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Research article

Rainbow trout rapidly recover from exposure to niclosamide: A piscicide and molluscicide used to control sea lamprey and snail populations

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ABSTRACT

Niclosamide (2′,5-dichloro-4′-nitrosalicylanalide) is a piscicide used to control invasive sea lamprey (*Petromyzon marinus*) in the Laurentian Great Lakes. It is also a molluscide used in tropical and sub-tropical freshwaters to control snail populations that are intermediate hosts to the blood flukes that causes schistosomiasis in humans. While the mechanism of niclosamide toxicity is known, its corresponding physiological effects on non-target fishes are not well-established. To better understand how niclosamide could adversely affect non-target fishes, rainbow trout (*Oncorhynchus mykiss*) were exposed to an environmentally relevant niclosamide concentration of 0.150 mg L^{-1} (measured = 0.12–0.18 mg L^{-1}) over 9 h, during which tissues were collected for measurement of energy stores and metabolites. Niclosamide exposure reduced brain ATP and glycogen by ~50 %, and liver glycogen by ~40 %. Reductions of ATP, phosphocreatine and glycogen were also observed in muscle, with corresponding increases in pyruvate and lactate, plus development of a metabolic acidosis (~0.2 unit decrease in intracellular pH). These disturbances were consistent with impaired mitochondrial oxidative phosphorylation and greater reliance on anaerobic glycolysis to generate ATP. Notably, physiological homeostasis was restored in the brain, liver, and muscle within 24 h after depuration in fresh, niclosamide-free water. We conclude that non-target fishes are susceptible to niclosamide, but at least in rainbow trout, the effects are readily reversed after exposure ceases. Similar approaches could be used to determine the susceptibility and resilience of other fishes to niclosamide in environments where it is required as a either a lampricide or a molluscicide.

1. Introduction

The invasion by sea lamprey (*Petromyzon marinus*) and overfishing devastated culturally significant Indigenous, commercial, and recreational fisheries in the Laurentian Great Lakes in the mid-20th century (Gaden et al., 2021). Sea lamprey populations were subsequently brought under control following the implementation of a sea lamprey control program in which the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) is applied to infested streams where it targets larval sea lamprey (ammocoetes) burrowed in the stream sediment (Applegate et al., 1966; Hubert, 2003; Siefkes, 2017). In the early 1960s, 2′,5-dichloro-4′-nitrosalicylanalide (niclosamide; aka. Bayluscide® or Bayer 73®) was added to the sea lamprey control program (Howell et al., 1964). Niclosamide is often co-applied with TFM, at 1–2 % the TFM concentration (Dawson, 2003), which reduces the amount of TFM needed by up to 40 % while maintaining its efficacy and selectivity to sea lampreys (Boogaard et al., 2003; Gutreuter and Boogaard, 2007). A granular formulation of niclosamide (granular Bayluscide®) is also 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 as effective (Dawson, 2003; Wilkie et al., 2019).

Niclosamide is also a widely used molluscicide recommended by the World Health Organization (WHO; 2017, 2019) for treatment of waters infested with snails that serve as intermediate hosts for parasitic blood flukes (flatworms) that cause the debilitating disease schistosomiasis in tropical countries, particularly in Africa and Asia (Joubert et al., 2001; Lardans and Dissous, 1998; McManus et al., 2018). Either an emulsifiable concentrate (EC) or wettable powder form of niclosamide are used to target snails, their eggs, and *Schistosoma* parasites in the water when used for schistosomiasis prevention (WHO, 2017). Niclosamide also has other therapeutic uses as an antihelmintic to treat intestinal parasites, and more recently as a potential treatment for cancer and type 2 diabetes

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in humans and animals, impairing cellular energy metabolism by uncoupling oxidative phosphorylation and causing cell cycle arrest and apoptosis (Alasadi et al., 2018; Tao et al., 2014).

Experiments using isolated mitochondria have demonstrated that both niclosamide and TFM impair oxidative phosphorylation in sea lamprey and rainbow trout (*Oncorhynchus mykiss*) (Birceanu et al., 2011; Borowiec et al., 2022; Huerta et al., 2020). This likely explains the energy store shortfalls reported in sea lamprey, trout and lake sturgeon (*Acipenser fulvescens*) following exposure to TFM, which are characterized by greater reliance on anaerobic pathways of ATP production such as glycolysis and phosphocreatine (PCr) mobilization and subsequent depletion of these energy stores (Birceanu et al., 2011, 2014; Ionescu et al., 2021). While much has been learned about the toxicological and physiological effects of TFM in non-target fishes in the last decade (Bussy et al., 2018a, 2018b; Hepditch et al., 2019; Ionescu et al., 2021; Lawrence et al., 2021, 2022; Middaugh et al., 2014; O'Connor et al., 2017; Sakamoto et al., 2016), physiological data on the effects of niclosamide have been restricted to its effects in embryonic zebrafish (*Danio rerio)*, and in juvenile lake sturgeon (*Acipenser fulvescens*) (Ionescu et al., 2022a; Zhu et al., 2020). There is little documentation of the acute physiological effects of niclosamide on non-target fishes at concentrations that bracket those used in actual sea lamprey or schistosomiasis control treatments. The WHO has noted a need to learn more about the effects of molluscicides on non-target organisms (WHO, 2017). Similarly, minimizing the non-target effects of lampricides remains a priority of the Great Lakes Fishery Commission (GLFC), which oversees sea lamprey control in the Great Lakes on behalf of Canada and the United States (WHO, 2017; GLFC, 2021).

Niclosamide is much more toxic than TFM to lamprey and other fishes and invertebrates (Newton et al., 2017; Wilkie et al., 2019). Indeed, we recently demonstrated that niclosamide is approximately 30–60 times a more potent inhibitor of mitochondrial oxidative phosphorylation than TFM (Borowiec et al., 2022). Based on these observations, we predicted that the short-term effects of niclosamide on anaerobic energy reserves would be identical but more severe than those of TFM in sea lamprey and non-target fishes. A limited number of studies have shown reductions in brain, liver and muscle tissue glycogen concentrations and phosphocreatine at much lower concentrations of niclosamide (~ 1/40th) than TFM in sea lamprey and lake sturgeon (*Acipenser fulvescens*; Ionescu et al., 2022a, 2022b). However, the adverse physiological effects of niclosamide exposure on energy metabolism in teleost fishes are poorly defined, despite their potential vulnerability to this pesticide in regions where it is used for controlling invasive sea lamprey or the prevention of schistosomiasis outbreaks due to snail infestations.

The objective of this study was to gain a better understanding of the physiological effects of niclosamide on juvenile rainbow trout (*Oncorhynchus mykiss*), a common and widely used teleost fish for aquatic toxicology studies, with a wide distribution in the Great Lakes and a relatively high tolerance to lampricides (Boogaard et al., 2003; Wilkie et al., 2019). In the present study, juvenile rainbow trout were exposed to niclosamide for up to 9 h, during which muscle, brain, liver, and blood samples were collected at fixed times to determine how niclosamide affected tissue high-energy phosphates (ATP, phosphocreatine), glycogen, lactate, and associated metabolites. Muscle intracellular pH (pHi) was also measured to quantify disturbances to acid-base balance that may have resulted from greater reliance on anaerobic glycolysis during exposure. The resilience of rainbow trout to niclosamide exposure was also determined by measuring the same tissue energy stores and metabolites in animals following a 24 h post-exposure recovery period.

2. Materials and methods

2.1. Animal husbandry`

Juvenile rainbow trout (total length 163.0 ± 1.5 mm; mass 45.0 ± 1.1 g; $N = 83$) were acquired from Rainbow Springs Hatchery, Thamesford, Ontario, Canada and housed 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 × 15 cm diameter) filled with biological beads suspended above the water surface. Water quality was monitored daily for temperature (15 ± 0.5 °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), respectively. Titratable alkalinity averaged 238 ± 5 mg L^{-1} as $CaCO_3$ (Test kit model AL-AP, HACH Limited, London, Ontario, Canada). Water hardness was ~350 mg L^{-1} as $CaCO_3$, with a water Na^+ concentration of ~1.1 mmol L^{-1}. 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 the start of experiments. All experiments followed Canadian Council of Animal Care (CCAC) guidelines and were approved by the WLU Animal Care Committee (Institutional Animal Use Protocol No. R18004).

2.2. Experimental procedures

Prior to experiments, the rainbow trout were fasted for 24 h, then transferred one at a time to one of 24 individual experimental glass aquaria (1 fish per aquarium). Each aquarium contained 8 L of the same WLU well-water described above (for water chemistry see Table S1), under static conditions, and were allowed to acclimate overnight. The aquaria were placed in a flow-through water bath, which maintained a constant temperature of 15 °C. Due to limited numbers of aquaria ($N = 24$), practical and space limitations, 4 separate series of experiments (N = 24 animals or less), were conducted over approximately 1 week.

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. 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. After approximately 30 min, aquaria (except controls) were dosed with 10 mL of 120 mg L^{-1} niclosamide dissolved in 50 % methanol (0.625 mL methanol L^{-1} = 0.0625 % methanol) to achieve a nominal niclosamide concentration of 0.15 mg L^{-1}, which was near the measured 12-h LC_{50} of niclosamide (0.134 mg L^{-1}) determined in preliminary experiments done using the same Wilfrid University well water (D. Foubister and M.P. Wilkie, unpublished observations). This is near the concentrations of the liquid formulation of niclosamide (emulsifiable concentrate) which is frequently combined with TFM (1–2 % niclosamide) during sea lamprey control operations in order to lower the 12-h $LC_{99.9}$ of TFM, which is the concentration of TFM required to kill 99.9 % of sea lamprey during lampricide treatments of infested rivers (Boogaard et al., 2003; Gutreuter and Boogaard, 2007). As our intention was to determine the sub-lethal effects of niclosamide alone, it was not applied with TFM in these experiments. However, this concentration of niclosamide is lower than that typically used for snail control, which are typically applied at target concentrations of 1 mg L^{-1} in flowing and lentic waters (WHO, 2017).

Water samples (20 mL) were collected immediately after adding niclosamide to the tanks (0 h) and at the conclusion (9 h) of the exposure for measurements of niclosamide concentrations (described below). All aquaria were well-aerated, achieving DO saturation levels >95 %. A sub-

set of fish exposed to the same amount of 50 % methanol (10 mL) in 8 L of water, minus the niclosamide, served as "shams" to ensure that any physiological changes observed in niclosamide-exposed fish were not a result of the solvent exposure. Nothing was added to the control tanks.

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 depuration in niclosamide-free water (n = 12). This exposure time approximates those used for lampricide (~ 9–12 h) and snail control (~ 8 h) applications (McDonald and Kolar, 2007; Sullivan et al., 2021; WHO, 2017). Control fish were sampled at the beginning of the experiment and 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 a liquid slurry (10 mL) of tricaine methanesulfonate (Syndel, Nanaimo, British Columbia) buffered with 2 parts $NaHCO_3$ to each aquarium to achieve respective concentrations of 1.0 g L^{-1} and 2.0 g L^{-1}. After being euthanized, each fish was patted dry with paper towel, weighed, and measured for total length. Blood was collected in a drop-wise fashion by post-anal transection of the caudal peduncle, into a heparin-coated 1.5 mL microcentrifuge tube and then centrifuged at 10,000*g* for 4 min. The resultant plasma supernatant was transferred to a new heparin-coated tube and flash frozen in liquid nitrogen and stored at −80 °C for later ion analyses. The liver and brain were then collected, snap frozen in liquid nitrogen and stored at −80 °C for later analysis. A filet of white muscle (1–2 g) was then collected from the lateral trunk, immediately freeze-clamped using pre-chilled aluminum tongs in liquid nitrogen and stored at −80 °C (Wang et al., 1994b).

2.3. Metabolite extraction and analysis

Tissue metabolite extraction and analysis followed Bergmeyer (1983) and Wang et al. (1994b) with slight modifications as described in Ionescu et al. (2022a, 2022b) and Wilkie et al. (1997). Briefly, brain, liver and white muscle filets were ground to a fine powder under liquid nitrogen in a stainless steel mortar using a ceramic pestle, and the powder (~100 mg) added to 4 volumes 8 % perchloric acid containing 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA; Sigma-Aldridge Canada, Oakville, Ontario, Canada), mixed by vortexing, and left on ice for 10 min and then centrifuged at 10,000*g* (4 min). The supernatant was then neutralized (to pH 7) with either 2 N KOH (aliquot 1) or 3 mol L^{-1} K_2CO_3 (aliquot 2) and stored frozen at −80 °C. For muscle and brain tissues, aliquot 1 was used for ATP, phosphocreatine, lactate and pyruvate analysis, as well as creatine (white muscle only). Depending on the tissue, metabolites were analyzed enzymatically (Bergmeyer, 1983; Wilkie et al., 1997) for ATP (hexokinase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase (LDH)), PCr (creatine kinase), creatine (creatine kinase, pyruvate kinase, LDH), and lactate (LDH) and pyruvate (LDH) using a microwell plate spectrophotometer at a wavelength of 340 nm (Epoch 2; BioTek, Winooski, VT, USA). Aliquot 2 was used for glycogen and glucose quantification in brain, liver, and muscle following digestion of the extract using amyloglucosidase in 2 mol L^{-1} acetate buffer at 37 °C (based on Bergmeyer, 1974), followed by glucose determination (hexokinase) at 340 nm on the digested supernatant to yield glycogen concentration in μmol glucosyl units g^{-1} wet weight, after correcting for free glucose in undigested extract (cf. Clifford et al., 2012).

Muscle intracellular pH determination followed Pörtner et al. (1990) in which the tissue was ground to a fine powder under liquid nitrogen (as described above) and approximately 100 mg of powder added to 400 μL of ice-cold metabolic inhibitor cocktail comprised of 150 mmol L^{-1} potassium fluoride and 6 mmol L^{-1} nitrilotriacetic acid sodium salt (Na_2NTA). The resulting slurry was mixed using a vortex mixer and briefly centrifuged (10 s), and the pH of the supernatant measured with a micro pH probe (Hamilton Bonaduz AG, Bonaduz, Switzerland) and meter (ION85 Analyzer, Radiometer, Copenhagen, Denmark).

Plasma Na^+ concentration was measured using flame atomic absorption spectroscopy (AAS; PinAAcle 900 T, Perkin Elmer, Waltham, MA, USA) and Cl^- concentration by coulometric titration using a chloride analyzer (Chloride Analyzer 926, Cole Parmer, Vernon Hills, IL, USA) as previously described (Ionescu et al., 2022b).

2.4. Water niclosamide quantitation

Quantitation of niclosamide in water followed methods described in Ionescu et al. (2022b). Water samples were thawed at room temperature, vortexed for 20 s and then transferred to a 5 mL clean glass test tube and spiked with 200 μg L^{-1} of niclosamide-(2-chloro-4-nitrophenyl-$^{13}C_6$) hydrate (VETRANAL®), which served as an internal standard. Water samples (15 mL) were vacuum filtered through 0.45 μm glass fiber filters (Pall Corporation, Michigan, USA;) using a Supelco Visiprep SPE (solid-phase extraction) vacuum filtration apparatus (Sigma Aldrich), and the eluent (1 mL) transferred to a 2 mL amber glass vial for subsequent LC-MS/MS analyses (Agilent 1260 HPLC with 6460 Triple Quad and Agilent Jetstream ESI source in negative ionization mode). Sample (exactly 10 μL) was injected onto an Agilent Eclipse XDB-C18 column, to isolate the analyte using gradient flow elution. The calibration curve (0 to 500 μg niclosamide L^{-1}) was made-up in HPLC grade methanol (Optima, LC/MS grade, Fisher Scientific, Ottawa, Canada), and sample niclosamide concentrations determined using linear regression, after adjusting for background. Sample recovery rates were ≥ 97.8 %.

2.5. Statistical analyses

All statistical data analyses were performed using Prism® 10.0.2 (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 standard deviations were significantly different from one another or were not transformable, data were analyzed 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 $P < 0.05$. The LC_{50} for niclosamide was calculated using the Litchfield Jr. and Wilcoxon (1949) method using R studio software (https://rawgit.com/JVAdams/LW1949/master/vignettes/Intro.html; Adams, 2016).

3. Results

3.1. Measured water niclosamide concentrations & rainbow trout survival

LC-MS/MS analysis of water samples taken from experimental tanks at the start (0 h) and end (9 h) of experiments revealed that measured niclosamide concentrations were initially higher than nominal concentrations (0.150 mg L^{-1}), averaging 0.179 ± 0.007 mg L^{-1} at the start of the experiment, declining to an average concentration of 0.123 ± 0.004 mg L^{-1} at the end of the 9 h exposure (Table 1). Niclosamide concentrations were below detection following the 24 h depuration (recovery) period in clean water.

Table 1
Measured water niclosamide concentrations.
Water niclosamide concentrations were measured using LC-MS/MS, in samples collected from experimental tanks at the start (0 h) and end (9 h) of experiments. Water from recovery (24 h Rec.) tanks was measured to assess residual niclosamide that may have been excreted by the fish following transfer to clean water. Data are expressed as the mean ± SEM (n). BDL: Below detectable limits.

Treatment	Measured Water [niclosamide] (mg L^{-1})
0 h	0.179 ± 0.007 (22)
9 h	0.123± 0.004 (22)
24 h Rec.	BDL (12)

Of the sixty rainbow trout exposed to niclosamide, all but three survived exposure, with one mortality at 3 h and two after 9 h of exposure. Variation in sample sizes for subsequent metabolite analyses were due to a lack of sufficient tissue to perform all analyses.

3.2. Effects of sham exposure compared to controls

There were no differences in the respective concentrations of glucose, glycogen, lactate, ATP, and phosphocreatine measured in brain, muscle or liver (glycogen and glucose only) in control (not exposed to niclosamide) and sham treated fish (exposed to 10 mL 50 % methanol in vehicle only in 8 L) between 0 h (start) and following 9 h (Table S2). Nor were any differences in the concentration of metabolites measured in control samples collected at the start of experiments compared to those sampled after 9 h (Table S2). Accordingly, all control and sham data were pooled into a single group for comparison to niclosamide-treated fish.

3.3. Effects of niclosamide on energy stores and metabolites in rainbow trout brain

The concentration of ATP in the brain of rainbow trout held under

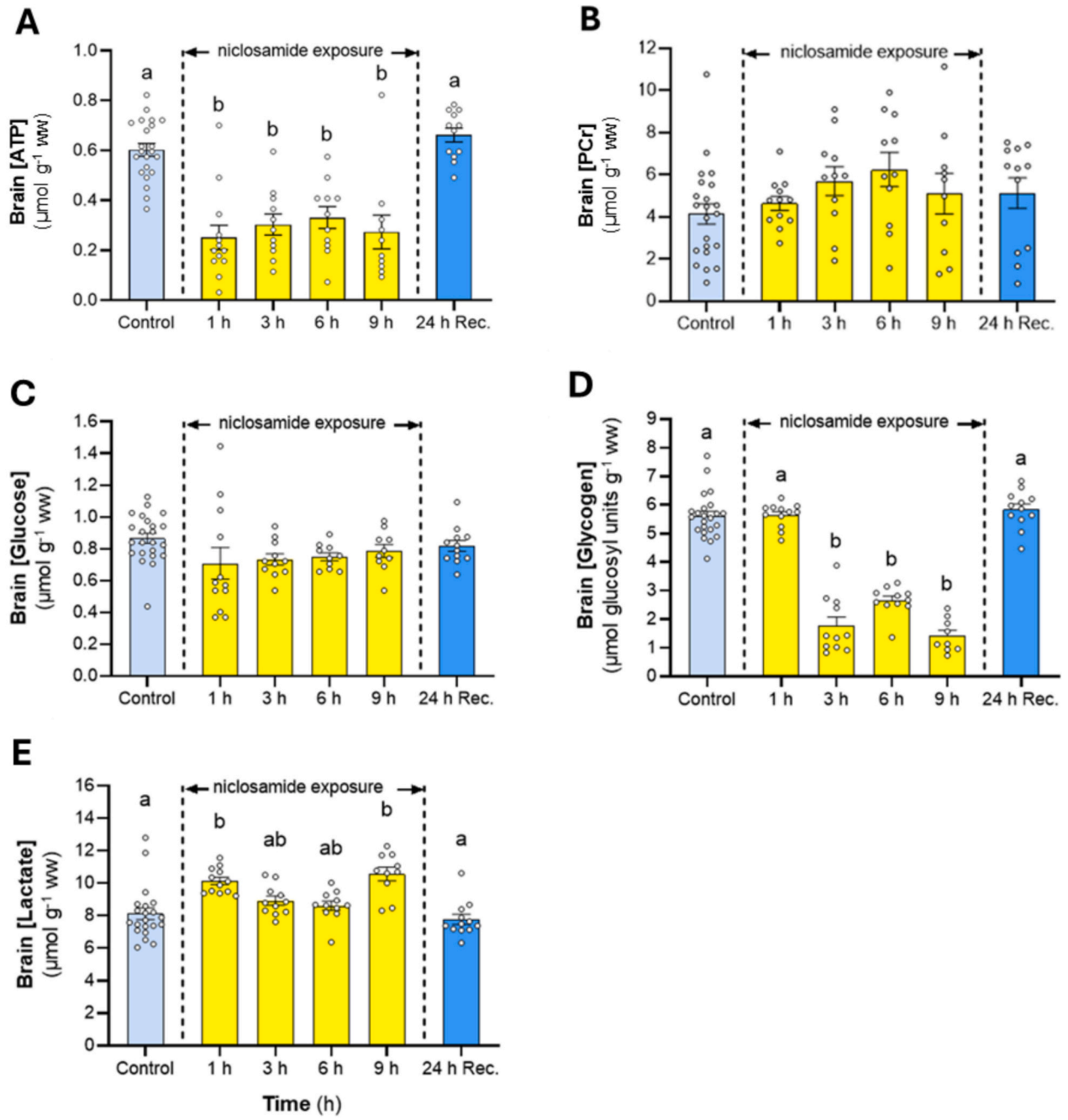

Fig. 1. Changes in the concentrations of (A) ATP, (B) PCr, (C) Glucose, (D) Glycogen and (E) Lactate in the brain of rainbow trout (*Oncorhynchus mykiss*) during (yellow bars), and following 9 h exposure to niclosamide (24 h recovery; $n = 12$; dark blue bar) at a nominal concentration of 0.15 mg L^{-1} (9 h LC_{50}) for 1 h (n = 12), 3 h ($n = 11$), 6 h ($n = 10$–11) and 9 h ($n = 9$–10), or held under control conditions (no niclosamide; $n = 21$–23; light blue bar). Data are expressed as the mean ± 1 SEM (Standard Error of the Mean). Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$), while open circles represent individual sample measurements.

control conditions averaged 0.60 ± 0.05 μmol g^{-1} ww, and PCr averaged 4.15 ± 0.93 μmol g^{-1} ww (Fig. 1A, B). 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 (Fig. 1A). Brain PCr concentrations were unchanged in the presence of niclosamide (Fig. 1B). Brain glucose, glycogen and lactate concentrations in rainbow trout controls averaged 0.87 ± 0.03 μmol g^{-1} ww, 5.61 ± 0.16 μmol glucosyl units g^{-1} ww and 8.17 ± 0.36 μmol g^{-1} ww, respectively (Fig. 1C). Brain glucose concentrations were unchanged in the presence of niclosamide (Fig. 1C), 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 (Fig. 1D). 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 (Fig. 1E).

3.4. Effects of niclosamide on energy stores in rainbow trout liver

Liver glucose concentrations in rainbow trout held under control conditions averaged 16.60 ± 1.24 μmol g^{-1} ww and glycogen concentrations averaged 43.08 ± 2.20 μmol glucosyl units g^{-1} ww (Fig. 2). Liver glucose remained unchanged in the presence of niclosamide relative to controls (Fig. 2A), 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 (Fig. 2B).

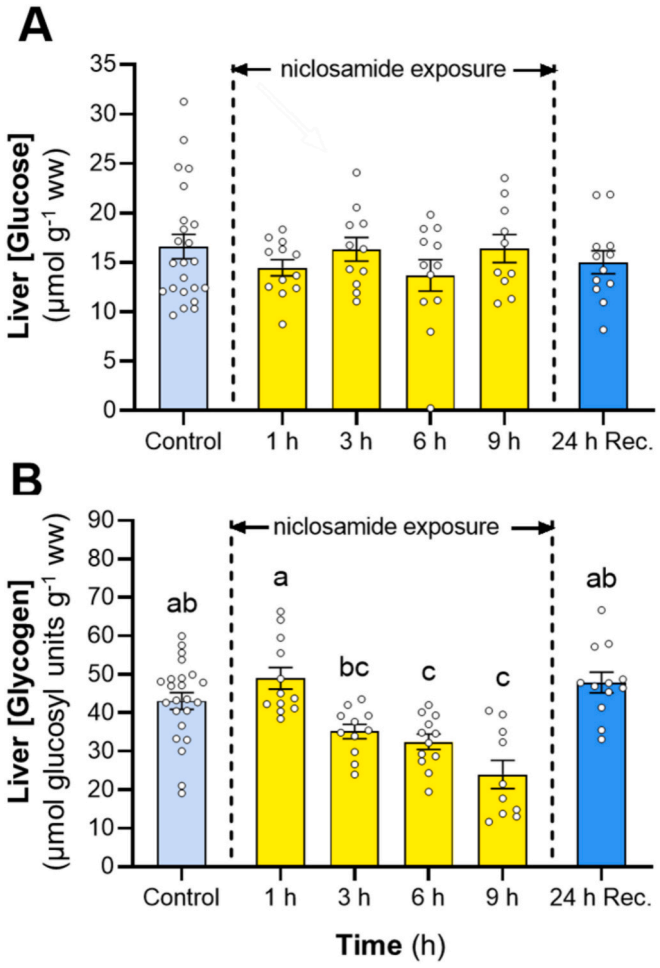

Fig. 2. Changes in the concentrations of (A) glucose and (B) glycogen in the liver of rainbow trout (*Oncorhynchus mykiss*) during (yellow bars) and following exposure to niclosamide (24 h recovery; n = 12; dark blue bar) at a nominal concentration of 0.15 mg L^{-1} for 1 h (n = 12), 3 h (n = 11), 6 h (n = 12) and 9 h (n = 10), or held under control conditions (no niclosamide; n = 23; light blue bar). Data are expressed as the mean ± 1 SEM. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$), while open circles represent individual sample measurements.

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

White muscle ATP concentrations in rainbow trout controls averaged 7.28 ± 0.30 μmol g^{-1} (Fig. 3A). 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 (Fig. 3A). White muscle PCr and creatine concentrations in rainbow trout controls average 25.90 ± 0.67 μmol g^{-1} ww and 21.00 ± 1.29 μmol g^{-1} ww, respectively (Fig. 3B, C). 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 (Fig. 3B). Creatine concentration in white muscle was significantly reduced by approximately 35 % at 3, 6 and 9 h compared to controls, returning to pre-exposure concentrations after 24 h recovery (Fig. 3C).

Concentrations of glucose and glycogen in white muscle of rainbow trout controls averaged 0.86 ± 0.02 μmol g^{-1} ww and 13.61 ± 0.20 μmol glucosyl units g^{-1} ww (Fig. 4A, B). No changes were observed in muscle glucose concentration in the presence of niclosamide (Fig. 4A), 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 (Fig. 4B).

Pyruvate and lactate concentrations in white muscle of rainbow trout controls averaged 0.25 ± 0.02 μmol g^{-1} ww and 2.21 ± 0.14 μmol g^{-1} ww, respectively (Fig. 4C, D). In the presence of niclosamide, muscle pyruvate concentrations were significantly increased after 9 h, by approximately 45 % r relative to controls, before returning control levels after 24 h recovery (Fig. 4C). 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 (Fig. 4D). 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 9 h, respectively, relative to controls, returning to pre-experimental levels (pH = 7.42 ± 0.02) after 24 h of recovery (Fig. 4E).

3.6. 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, with no significant differences observed due to niclosamide exposure (Table 2).

4. Discussion

4.1. Effects of niclosamide on energy stores and metabolites

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 in mammals (Alasadi et al., 2018; Jurgeit et al., 2012; Park et al., 2011) and recently reported in mitochondria isolated from sea lamprey (Borowiec et al., 2022). These observations are similar to those reported in rainbow trout exposed to TFM, also an uncoupler of mitochondrial oxidative phosphorylation that

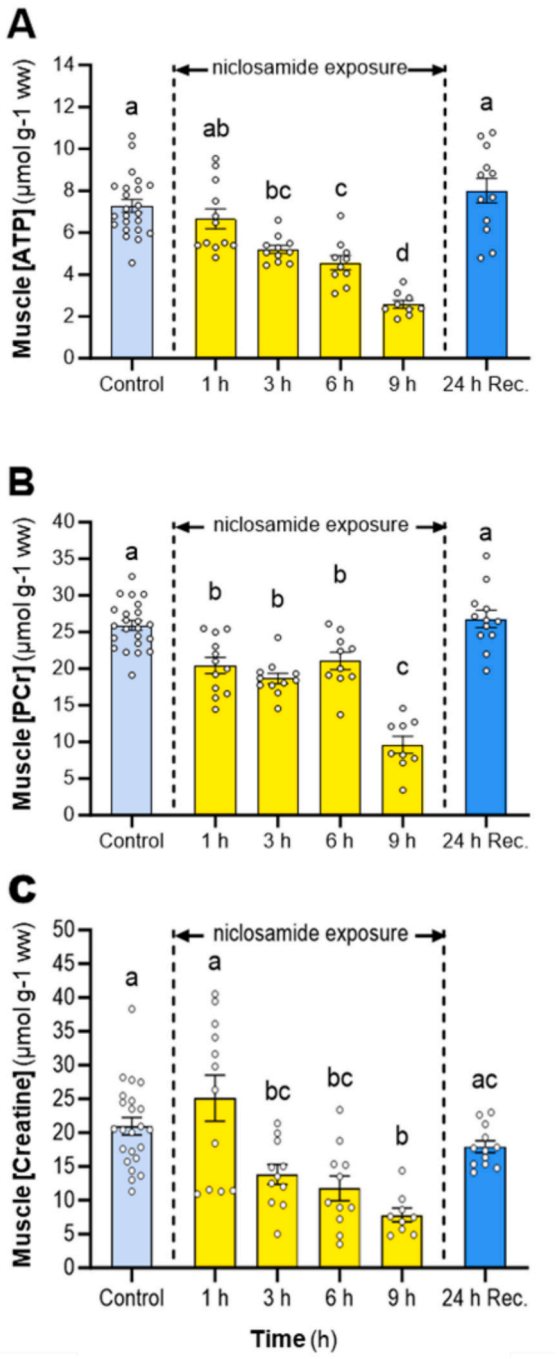

Fig. 3. Changes in the concentrations of (A) ATP, (B) PCr and (C) creatine in the muscle of rainbow trout (*Oncorhynchus mykiss*) during (yellow bars) and following exposure to niclosamide (24 h recovery; n = 12; dark blue bar) at a nominal concentration of 0.15 mg L^{-1} for 1 h (n = 12), 3 h (n = 11), 6 h (n = 10–11) and 9 h (n = 9), or held under control conditions (no niclosamide; n = 23; light blue bar). Data are expressed as the mean ± 1 SEM. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$), while open circles represent individual sample measurements.

lowers 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 or due to hypoxia or anoxia, ATP is temporarily generated from high energy phosphagens such as PCr and by anaerobic glycolysis (see Hochachka and Somero, 2002; for reviews). PCr buffers ATP stores via the creatine phosphokinase (CPK) mediated dephosphorylation of PCr transferring a phosphate group to ADP to sustain ATP levels in tissues during hypoxia and vigorous exercise (McLeish and Kenyon, 2005; Wallimann et al., 2011; Wood, 1991). This did not appear to be the case in the in the trout brain, however, as PCr concentrations were unchanged throughout niclosamide exposure despite the sustained reduction in ATP supply. Nor were significant reductions in PCr observed in the brain of trout exposed to TFM (Birceanu et al., 2014), which again suggests that the role of PCr in buffering ATP concentrations may be limited in this fish. This was unlike observations previously reported in larval sea lamprey exposed to niclosamide or TFM, in which resting PCr concentrations were approximately 75 % greater and readily mobilized in response to several hours of TFM and niclosamide exposure (Birceanu et al., 2009; Clifford et al., 2012; Ionescu et al., 2022b). It is possible that PCr turnover via CPK was simply higher during niclosamide exposure, but this is hard to imagine because replenishment of the PCr pool would depend upon a ready supply of ATP and restoration of intracellular pH (Hochachka and Somero, 2002; Wood, 1991; Yquel et al., 2002), each of which were likely lower due greater reliance on anaerobic glycolysis which was needed to sustain ATP production in the face of lower aerobic mitochondrial ATP production using oxidative phosphorylation.

Due to drops in O_2 levels caused by restricted blood flow (e.g. ischemia) in mammals, or under hypoxic/anoxic environmental conditions in many fishes, PCr concentrations typically undergo declines in response to drops in ATP (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 comparatively low brain anaerobic capacity characterized by relatively low PCr concentration and glycogen stores 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; Rovainen et al., 1969; van Raaij et al., 1994). 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 phosphokinase (CPK)/PCr system could be informative.

Wilkie et al. (2007) suggested that in sea lamprey, death from TFM was likely to occur when the glucose supply to the brain was depleted, making it impossible to use anaerobic glycolysis to meet the demands of the nervous system, ultimately leading to loss of homeostasis and death. Notably, the reduction in brain ATP and glycogen observed in rainbow trout were more pronounced with niclosamide exposure than those observed in rainbow trout exposed to TFM (Birceanu et al., 2014). This may reflect the greater potency of niclosamide compared to TFM, as recently demonstrated using isolated mitochondria from adult sea lamprey (Borowiec et al., 2022). 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 of most fishes under typical, normoxic conditions (Polakof et al., 2007, 2012; Soengas and Aldegunde, 2002). 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. Fig. 1; Birceanu et al., 2014; DiAngelo and Heath, 1987). In contrast, the brain of larval sea lamprey has glycogen concentrations that typically exceed 100 µmol glucosyl units g^{-1} ww (Clifford et al., 2012; Henry et al., 2015; Foster et al., 1993; Rovainen et al., 1969). In larval sea lamprey, such high brain glycogen concentrations could be an adaptation that enables them to temporarily tolerate hypoxic conditions, which may 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-

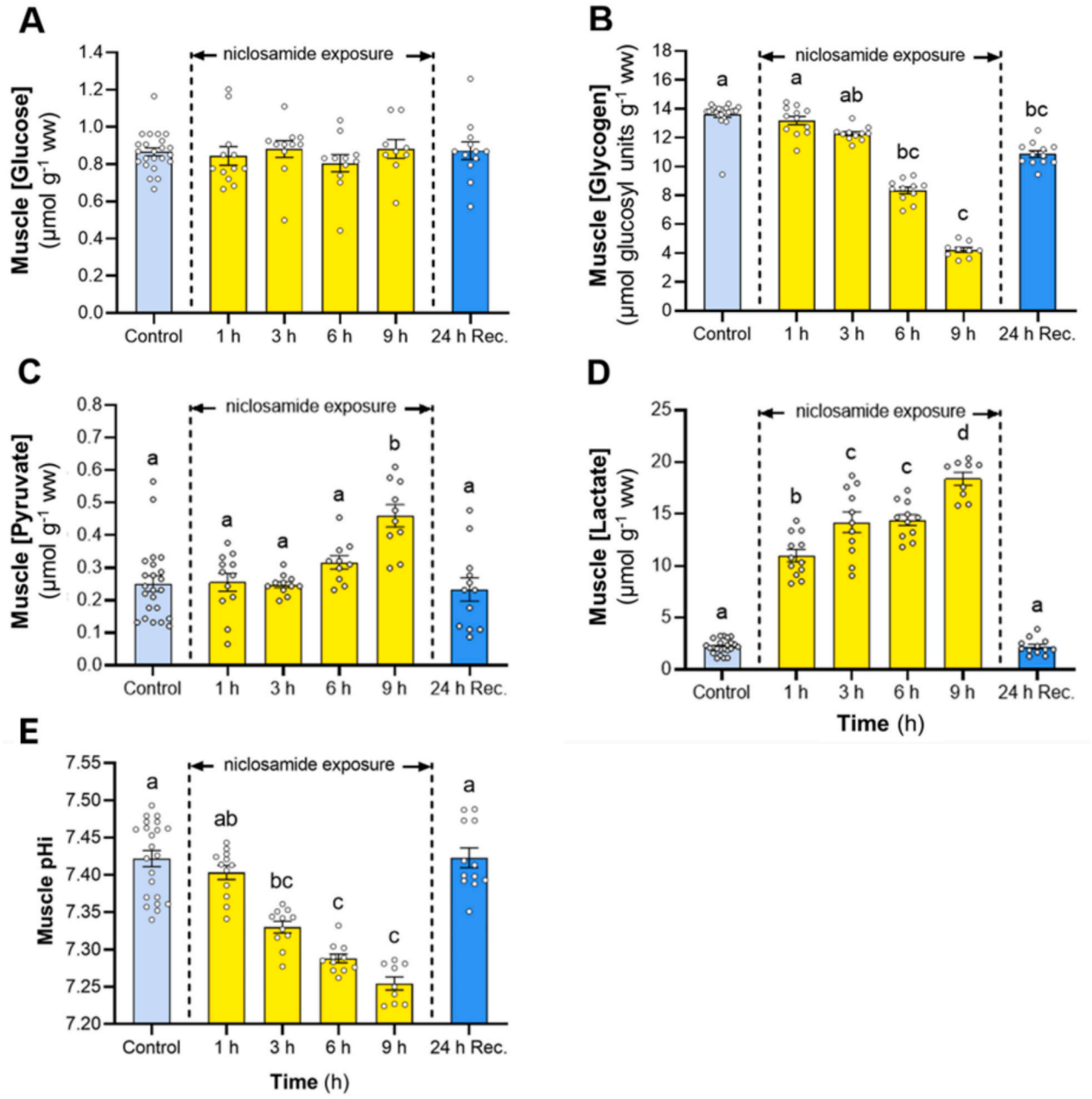

Fig. 4. Changes in the concentrations of (A) glucose, (B) glycogen, (C) pyruvate and (D) lactate, and changes in (E) pHi in the white muscle of rainbow trout (*Oncorhynchus mykiss*) during (yellow bars) and following exposure to niclosamide (24 h recovery; n = 12; dark blue bar) at a nominal concentration of 0.15 mg L^{-1} for 1 h (n = 12), 3 h (n = 11), 6 h (n = 10–11) and 9 h (n = 9–10), or held under control conditions (no niclosamide; n = 23; light blue bar). Data are expressed as the mean ± 1 SEM. Different lowercase letters indicate significant differences between each treatment group and controls ($P \leq 0.05$), while open circles represent individual sample measurements.

covered shallow lakes and ponds (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 (but these are much lower than in crucian carp or goldfish) to meet glucose demands of nervous tissues, achieved by increases in glycogenolysis in response to glucagon release in the face of reduced glucose supply or increased demand (Polakof et al., 2012; Soengas and Aldegunde, 2002). 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.

With greater reliance on anaerobic glycolysis and the subsequent hydrolysis of ATP, rates of metabolic acid production also increase (H^+; Hochachka and Mommsen, 1983), which may further disturb the central nervous system (CNS) function. There was insufficient brain tissue to determine how niclosamide affected intracellular pH (pHi) in the brain of the trout. 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, 1986a, 1986b), suggesting that there may have been a comparable or even 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. A goal of future studies should be to examine pHi in not only the brain, but also the blood to better understand how niclosamide exposure affects systemic acid-base balance in trout and other non-target fishes.

Table 2
Effects of niclosamide on rainbow trout blood plasma ions.
Concentrations of Na^+ and Cl^- in the blood plasma of rainbow trout (*Oncorhynchus mykiss*) during and following 9 h exposure (24 h recovery) to niclosamide at a nominal concentration of 0.15 mg L^{-1} (9 h LC_{50}) for up to 9 h or held under control conditions (no niclosamide). Data are expressed as mean ± SEM (n). No significant differences were observed.

Treatment	Na^+ mmol L^{-1} (± SEM; n)	Cl^- mmol L^{-1} (± SEM)
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)

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 (Barros et al., 2020; Hochachka and Somero, 2002; Soengas and Aldegunde, 2002), however, the lactate was likely oxidized in the mitochondria. Although, niclosamide impairs ATP production by uncoupling oxidative phosphorylation, there is no established 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 ultimately 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 readily available 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 (aka: leak respiration) of trout isolated liver mitochondria were enhanced by approximately 2- to 3-fold in the presence of TFM and 2,4-dinitrophenol (DNP), the latter also a well-established uncoupler of oxidative phosphorylation (Birceanu et al., 2011). Similar increases in State IV respiration, were observed in mitochondria isolated from sea lamprey when exposed to niclosamide (Borowiec et al., 2022) and in mouse liver (Tao et al., 2014).

The resting values and reductions in energy stores observed 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 (e.g. Kieffer et al., 1994; Milligan and Wood, 1986a, 1986b; Wang et al., 1994a, 1994b). 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 (Milligan and Wood, 1986a, 1986b; Wang et al., 1994a, 1994b).

The combined changes in the concentration of pyruvate, lactate and pHi were used to calculate the redox state of the trout's white muscle to examine the underlying mechanisms driving the observed metabolic disturbances caused by niclosamide (Wang et al., 1994a; Ionescu et al., 2022b). The observed decline in the pyruvate/lactate concentration ratio was expected based on the large increase in muscle lactate that was observed due to the need to rely on glycolysis to produce ATP (Table 3). The observed increase (> 85 %) was comparable to values reported in trout immediately following exhaustive exercise (Wang et al., 1994a). Calculations of the NAD^+/NADH ratio, which considers changes in muscle intracellular pH (Wang et al., 1994a), was reduced by 80 % after 1 h and remained suppressed through the entire niclosamide exposure

Table 3
Effects of niclosamide exposure on the [pyruvate]/[lactate] ratio and the estimated cytoplasmic redox state ([NAD+]/[NADH] ratio) of rainbow trout muscle. Data are expressed as the mean ± SEM (n). Different letters designate values that significantly differ from one another ($P < 0.05$).

Exposure Time (h)	[Pyruvate]/[Lactate]	[NAD+]/[NADH]
Control	0.13 ± 0.02 (9)^a	2129 ± 310 (9)^a
1 h	0.02 ± 0.00 (9)^b	383 ± 44 (9)^b
3 h	0.02 ± 0.00 (9)^b	433 ± 42 (9)^b
6 h	0.02 ± 0.00 (9)^b	562 ± 31 (9)^b
9 h	0.03 ± 0.00 (9)^b	698 ± 65 (9)^b
24 h Recovery	0.10 ± 0.02 (9)^a	1854 ± 371 (9)^a

period (Table 3). These observations suggest there may have been O_2 supply limitations and/or a decreased capacity of the white muscle mitochondria to oxidize NADH using O_2, the final electron acceptor in the electron transport chain (Wang et al., 1994a; Hochachka and Somero, 2002). These observations contrast those reported in exhaustively exercise salmonids, in which the NAD^+/NADH ratio is unchanged and O_2 supply is thought to be sufficient to permit recovery of post-exercise redox (Wang et al., 1994a; Wilkie et al., 1997). The differences could be related to the prolonged nature of niclosamide exposure (> 9 h), plus its potent uncoupling of mitochondrial oxidative phosphorylation, which could be compromising other aspects of mitochondrial function. The relatively low amount of vascularization and mitochondrial density of the white muscle, also referred to as fast twitch (type IIb) muscle in vertebrates (McClelland and Scott, 2014), compared to tissues with a higher oxidative capacity such as red muscle or cardiac muscle, may also be important. Notably, muscle redox state is unaltered by niclosamide in the carcass of larval sea lamprey (Ionescu et al., 2022b), in which the muscle is a mix of red and white muscle fibers which are better perfused with blood and contain more mitochondria (Meyer, 1979; Peters and Mackay, 1961). Hence, the effects of niclosamide on the muscle of fishes could vary from species to species based on variation in red vs white muscle fiber composition, distribution and vascularization. Importantly, muscle redox status, as with other metabolic measures we made, was restored to pre-exposure levels within 24 h, further indicating the reversible nature of sub-lethal niclosamide exposure on non-target organisms.

The post-niclosamide recovery period was also notable for the complete restoration of liver, and muscle, glycogen stores within 24 h, even though the animals were not feeding. A possible explanation is that insulin was mobilized during this time and promoted glycogen synthesis, when there would be less demand for glucose and anaerobic ATP production (see Hemre et al., 2002; Nelson and Sheridan, 2006 for reviews). Exploring what role endocrine processes play in mediating glycogenolysis and glycogen synthesis would be a fruitful avenue of investigation, particularly the relationship between glucagon and insulin in restoring glucose homeostasis in trout and other non-target organism following niclosamide application. Nevertheless, the rapid recovery and lack of longer-term physiological disturbances following niclosamide and TFM exposure observed in trout, sturgeon and lamprey appears to be a characteristic feature of these pesticides. These findings therefore suggests that niclosamide would likely have minimal long-term effects on non-target fish populations following its use for snail or sea lamprey population control.

4.2. Effects of niclosamide on blood plasma ions

Plasma Na^+ and Cl^- concentrations were not altered in response to niclosamide exposure (Table 2), which is similar to results observed following exposure of rainbow trout and larval sea lamprey (Birceanu et al., 2009, 2014; Henry et al., 2015) to TFM. Collectively, these observations also suggests that gill damage due to niclosamide exposure was unlikely. This interpretation is supported by transmission electron microscopy (TEM) analysis, which revealed no pathological changes to

gill ultrastructure in rainbow trout fry exposed to niclosamide-TFM mixtures at concentrations commonly used in sea lamprey control operations (Mallatt et al., 1994). Similarly, no histopathological damage was observed when trout fry were exposed to higher concentrations of niclosamide (LC_{100}; 0.2 mg L^{-1} niclosamide), which were 10–60 % greater than those used in the present study (Mallatt et al., 1994).

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. Gill ionocytes, also known as chloride cells or mitochondria (MR) cells, actively take-up Na^+, Cl^-, and Ca^{2+} from fresh water to counter the diffusive ion loss across the gills of fishes in freshwater (e.g. Dymowska et al., 2012). Due to the high ATP demands of ionocytes, we hypothesized that these cells would be targeted by niclosamide leading to decreased ATP production and impaired ion uptake. Although we had not previously found that TFM interferes with ion uptake by fish gills, we reasoned that because niclosamide is a much more potent inhibitor of oxidative ATP production by mitochondria ($\geq$ 40 times; Borowiec et al., 2022), that the gills could be more sensitive to niclosamide than TFM. While no changes in plasma Na^+ or Cl^- were observed, we did not directly measure rates of Na^+, Cl^- or Ca^{2+} uptake by the fish, which can be done using radiolabelled ions, which could be addressed in future studies. The time of exposure (9 h) may have also been too brief to elicit measurable changes in plasma Na^+ and Cl^- balance, which often take days to develop following insults such as exposure to acid or alkaline pH (e.g. Wilkie and Wood, 1991; Wood et al., 1988). Examining niclosamide exposure over a longer time-course and/or at higher concentrations could therefore be informative to better understand its potential effects on ion and acid-base physiological processes mediated by the gills.

4.3. Conclusions and implications for lampricide and molluscicide treatments

Exposure of rainbow trout to niclosamide, at concentrations near its nominal 9-h LC_{50} of 0.15 mg L^{-1}, resulted in adverse physiological effects within 1–3 h of exposure including disturbances to energy metabolism, muscle redox state and acid-base balance. However, the recovery of the trout's metabolic status, within 24 h after withdrawal from niclosamide exposure, demonstrates that effects are short-lived and reversible. Indeed, recovery may have been faster than 24 h, due an ability to rapidly eliminate niclosamide due to the generation of outward gradients to rapidly facilitate unloading of the highly lipophilic parent niclosamide ($\log K_{ow} = 10$ at pH 9.6; PubChem, 2024) across the gills. Phase 2 biotransformation via conjugation reactions in the liver that generate more water soluble sulfated- and glucuronidated-niclosamide forms could have also contributed to rapid elimination of niclosamide via renal pathways during and following exposure (Allen et al., 1979; Statham and Lech, 1975). There is variation in niclosamide tolerance amongst teleost fishes in North America, with ictalurid catfishes amongst the most sensitive, and the centrarchid fishes such as bluegill (*Lepomis macrochirus*), and cyprinid fishes such as common carp (*Cyprinus carpio*) and goldfish, approximately 5-fold more tolerant based on LC_{50} values (Dawson, 2003). Differences in niclosamide tolerance could be related to variation in the capacity of sea lamprey, fishes, and other non-target organisms, to use Phase 2 biotransformation as reported for TFM (Kane et al., 1994). Hence glucuronidation or sulfation capacity could be used as a possible predictor of niclosamide sensitivity in non-target fishes in jurisdictions where this compound is used as a molluscicide.

Sea lamprey control personnel are continually presented with the challenge of ensuring the protection of non-target fishes while administering lampricide treatments to waterways. When the fast sinking, granular form of niclosamide (granular Bayluscide®) is used in lentic areas or in very large fast-flowing rivers, it is much less likely that non-target fishes will be exposed to high concentrations of niclosamide, such as those used in this study, for very long periods because they can escape the niclosamide by moving up in the water column to evade the lampricide, or the niclosamide will quickly dissipate. When the emulsifiable concentrate of niclosamide (1–2 %) is used in combination with TFM to treat long stretches of rivers or streams over several hours, prolonged exposure and its associated metabolic disturbances are more likely due to its greater than additive (synergistic) interactions with TFM (Hepditch et al., 2019).

Non-target exposure is also a concern in situations where niclosamide is administered as a molluscicide for snail population control to reduce the risk of schistosomiasis transmission in tropical waters in Asia, Africa and South America (WHO, 2019). Powdered and emulsifiable concentrates of niclosamide, similar to those previously used or currently used for sea lamprey control, are used for snail control (WHO, 2017). However, application procedures differ, including the use of much higher concentrations of niclosamide to treat snail infested waters. In flowing waters, niclosamide is applied at approximately 1 mg L^{-1} over a period of approximately 8 h (WHO, 2017). However, focal applications of niclosamide may also be required over smaller and more restricted areas such as stagnant or very low flow areas (WHO, 2017). Because niclosamide targets mitochondrial function by uncoupling oxidative phosphorylation (Borowiec et al., 2022; Tao et al., 2014), the metabolic disturbances arising from niclosamide exposure reported in rainbow trout would likely be observed in other non-target organisms, but the severity could vary due to differences in treatment dose and duration of exposure, local environmental conditions and species.

It is also worth noting that the ETC in many invertebrates (including molluscs) is known to contain an alternative oxidase (AOX) (Weaver and McDonald, 2023). In many species AOX provides metabolic flexibility in energy transduction and carbon metabolism during various types of stress (Weaver and McDonald, 2023). Recent work has demonstrated that after niclosamide treatment AOX mRNA and protein levels increase while reactive oxygen species production decreased in the gonad and liver in the snail *Oncomelania hupensis* (Jiang et al., 2022). Inhibition of AOX with salicylhydroxamic acid during niclosamide treatment led to a 56–76 % increase in snail mortality (Jiang et al., 2022).

Where there is a need to more accurately determine how sub-lethal niclosamide adversely affects non-target organisms for snail control, a knowledge of their physiological responses and capacity to recover from exposure could assist program managers when planning treatments. We propose that similar approaches to those used in the present study on rainbow trout could be used to learn more about the sensitivity, responses and resilience of other, locally relevant non-target organisms to niclosamide when it is used as a molluscicide for snail population control.

CRediT authorship contribution statement

R. Adrian Ionescu: Writing – original draft, Methodology, Investigation, Formal analysis. **Dejana Mitrovic:** Writing – review & editing, Methodology, Investigation. **Oana Birceanu:** Writing – review & editing, Funding acquisition, Conceptualization. **Allison E. McDonald:** Writing – review & editing, Funding acquisition, Conceptualization. **Jonathan M. Wilson:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Mark R. Servos:** Writing – review & editing, Resources, Methodology, Data curation. **Michael P. Wilkie:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

Co-author J.M. Wilson is an Associate Editor with CBP, and was not involved in the review process. We declare no other competing or financial interests. Nor have we co-authored or collaborated with any of the recommended referees in the last 3 years.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2025.110199.

Data availability

Data will be made available on request.

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