The Mechanism of Increased Tolerance to the Pesticide, 3-Trifluoromethyl-4-Nitrophenol (TFM), in Invasive Sea Lamprey (*Petromyzon marinus*) at Warmer Water Temperatures

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Abstract

The phenolic compound 3-trifluoromethyl-4'-nitrophenol (TFM) is used as a lampricide, applied to rivers and streams to control populations of invasive sea lamprey (*Petromyzon marinus*) in the Great Lakes. 3-Trifluoromethyl-4'-nitrophenol is used to selectively target larval sea lamprey because of their limited capacity to detoxify it. The tissue TFM accumulation impairs mitochondrial adenosine triphosphate production by uncoupling oxidative phosphorylation, leading to energy depletion and death. Sea lamprey tolerance to TFM is greater in the summer, but the underlying mechanism(s) are unresolved. The present study tested the hypothesis that an increased capacity of sea lamprey to eliminate TFM at warmer temperatures increases their tolerance to TFM. Acute toxicity tests demonstrated that the 12-hr median lethal concentration (LC50) of TFM steadily increased by approximately 1.5-fold as water temperature rose from 7 to 28°C. When lamprey were acclimated to one of three temperatures (6, 12, 24°C) and exposed to an identical TFM concentration (i.e., 12-hr LC25 at 12°C), muscle and liver TFM concentrations were approximately 30% and 36% lower in lamprey acclimated to 24°C, suggesting more effective elimination of TFM at warmer temperatures. Calculations of the TFM steady state concentration and elimination half-life ($t_{1/2}$) in the liver and muscle following TFM exposure suggested that they have a greater capacity to eliminate TFM in warmer water. We propose that the sea lamprey's capacity to detoxify TFM is greater at higher temperatures, preventing internal concentrations from reaching lethal levels during acute (9–12 hr) TFM exposure of similar duration to field applications. We also propose that water temperature, in addition to water pH and alkalinity, be considered when determining TFM application concentration used to optimize sea lamprey treatment effectiveness.

Key Words: Aquatic Invasive Species, Phase II Metabolism, Phenol toxicity, Climate change, Laurentian Great Lakes

Introduction

The phenolic compound 3-trifluoromethyl-4-nitrophenol (TFM) has been used as a method to control invasive sea lamprey (*Petromyzon marinus*) populations in the Laurentian Great Lakes for over sixty years (Siefkes 2017; Wilkie et al., 2019). Sea lampreys are jawless fish that invaded the Great Lakes in the mid-late 1800s and early 1900s via man-made canals (Eshenroder, 2014; Smith, 1980). Sea lamprey metamorphose from suspension-feeding larvae (also called ammocoetes) into blood sucking parasites (Manzon et al., 2015). Their parasitism of top predatory fishes in the Great Lakes resulted in massive reductions in the populations of commercial, recreational and Indigenous fisheries by the mid-twentieth century (Siefkes, 2017; Smith, 1980). Populations were subsequently brought under control following the implementation of a binational (USA and Canada) sea lamprey control program (SLCP) that uses a combination of barriers to block the upstream migration of adult lampreys and application of TFM to nursery streams containing larval lamprey (Hubert, 2003; Siefkes 2017; Wilkie et al., 2019).

In the SLCP, field applications of TFM (typically < 12 hr) target the larval sea lamprey, which have a greater sensitivity than non-target fishes to the compound due to their relatively low capacity to metabolize TFM using phase II metabolism (e.g., glucuronidation) and eliminate the compound (Kane et al., 1994; Lawrence et al., 2021; Lech & Statham, 1975). 3-Trifluoromethyl-4-nitrophenol induces toxicity by interfering with aerobic (oxygen-dependent) adenosine triphosphate (ATP) production through the uncoupling of oxidative phosphorylation in the mitochondria, resulting in ATP starvation and death (Birceanu et al., 2011; Huerta et al., 2020; Niblett & Ballantyne, 1976). As a weak acid, with a pKa of 6.38, the ionization state of TFM varies with changes in water pH (Hubert, 2003; McConville et al., 2016). Hence, the

toxicity of TFM to both sea lamprey and non-target fishes changes with pH by altering its bioavailability. As the pH is lowered, the proportion of TFM in its lipophilic, un-ionized form (TFM-OH) increases, thereby increasing the transport across the gill membranes (Armitage et al., 2017; Figure 1a). Similarly, higher alkalinity leads to lower TFM toxicity, most likely due to increased buffering capacity in the gill microenvironment, resulting in reduced availability of the unionized form (TFM-OH; Armitage et al., 2017; Wilkie et al., 2021).

The season has also been shown to play a role in the sensitivity of larval sea lamprey to TFM, with greater TFM tolerance in summer compared to spring (Applegate et al., 1961; Muhametsafina et al., 2019; Scholefield et al., 2008; Schueller et al., 2024). Recent studies suggest that these differences may be primarily related to higher water temperatures (Hlina et al., 2021; Muhametsafina et al., 2019). Other phenolic compounds, including the uncoupler 2,4-dinitrophenol and pure phenol, have also been observed to be less toxic to fish at warmer temperatures (Howe et al., 1994; Patra et al., 2015), but the underlying mechanisms remain unclear.

The present study aimed to assess the effects of temperature on the accumulation and toxicity of TFM in larval sea lamprey. Accordingly, 12-hr median lethal concentration (LC50) values were determined for TFM in larval sea lamprey across a wide range of acclimation temperatures (7–30°C) to test the hypothesis that TFM tolerance increases with temperature. Another group of sea lamprey acclimated to 6°C, 12°C and 24°C, were subsequently exposed to a common concentration of TFM (LC25 at 12°C) to determine how patterns of TFM accumulation and elimination were affected by temperature alone, while minimizing TFM-induced mortality. We hypothesized that TFM toxicity would decrease with increased temperature and that its accumulation in the liver and muscle tissue would be reduced at warmer temperatures. A better understanding of how temperature alters the bioaccumulation and toxicity of TFM in sea lamprey will inform future management decisions for lamprey control

and contribute to a better understanding of the implications of temperature for the assessment of phenolic organic contaminants.

Material and methods

Experimental animals and holding

Larval sea lamprey (*n* = 595, mass = 1.94 ± 0.03 g and total length = 11.42 ± 0.6 cm at the time of the experiments) were provided by the US Geological Survey, Hammond Bay Biological Station (HBBS), Millersburg, Michigan, USA. The sea lamprey were held for approximately 2 months prior to experiments, under a 12:12-hr light: dark photoperiod in aerated flow-through 75-L tanks fed with City of Waterloo dechlorinated tap water (temperature 12°C; pH 7.9-8.1; titratable alkalinity ~ 221 mg L^{-1} as $CaCO_3$) with a replacement rate of 0.5 L min^{-1}. The tanks were filled with 3 to 5 cm of Quikrete Play sand (Home Depot, Waterloo, ON, CA) to provide burrowing substrate (Birceanu et al., 2009). The larval lamprey larvae were fed a slurry of wet bakers' yeast (Fleischmanns®) once a week at a ration of 1 g per larva per week (Holmes & Youson, 1994). All experiments were approved by the Wilfrid Laurier University Animal Care Committee (Animal Use Protocol Number R22003) and conducted in accordance with guidelines of the Canadian Council on Animal Care.

TFM

Field formulation TFM (35% dissolved in isopropanol; Clariant SFC GMBH WERK, Griesheim, Germany), provided courtesy of the Sea Lamprey Control Centre, Fisheries and Oceans, Canada, Sault Ste. Marie, Ontario, was used for all TFM exposure experiments. Working concentrations of TFM were prepared using dechlorinated laboratory water and verified against standards prepared using analytical-grade TFM (99%, CAS No: 88-30-2, Millipore Sigma, Oakville, ON). To ensure that TFM exposures during the experiments were stable, the appropriate amounts of working stock (1 g L^{-1}) were added to each aerated glass test aquarium approximately 12 hr prior to each toxicity test. Water samples (10 mL) were

collected from each tank for TFM measurements immediately after TFM working stock addition, the next day and at 0 hr, 6 hr, and 12 hr after sea lamprey were added to the tanks for toxicity tests.

Experimental protocols

Approximately 2 months after capture, the sea lamprey were acclimated to different temperatures ranging from 6 to 28°C, two series of experiments were performed. The first series consisted of acute toxicity experiments to quantify the effect of temperature on larval sea lamprey sensitivity to TFM (Experiment 1). A second series subjected larval sea lamprey to an identical nominal TFM exposure concentration (the LC25 of TFM at 12°C) at one of three temperatures (6, 12, and, 24°C) to determine how TFM accumulation in liver and muscle differed with temperature, and to measure muscle energy stores and energy metabolite concentrations (Experiment 2). All TFM exposures were conducted for 12 hr to approximate typical field applications.

Experiment 1 - Acute toxicity tests

After an initial 2-month acclimation to laboratory conditions, larval sea lamprey were separated into seven aquaria (n = 80 animals per 70-L aquaria) with the rate of partial water replacement of approximately 1 L min^{-1}. Each tank was equipped with either a heater (Finnex Titanium Heater TH-800 plus, JSK Merchandise Inc. USA) or a chiller (Coralife ¼ hp Aquarium Chiller, Franklin, WI, USA) and temperature controllers (Inkbird ITC-308 Temperature Controller, Ink Bird Tech. C.I., China). The water temperature of each tank was gradually adjusted at a rate of 2°C per 24 hr, to one of seven target temperatures: 7, 12, 18, 21, 25, 28, and 30°C. Lamprey larvae were acclimated at their respective target temperatures for at least three weeks (no more than 30 days), and fish were not fed for at least 48 hr before beginning experiments.

Sea lamprey larvae (*n* = 70–80 per temperature, *n* = 10 per TFM concentration) were exposed to a range of nominal TFM concentrations (0.5–10.0 mg L^{-1}) to generate concentration-toxicity curves. From these curves, the 12-hr LC50 and 12-hr LC99.9 (minimal lethal concentration, MLC) were calculated to compare the variation in TFM toxicity across seven temperatures (7, 12, 18, 21, 25, and 28°C). The tests followed American Standard and Testing Methods guidelines for static toxicity tests on aquatic organisms (ASTM International, 2014). Each set of lamprey were tested at their respective acclimation temperature in glass aquaria filled with 8 L of the same well-aerated, dechlorinated water described above. The aquaria did not contain sand burrowing substrate, which can lead to greater TFM survival, presumably by interfering with TFM flow through the substrate (Bills et al., 2003), in keeping with previous TFM toxicity studies (e.g. Bills et al., 2003; Hlina et al., 2021; Schueller et al., 2024). Each aquarium was immersed in a recirculating water bath to maintain the appropriate temperature for the duration of the experiment.

On the day of the toxicity test, sea lamprey were collected from the appropriate acclimation tank using a dip net and arbitrarily distributed to each test tank containing TFM at one of eight concentrations (*n* = 5 lamprey per aquarium; *n* = 10 per TFM concentration). Lamprey acclimated to low temperatures (7, 12, 18°C) were exposed to nominal TFM concentrations ranging from 0.5 to 9.0 mg L^{-1}. In contrast, lamprey acclimated to high temperatures (21, 25, 28°C) were exposed to nominal TFM concentrations ranging from 1 to 10 mg L^{-1} (see online supplementary material, Table S1). 3-Trifluoromethyl-4'-nitrophenol concentrations were then measured in water samples (10 mL) collected at 3, 6, and 12 hr to determine whether TFM concentrations remained stable throughout each experiment (see online supplementary material, Table S1).

Water temperature remained within 0.4°C of target values, while dissolved oxygen (DO) exceeded 90% saturation across treatments. The water pH was measured at the onset (0 hr),

6 hr and 12 hr of the TFM exposures, and was relatively stable, with average water pH values falling between pH 8.0 to 8.1 for all treatments (Table 1). Water alkalinity ranged from 221 to 238 mg L^{-1} as $CaCO_3$ (Table 1).

The larval sea lamprey (*n* = 10 per TFM concentration, in duplicate 5 per tank) were monitored hourly during each toxicity test. When no movement or ventilation was visually observed, mortality was confirmed by a light tail pinch with forceps to ensure the animal was unresponsive. Mortalities were quickly removed, and the time of death, body length and mass were recorded (see online supplementary material, Table S1). After the 12-hr exposure, any survivors were euthanized in an aerated bath containing a lethal dose of buffered tricaine methanesulfonate (MS-222, CAS No. 886-86-2, Syndel, Nanaimo, BC, Canada; 1.5 g L^{-1} MS-222 plus 3.0 g L^{-1} $NaHCO_3$).

Experiment 2 - Effects of temperature on TFM accumulation and tissue energy stores and metabolites

To quantify how temperature affected patterns of TFM tissue accumulation and energy reserves, separate groups of larval sea lamprey (*n* = 60 lamprey per temperature) were exposed to a single concentration of TFM (3.74 mg L^{-1}, the 12-hr LC25 at 12°C determined in Experiment 1) at temperatures of 6, 12, or 24°C. Water chemistry was measured over the 12-hr exposure with minimal variations in measured temperature, pH, dissolved oxygen and alkalinity (Table 2). 3-Trifluoromethyl-4'-nitrophenol concentration was stable over the 12-hr of exposure across the temperatures (Table 2).

For each temperature group, control lamprey (*n* = 10–15) were held under the same conditions, minus the TFM exposure with half the animals sampled within the first hour and half at the end of the 12-hr exposure for measurement of baseline levels of energy stores and metabolites and to ensure there were no changes in these parameters over the duration of the experiment. At 1, 3, 6, 9, or 12 hr, a sub-sample of lamprey (*n* = 8–10, per treatment) were

euthanized as described above, followed by the rapid collection of liver and muscle, taken from both sides of the animal and approximately 3 cm below the gill arches, which were freeze-clamped using liquid nitrogen-cooled aluminum tongs, and stored at −80°C until processed. Individual lamprey weight and lengths were recorded and used to calculate Fulton's condition factor (CF = mass (g)/length $(mm)^3 \times 10^6$; Holmes & Youson, 1994; Schueller et al., 2024).

Analytical Methods

Water TFM concentrations were confirmed spectrophotometrically using 1.5-mL polystyrene cuvettes on a NovaSpec II spectrophotometer (Pharmacia Biotech, Cambridge, UK) at a wavelength of 395 nm (Fisheries and Oceans Canada Instrument Operating Procedure – IOP # 012.4). The TFM standards were established according to the micro-modified GLFC Standard Operating Procedure 43.1.0 (Hammond Bay, MI, USA), and dilutions were prepared using the experimental water.

The liver and muscle samples collected in Experiment 2 were processed for TFM quantification as initially outlined by Lawrence et al. (2021) with modifications and optimizations (for complete method details see online supplementary material, Table S1). Briefly, approximately 100 mg of finely ground tissue was extracted using Captiva EMR-Lipid 96 well plates (Agilent Technologies, Santa Clara, CA). The eluted sample was then analyzed by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). The concentration of TFM in each sample was quantified using an Agilent 1260 HPLC coupled to a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). An Agilent Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm) was used to separate the analytes chromatographically. All data were processed using Mass Hunter Quantitative Analysis software version 10.1 (Agilent, Santa Clara, USA), and TFM tissue concentration was expressed as nmol g^{-1} wet weight. The calibration curve ranged from 0 µg L^{-1} to 5000 µg L^{-1},

and the instrument detection limit was 0.04 μg L^{-1}, while the method detection limit was 0.25 μg L^{-1}. The sample recoveries were between 85.9 and 103.8% based on our calibration curve.

Muscle energy metabolites

Tissue metabolite extraction procedures were described by Clifford et al. (2012). Briefly, sea lamprey muscle tissue samples were ground to a fine powder under liquid nitrogen and homogenized with 4 volumes of 8% PCA (perchloric acid) containing 1 mmol L^{-1} EDTA, using a 30 s pulses of a bead homogenizer (Bead Ruptor Elite, Omni International, Kennesaw, GA, USA). After centrifugation (at 4°C and 10,000*g* for 5 min), the resulting supernatant was divided into 2 aliquots: one for tissue glycogen measurements and the other for lactate, ATP, and phosphocreatine (PCr) analysis. After neutralization with potassium carbonate (glycogen aliquot) or potassium hydroxide (ATP/PCr aliquot), both aliquots were flash-frozen and stored at –80°C for later analysis.

The muscle tissue samples were analyzed using enzyme-linked assays outlined in Bergmeyer (1983) for ATP (hexokinase, HK), PCr (creatine kinase; CK), and lactate (lactate dehydrogenase; LDH). The second aliquot was used to enzymatically determine glycogen content by breaking it down into glucose using amyloglucosidase digestion, followed by enzymatic quantification of glucose concentrations using HK (Bergmeyer, 1983). All enzyme-linked metabolite concentrations were determined spectrophotometrically at 340 nm wavelength and using a microwell plate spectrophotometer (SpectraMax 190, Molecular Devices, CA, USA). All concentrations were expressed as μmol g^{-1} wet weight of tissue.

Calculations & statistical analysis

Experiment 1 – Acute toxicity tests:

Mortality data collected was analyzed in R (version 4.5.0, for R Code see online supplementary material), without transformation. The concentration-response relationship was determined by the generation of concentration-survival curves, where each tank (replicate or

not) was treated as an individual exposure. A generalized linear model (GLM) with a Bernoulli distribution and a logit link was applied to determine the effects of TFM dose, acclimation temperature, and their interaction on the probability of death (mortality) as follows:

$$y = \alpha + \beta_1 \times dose + \beta_2 \times T + \beta_3 \times dose \times T \quad (1)$$

$$m = \exp(y) / (1 + \exp(y)) \quad (2)$$

where α is equal to −15.2984, β_1 is equal to 3.8520, β_2 is equal to 0.2004, and β_3 is equal to −0.0846. R^2 is equal to 0.7233

For the R code, details of model generation and leave-one-out validation results, please see online supplementary material (Table S2).

The above equations were then reworked to determine the 12-hr LC50 and MLC (minimal lethal concentration; LC99.9) for different temperatures, as follows:

$$dose = (\ln(m / (1 - m)) - \alpha - \beta_2 * T) / (\beta_1 + \beta_3 \times T) \quad (3)$$

Confidence intervals (CIs) for the LC doses were estimated from the model by determining the TFM concentrations with a 5% chance of achieving the desired mortality (lower concentration bound) and those with a 95% chance of achieving the desired mortality (upper concentration bound). Differences in the respective 12-hr LC50 or MLC values were considered statistically significant if the 95% confidence intervals did not overlap (Finney, 1971). Toxicity data are reported as the 12-hr LC50 or 12-hr LC99.9 (MLC) ± 95% confidence interval (CI).

Experiment 2 – TFM tissue accumulation

Larval length and weight, TFM tissue concentrations, and muscle energy metabolites were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests to compare values between temperatures and within treatment groups (time periods) at a given temperature. In cases where the assumptions of normality and homogeneity were not met, non-parametric (Brown-Forsythe and Welch) ANOVA was used, followed by Dunn's Test of Multiple Comparisons test. Statistical analysis was performed using GraphPad Prism version

10.4.1 (GraphPad Software Inc, La Jolla, CA, USA). The morphometric and physiological data are reported as the mean ± S.E.M. The level of statistical significance was set at α = 0.05.

Results

Effects of acclimation temperature on body condition

As acclimation temperature increased from 7°C to 28°C (Experiment 1), the CF of the larval sea lamprey steadily increased from 1.19 ± 0.02 at 7°C to 1.29 ± 0.02 at 28°C, with a peak CF of 1.30 ± 0.02 at 25°C (Table 3). These differences in CF were primarily due to decreases in body length with acclimation temperature, which ranged from 117.2 ± 0.16 mm at 6°C to 109.2 ± 0.13 mm at 28°C (p = 0.002; Table 3). There were no significant changes in body mass across the 6 temperature (p > 0.05), however.

Similar observations were made in Experiment 2 (TFM accumulation), in which CF significantly increased from 1.09 ± 0.02 at 6°C to 1.27 ± 0.03 at 24°C (p < 0.0001). Again, these differences were due to the significantly lower (p = 0.015) body lengths, but not body mass, of the animals at 24°C (Table 3).

The measured mean TFM concentrations in exposure aquaria during acute toxicity tests were close to the nominal (target) concentrations (see online supplementary material, Table S1). Water pH was relatively stable (8.08 ± 0.04) across treatment concentrations and temperatures, while dissolved oxygen averaged 98.7 ± 0.5 and alkalinity during exposures was measured to be 221-238 mg L^{-1} as $CaCO_3$ (Table 1).

As water temperature increased incrementally from 7 to 28°C, the concentration-survival curves generated from the GLM model (see methods) underwent an overall rightward shift (Figure 2), reflecting a progressive increase in the 12-hr LC50 with temperature. The 12-hr LC50 increased from 4.3 mg L^{-1} (95% CI = 3.9 - 4.6 mg L^{-1}) at 7°C to 6.5 mg L^{-1} (6.1–7.2 mg L^{-1}) at 28°C, a more than 1.5-fold increase (Figure 3). Similarly, the MLC increased from an average of 5.7 mg L^{-1} (5.2–6.5 mg L^{-1}) at 7°C to 9.6 mg L^{-1} (8.5–12.1 mg L^{-1}) at 28°C, again a

greater than 1.5-fold increase between the low and high temperature exposures (Figure 3). Acclimations at 30°C were also attempted; however, sea lamprey were not able to survive at this temperature for more than a few days (3–4).

Effects of temperature on TFM tissue accumulation

All larvae survived the TFM exposure in cold (6°C) and warm (24°C) waters, but there were 2 mortalities out of 60 animals in the moderate 12°C treatment group (one at 9 hr and one at 12 hr), which were excluded from subsequent measurements and analysis. After the first hour of exposure, TFM accumulation in the liver was highest in the 12°C group, where liver TFM concentrations averaged 32.8 (± 3.9) nmol g^{-1} wet weight, which was significantly higher (p = 0.003) by approximately 39% than the TFM concentration measured at 24°C, averaging 19.9 (± 1.1) nmol g^{-1} wet weight. Accumulation was lowest in the 6°C group, which averaged 9.3 (± 1.0) nmol g^{-1} wet weight after 1 hr (Figure 4A). The TFM continued to accumulate in the liver at 12°C, before stabilizing after 6 hr of exposure, at which time the mean TFM concentration was 52.6 (± 9.1) nmol g^{-1} wet weight and did not significantly change thereafter (Figure 4A). In contrast to the 12°C lamprey, the concentration of liver TFM stabilized after only 3 hr in the 24°C group, at which time the concentration averaged 33.5 (± 2.1) nmol g^{-1} wet weight. The liver TFM concentration took the longest to stabilize at 6°C, reaching a concentration of 48.2 (± 6.3) nmol g^{-1} wet weight after 9 hr (Figure 4A). At 12 hr, the lowest TFM concentrations were observed in the 24°C lamprey (38.1 ± 2.5 nmol g^{-1} wet weight), which were 30% lower than in the 12°C lamprey (p = 0.002). However, the concentration of TFM in the liver of the 6°C lamprey (48.34 ± 6.4) fell between the measured concentrations of lamprey exposed to TFM at 12°C (54.73 ± 2.88) and 24°C lamprey, respectively, and were not different from either one (p = 0.75 and p = 0.41; Figure 4A).

Similar results were observed in the muscle tissue where, over the first hour of exposure, TFM accumulation was highest in the 12°C group, averaging 11.6 (± 0.9) nmol g^{-1} wet weight, while the TFM concentration in the 24°C group averaged 8.4 (± 0.5) nmol g^{-1} wet weight, which was significantly lower by approximately 28% (p = 0.028) than the moderate 12°C group (Figure 4B). As in liver, muscle TFM accumulation was lowest in the 6°C group after 1 hr, averaging 3.0 (± 0.2) nmol g^{-1} wet weight (Figure 4B). As observed in the liver, TFM concentration in the 24°C lamprey stabilized the fastest, after approximately 3 hr of exposure, averaging between 11.8 and 12.9 nmol g^{-1} wet weight thereafter (Figure 4B). In the 12°C lamprey, TFM continued to increase in the muscle through 9 hr of exposure, averaging 24.1 (± 1.0) nmol g^{-1} wet weight after 9 hr, then falling slightly to 20.1 (± 0.9) nmol g^{-1} wet weight after 12 hr (Figure 4B). The TFM increased steadily in muscle at 6°C over the entire 12 hr exposure period, but did not stabilize, reaching mean TFM concentrations of 16.5 (± 0.8) nmol g^{-1} wet weight after 12 hr (Figure 4B). In the 12°C group, the TFM muscle concentration was 18 to 51% higher than in the 6°C and 24°C lamprey at all measured time points. Over the 12 hr experimental period, the concentrations of TFM in the muscle at 6°C and 24°C overlapped at 6 hr (Figure 4B). However, concentrations were higher after 1 hr and 3 hr of exposure at 24°C (8.4 ± 0.5 and 12.1 ± 0.5 nmol g^{-1} wet wt) than at 6°C (3 ± 0.2 and 7.4 ± 0.9 nmol g^{-1} wet wt; p < 0.001; Figure 4B). After 6 hr of exposure at 6°C, TFM concentrations at 9 hr and 12 hr (14.6 ± 1.0 and 16.5 ± 0.8 nmol g^{-1} wet wt) were significantly higher (p = 0.038 and p = 0.013, respectively) than at 24°C (11.8 ± 0.7 and 12.9 ± 0.8 nmol g^{-1} wet wt). After 12 hr exposure, the muscle TFM concentration in the 24°C lamprey (12.9 ± 0.8 nmol g^{-1} wet wt) was significantly lower by approximately 22% (p = 0.001) and 36% (p < 0.001) than at 6°C (16.5 ± 0.8 nmol g^{-1} wet wt) and 12°C (20.1 ± 0.9 nmol g^{-1} wet wt) lamprey, respectively. Unlike in the

liver, TFM concentrations in the muscle at 6°C and 12°C were significantly different (p = 0.018) from one another (Figure 4B).

Effects of TFM exposure on muscle energy stores at different temperatures

Acclimation temperature had limited effects on the sea lamprey muscle energy stores during the 12-hr LC25 TFM exposure, with minimal changes in glycogen and lactate concentrations within temperature treatments, while ATP and PCr concentrations were only affected at 6 and 24°C. Following acclimation, muscle glycogen concentrations were not significantly different amongst the control (no TFM) lamprey acclimated to 6, 12, or 24°C, with respective no TFM exposure values averaging 16.7 (± 1.6) μmol g^{-1} wet weight, 15.9 (± 2.4) μmol g^{-1} wet weight and 10.3 (± 1.5) μmol g^{-1} wet weight (Table 4). The glycogen concentration in the 24°C group (5.9 ± 1.5) was significantly reduced (p < 0.01 and p = 0.02) from 6°C and 12°C (20.7 ± 3.6 and 15.1 ± 2.6 μmol g^{-1} wet wt) at 1 hr and thereafter.

Glycogen concentration in the 6°C and 12°C treatments changed minimally during TFM exposure, and only statistically significant in the 6°C group by 12 hr of exposure, when concentrations were 2.1 times higher than in muscle of the control animals (no TFM; 34.8 ± 4.8; Table 4). In contrast, at 24°C the muscle glycogen concentration trended downward from 10.3 (± 1.5) to 5.9 (± 1.5) μmol g^{-1} wet weight, but was not significant (p = 0.37).

Initial (control, no TFM) lactate levels in 24°C sea lamprey muscle tissue were significantly different from those at 6°C (p < 0.01) and 12°C (p = 0.02), increasing from 0.9 (± 0.2) μmol g^{-1} wet weight in the 6°C group, to 2.2 (± 0.7) at 12°C, and to 5.6 (± 0.9) μmol g^{-1} wet weight in the 24°C group (Table 4). During the 12-hr TFM exposure there was an increasing trend in muscle lactate only observed in the 6°C group, approximately 5-fold at 1, 3, and 6 hr, followed by a further and significant jump in lactate concentration at 9 hr and 12 hr when lactate was approximately 10-fold higher than the control values (both p < 0.01, Table 4). Notably, no

significant changes in muscle lactate concentration were observed in the lamprey exposed to TFM at 12°C or 24°C (Table 4).

The concentrations of ATP in control (no TFM) larval sea lamprey at 12°C were significantly lower than measurements made at 6°C and 24°C (both $p < 0.01$), while they were not significantly different from one another ($p = 0.65$; Table 4). This pattern of significant difference in ATP concentrations within the 12°C treatment when compared to the other 2 temperatures (6 and 24°C) persisted over most of the exposure periods (Table 4). In both 6°C and 24°C treatments, apart from 6 hr, there was a significant ($p < 0.05$) downward trend in the ATP concentrations across the 12-hr exposure. However, there was an inexplicable increase at 6 hr in both treatments, when ATP concentrations were 25% higher than in the unexposed control animals at this temperature (Table 4). 3-Trifluoromethyl-4'-nitrophenol exposure did not affect muscle ATP at 12°C, as none of the values obtained over the 12-hr exposure period significantly differed from the controls.

As with ATP, PCr concentrations were approximately 60% greater than control values at the 6 hr mark (Table 4). In the control levels (no TFM), PCr averaged 5.4 ± 0.2 μmol g^{-1} wet weight at 6°C, 8.9 ± 0.7 μmol g^{-1} wet weight at 12°C and 8.1 ± 0.9 μmol g^{-1} wet weight at 24°C, with only significant ($p < 0.01$) change between 6°C and 12°C (Table 3). At 6°C, muscle PCr concentrations were approximately 2-fold greater ($p = 0.04$ or $p < 0.01$) than those in the control animals but remained relatively stable throughout TFM exposure (Table 4). At 12°C, PCr concentration was relatively stable compared to controls (Table 4). At 24°C group, however, PCr gradually and significantly (p between < 0.05 and < 0.01) decreased during exposure and by 9 hr, PCr concentrations were 50% lower than observed in control animals (Table 4).

Discussion

The present study provides further evidence that the tolerance of larval sea lamprey to TFM increases with water temperature. Exposure to TFM in warmer water also led to less accumulation of TFM in the liver and muscle, suggesting that larval sea lamprey have a greater capacity to eliminate TFM from the body than at cooler temperatures. As we discuss below, we propose that the higher metabolic rates of larval sea lamprey at warmer temperatures (Holmes & Lin 1994; Lewis, 1980) increased their ability to eliminate TFM. These findings provide further insight into why the tolerance of larval sea lamprey to TFM changes seasonally, with higher TFM tolerance in the late summer when water temperatures are typically much warmer than in the spring (Hlina et al., 2021; Muhametsafina et al., 2019; Scholefield et al., 2008; Schueller et al., 2024).

Relationship between TFM toxicity and water temperature in larval sea lamprey

The progressive increase in the TFM 12-hr LC50 and 12-hr MLC values of larval sea lamprey as water temperature progressively increased from 7°C to 28°C provides further evidence that greater TFM tolerances in the summer are mainly a function of temperature (Muhametsafina et al., 2019). The most important metric of TFM toxicity used in sea lamprey control is the 9-hr LC99.9 (MLC; Bills et al., 2003), at which TFM concentrations need to be sustained (or higher) for a minimum of 9 hr in treated reaches of streams (Sullivan et al., 2021). Since the toxicity of TFM is dependent on water pH and alkalinity, treatment MLCs are routinely determined using pH-alkalinity charts based on prior comprehensive laboratory toxicity testing (Bills et al., 2003; Sullivan et al., 2021). More recently, lab and field studies have shown that the MLCs observed in the field typically exceed pH-alkalinity chart values at warmer temperatures, which was also the case in this study (Table 5; Muhametsafina et al., 2019; Hlina et al., 2021; Scholefield et al., 2008; Schueller et al., 2024). These findings lend additional support to previous observations that reliance on the pH-alkalinity chart concentrations alone can greatly underestimate the MLCs needed for effective sea lamprey

eradication. If strictly followed, use of these chart values would result in increased numbers of residual larval sea lamprey that survive treatment and subsequently metamorphose into parasitic sea lamprey (Muhametsafina et al., 2019; Schueller et al., 2024).

Previously, the tolerance of larval sea lamprey to TFM was shown to steadily increase as water temperatures increased from 6°C to 12°C to 24°C (Muhametsafina et al., 2019). Curiously, the relative increase in the respective 12-hr LC50 and 12-hr MLC of TFM over a similar temperature range (7–25°C) in the present study, increasing by approximately 1.3-fold and 1.5-fold, were lower than the 1.9-fold and 2.2-fold increases reported by Muhametsafina et al. (2019). At first glance, these apparent discrepancies suggest that the effects of temperature on TFM toxicity may be less predictable than thought. However, such an interpretation is misleading because the actual concentration changes in the MLC and LC50 values we observed were similar or greater than those reported by Muhametsafina et al. (2019; Table 6). For instance, the actual increase in the MLC between 7°C and 25°C in the present study was 2.9 mg L^{-1}, compared with approximately 2.2 mg L^{-1} observed between 6°C and 24°C in the earlier study. Thus, when interpreting temperature-TFM toxicity relationships, it is more appropriate to measure the actual (absolute) changes in MLC and LC50 at different temperatures rather than relative changes.

A likely explanation for the differences in the relative toxicity of TFM were the marked differences in water (titratable) alkalinity between the two studies. Water pH was more or less similar (Muhametsafina et al. - pH ~7.9 - 8.2; Present study - pH ~ 8.0 - 8.1), but alkalinity was much lower in the earlier study (~ 85 mg L^{-1} as $CaCO_3$), resulting in relatively lower MLC and LC50 values at each respective temperature (Table 6). On the other hand, alkalinity was 3 to 4 times higher in the present study (221–238 mg L^{-1} as $CaCO_3$), resulting in much higher MLC and LC50 values. As noted above, the MLC and LC50 values for TFM are much smaller at lower alkalinity (Bills et al., 2003; Wilkie et al., 2021). Hence, the increases in actual MLC with

temperature were proportionately greater at the lower alkalinities reported by Muhametsafina et al. (2019) than in the higher-alkalinity waters used in the present study.

The underlying mechanisms of why higher alkalinity attenuates TFM toxicity are unclear. One possibility is that the greater buffer capacity at higher alkalinity results in less acidification of the water next to the gill due to the excretion of respiratory CO_2 and metabolic acid (Lloyd & Herbert, 1960; Playle & Wood, 1989). This was demonstrated using rainbow trout (*Oncorhynchus mykiss*) fitted with opercular catheters, in which decreases in the pH of expired gill water were attenuated as water alkalinity increased (Wilkie et al., 2021). The uptake of TFM is directly related to water pH, with rates of TFM uptake increasing as water pH decreases due to increases in the relative amounts of the un-ionized species of TFM (TFM-OH) in the water (Figure 1; Hlina et al., 2017). Similarly, with less acidification, there would be less un-ionized TFM in the gill microenvironment than in a bulk water of a given TFM concentration, which would result in a lower inwardly directed TFM-OH gradient across the gills and lower TFM accumulation (Wilkie et al., 2021). Similar acidification in the gill micro-environment of larval lampreys likely takes place, but further studies are needed to test this hypothesis.

Dietary factors or energy status of the lamprey may have also contributed to differences in sensitivity but were likely relatively minor (Hlina et al., 2021; Schueller et al., 2024). The animals in the present study had been in the lab for 2 to 3 months and were fed a maintenance diet of yeast prior to toxicity testing, as opposed to being held for 7 to 10 days following capture in mid-summer after feeding on their natural diet of detritus (Hlina et al., 2021; Sutton & Bowen, 1994). The latter had substantially higher condition factors, falling between approximately 1.6 and 1.7 (Hlina et al., 2021), compared to 1.2 and 1.3 in the present study, which is an important index of energy stores in fishes (e.g. Busacker et al., 1990; Schloesser & Fabrizio, 2017), and other studies have suggested that higher body condition and liver

glycogen could be important determinants of TFM sensitivity in freshly caught sea lamprey (Scholefield et al., 2008; Schueller et al., 2024).

It is unlikely that the narrow differences in CF, which were due to shorter body lengths in the warm water larvae, had a significant impact on either TFM survival or TFM accumulation. Nalesnik et al. (2025) showed that there was a significant relationship between survival time, body length and body mass in larval sea lamprey exposed to TFM. The present study, however, was designed to examine the LC50 and MLC, not survival time. Along with water, TFM concentration (dose) and temperature, we tested mass and length as co-variables in our toxicity curve models (Figure 2). However, the strongest relationship (goodness of fit) based on lower Akaike Information Criterion (AIC) value, where for temperature and TFM concentration only. Inclusion of mass and length, alone or together, did not significantly improve the fit of our temperature versus survival model (Figure 2; online supplementary material, Table S2). Moreover, the McFadden pseudo-R^2 values were virtually identical when body mass and length were included in the calculation, indicating no additional influence of these parameters on the TFM toxicity-temperature relationship. It is also worth noting that both length and mass decreased with increasing acclimation temperature in the present study, when sea lamprey were more tolerant of TFM. Given the information above, plus the narrow range in the mean body mass and body length, less than 10 % in both cases, we are confident that differences in TFM tolerance with temperature were not influenced by variation in body size in these experiments.

Differences in body size also likely had little effect on the TFM uptake rates in the present study. Rates of TFM uptake are inversely related to body size in larval sea lamprey, as described by the allometric power equation: TFM uptake rate = $7.24\ M^{0.34}$ (Tessier et al., 2018). Because this relationship was established in water with a similar chemical make-up as the present study, we were able to use this formula to calculate how much mean TFM uptake

rates could have varied in the present experiment using the mean body mass data from Table 3. As expected, the mean rates of TFM uptake would have varied little, between 4.55 to 4.88 nmol g^{-1} hr^{-1}, or less than 10%, further suggesting that body mass had little effect on our findings.

The range of temperatures examined in the present study (7°C to 28°C) span those observed in Great Lakes rivers and streams between April and November that are infested with larval sea lamprey and likely to be treated with TFM (e.g., Hlina et al., 2021; Schueller et al., 2024). However, it should be noted that the likelihood of larval sea lamprey being present in streams in which water temperatures exceed their thermal niche (17.8–21.8°C) is reduced as water temperatures approach 25°C (Dawson & Jones, 2009; Holmes & Lin, 1994). It is unlikely that sea lamprey would be found living at 28°C, but temperatures approaching 24°C are not unusual in lamprey infested streams (Hlina et al. 2021).

A meta-analysis by Wang et al. (2019) demonstrated similar increases in tolerance with increasing temperature to phenol and pentachlorophenol in aquatic animals, including fishes, which was generally followed by a rapid decline as the temperature surpassed the thermal optima (an inverse V-shaped toxicity curve with temperature). Similarly, the toxicity of 2,4-dinitrophenol, another well known uncoupler of oxidative phosphorylation in mitochondria (Grundlingh et al., 2011), decreases with temperature in rainbow trout (Howe et al., 1994), as does the toxicity of phenol to trout before sharply declining as temperatures increase beyond the thermal preference zone (Patra et al., 2015).

Mechanisms of greater TFM tolerance in warmer waters based on TFM tissue accumulation

While several studies have shown that freshwater fishes exhibit increasing tolerance to phenolic compounds such as phenol, 2,4-dinitrophenol, and TFM with increasing temperature (e.g., Howe et al., 1994; Muhametsafina et al., 2019; Patra et al., 2015), the underlying mechanisms remain poorly understood. For this reason, patterns of TFM accumulation in the

liver and the muscle of larval sea lamprey were measured at 6°C, 12°C, and 24°C to provide insight into how TFM tolerance increases with temperature.

The primary site of TFM uptake is believed to be the gills, which (as noted above) is driven by the inward diffusion of TFM-OH down its water-to-blood concentration gradient (Wilkie et al., 2021). The rate of uptake is also proportional to metabolic rate, increasing as oxygen consumption rates increase, further demonstrating that the gill is the predominant site of TFM uptake (Tessier et al., 2018). As metabolic rate increases with ambient temperature in poikilothermic animals, including lampreys (Holmes & Lin, 1994; Lewis, 1980), it follows that elevated ventilation rates and gill irrigation also increase, resulting in higher uptake at the gill as demonstrated in larval sea lamprey using ^{14}C-TFM (Hlina et al., 2021; Tessier et al., 2018). Yet, after 12 hr of TFM exposure the measured concentration of TFM in the muscle and liver was lower in the larvae held at 24°C compared to those held at 12°C and 6°C (muscle only).

To explain these observations and provide further insight into how larval sea lamprey handled TFM at different temperatures we used a classical pharmacokinetics approach to calculate the steady state concentration (Css), or plateaus, at which the rated of accumulation is equal to the rate of elimination, and the half-life ($t_{1/2}$) of elimination (Gupta, 2016; Newman, 2020) for TFM from the liver and muscle at each temperature. Both of these organs are well perfused with blood (Percy & Potter, 1979; Peters & MacKay, 1961) in lamprey, and therefore representative of the relative TFM loads in the animals at the different temperatures. Each parameter was calculated using kinetic curves of the respective TFM accumulation versus time by liver and muscle using the data presented in Figure 4. The curves were fitted by non-linear regression using a one-compartment, least squares model (GraphPad Prism, version 10).

The analysis demonstrated that the Css in the liver at 24°C was 34.0 nmol g^{-1}, which was approximately 44% and 32% lower than the Css calculated at 6°C and 12°C, respectively (Figure 5A). The lower Css of the warm water lamprey, exposed to the same concentration of

TFM as those at 6°C and 12°C, supports our hypothesis that their capacity to detoxify TFM increases with temperature. A similar pattern was observed in the muscle at 24°C, where Css was approximately 40% lower than 6°C and 12°C (Figure 5B).

Because the rates of absorption of xenobiotics is equal to the rate of elimination under Css conditions (Gupta, 2016; Kalant & Roschlau, 1998; Newman, 2020) we used the kinetic curves to calculate the half-life for TFM elimination ($t_{1/2}$), the time required to clear 50% of the TFM load by the animals, treating both the liver and muscle as single compartments. The $t_{1/2}$ of TFM in the liver was 5.18 hr at 6°C, approximately 7-fold greater than that observed at 24°C, where $t_{1/2}$ was 0.77 hr, indicating that the rate of elimination and uptake of TFM were much slower in colder water (Figure 5A). The $t_{1/2}$ of TFM in the liver at 12°C and 24°C were not significantly different, suggesting that each had a similar rate of elimination. The differences in $t_{1/2}$ in the muscle were more clear-cut, decreasing in a stepwise manner as temperature increased (Figure 5B). The slower $t_{1/2}$ in the cold water also explains why there was no significant difference between the liver TFM concentration after 12 hr of exposure at 6°C and 24°C (Figure 4A). With a slow $t_{1/2}$ (5.18 hr – see above) at 6°C, the concentration of TFM in the liver would not have reached 95% of the Css of 60.1 nmol g^{-1} until approximately 22 hr, after 4.3 half-lives would have elapsed (Gupta, 2016).

We propose that as waters warm that sea lamprey increase their capacity to eliminate TFM through temperature dependent increases in the rates of enzymatically mediated TFM-biotransformation in the liver. In poikilotherms, metabolic rates and enzyme activities are known to increase with temperature, as characterized by greater maximal enzyme activities (V_{max}) which can result from changes in protein structure (e.g., folding-unfolding), and either enhance or inhibit enzyme function through changes in substrate-enzyme affinity (Somero, 2004). Future enzyme kinetic studies and the quantification of mRNA and proteins would shed further light on the underlying mechanisms of increased TFM tolerance in warmer waters.

The Phase II metabolic pathways are thought to be the primary method of enzymatic TFM biotransformation in fishes, including sea lamprey (Kane et al., 1994; Lech & Statham, 1975). These include glucuronidation, during which a glucuronic acid functional group is added to TFM to generate TFM-glucuronide (Figure 1B; Kane et al., 1994; Lech & Stathan, 1975), and sulfation, in which a sulphate functional group is used to form TFM-sulphate (Figure 1B; Bussy et al., 2018a, b). Kane et al. (1994) demonstrated that the activity of UDP-glucuronylsyltransferase (UDPGT), the enzyme required to convert parent TFM into TFM-glucuronide, was much higher in non-target fishes such as bluegill (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*), compared to sea lamprey. The efficiency of UDPGT (Vmax/Km) was also much lower in sea lamprey, which have since been found to also express fewer isoforms of the enzyme compared to bluegill (Kane et al., 1994; Lawrence et al., 2023). While the capacity of larval sea lamprey to produce TFM-glucuronide is limited, there is also recent evidence that sea lamprey use phenol sulfotransferase to biotransform parent TFM into TFM-sulfate, which may be quantitatively more important for TFM detoxification than UDPGT (Bussy et al., 2018a, b). Hence, an increase in the activities of UDPGT and/or phenol sulfotransferase with water temperature may explain why TFM accumulation is lower in warmer waters.

Effects of warmer water on muscle energy stores

Reduced rates of mitochondrial oxidative phosphorylation during TFM exposures lead to the consumption of tissue anaerobic energy reserves (glycogen and PCr) to sustain ATP production (Birceanu et al., 2011). An increased reliance on anaerobic glycogen stores for ATP supply in the tissues of sea lamprey and non-target fish also results in the accumulation of lactate (Birceanu et al., 2014; Clifford et al., 2012). Consistent with greater reliance on anaerobic metabolism, significant reductions in PCr were observed at 24°C, but not at 6°C or 12°C, where no significant declines were observed (Table 4). The decrease in PCr at 24°C

was likely related to the need to increase rates of PCr hydrolysis via the creatine phosphokinase reaction to buffer ATP stores (e.g., Clifford et al., 2012; Ionescu et al., 2021), compared to the colder 12°C and 6°C conditions, where ATP demands would be less. The significant increase in PCr with TFM at 6°C was unexpected, but we speculate that it may be due to their much lower metabolism at these temperatures, which in turn could be linked to lower ATP demand. The absence of significant changes in either glycogen or lactate at 12°C or 24°C were likely due to the relatively low concentration of TFM to which they were exposed (12-hr LC25 of TFM at 12°C; 3.74 ± 0.02 mg L^{-1}) compared to previous studies where sea lamprey were exposed to the 12-hr LC50 of TFM (Table 4). Had the TFM exposure periods been longer, particularly at 12°C or 24°C, we speculate we would have observed the anticipated drops in glycogen and higher lactate in the muscle (Wilkie et al., 2019). It should also be noted that the brain is more sensitive to TFM-induced reductions in glycogen and lactate, which were not examined in this study (Clifford et al., 2012).

Conclusion and Implications

The present study supports the hypothesis that the underlying mechanisms of increased TFM tolerance in larval sea lamprey at warmer temperatures are related to an enhanced capacity to increase rates of TFM elimination stemming from either greater rate of TFM metabolism and/or excretion. Future studies focused on measuring Phase II biotransformation products such as TFM-glucuronide or TFM-sulphate, enzyme kinetics, and enzyme gene and protein expression are needed to ascertain the underlying details that increase the sea lamprey's capacity to eliminate TFM at higher temperatures.

Our findings also provide further evidence of how warming temperatures later in the summer, or more sustained or transient elevations of water temperature due to climate change, could potentially undermine sea lamprey control measures in the Great Lakes (Lennox et al., 2020). We propose that temperature be considered when establishing TFM treatment

concentrations, as routinely done with water pH and alkalinity (Bills et al., 2003; Schueller et al., 2024), to compensate for temperature-induced effects on TFM toxicity. Although most teleosts and invertebrate species of concern (e.g., native mussels) are less sensitive to TFM than the sea lamprey, it is not known how they will respond to TFM at higher temperatures, when metabolic demand is greater and oxygen availability is lower. How non-target species respond to TFM at warmer temperatures, and how to mitigate any adverse effects of lampricides under such conditions, should be a priority of future studies.

Beyond sea lamprey control, we propose that the simple phenolic structure of TFM may make it an ideal analog to model the uptake, distribution, detoxification and elimination of more toxic phenols in aquatic ecosystems. There are several phenolic compounds that are highly toxic even at very low concentrations and can be carcinogens (e.g., pentachlorophenol; International Agency for Research on Cancer, 2025), endocrine disruptors such as bisphenol A (e.g., BPA; Surana et al., 2022) or can cause genetic malformations (du Plessis et al., 2023). Further research into the effects of temperature on the pharmacokinetics of TFM and other phenolic compounds is needed to expand our understanding of how temperature influences their acute and chronic toxicity to various aquatic organisms. This research may be particularly important as the average and peak temperatures of aquatic ecosystems increase due to climate change.

Figure 1. **Structure and detoxification of 3-trifluoromethyl-4-nitrophenol (TFM).** (A) Structure of un-ionized TFM and ionized TFM, and corresponding dissociation constant (pKa). (B) The predominant Phase II TFM biotransformation products, TFM-glucuronide and TFM-sulphate. UDP-glucuronylsyltransferase (UDPGT) enzyme.

Figure 2. Shifts in the 3-trifluoromethyl-4-nitrophenol (TFM) concentration-mortality curves of sea lamprey with increasing temperature. Curves depicting changes in the toxicity of TFM with increasing temperature. Fitted survival curves (solid line) based on % mortality per test tank (*n* = 5 per tank; 2 tanks per concentration; solid dots). Grey (light) shading depicts 95% confidence intervals. Dotted lines represent the curve at the preceding temperature. *n* = 480 (*n* = 10 fish per concentration x 8 concentrations x 6 temperatures).

Concentration-mortality curves were generated from a generalized linear model (GLM) with a Bernoulli distribution and a logit link (for R code, see online supplementary material).

Figure 3. Effects of temperature on the acute toxicity of 3-trifluoromethyl-4-nitrophenol (TFM) to larval sea lamprey. Data depicted as the corresponding 12-hr LC50 (blue bars) and minimum lethal concentration (12-hr LC99.9; MLC, red bars) of TFM to larval sea lamprey (based on the results of Figure 2). Acclimation above 28°C was attempted, but survival was poor and required no further analysis. The 95% confidence intervals (CI) values sharing an identical lowercase letter are not significantly different between LC50 values, while identical uppercase letters denote no significant differences between MLC values across temperatures. Values where the 95% CI do not overlap are considered significantly different (Finney, 1971).

Figure 4. Effects of temperature on 3-trifluoromethyl-4-nitrophenol (TFM) accumulation in larval sea lamprey. The TFM concentration in the (A) liver and (B) muscle tissues of larval lamprey exposed to a TFM concentration (3.74 mg L^{-1} ± 0.02) at temperatures of 6°C (blue), 12°C (yellow) and 24°C (red). Significantly different mean values within a single temperature are denoted by different lowercase letters. Significantly different mean values between the temperatures at a single time period are denoted by different uppercase letters. Data presented as mean ± SEM (n = 8-10; $p < 0.05$).

Figure 5. Relationship between temperature and 3-trifluoromethyl-4-nitrophenol (TFM) accumulation and elimination. The TFM concentration in the (A) liver and (B) muscle of larval sea lamprey exposed to a common concentration of TFM (3.74 mg L^{-1} ± 0.02). Curves were fitted by non-linear regression using least squares analysis from which the apparent steady state concentration (Css) and elimination half-life ($t_{1/2}$) were calculated. The $t_{1/2}$ elimination was calculated from the rate constant of TFM accumulation (k) to reach Css, based on the assumption that at Css the rate of TFM elimination was equal to its rate of accumulation. Calculations and curve fitting completed using GraphPad Prism 10. Data re-plotted from Figure 4. n = 7-10 samples at each time period. Light dashed lines: 95 % confidence intervals.

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Table 1. **Measured water quality during 12-hr toxicity tests of larval sea lampre**

Temperature (°C)			**Alkalinity**		
Nominal	Measured	pH	(mg L^{-1} as $CaCO_3$)	Dissolved O_2	#*n*
7	7.3 ± 0.1	8.08 ± 0.01	221	95.7 ± 1.8	28
12	11.9 ± 0.2	8.11 ± 0.03	221	99.1 ± 0.8	40
18	18.2 ± 0.1	8.08 ± 0.02	221	99.9 ± 0.4	40
21	21.3 ± 0.1	8.06 ± 0.01	221	98.1 ± 0.4	40
25	24.9 ± 0.1	8.08 ± 0.03	238	97.8 ± 0.3	30
28	27.0 ± 0.4	8.09 ± 0.03	238	96.5 ± 0.6	30

Data presented as the mean ± S.E.M. #*n* denotes the number of total measurements

y to a range of 3-trifluoromethyl-4-nitrophenol (TFM) concentrations at different

s per treatment. Nominal and measured TFM concentrations across temperature and f

temperatures.

for associated mortalities see online supplementary material.

Table 2. Measured water quality and 3-trifluoromethyl-4-nitrophenol (TFM) conce

Temperature (°C)			**Alkalinity**			**TFM** (mg L^{-1})	
Nominal	Measured	pH	(mg L^{-1} as $CaCO_3$)	Dissolved O_2	#*n*	Nominal	Measured
6	6.2 ± 0.1	8.01 ± 0.02	221	98.4 ± 0.5	22	3.74	3.74 ± 0.04
12	12.3 ± 0.1	8.00 ± 0.02	204	94.9 ± 1.2	22	3.74	3.75 ± 0.04
24	23.9 ± 0.1	7.99 ± 0.01	204	94.2 ± 0.6	22	3.74	3.72 ± 0.02

Data presented as the mean ± S.E.M. *n* denotes the number of total measurements pe

entrations during the 12-hr exposure of larval sea lamprey to a nominal (3.74 m

er treatment.

g L^{-1}) TFM concentration across temperatures.

Table 3. Effects of acclimation temperature on body length, mass and c

Temperature (°C)	**CF**	**Mass** (g)
Experiment 1 - TFM Toxicity		
7	1.19 ± 0.02[a] (75)	2.00 ± 0.08[a] (80)
12	1.22 ± 0.01[a] (80)	2.02 ± 0.07[a] (80)
18	1.23 ± 0.02[ab] (70)	1.91 ± 0.08[a] (80)
21	1.26 ± 0.02[ab] (70)	1.96 ± 0.09[a] (80)
25	1.30 ± 0.02[b] (70)	1.91 ± 0.09[a] (79)
28	1.29 ± 0.02[b] (69)	1.82 ± 0.08[a] (80)
Experiment 2 - TFM Accumulation		
6	1.09 ± 0.02[a] (48)	1.92 ± 0.07[a] (48)
12	1.13 ± 0.02[a] (60)	1.84 ± 0.09[a] (60)
24	1.27 ± 0.03[b] (60)	1.83 ± 0.07[a] (60)

Larval sea lamprey were acclimated for a minimum of 3 weeks. Number (*n*)

condition factor (CF).

Length (mm)
117.2 ± 0.16[a] (75)
117.5 ± 0.15[a] (80)
114.4 ± 0.14[a] (70)
114.7 ± 0.15[a] (80)
111.2 ± 0.14[b] (70)
109.2 ± 0.13[b] (69)
116.6 ± 0.15[a] (48)
114.6 ± 0.17[a] (60)
108.4 ± 0.13[b] (60)

represents the number of larval sea lamprey per temperature treatment and variable. I

Data presented as mean ± SEM. Data sharing a common letter are not significantly di

ifferent from one another ($p < 0.05$). CF = [Mass / (Length)^3] x 10^6 (Holmes & Youson

ı, 1994).

Table 4. Changes in muscle energy stores and metabolite concentrat

	6°C	
Time (hr)	Mean ± SEM	Significance
[Glycogen] (μmol g-¹ wet weight)		
Control	16.65 ± 1.58a	
1	20.7 ± 3.61a	0.96
3	22.31 ± 3.36a	0.85
6	28.79 ± 3.31a	0.12
9	26.41 ± 3.75a	0.36
12	34.77 ± 4.82*a	**<0.01**
[Lactate] (μmol g^{-1} wet weight)		
Control	0.89 ± 0.18a	
1	4.23 ± 0.92ab	0.08
3	4.65 ± 1.16a	0.14
6	4.20 ± 0.81*a	0.05
9	8.82 ± 0.75*a	**<0.01**
12	8.34 ± 1.45*a	**0.02**
[ATP] (μmol g^{-1} wet weight)		
Control	3.54 ± 0.03a	
1	3.23 ± 0.11a	0.24
3	3.32 ± 0.05a	**0.04**
6	5.25 ± 0.09*a	**<0.01**
9	3.43 ± 0.08a	0.32
12	3.25 ± 0.06*a	**0.01**
[PCr] (μmol g^{-1} wet weight)		
Control	5.37 ± 0.16a	
1	9.67 ± 1.06*a	0.05
3	10.43 ± 0.72*a	**<0.01**
6	11.54 ± 0.21*a	**<0.01**
9	8.21 ± 0.64*a	**0.04**
12	10.86 ± 0.86*a	**<0.01**

Larval sea lamprey were exposed to the same concentration of TFM (3.74

tions in larval sea lamprey exposed to 3-trifluoromethyl-4-nitrophenol (TFM) at diff

12°C		24°C	
Mean ± SEM	Significance	Mean ± SEM	Significance
15.86 ± 2.39a		10.33 ± 1.51a	
15.13 ± 2.26a	> 0.99	5.91 ± 1.54b	0.37
15.81 ± 2.25a	> 0.99	5.93 ± 1.24b	0.37
15.23 ± 2.83b	> 0.99	4.95 ± 1.34c	0.14
16.11 ± 2.64b	> 0.99	6.64 ± 1.55c	0.64
20.73 ± 1.73b	0.71	6.59 ± 1.36c	0.58
2.19 ± 0.68a		5.55 ± 0.86b	
2.77 ± 0.79a	0.99	6.16 ± 0.86b	0.86
4.22 ± 0.89a	0.57	7.07 ± 1.05a	1.05
5.29 ± 0.85a	0.14	6.39 ± 0.44a	0.44
5.84 ± 1.06a	0.06	6.29 ± 0.84a	0.84
5.74 ± 0.90a	0.07	6.53 ± 0.86a	0.86
1.92 ± 0.18b		3.29 ± 0.23a	
2.04 ± 0.30b	0.91	0.83 ± 0.18*c	**<0.01**
1.86 ± 0.21b	0.91	0.94 ± 0.24*c	**<0.01**
1.30 ± 0.20b	0.16	4.46 ± 0.47a	0.41
1.21 ± 0.19b	0.12	0.84 ± 0.14*b	**<0.01**
1.66 ± 0.30b	0.27	0.34 ± 0.08*c	**<0.01**
8.37 ± 0.60b		8.09 ± 0.88ab	
5.93 ± 0.33*b	**0.03**	5.07 ± 0.38*b	**<0.05**
7.99 ± 0.56a	0.44	5.23 ± 0.76b	0.12
7.17 ± 0.63b	0.44	13.46 ± 1.25*a	**0.02**
7.18 ± 0.78a	0.24	3.60 ± 0.32*b	**<0.01**
6.86 ± 0.77b	0.18	4.20 ± 0.60*c	**0.01**

1 mg L^{-1} ± 0.02) at 6°C,12°C and 24°C. Asterisks denote significant differences within the

erent temperatures.

e temperature treatment compared to control values ($p < 0.05$), while a unique lower-c

case letter denote significant differences between the treatment temperatures at a spe

ecific time point ($p < 0.05$). N = 8 - 10 (10 - 15 control) lamprey per timed exposure ac

:ross temperatures.

Table 5. **Differences in the observed and predicted minimal lethal concentra**

Temperature (°C)	**pH**	**Alkalinity** (mg L^{-1} as CaCO3)	**Observed MLC**	**Predicted MLC**
7	8.08 ± 0.01	221	5.67 (5.23 – 6.51)	3.4
12	8.11 ± 0.03	221	6.16 (5.69 – 7.03)	3.4
18	8.08 ± 0.02	221	6.99 (6.48 – 7.83)	3.4
21	8.06 ± 0.01	221	7.56 (7.04 – 8.39)	3.4
25	8.08 ± 0.03	238	8.57 (7.91 – 9.73)	3.6
28	8.09 ± 0.03	238	9.63 (8.52 – 12.07)	3.6

Predicted 12-hr MLC values obtained from charts depicting expected lethal conc

ation (MLC) of 3-trifluoromethyl-4-nitrophenol (TFM) to larval sea lamprey accli

Difference (fold)
1.67
1.81
2.06
2.22
2.38
2.68

entrations of TFM to produce 99.9% mortality in larval sea lamprey at selected water

imated to different water temperatures.

pH and alkalinities (Bills et al., 2003).

Table 6. Differences between the present study and Muhametsafina et al., 2019 in rela

Experimental Study	Temp Range	ΔMLC	
		Relative change (fold)	Actual change (mg L^{-1})
Present Study	7 - 25°C	1.3 - 1.6	2.9
Muhametsafina et al., 2019	6 - 24°C	1.8 - 2.5	2.2

ative and actual changes of minimal lethal concentration (ΔMLC) and LC50 (ΔLC50)

ΔLC50	
Relative change (fold)	**Actual change (mg L^{-1})**
1.3 - 1.5	1.7
1.8 - 2.0	1.5

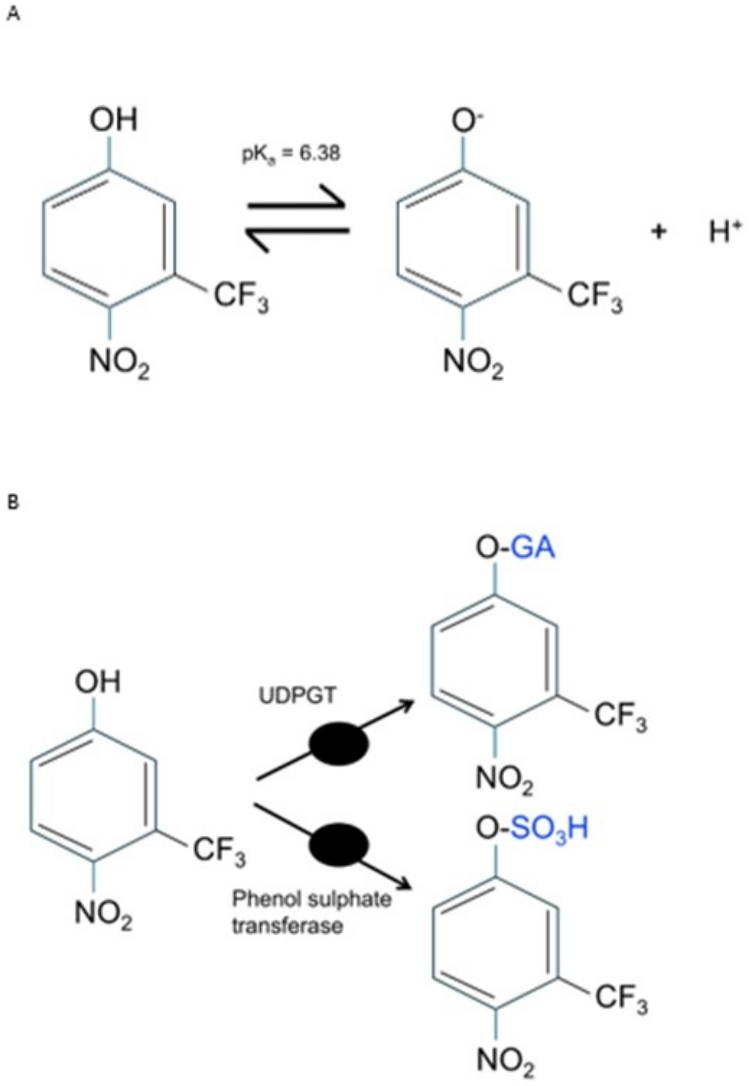


Figure 1. Structure and detoxification of 3-trifluoromethyl-4-nitrophenol (TFM). (A) Structure of un-ionized TFM and ionized TFM, and corresponding dissociation constant (pKa). (B) The predominant Phase II TFM biotransformation products, TFM-glucuronide and TFM-sulphate. UDP-glucuronylsyltransferase (UDPGT) enzyme.

Alt text: Graphical representation of TFM chemical structure. (A) nitro- and trifluoromethyl-substituted phenol can deprotonate to form the corresponding phenoxide ion, and the equilibrium between the protonated and deprotonated forms is governed by a pK_a of 6.38. (B) The substituted phenol can be metabolized by phase II enzymes: UDP-glucuronosyltransferases attach a glucuronic acid to form a glucuronide conjugate, while phenol sulfate transferases attach a sulfate to form a sulfate conjugate.

160x219mm (96 x 96 DPI)

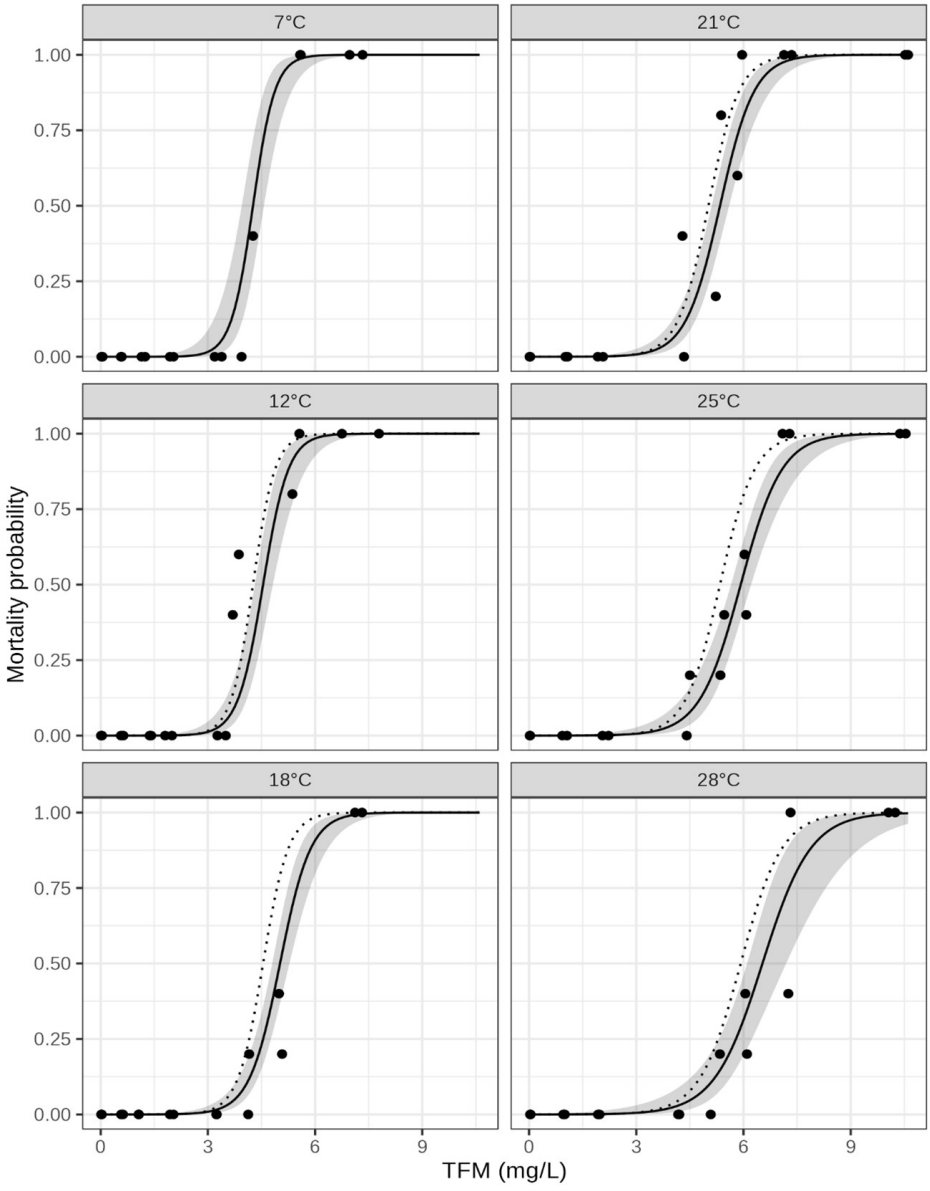


Figure 2. Shifts in the 3-trifluoromethyl-4-nitrophenol (TFM) concentration-mortality curves of sea lamprey with increasing temperature. Curves depicting changes in the toxicity of TFM with increasing temperature. Fitted survival curves (solid line) based on % mortality per test tank (n = 5 per tank; 2 tanks per concentration; solid dots). Grey (light) shading depicts 95% confidence intervals. Dotted lines represent the curve at the preceding temperature. n = 480 (n = 10 fish per concentration x 8 concentrations x 6 temperatures). Concentration-mortality curves were generated from a generalized linear model (GLM) with a Bernoulli distribution and a logit link (for R code, see online supplementary material).
Alt text: All temperatures show a sigmoid (S-shaped) dose–response: low mortality at low dose, rapid rise at mid doses, high mortality at upper doses. The individual black points represent observed mortality data; the solid curve is a fitted model, and the shaded area (and dotted line) indicates uncertainty. Across temperatures from 7°C to 28°C, the TFM dose–response curves shift and change shape.

152x193mm (220 x 220 DPI)

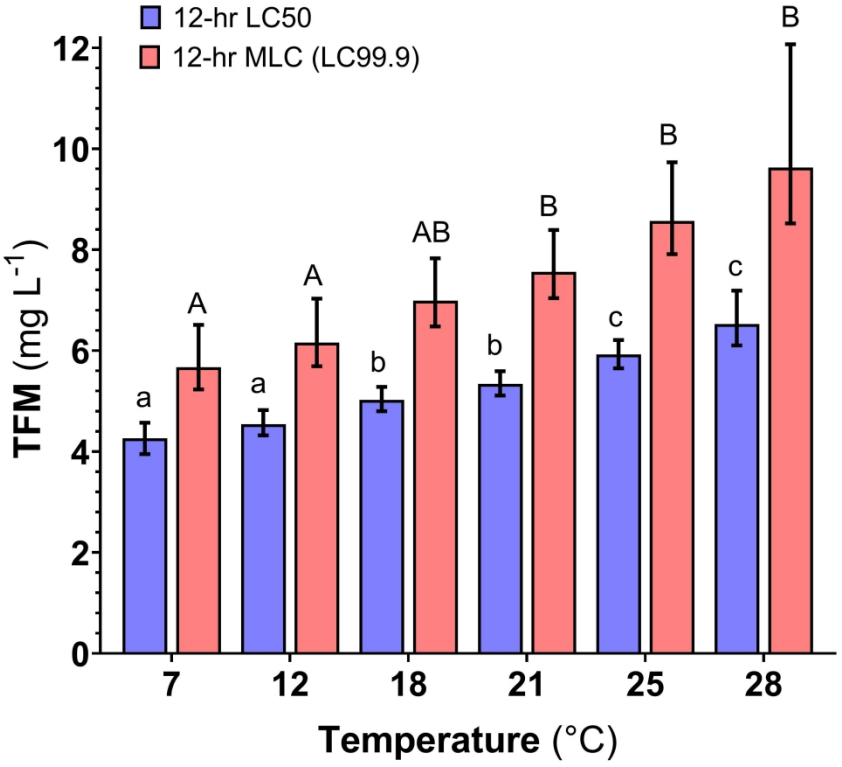


Figure 3. Effects of temperature on the acute toxicity of 3-trifluoromethyl-4-nitrophenol (TFM) to larval sea lamprey. Data depicted as the corresponding 12-hr LC50 (blue bars) and minimum lethal concentration (12-hr LC99.9; MLC, red bars) of TFM to larval sea lamprey (based on the results of Figure 2). Acclimation above 28°C was attempted, but survival was poor and required no further analysis. The 95% confidence intervals (CI) values sharing an identical lowercase letter are not significantly different between LC50 values, while identical uppercase letters denote no significant differences between MLC values across temperatures. Values where the 95% CI do not overlap are considered significantly different (Finney, 1971).
Alt text: The figure displays TFM concentrations (mg L^{-1}) needed to kill fish at different temperatures. Two metrics are shown for each temperature: 12-hr LC50 (blue bars) — the TFM concentration that kills 50% of the fish in 12 hours. 12-hr MLC (LC99.9) (red bars) — the TFM concentration that kills ~99.9% in 12 hours (practically almost all).

264x208mm (300 x 300 DPI)

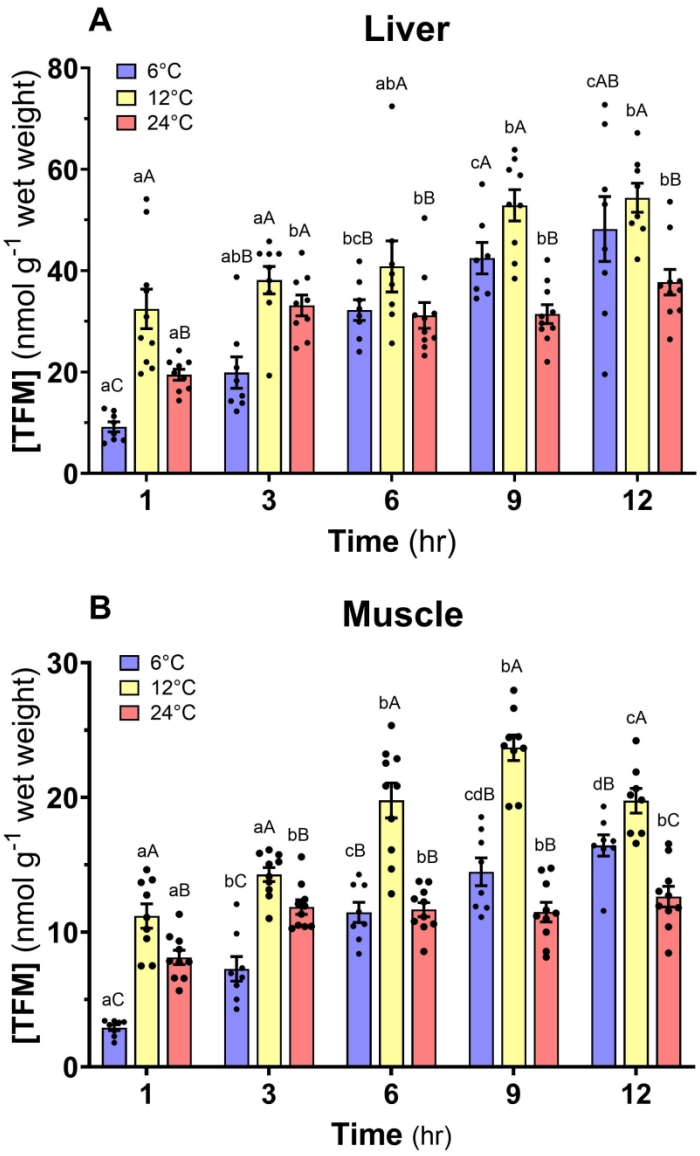
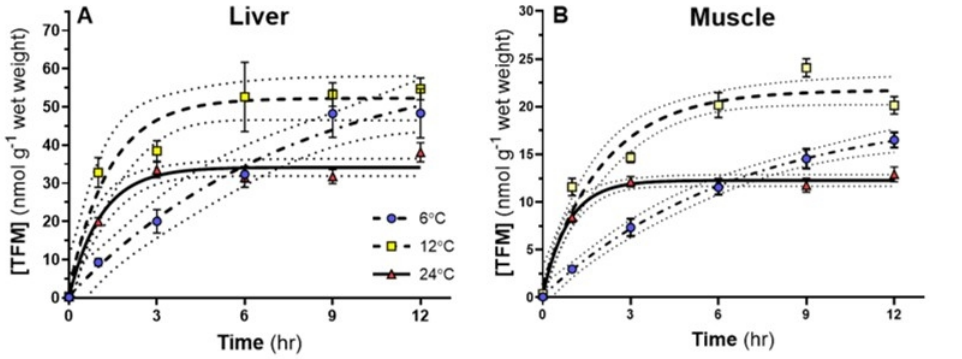


Figure 4. Effects of temperature on 3-trifluoromethyl-4-nitrophenol (TFM) accumulation in larval sea lamprey. The TFM concentration in the (A) liver and (B) muscle tissues of larval lamprey exposed to a TFM concentration (3.74 mg L-1 ± 0.02) at temperatures of 6°C (blue), 12°C (yellow) and 24°C (red). Significantly different mean values within a single temperature are denoted by different lowercase letters. Significantly different mean values between the temperatures at a single time period are denoted by different uppercase letters. Data presented as mean ± SEM (n = 8-10; $p < 0.05$).

Alt text: (A) This bar chart shows how much TFM (in nmol per gram of wet weight) accumulated in the liver of fish over time (1, 3, 6, 9, 12 hours) at three temperatures: 6°C (blue), 12°C (yellow), 24°C (red). There are statistical letters above the bars indicating differences: bars with different letters (capital and lowercase) are significantly different from one another within that time point.

(B) This bar chart shows TFM in muscle tissue over the same time points and temperatures. Again, statistical letters indicate which values differ at each time point.



Temp (°C)	Css (nmol g^{-1} wet weight)	$t_{1/2}$ Elimination (hr)	k (hr^{-1})	R^2
6°C	60.1 (48.1-109.9)	5.18 (2.95-13.04)	0.134 (0.053-0.235)	0.800
12°C	48 (43.7-52.7)	0.73 (0.42-1.25)	0.945 (0.341-1.660)	0.742
24°C	33.8 (31.1-36.1)	0.77 (0.55-1.07)	0.898 (0.647-1.271)	0.811

Temp (°C)	Css (nmol g^{-1} wet weight)	$t_{1/2}$ Elimination (hr)	k (hr^{-1})	R^2
6°C	19.5 (16.6-25.0)	4.55 (3.21-7.12)	0.152 (0.097-0.216)	0.906
12°C	20.7 (19.1-22.6)	1.29 (0.88-1.82)	0.536 (0.380-0.788)	0.86
24°C	12 (11.4-12.6)	0.6 (0.46-0.78)	1.148 (0.882-1.536)	0.861

Figure 5. Relationship between temperature and 3-trifluoromethyl-4-nitrophenol (TFM) accumulation and elimination. The TFM concentration in the (A) liver and (B) muscle of larval sea lamprey exposed to a common concentration of TFM (3.74 mg L-1 ± 0.02). Curves were fitted by non-linear regression using least squares analysis from which the apparent steady state concentration (Css) and elimination half-life (t1/2) were calculated. The t1/2 elimination was calculated from the rate constant of TFM accumulation (k) to reach Css, based on the assumption that at Css the rate of TFM elimination was equal to its rate of accumulation. Calculations and curve fitting completed using GraphPad Prism 10. Data re-plotted from Figure 4. n = 7-10 samples at each time period. Light dashed lines: 95 % confidence intervals.
Alt text: The graph has two side-by-side plots showing changes in internal TFM concentration over time for three temperature treatments: low (blue circles), medium (yellow squares), and high (red triangles). Time points include multiple hours during exposure. Nearer Table: The Css, t1/2 Elimination, k constant and R2 value for TFM accumulation in liver across temperatures. Further Table: The Css, t1/2 Elimination, k constant and R2 value for TFM accumulation in muscle across temperatures.

220x165mm (96 x 96 DPI)