lethal exposure to the lampricide.

In the last 10–15 years, several studies have improved our understanding regarding the toxicity and physiological effects of TFM in lake sturgeon (Boogaard et al., 2003; Bussy et al., 2017a, 2017b; Hepditch et al., 2021; Ionescu et al., 2021; Middaugh et al., 2014; O'Connor et al., 2017; Sakamoto et al., 2016). Only a few, on sea lamprey (Lawrence et al., 2021; Ionescu et al., 2021) and in zebrafish (*Danio rerio*; Zhu et al., 2019), have explored the physiological effects of niclosamide exposure. In lake sturgeon, such information could prove helpful in developing lampricide application strategies to effectively control sea lamprey without compromising lake sturgeon conservation efforts in the Great Lakes.

The goal of this study was to acquire a better understanding of the physiological effects that sub-lethal exposure to niclosamide had on young-of-the-year (YOY) lake sturgeon and their ability to restore homeostasis after exposure. We, therefore, exposed juvenile lake sturgeon to their pre-determined 9-h LC_{50} of niclosamide for 9 h, during which time, brain, muscle, liver and blood samples were collected at different time intervals for analysis of tissue ATP, phosphocreatine (PCr), glycogen, lactate, and related metabolites. Because high rates of glycolysis often results in accumulation of H^+ derived from ATP hydrolysis, intracellular pH (pHi) was also measured in the muscle (carcass) to quantify any disturbances to acid-base balance. The resiliency of lake sturgeon to sub-lethal niclosamide exposure was assessed by collecting and analyzing samples following a 24 h post-niclosamide exposure recovery period.

2. Methods and materials

2.1. Animal husbandry

Lake sturgeon fertilized eggs, in the eyed stage, were provided courtesy of Joe Hunter, Sustainable Sturgeon Culture, Emo, Ontario, Canada, and reared at the Alma Aquaculture Research Station, University of Guelph, 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 (WLU) on March 15, 2019, where they were housed in a circular tank (water volume ~ 400 L) continuously receiving aerated WLU well water (temperature = 14.5 ± 0.5 °C; alkalinity = 280 ± 5 mg L^{-1} $CaCO_3$; pH = 7.93 ± 0.1; dissolved O_2 = 95 ± 2%) at a rate of 1 L min^{-1} at a stocking density of 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 and alkalinity] were checked daily whereas chlorine and total ammonia were checked weekly. Experiments were completed following a minimum of 2 weeks holding and followed Canadian Council of Animal Care guidelines and were approved by the WLU Animal Care Committee (Animal Use Protocol No. R18005).

2.2. Niclosamide toxicity tests

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 WLU 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 the field formulation of niclosamide (Bayluscide® emulsifiable concentrate, containing 16.9% active ingredient; Coating Place Inc., Verona, WI, USA), which was provided courtesy of the Sea Lamprey Control Centre, Sault St. Marie, Ontario, Canada. Separate groups of lake sturgeon (N = 12 each) were exposed to different concentrations of niclosamide (0.05, 0.075, 0.10, 0.11, 0.13, 0.17, 0.25, 0.50 or 1.00 mg L^{-1}), 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 Litchfield and Wilcoxon (1949) using online free-ware (https://jvadams.shinyapps.io/LW1949demo/).

2.3. Experimental procedures

All experiments were conducted using the same stock of field formulation niclosamide, dissolved in 50% methanol, as described above. Experimental aquaria (N = 24; 10 L maximum volume) were filled with 8 L of WLU well water 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, brain, liver and carcass (whole body minus the head and viscera) 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-exposure recovery, after which they were euthanized, followed by blood and tissue collection.

Water samples (10 mL) were collected from sub-sets of aquaria at the beginning (0 h) and at the end of the experiment (9 h) for later determination of niclosamide concentration, which did not change from the target concentration of 0.11 mg L^{-1} over the 9 h exposure period. The relatively small total biomass of animals (3 animals per aquaria; total biomass ~ 10 g) compared to the total volume of water containing niclosamide in the system (8 L) likely minimized changes in water niclosamide concentration due to uptake by the animals. In addition, continual mixing of the water via aeration helped to ensure that the dissolved niclosamide was evenly distributed in the water.

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% perchloric acid (PCA) containing 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), centrifuged at 10, 000 *g* for 5 min 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 − 80°C.

2.4. Quantification of niclosamide in water

Water samples (10 mL) were collected at 0 h and 9 h (conclusion of exposure) from a sub-set of aquaria, transferred into 20 mL glass scintillation vials and stored at − 20 °C until analysis. Prior to analysis, samples were completely thawed at room temperature vortexed for 20 s, with 5 mL of each sample, standard or blank transferred to a new, clean glass test tube and spiked with niclosamide-(2-chloro-4-nitrophenyl-13C6) hydrate (200 μg L^{-1}) internal standard and vortexed for an additional 20 s. Samples were then filtered through 0.45 μm glass fiber filters (Pall Corporation, Michigan, USA) using vacuum filtration (15 mL) and collected into clean glass test tubes from which 1 mL of the eluent was transferred into a 2 mL amber glass vial for subsequent LC-MS/MS analysis using an Agilent 1260 HPLC with 6460 Triple Quad and Agilent Jetstream and electrospray ionization source in negative ionization mode. An Agilent Eclipse XDB-C18 column (4.6 ×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). Instrument source parameters were set as follows: temperature = 400 °C for evaporation, gas temperature = 230 °C, gas flow = 12 L min^{-1}, nebulizer set to 275.8 kPa, and capillary voltage = 2500 V.

All data was processed using Mass Hunter Quantitative Analysis software (Agilent, Santa Clara, USA) version B.05.02. Niclosamide sample recoveries were 88.1 – 99.4%. The calibration curve ranged from 0 to 500 μg L^{-1} niclosamide, made up in HPLC grade methanol (Optima, LC/MS grade, Fisher Scientific, Ottawa, Canada), with a detection limit of 0.08 μg L^{-1}. Sample niclosamide concentrations were determined by quadratic regression (y = 9.374034E-0.005 ×2 + 0.123684x + 0.034079; R^2 = 0.9992663), after adjusting for background.

2.5. Tissue preparation for analyses

Procedures for tissue metabolite extraction followed those outlined in Bergmeyer (1983), and modified as described in Wilkie et al. (2001, 1997). Briefly, using an insulated mortar and pestle, lake sturgeon carcasses were ground to a fine powder under liquid N_2 and samples acidified for 10 min with 4 volumes of 8% PCA containing 1 mmol L^{-1} EDTA, 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, phosphocreatine (PCr), 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 and liver were similar to carcass but due to the smaller amounts of tissue available, brain (< 50 mg) or liver (<100 mg) was added to 1.5 mL microcentrifuge tubes and homogenized on ice using a hand-held, motorized, plastic pestle homogenizer (Gerresheimer Kimble Kontes LLC, Dusseldorf, Germany) in 4 volumes 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.

2.6. Analytical techniques

Tissue (carcass, brain 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 37 °C. 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 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 was analyzed in the same manner as described above for other tissues.

2.7. Intracellular pH determination

Carcass intracellular pH (pHi) measurements followed methods described by Pörtner (1990). Briefly, carcass (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 s and pulsed in a centrifuge for ~10 s 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 min before final readings were recorded.

2.8. 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).

2.9. 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 ± 1 SEM, with the level of significance set to $P < 0.05$.

3. Results

3.1. 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), nominal niclosamide concentrations of 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 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 died after 3 h and 2 after 6 h exposure. At a 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. The corresponding 9-h LC_{50} for niclosamide was calculated to be 0.111 mg L^{-1} (95% CI = 0.105–0.118 mg L^{-1}).

3.2. Physiological responses to niclosamide exposure

3.2.1. Water niclosamide concentration

Based on the acute toxicity test, the nominal concentration of niclosamide (9-h LC_{50}) to which the sturgeon were exposed was 0.11 mg L^{-1}. The measured niclosamide concentrations were maintained near this value, averaging 0.12 ± 0.005 mg L^{-1} (Standard deviation).

3.2.2. Survival

Mortality was observed when the lake sturgeon were exposed to a nominal niclosamide concentration of 0.11 mg L^{-1}, but not until the latter stages of exposure when 1 of 3 fish from 3 different tanks died after 6 h. Similarly, 2 of 3 fish in one tank and 1 of 3 fish in another tank died following 9 h of exposure. Hence, the physiological responses reported reflect the responses of lake sturgeon that survived exposure, not those that died.

3.2.3. 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, or carcass control fish (not exposed to niclosamide) sampled at the beginning of the experiment (time 0 h) and those sampled after 9 h. Nor were any differences in plasma lactate or ion concentrations observed. 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.

3.2.4. Effects of niclosamide 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 (Fig. 1). Exposure to the measured 9-h LC_{50} of niclosamide, 0.1mg 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 (Fig. 1A). Brain PCr concentrations were not significantly affected by niclosamide exposure (Fig. 1B).

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 (Fig. 2). 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 (Fig. 2A). Brain glycogen levels remained unchanged throughout the experiment (Fig. 2B). 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 (Fig. 2C).

3.2.5. Effects of niclosamide 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 (Fig. 3). Liver glucose remained unchanged in the presence of niclosamide relative to

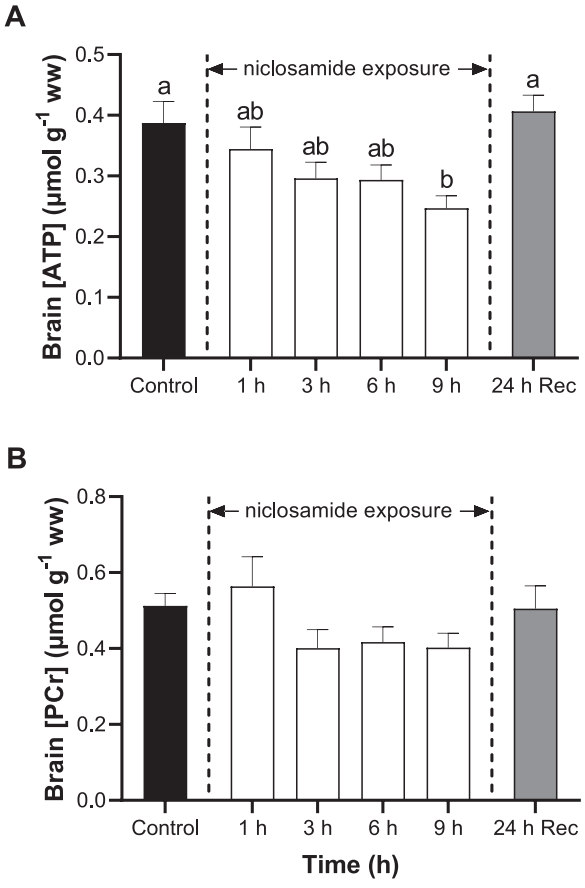

Fig. 1. Effects of niclosamide on ATP and phosphocreatine in the brain. Changes in the concentrations of (A) ATP and (B) PCr in the brain of lake sturgeon (*Acipenser fulvescens*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 11; gray bars) at a concentration of 0.12 mg 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 ($P \leq 0.05$).

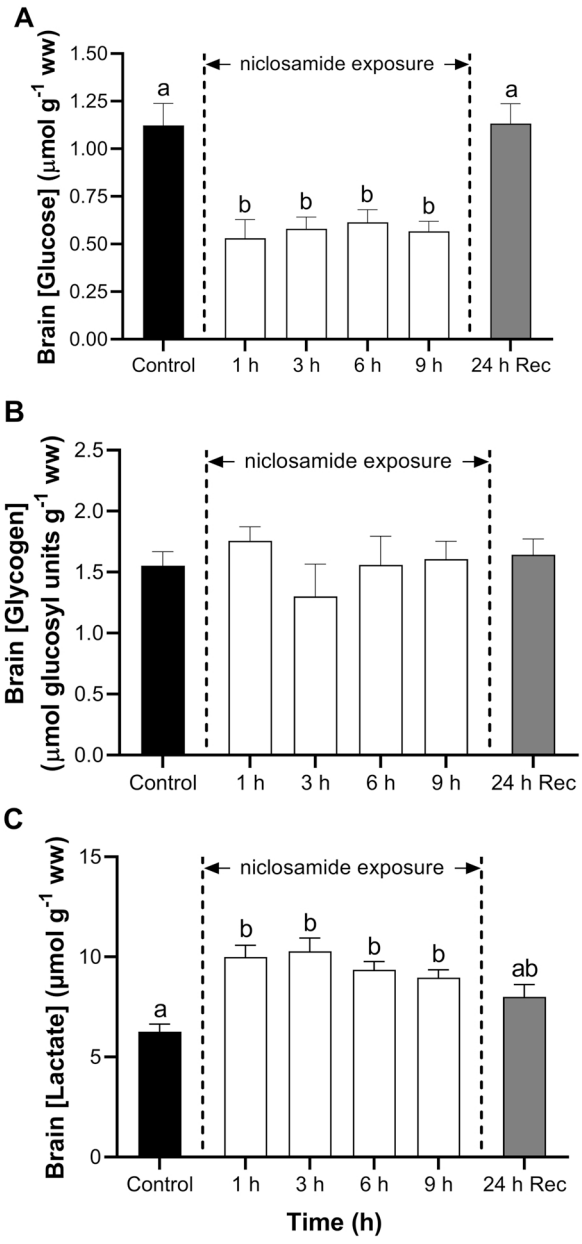

Fig. 2. Effects of niclosamide on brain glycogen. Changes in the concentrations of (A) glucose, (B) glycogen and (C) lactate in the brain of lake sturgeon (*Acipenser fulvescens*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 11; gray bars) at a concentration of 0.12 mg 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 ($P \leq 0.05$).

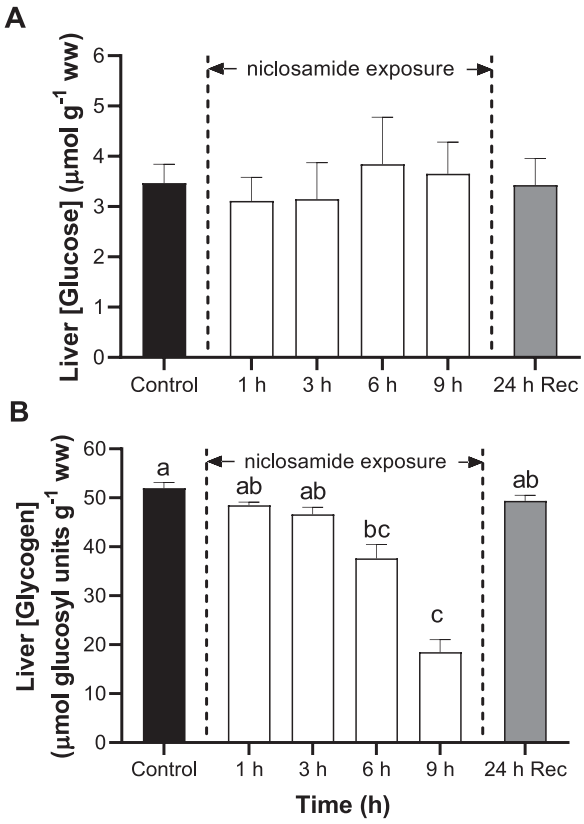

Fig. 3. Effects of niclosamide on hepatic glycogen. Changes in the concentrations of (A) glucose and (B) glycogen in the liver of lake sturgeon (*Acipenser fulvescens*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 12; gray bars) at a concentration of 0.12 mg L^{-1} (9 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 ($P \leq 0.05$).

controls (Fig. 3 A) 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 (Fig. 3B).

3.2.6. *Effects of niclosamide 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 (Fig. 4). 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. Carcass ATP was not significantly different from controls following 24 h recovery (Fig. 4 A). Carcass PCr concentrations experienced an immediate and sustained reduction of approximately 60% in the presence of niclosamide relative to controls, returning to pre-exposure levels after 24 h recovery (Fig. 4B).

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 (Fig. 5). No changes were observed in carcass glucose concentration during exposure to niclosamide (Fig. 5 A). Carcass glycogen concentrations in the niclosamide treated sturgeon were not significantly different from controls during the exposure or post-exposure recovery period, but were highly variable, with a significant difference between 3 h (Fig. 5B).

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 (Fig. 5C, D). In the presence of niclosamide carcass pyruvate concentrations experienced an immediate and sustained elevation of approximately 50% relative to controls but returned to pre-exposure levels after 24 h recovery (Fig. 5C). In the presence of niclosamide, carcass lactate significantly increased by 2-fold at 1, 6 and 9 h, and by 3-fold after 3 h, but returned to control concentrations after 24 h recovery (Fig. 5D). The elevation of carcass lactate was mirrored in the plasma, in which pre-exposure lactate concentrations averaged 0.5 ± 0.1 µmol but increased by approximately 2-fold during niclosamide exposure, before declining towards pre-experimental concentrations after 24 h recovery (Fig. 5E).

Carcass pHi averaged 7.12 ± 0.002 in control, with niclosamide exposure resulting in a slight acidosis characterized by stepwise

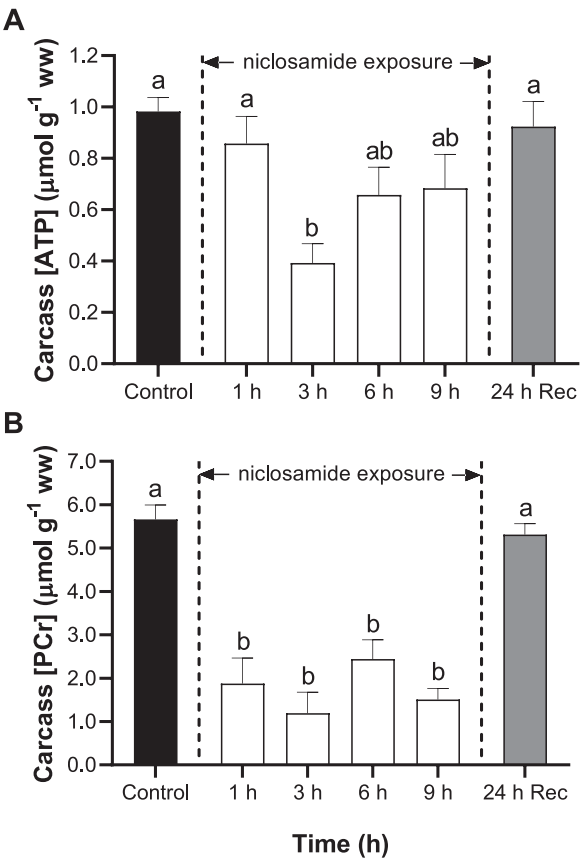

Fig. 4. Carcass energy stores and metabolites in niclosamide-exposed lake sturgeon. Changes in the concentrations of (A) ATP and (B) PCr in the muscle of lake sturgeon (*Acipenser fulvescens*) during (open bars) and following exposure to niclosamide (24 h recovery; n = 12; gray bars) at a nominal concentration of 0.12 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 ($P \leq 0.05$).

decreases in pHi of 0.03, 0.04 and 0.10 pH units at 3 h, 6 h and 9 h, respectively. However, pHi had returned to pre-exposure values after 24 h of recovery (Fig. 5F).

3.2.7. *Effects of niclosamide on plasma ions*

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, and were unaffected by niclosamide exposure (Table 1).

4. Discussion

4.1. *Niclosamide interferes with ATP production in lake sturgeon*

Young-of-the-year lake sturgeon that survived exposure to niclosamide at a concentration near its 9-h LC_{50} (0.12 mg L^{-1}), experienced significant decreases in brain ATP and glucose, and marked reductions in liver glycogen. As with TFM (Birceanu et al., 2011), niclosamide is thought to target the mitochondria, uncoupling oxidative phosphorylation, leading to a decrease in ATP production (e.g. Li et al., 2003; 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; Birceanu et al., 2009; Clifford et al., 2012). The situation is similar in lake sturgeon exposed to TFM (Ionescu et al., 2021) 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., 2014, 2009; 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 ATP demands increase, such as during bursts of muscular activity (McLeish and Kenyon, 2005; Moyes and West, 1995).

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 a very low anaerobic capacity characterized by very low glycogen concentrations, less than 1 µmol g^{-1} ww, which is about 1/10 the concentrations measured in rainbow trout and 1/100 than those 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). The lake sturgeon brain also has very low concentrations of phosphocreatine, less than 1 µmol g^{-1} ww, which are about 1/5–1/10 the concentrations measured in the brain of sea lamprey and rainbow trout (Birceanu et al., 2014, 2009; 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, 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 in this critical organ.

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 in sturgeon suggests that its 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 strongly suggests that this need for glucose was met by increased reliance on hepatic glycogenolysis.

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. 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 because the brain would be starved of its primary energy reserve, glucose.

Despite the adverse physiological effects of niclosamide exposure, even at sub-lethal concentrations, lake sturgeon readily recovered within 24 h following exposure. Of particular interest was the recovery of glycogen in the liver and the muscle. Numerous studies have shown that glycogen recovery in the muscle following exhaustive exercise in fish may take up to 12–24 h but recovery is variable and species dependant (Kieffer, 2000). In mammals it was long believed that glycogen regeneration took place via the Cori cycle in which muscle

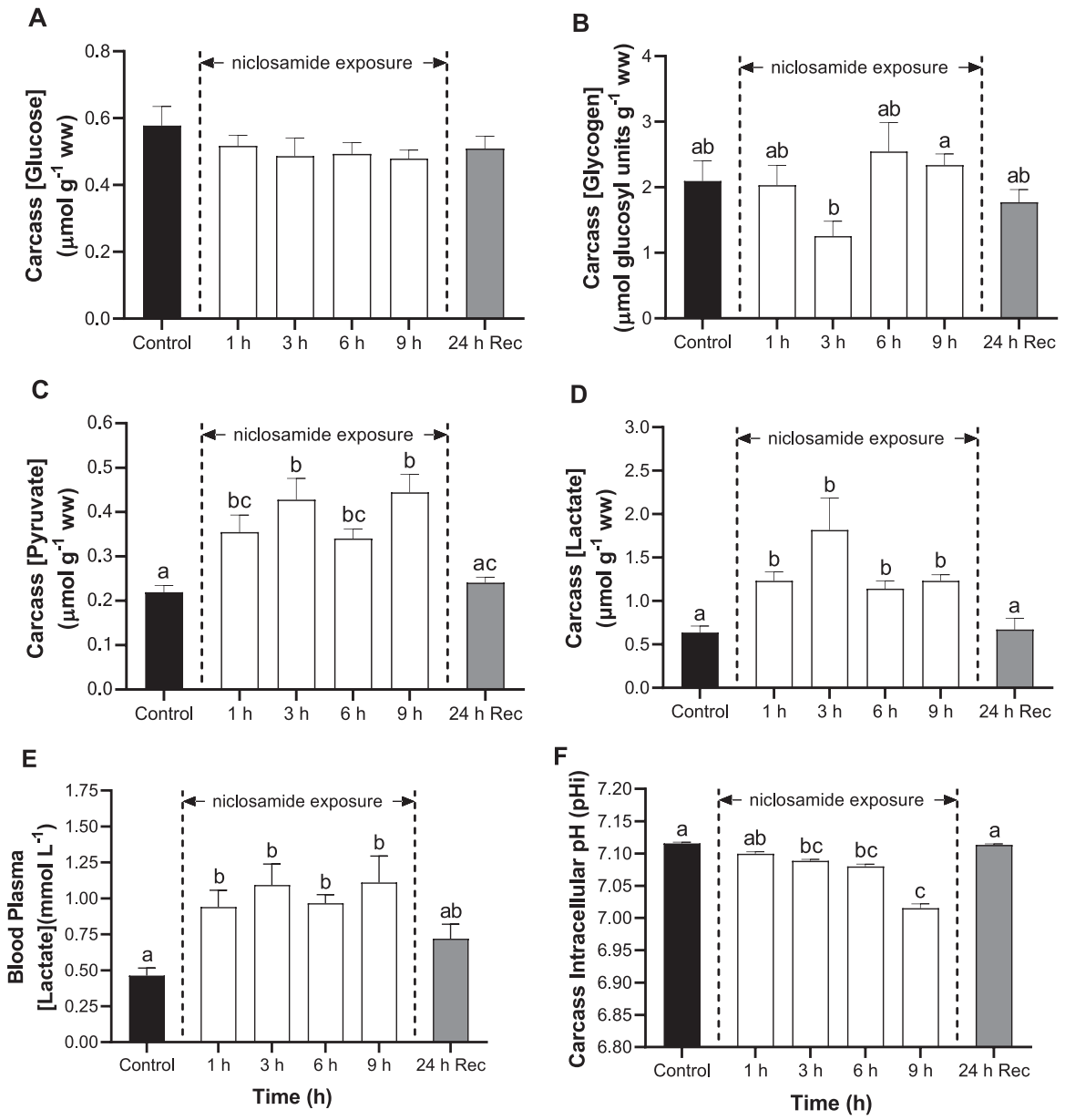

Fig. 5. Effects of niclosamide on carcass glycogen and metabolites. Changes in the concentrations of (A) glucose, (B) glycogen, (C) pyruvate, (D) lactate in the carcass, (E) lactate in blood plasma, and (F) pHi in the carcass of lake sturgeon (*Acipenser fulvescens*) during (open bars) and following exposure to niclosamide (carcass 24 h recovery; n = 12; plasma 24 h recovery; n = 6; gray bars) at a concentration of 0.12mg 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 ($P \leq 0.05$).

lactate was transported to the liver via the blood, where it was converted to glucose, then transported back to the muscle where it was 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 was retained in the muscle and that is clearance coincided 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, with the concomitant restoration of lactate, during recovery from niclosamide exposure.

4.2. *Effects of niclosamide on blood plasma ions*

Uncoupling of mitochondrial oxidative phosphorylation by niclosamide might also be expected to impair gill-mediated ion- and osmoregulation, which require ATP for primary and secondary active transport to take-up ions from fresh water via gill mitochondria rich cells (aka. MRCs or ionocytes; see Evans et al., 2005 for review). Using transmission electron microscopy, 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, as characterized by cell rounding, vacuolization,

Table 1
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 concentration of 0.12mg 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)

enlargement of mitochondria, and swelling of the intracellular space of MRCs. While both lampricides caused similar damage, niclosamide exposure also resulted in a higher incidence of necrosis to MRCs (Mallatt et al., 1994). The authors suggested that the damage observed may have compromised iono- and osmo-regulation, ultimately contributing to death by impairing 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 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 exposed to TFM, and to sea lamprey exposed to TFM and niclosamide (Birceanu et al., 2009, 2014; Ionescu et al., 2021). 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, 2014), or on plasma ion (Na^+, K^+) concentrations in these fishes (Birceanu et al., 2009, 2014; Henry et al., 2015).

4.3. Impacts of niclosamide on the ecophysiology of lake sturgeon

As a consequence of metabolic impairment, niclosamide exposure could also adversely affect a variety of physiological processes. For example, it has been recently demonstrated that fishes experience olfactory impaiment when exposed to certain environmental toxicants such as metals and pesticides (Dew et al., 2014; Green et al., 2010; Mirza et al., 2009; Tierney et al., 2010). Impaired olfaction can lead to failure in perception of chemosensory cues and response, in turn resulting in maladaptive behavior (Norris et al., 1999; Sovová et al., 2014; Tierney et al., 2010; Weis and Weis, 1995). Using electro-olfactography (EOG), Sakamoto et al. (2016) showed that in YOY lake sturgeon, TFM impaired olfactory sensory neurons (OSNs) and ciliated sensory neurons used to recognize food cues, and migration and alarm cues. Because TFM and niclosamide are both halogenated phenolic compounds which are thought to impart toxicity in a similar fashion, it seems likely that niclosamide could similarly impair olfaction and behavior in lake sturgeon. For instance, diminished brain ATP and glucose stores in niclosamide-exposed lake sturgeon could 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 (Dew et al., 2014; Green et al., 2010; Mirza et al., 2009; Tierney et al., 2010) and contributing to the impairment of electric signals from the OSNs. It would be worthwhile to investigate if niclosamide exposure significantly alters EOGs, as it does in TFM-exposed lake sturgeon (Sakamoto et al., 2016).

The persistent reduction in PCr in the carcass (mainly muscle) suggests that lake sturgeon could experience greater predation vulnerability due to a reduced burst swim performance capacity. Evidence in support of this hypothesis includes observations that lake sturgeon were 79% more active (hyperactive) but swim performance was impaired, with 19% slower acceleration and 16% slower peak velocity when exposed to TFM (Sakamoto et al., 2016). However, it should be noted that sturgeon likely do not rely on burst swimming as much as other fishes, particularly salmonids (Kieffer et al., 2001; Zhang et al., 2017). In other Acipinseridae such as the shortnose (*Acipenser brevirostrum*) and Atlantic sturgeon (*Acipenser oxyrhynchus*), with virtually identical body designs to lake sturgeon, burst swim performance is not likely an important component of these animal's locomotory repertoire due to low levels of glycogen and PCr in the muscle, which is manifested by relatively mild metabolic disturbances in the muscle after burst exercise (6 min of chasing to exhaustion; Kieffer et al., 2001). In the present study, lake sturgeon had even lower levels of PCR and glycogen in the carcass (mainly muscle) than Atlantic and shortnose sturgeons, suggesting that any impact of niclosamide exposure on their burst swimming capacity would have been negligible. This also likely explains why the reduction in muscle pHi, though statistically significant, was minimal (~ 0.1 pH units).

Aerobic swimming performance, on the other hand, was likely impaired, which could have important ecophysiological implications. Aerobic modes of swimming are more important in the sturgeons. For instance, Kieffer and May (2020) reported that while maximum sustainable swimming speeds are much lower in shortnose sturgeon than other species of fishes, Ucrit was sustained following repeated bouts of swimming, suggesting that sturgeons are "slow and steady" swimmers, implying that they mainly rely on aerobic respiration to fuel their ATP demands. By interfering with oxidative phosphorylation, we speculate that niclosamide, not to mention TFM, significantly impairs aerobic swim performance in lake sturgeon. Studies examining sustained swimming performance, particularly multiple swimming trials (*c.f.* Kieffer and May, 2020), would be particularly useful for testing this hypothesis. The effects of niclosamide on swim performance would also be affected by how quickly any accumulated niclosamide is detoxified and eliminated following exposure, but to date this data is not available in lake sturgeon.

4.4. Implications for lake sturgeon population enhancement and sea lamprey control

The potential for sea lamprey parasitism to cause serious harm to Great Lakes fisheries, worth in excess of 7 billion dollars annually, remains (Krantzberg and De Boer, 2008; GLFC, 2011), and the use of lampricides such as TFM and niclosamide is integral to controlling sea lampreys (Siefkes, 2017; Wilkie et al., 2019). However, 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, 2017). On the other hand, government agencies and First Nations are concerned that lampricide applications could undermine these efforts, particularly TFM which has been identified as a source of age-0 lake sturgeon mortality (Boogaard et al., 2003; Johnson et al., 1999; 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 the concentration of lampricide (TFM) 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, and were subsequently 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 shown using a generalized Great Lakes' sturgeon population model relating lake sturgeon populations changes to sea lamprey-associated mortality (Dobiesz et al., 2018). Their analysis demonstrated that sea lamprey parasitism on sub-adult (ages 7–24 years) lake sturgeon can result in 32% mortality, which far outweighed the marginal increase (5.7%) in adult lake sturgeon abundance that could result from eliminating TFM use in streams occupied by the fish.

Although the present study demonstrates that lake sturgeon experience marked physiological disturbances following niclosamide exposure, it is equally important to note that they readily recover (within 24 h). Nevertheless, it would still be prudent to develop or modify application procedures 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., 2021), delaying treatments until later in the summer or early autumn when the sturgeon are larger, and accumulate less lampricide, would also be an effective strategy to protect sturgeon from episodic lampricide exposure. Indeed, such a strategy has already been implemented to protect sensitive sturgeon populations in the Great Lakes (Dobiesz et al., 2018). It would also be beneficial to learn more about how niclosamide and/or TFM effects olfaction and behavior, 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.

CRediT authorship contribution statement

Adrian Ionescu: Conceptualization, Validation, Formal Analysis, Investigation, Writing-Original Draft, Visualization, Project Administration. **Dejana Mitrovic:** Validation, Investigation. **Michael P. Wilkie:** Conceptualization, Validation, Resource, Data Curation, Writing-Review & Editing, Supervision, Project Administration, Funding Acquisition.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112969.

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