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# The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) stimulates oxygen consumption by larval sea lamprey in a dose-dependent manner

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ABSTRACT

Sea lamprey (*Petromyzon marinus*) are an invasive species in the Laurentian Great Lakes, where parasitism by blood-feeding juvenile lampreys greatly reduced populations of economically and culturally important native fishes in the early-mid 1900s. To control sea lamprey populations, the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) is added to streams infested with larval sea lamprey. Sea lamprey have a lower capacity to detoxify TFM than most non-target fishes, making it a highly effective pesticide. TFM decreases ATP production by disrupting oxidative phosphorylation in the mitochondria, leading to an increase in mitochondrial oxygen consumption. However, little is known about how TFM affects whole animal oxygen consumption in sea lamprey and other fishes. To test the hypothesis that TFM has dose-dependent effects on larval sea lamprey metabolic rate, we measured the mass-specific oxygen consumption rates ($\dot{M}_{O_2}$) of larval sea lamprey using intermittent-flow respirometry during TFM exposure. Exposure to increasing concentrations of TFM led to stepwise increases in $\dot{M}_{O_2}$ in larval sea lamprey, resulting in death after $\dot{M}_{O_2}$ reached levels equivalent to their known maximum metabolic rates. Similar measurements of $\dot{M}_{O_2}$ could be used to determine the relative TFM sensitivity of non-target species to better assess the potential impacts of TFM on resident fisheries.

## 1. Introduction

The sea lamprey (*Petromyzon marinus*) is an invasive species in the Laurentian Great Lakes, gaining access from the Atlantic Ocean to the Great Lakes through man-made shipping canals (Eshenroder, 2014; Morman et al., 1980). Sea lamprey start their lives as filter-feeding larvae and live burrowed in the soft sediment of streams (Sutton and Bowen, 1994; Wilkie et al., 2022). After 3–7 years, the larvae metamorphose into juveniles that migrate downstream and feed on the blood of other fishes using their toothed oral disc and tongue (Farmer, 1980; Renaud et al., 2009). After approximately 12–20 months, the much larger, maturing sea lamprey cease feeding and migrate up streams where they spawn and die (Applegate, 1951; Bergstedt and Swink, 1995).

Sea lamprey parasitism of large native fishes in the Great Lakes, along with commercial overfishing, contributed to massive reductions in the populations of commercial, recreational, and culturally significant fishes (Applegate, 1951; Gaden et al., 2021; Smith and Tibbles, 1980; Sullivan et al., 2021). This led Canada and the United States to establish the Great Lakes Fishery Commission (GLFC) in 1955, who is mandated to manage fisheries and to implement a control program to eradicate sea lamprey from the Great Lakes (Gaden et al., 2021). Sea lamprey were not eradicated, but populations were reduced by more than 90% from historic highs in the 1950s using an extensive network of barriers to prevent adults from reaching their spawning grounds, and through the application of 3-trifluoromethyl-4-nitrophenol (TFM) to streams infested with larval sea lamprey (Siefkes, 2017; Sullivan et al., 2021).

TFM is typically applied to sea lamprey-infested streams every 2–4 years based on the likelihood of larvae metamorphosing into juveniles the following year (McDonald and Kolar, 2007). Once a stream is selected for treatment, the amount of TFM applied is based on the minimum lethal concentration (MLC) of TFM (i.e., the concentration needed to kill 99.9% of larval sea lamprey over a 9–12h exposure period; Bills et al., 2003; Sullivan et al., 2021). Lampreys are more susceptible to

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TFM than most other aquatic organisms (Boogaard et al., 2003; Wilkie et al., 2019), with Boogaard et al. (2003) showing toxicity ratios ranging between 1.27 and 8.78 for select non-target comparisons. This is due to their lower capacity to biotransform and detoxify TFM using phase II biotransformation pathways in the liver (Bussy et al., 2018; Kane et al., 1994; Lech and Statham, 1975). Recent studies suggest that this is because lampreys express a limited number of genes coding for different isoforms of two phase II biotransformation pathways, UDP-glucuronosyltransferase enzymes (UDP-GTs), along with genes coding for sulfotransferase enzymes, which conjugate TFM to TFM-sulfate (Lawrence et al., 2022, 2023).

A defining feature of uncoupled oxidative phosphorylation is an increase in the rate of mitochondrial state 4 oxygen consumption (leak respiration). Under steady-state conditions, transmembrane potential across the inner mitochondrial membrane is maintained by complexes I, III, and IV of the electron transport chain (ETC). Each translocates $H^+$ from the mitochondrial matrix, across the inner mitochondrial membrane, into the intermembrane space. The net accumulation of $H^+$ in the intermembrane space generates a $H^+$-gradient (the proton-motive force), across the inner membrane that is needed to drive ATP synthesis via the ATP synthase. As $O_2$ is the final electron acceptor of the ETC, this process consumes $O_2$ at relatively low (basal) rates under state 4 conditions. In the presence of ADP substrate and higher ATP demand, ATP synthesis increases, leading to greater oxygen consumption (called state 3 or OX-PHOS respiration) due to the need to increase $H^+$-pumping to counter increased flux of $H^+$ into the matrix via the ATP-synthase.

Exposure of mitochondria to TFM results in elevated state 4 respiration because, like other uncouplers, TFM acts as a protonophore leading to increased transmembrane $H^+$ permeability, which decreases the transmembrane $H^+$ gradient (Birceanu et al., 2011; Borowiec et al., 2022; Huerta et al., 2020). This corresponding decrease in the proton-motive force not only decreases ATP production via the ATP-synthase under state 3 conditions, it triggers a negative feedback response resulting in increased $H^+$-pump activity by the ETC protein complexes to restore the transmembrane $H^+$-gradient under state 4 conditions (Wilkie et al., 2019). As $O_2$ is the final electron acceptor of the ETC, rates of state 4 mitochondrial oxygen consumption increase in the presence of TFM (Birceanu et al., 2011; Borowiec et al., 2022; Huerta et al., 2020). When mitochondrial ATP supply no longer meets metabolic demands under these conditions, anaerobic pathways of ATP production are activated, such as glycolysis and phosphocreatine hydrolysis, to temporarily make up the ATP shortfall (Birceanu et al., 2009, 2014; Clifford et al., 2012; Ionescu et al., 2021). However, when anaerobic fuel sources are depleted, homeostasis deteriorates leading to death (Wilkie et al., 2019).

In the present study, we hypothesized that increases in mitochondrial oxygen consumption caused by TFM exposure translate into an overall increase in mass-specific oxygen consumption ($\dot{M}_{O_2}$) at the whole animal level. Kawatski et al. (1974) reported greater $\dot{M}_{O_2}$ of midge larvae exposed to TFM using classical static respirometry, consistent with uncoupled oxidative phosphorylation. Surprisingly, little else is known about the effects of TFM upon whole animal $\dot{M}_{O_2}$, both in sea lamprey or non-target organisms, during and following TFM exposure. In the present study, $\dot{M}_{O_2}$ was quantified in larval sea lamprey using automated, intermittent-flow respirometry during and following TFM exposure. We predicted that exposure to stepwise increases in TFM concentrations would lead to dose-dependent increases in the $\dot{M}_{O_2}$ of larval sea lamprey that could serve as a non-lethal measure of TFM toxicity.

## 2. Methods

### 2.1. Animal holding

Larval sea lamprey were obtained from a stock maintained at the Hammond Bay Biological Station (US Geological Survey, Millersburg, MI), following their capture by US Fish and Wildlife Service personnel using pulsed-DC electrofishing. The animals were subsequently shipped to Wilfrid Laurier University (WLU) by overnight courier in plastic bags (N = 200–250 larvae per bag) filled with ice-cold, $O_2$-saturated water packed in hard-sided coolers. At WLU, the animals were transferred into a 110 L fibreglass aquarium supplied with dechlorinated, aerated City of Waterloo water, with the bottom lined with 4–5 cm sand to provide the larvae with burrowing substrate. The tanks were on partial recirculation, with biological filtration taking place in a lower sump and temperature maintained at 13 ± 1°C. The larvae were fed a slurry of wet baker's yeast on a weekly basis (approx. 1 g yeast per larva). Water pH (pH 8.4–8.5) and alkalinity (~210 mg $L^{-1}$ as $CaCO_3$) was regularly monitored as part of our standard animal husbandry practices. All experimental procedures were approved by the WLU Animal Care Committee (Animal Use Protocol No. R22003) and adhered to the guidelines of the Canadian Council of Animal Care (CCAC).

### 2.2. Experimental setup

$\dot{M}_{O_2}$ was determined using intermittent-flow respirometry (Svendsen et al., 2016). The setup consisted of two separate recirculation systems (one for clean water and another for water dosed with TFM), both of which could be connected to a water bath (100×45×15 cm; containing approx. 43 L of water) where the respirometers were placed (schematics and photos provided in Electronic Supplementary Material (ESM) Appendix S1). This allowed for a quick transition between clean water (TFM-free) and TFM-treated water with minimal disturbance to the animals. Water temperature during experiments was controlled using two ½ HP-aquarium chillers (EcoPlus, USA) and two temperature regulators (TMP-REG, Loligo Systems, Denmark), thus avoiding any temperature-induced changes in TFM tolerance in the animals (Hlina et al., 2021).

The custom-built respirometers were constructed from clear PVC piping (inner diameter = 20 mm; length = 100 mm) with a total volume of 45 mL including the recirculation tubing. Loose polyester fibres ($\bar{x}$ = 51 mg, $\sigma_{\bar{x}}$ = 0.5 mg) were added to the respirometer to act as an inert burrowing substrate, which aided in calming the larvae (Tessier et al., 2018; Wilkie et al., 1999). Preliminary tests using water-soluble food colouring showed that the low-density polyester fibres did not interfere with the water flow patterns through the chamber. The fibres were replaced in-between runs to avoid biofilm accumulation, which could have influenced background oxygen concentrations. Each respirometer was rinsed with 5% HCl followed by a rinse with water; then sprayed with 70% ethanol and left to dry between every second run. Respirometers were allowed to completely dry before reuse.

The respirometers were equipped with $O_2$ probes (OXFLOW-HS; PyroScience GmbH, Aachen, Germany) and pH probes (PHFLOW-PK8; PyroScience GmbH). A single temperature probe (TDIP15; PyroScience GmbH) was also connected to the recirculation loop of one respirometer to measure water temperature within the chambers. The probes were in turn connected to a PyroScience Firesting $O_2$ sensor (FSO2-C4; PyroScience GmbH) and a PyroScience Firesting Pro sensor (FSPRO-4; PyroScience GmbH). In-chamber oxygen concentration and temperature were measured every second, whereas pH was measured every 10 s.

The recirculation pumps (model AD20P-0510A, Shenzhen Giant Electric Tech Inc) were connected to a custom-made recirculation pump controller (powered by an Arduino microcontroller board) through which the speed of the pumps could be controlled. Water flow was maintained at a level that allowed for accurate $O_2$ measurements but without causing water turbulence and disturbing the larvae inside the chamber. One flush pump was used to flush all four respirometers. This pump was connected to a custom-made flush controller which flushed each respirometer for 3 min following each 5 min $O_2$ measurement cycle. The data from the first 30 s of each $O_2$ measurement were discarded (wait phase).

### 2.3. Water chemistry

For this experiment, water was prepared with a target pH of 8.4–8.5 and target alkalinity of 210 mg $L^{-1}$ as $CaCO_3$. A 1000 L tank was filled with dechlorinated water and vigorously aerated to ensure the removal of unwanted $CO_2$ and sufficient $O_2$ saturation. Water from this tank was used to fill up the recirculation systems when needed. pH and alkalinity measurements were regularly taken. To correct pH and alkalinity to target levels, reagent grade sodium bicarbonate (1–8 g; BioShop, Canada) or 5% HCl (1.46 M BioShop, Canada) were added to the tank and to the recirculation systems as appropriate.

Water quality was maintained close to the intended targets for pH, alkalinity and TFM (Table 1). Summarily, pH variation of the water bath during TFM exposure ranged from 8.43 to 8.60 (ESM Appendix S2), while alkalinity ranged from 140 to 220 mg $L^{-1}$ as $CaCO_3$, but never varied by more than 60 mg $L^{-1}$ as $CaCO_3$ within a trial. The first control run experienced a lower alkalinity, but since no TFM was applied during this run, this lower alkalinity bears no effect on the final results (Table 1). Temperature was maintained near 13°C for each run, consistently ranging between 12.8 and 12.9°C (ESM Appendix S2). Measured TFM concentrations were always within ± 5% of the nominal TFM concentration (Table 1). The range of TFM concentrations to use in this study was determined by consulting the reference tables presented in Bills et al. (2003).

### 2.4. Procedure

Larval sea lamprey (N = 28, mass: 1.46 g ± 0.09, length: 10.85 cm ± 0.19, condition factor, K: 0.11 ± 0.02; mean ± SEM) were not fed for 48h prior to experiments. At the start of each trial, the larvae were removed from the substrate, weighed to the nearest 0.01 g, and then placed in their respective individual respirometer, in the afternoon. Measurements of $\dot{M}_{O_2}$ were initiated immediately following the transfer of each animal into the respirometer, and the animals were allowed to remain in clean (TFM-free) water overnight. The following morning, the animals were exposed to one of six different TFM concentrations: 0 (control, N = 8), 2, 3, 4, 5, and 6 mg $L^{-1}$ TFM (N = 4 per test concentration). The data from one control was ultimately excluded due to a chamber malfunction, reducing the number of controls to 7. The animals were then left at the target TFM concentration for 12h. During TFM treatment, in-chamber TFM concentrations were measured regularly (every 1–2 h) by collecting water samples from the respirometer outflows during flush intervals, followed by TFM quantification using a NovaSpec II spectrophotometer (Pharmacia Biotech, Cambridge, England UK) set to a wavelength of 395 nm, following procedures of the US Geological Survey, Hammond Bay Biological Station (SOP 431). After 12h exposure, during the final measurement period (i.e., while the respirometers were closed), the TFM-treated water was drained from the bath, rinsed and replaced with clean (TFM-free) water, and a new cycle of $\dot{M}_{O_2}$ measurements initiated by flushing and re-directing the TFM-treated water to waste so as not to contaminate the clean-water bath. TFM measurements performed on the water coming out of a flush tube at the end of the first flush showed no residual TFM remained inside the chambers. After this transition, post-TFM exposure $\dot{M}_{O_2}$ was measured for another 12h. On the morning of the following day, the animals were euthanized using 1 g $L^{-1}$ tricaine methanesulfonate (MS-222; Syndel Inc., Nanaimo, BC), buffered with 2 g $L^{-1}$ sodium bicarbonate, and the length (nearest mm) was recorded. Background oxygen consumption was measured prior to the addition of animals into the respirometry chambers, and after their removal, to correct for any microbial consumption of $O_2$ that might have otherwise confounded measurements.

Animals were checked every hour to determine mortality. At higher TFM concentrations, mortality was preceded by an abrupt decrease in $\dot{M}_{O_2}$ during the TFM exposure period. Mortality was confirmed by lack of reaction by the animal to a pinch of the tail with forceps.

### 2.5. Calculations, statistics and data analysis

#### 2.5.1. $\dot{M}_{O_2}$ calculations

The $\dot{M}_{O_2}$ for each cycle was determined using a modified version of the FishResp R package (Morozov et al., 2019 as modified by H. Flávio, available at: https://github.com/hugomflavio/pyroresp), using R v4.4.1 (R Core Team, 2024). Recorded $O_2$ values (hPa) were converted to µmol $O_2$ $L^{-1}h^{-1}$ using the respirometry R package (Birk, 2024). Changes in background respiration were linearly modelled over time using the pre- and post-background readings. This linear model was then used to estimate the background $\dot{M}_{O_2}$ at the time of each cycle and to correct the recorded oxygen readings. The only exception to this method was following the 2 mg $L^{-1}$ TFM run, where only the pre-background was used to correct for background $\dot{M}_{O_2}$ because post-background measurements were deemed unusable due to unexpected fluctuations. Linear models were then applied to the corrected $O_2$ readings to determine the slope and $R^2$ of the lines of best fit for each cycle. Cycles with an $R^2$ of or above 0.9 were considered valid for $\dot{M}_{O_2}$ determination. The respective slopes were converted into $\dot{M}_{O_2}$ (µmol $O_2$ $g^{-1}h^{-1}$) by taking into account the corrected volume of the respirometer and the mass of the animal, as follows: $\dot{M}_{O_2} = S \times V \times M^{-1}$, where: $S$ = oxygen decrease slope (µmol $O_2$ $L^{-1}h^{-1}$; corrected for background $O_2$ consumption), $V$ = respirometer volume (corrected for the mass of the animal, assuming a 1 g:1 mL animal density), $M$ = mass of the animal (g). All the $\dot{M}_{O_2}$ values used for the data analyses are provided in spreadsheet format in ESM Appendix S3.

#### 2.5.2. Pre-exposure SMR calculation

Pre-exposure SMR was determined by calculating the quantile 0.2 for the pre-exposure $\dot{M}_{O_2}$ values of each animal (Chabot et al., 2016). A Generalized Linear Model (GLM) with Gamma distribution and log link function was applied to test for the effects of the different test groups (factorial, six levels) on the SMR, to confirm the absence of time- or trial-related changes in the measurements.

**Table 1**
TFM concentration, pH, and alkalinity throughout the course of the various TFM exposures performed. Note: Alkalinity was only measured at the start and at the end of the experiments, which encompasses a period of 36 h (of which the TFM exposure encompassed the middle 12 h), while pH and TFM were monitored throughout the exposure. The bath pH was determined through collection of water samples, while the in-chamber pH was measured using flow-through sensors. Expected LC99.9 range for the measured pH and alkalinity conditions, as per Bills et al. (2003), are also displayed.

| Target TFM (mg $L^{-1}$) | Measured TFM (mg $L^{-1}$) | Temperature (°C) | Bath pH (range) | In-chamber pH (quant. 0.025, 0.5, 0.975) | Alkalinity (mg $L^{-1}$ as $CaCO_3$) | Expected 12-h $LC_{99.9}$ (mg $L^{-1}$) |
|---|---|---|---|---|---|---|
| 0 | – | 12.8–12.9 | 8.40–8.45 | 8.407, 8.429, 8.447 | 85–119 | – |
| 0 | – | 12.8–12.9 | 8.33–8.47 | 8.357, 8.405, 8.455 | 153–204 | – |
| 2 | 1.90–2.08 | 12.8–12.9 | 8.45–8.56 | 8.496, 8.517, 8.553 | 187–204 | 6.5–7.2 |
| 3 | 2.95–3.01 | 12.8–12.9 | 8.51–8.58 | 8.524, 8.540, 8.562 | 140–200 | 5.8–8.4 |
| 4 | 4.03–4.06 | 12.8–12.9 | 8.52–8.60 | 8.540, 8.558, 8.573 | 200–220 | 7.2–8.4 |
| 5 | 4.95–5.13 | 12.8–12.9 | 8.41–8.59 | 8.474, 8.564, 8.588 | 220–240 | 6.1–8.9 |
| 6 | 5.93–5.98 | 12.8–12.9 | 8.43–8.54 | 8.441, 8.491, 8.538 | 180–220 | 5.6–7.6 |

#### 2.5.3. Calculation of changes induced by TFM exposure

$\dot{M}_{O_2\ peak}$ was determined as the maximum $\dot{M}_{O_2}$ recorded during the 12h exposure period. $\Delta\dot{M}_{O_2\ peak}$ was calculated as the $\dot{M}_{O_2\ peak}$ subtracted by the SMR of the respective animal. The time taken to reach $\dot{M}_{O_2\ peak}$ was also determined. Gaussian GLMs were applied to test for effects of TFM concentration (continuous) on 1) the $\Delta\dot{M}_{O_2\ peak}$ (log link) and 2) the time to reach $\dot{M}_{O_2\ peak}$ during exposure (identity link). The second model was only applied to the animals exposed to TFM, as controls did not exhibit an increase in $\dot{M}_{O_2}$ (as expected).

#### 2.5.4. Post-exposure recovery $\dot{M}_{O_2}$ calculation

Because $\dot{M}_{O_2}$ during recovery was expected to decrease as a function of the rate of TFM elimination, the half-time of recovery (akin to half-time of elimination; $t_{1/2}$) was calculated for $\dot{M}_{O_2}$ using the formulas provided by Endrenyi (1998). Summarily, the $\dot{M}_{O_2}$ was log-transformed and fitted with a linear regression. The slope of the linear regression ($m$) was then used to calculate $k$, which in turn allows the calculation of $t_{1/2}$, as follows: $k = -2.303 \times m$, and $t_{1/2} = ln(2) \times k^{-1}$.

For each animal, the recovery $\dot{M}_{O_2}$ data used for this calculation was trimmed to the initial decline in $\dot{M}_{O_2}$ up to 1.1 times the pre-exposure SMR. This was done to ensure a good fit to the linear proportion of the log-decline. Because the animals exposed to 3 mg $L^{-1}$ TFM exhibited high activity and did not return readily to an SMR state, only the first 10 recovery $\dot{M}_{O_2}$ values were used to calculate $t_{1/2}$ for those animals. A Gamma GLM with log link function was applied to test for the effects of TFM concentration (continuous) on $t_{1/2}$.

## 3. Results

### 3.1. Pre-exposure SMR

After a period of initial elevated $\dot{M}_{O_2}$ (averaging approx. 3 μmol $O_2$ $g^{-1}h^{-1}$), the animals quickly calmed down. Activity tended to increase again during the night, between 19:30 and 05:00, with the mean $\dot{M}_{O_2}$ then decreasing again to approximately 1–1.5 μmol $O_2$ $g^{-1}h^{-1}$ (Fig. 1A). All animals tested were observed to have a similar pre-exposure SMR (GLM, N = 27, F = 1.54, p-value = 0.222), with predicted values ranging between 0.96 and 1.37 μmol $O_2$ $g^{-1}h^{-1}$ across the different trials (Fig. 1B; Table 2).

### 3.2. Changes induced by TFM exposure

TFM exposure stimulated the $\dot{M}_{O_2}$ of larval sea lamprey in a dose-dependent manner as concentrations increased from 2 to 6 mg $L^{-1}$ (Fig. 2; Table 2). TFM concentration had a significant effect on $\Delta\dot{M}_{O_2\ peak}$ (the difference between the SMR and the $\dot{M}_{O_2\ peak}$) observed during TFM exposure, with animals exposed to higher concentrations exhibiting a larger $\Delta\dot{M}_{O_2\ peak}$ (Fig. 3A; Table 2; GLM, N = 27, F = 120.56, p-value = $4.7\times10^{-11}$). Further, $\dot{M}_{O_2\ peak}$ was reached faster in animals exposed to higher TFM concentrations (~6.5h at 6 mg $L^{-1}$ compared to ~11h at 2 mg $L^{-1}$; Fig. 3B; Table 2; GLM, N = 20, F = 25.60, p-value = $8.16\times10^{-5}$).

Notably, two animals exposed to 5 mg $L^{-1}$ of TFM died after 9.4h and 11.9h of exposure (noted by daggers in Fig. 2), along with all four animals exposed to 6 mg $L^{-1}$ of TFM (5.3–8.5h into the exposure period; Fig. 2). Postmortem $\dot{M}_{O_2}$ data for each of these animals were removed from analyses.

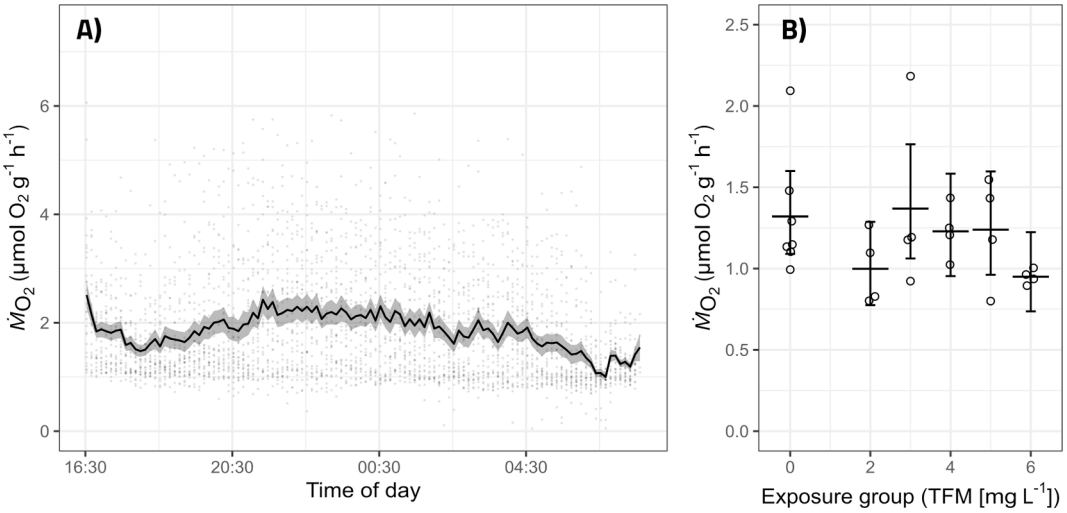


**Fig. 1.** $\dot{M}_{O_2}$ and SMR of larval sea lamprey prior to TFM exposure. A) Mean pre-exposure $\dot{M}_{O_2}$ of all animals (solid line) and respective SEM (grey ribbon), with individual values marked as semi-transparent dots. B) Pre-exposure SMR of all tested animals along with the model's fitted values and respective confidence intervals.

**Table 2**
Summary of the metrics calculated. Data shown as mean ± SEM. Mortality shown as number of deaths during exposure over number of tested animals. Post-exposure $t_{1/2}$ could not be calculated for the highest concentration tested as all animals died during exposure.

| Target TFM (mg $L^{-1}$) | SMR ($O_2$ $g^{-1}h^{-1}$) | $\dot{M}_{O_2\ peak}$ ($O_2$ $g^{-1}h^{-1}$) | $\Delta\dot{M}_{O_2\ peak}$ ($O_2$ $g^{-1}h^{-1}$) | Time to $\dot{M}_{O_2\ peak}$ (h) | $t_{1/2}$(h) | Mortality |
|---|---|---|---|---|---|---|
| 0 | 1.32 ± 0.14 | 2.12 ± 0.21 | 0.80 ± 0.10 | – | – | 0/7 (0 %) |
| 2 | 1.00 ± 0.11 | 2.35 ± 0.31 | 1.35 ± 0.21 | 11.02 ± 0.33 | 1.78 ± 0.38 | 0/4 (0 %) |
| 3 | 1.37 ± 0.28 | 4.44 ± 0.46 | 3.08 ± 0.25 | 9.65 ± 0.43 | 1.63 ± 0.12 | 0/4 (0 %) |
| 4 | 1.23 ± 0.08 | 5.68 ± 0.65 | 4.45 ± 0.59 | 9.72 ± 0.70 | 2.00 ± 0.15 | 0/4 (0 %) |
| 5 | 1.24 ± 0.17 | 6.92 ± 0.88 | 5.68 ± 0.76 | 8.58 ± 0.95 | 2.14 ± 0.02 | 2/4 (50 %) |
| 6 | 0.95 ± 0.02 | 8.15 ± 1.03 | 7.20 ± 1.02 | 6.28 ± 0.66 | – | 4/4 (100 %) |

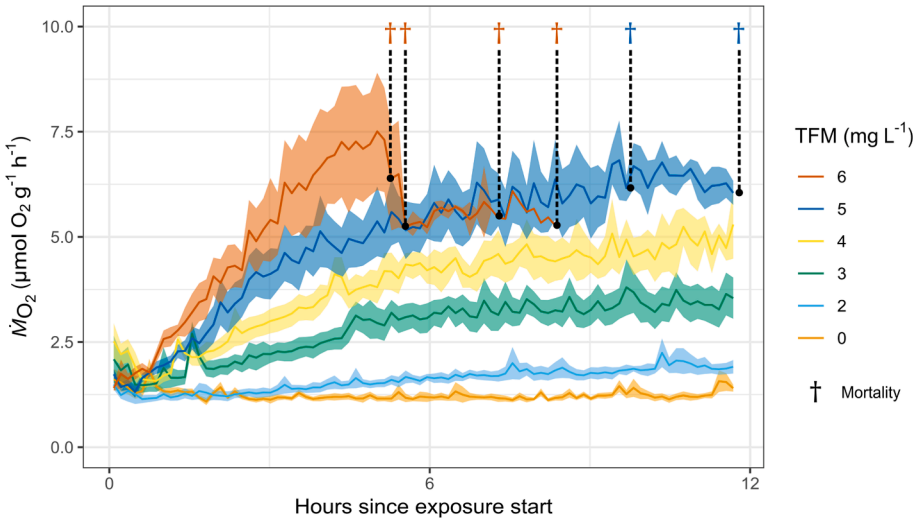


**Fig. 2.** Changes in the $\dot{M}_{O_2}$ of larval sea lamprey during 12 h exposure to increasing concentrations of TFM. Data depicted as the mean $\dot{M}_{O_2}$ (solid lines) ± SEM (shaded ribbons). N = 4 per at each TFM exposure concentration; N = 7 for controls (no TFM). Note: Mortalities (†) were observed at 5 mg $L^{-1}$ (N = 2 of 4 animals) and 6 mg $L^{-1}$ (N = 4 of 4).

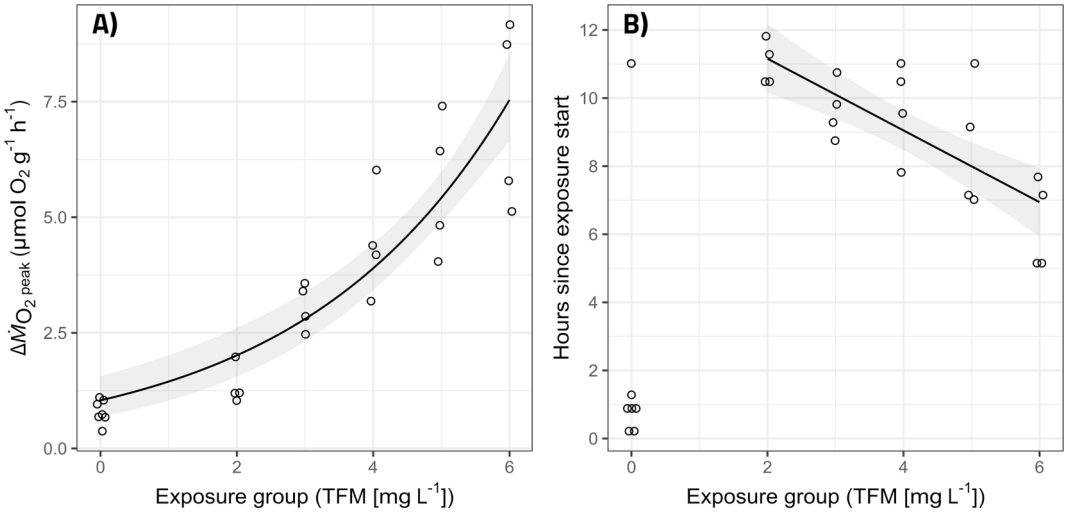


**Fig. 3.** Changes in $\dot{M}_{O_2}$ induced by exposure to increasing concentrations of TFM. A) Maximum change in $\dot{M}_{O_2}$ ($\Delta\dot{M}_{O_2\ peak}$) induced by exposure to TFM. B) Time to reach $\dot{M}_{O_2\ peak}$ during TFM exposure.

### 3.3. Post-exposure recovery

Upon return to clean water, the $\dot{M}_{O_2}$ of all larvae that survived TFM exposure (2–5 mg $L^{-1}$) rapidly declined towards the SMR recorded prior to exposure (Fig. 4). The half-time for $\dot{M}_{O_2}$ to recover ($t_{1/2}$) ranged between 1.78 and 2.14h (Table 2), with no significant effect of exposure TFM concentration (GLM, N = 14, F = 0.795, p-value = 0.39). Additionally, all surviving animals made a full recovery, returning to $\dot{M}_{O_2}$ values comparable to SMR within 2–4 h of being returned to clean water.

### 3.4. Background magnitude

Excluding the 2 mg $L^{-1}$ run, pre-background averaged 28.5% of SMR, while post-background averaged 58% of SMR. During the 2 mg $L^{-1}$, background levels were surprisingly high (100–350% of SMR), for reasons we were unable to determine. To ensure that this did not impact the results of this study, we reconducted the analyses without the 2 mg $L^{-1}$ data (ESM Appendix S4). Since the calculated $\dot{M}_{O_2}$ values for the 2 mg $L^{-1}$ data were within the expected range after background correction and the majority of the results presented here are not affected by the inclusion or exclusion of this data (ESM Appendix S4), we have decided to keep this run in the analyses. The only analysis where the conclusion would change with the inclusion or exclusion of the 2 mg $L^{-1}$ run is the $t_{1/2}$ to recovery. If using the whole dataset, TFM is deemed to have no significant effect on $t_{1/2}$ to recovery, while when the 2 mg $L^{-1}$ run is excluded, TFM is deemed to have a significant effect. However, the $t_{1/2}$ to recovery model has a very limited number of points (14 points with the 2 mg $L^{-1}$ run, 10 points without it) and is not the main focus of this study, so we do not believe this change in results justifies excluding the 2 mg $L^{-1}$ run. Finally, post-background levels for the 6 mg $L^{-1}$ run were particularly high compared to the average (96–135% of SMR). We expect this to be related to the fact that the animals died during the exposure, leading to quick bacterial proliferation within the chamber. In the event that we may have overestimated the post-background for this run, the true peak in $\dot{M}_{O_2}$ for the animals would have been even higher, which would further reinforce our conclusions.

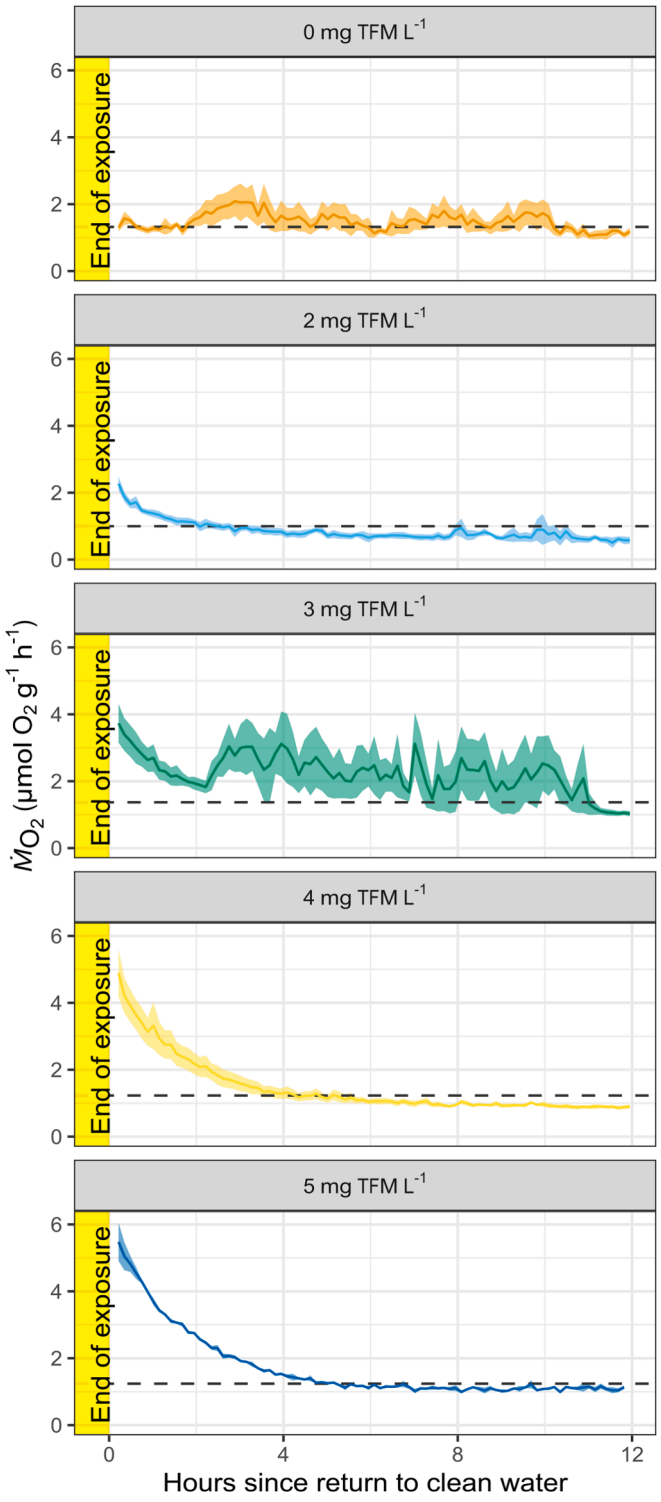


**Fig. 4.** Post-exposure recovery $\dot{M}_{O_2}$ (mean per exposure group ± SEM ribbon). The average SMR recorded before the start of the exposure is marked by the horizontal dashed lines.

## 4. Discussion

### 4.1. TFM exposure results in dose-dependent increases in $\dot{M}_{O_2}$

Exposure to increasing concentrations of TFM resulted in corresponding, stepwise increases in whole animal $\dot{M}_{O_2}$ in larval sea lamprey, supporting our hypothesis that TFM's effects on mitochondrial metabolism scale-up to the whole animal. The nature of the increase in $\dot{M}_{O_2}$ likely reflects increasing effects of TFM on mitochondrial metabolism, rather than a generalized stress response. These observations are consistent with TFM's known uncoupling effects on oxidative phosphorylation, which leads to an increase in mitochondrial oxygen consumption (Birceanu et al., 2011; Borowiec et al., 2022; Huerta et al., 2020; Niblett and Ballantyne, 1976).

The spike in mean $\dot{M}_{O_2\ peak}$ to 7–8 μmol $O_2$ $g^{-1}h^{-1}$ in the animals exposed to lethal TFM concentrations (5 and 6 mg $L^{-1}$), represents a value similar to the post-exercise maximum metabolic rates (MMR) for sea lamprey larvae at this test temperature (Wilkie et al., 2001; H. Flávio and M.P. Wilkie, unpublished data). This likely corresponds to the mitochondria's electron transport chain activities nearing their maximum capacity, a point at which they would be unable to maintain the proton gradient, thus prompting the mitochondria to consume $O_2$ at maximal rates. This similarity between the $\dot{M}_{O_2\ peak}$ recorded during exposure to lethal concentrations of TFM and the post-exercise MMR could be very valuable, as it could open the door to the determination of TFM sensitivity without requiring extensive lethal experimentation. That is, we could predict the lethal TFM concentration by testing several sub-lethal concentrations and extrapolating towards the known maximum metabolic rate of the animal (determined through exhaustive exercise). This opens an avenue for more extensive testing on non-target, at-risk species, where large-scale lethal experimentation is not desirable.

### 4.2. SMR

SMR values obtained during this study ranged from 0.96 to 1.37 μmol $O_2$ $g^{-1}h^{-1}$. This low SMR is consistent with previous findings, notably Wilkie et al. (2001), in which larvae had an SMR of around 1.0 μmol $O_2$ $g^{-1}h^{-1}$, as measured using static respirometers containing cotton as artificial burrowing substrate. Similarly, Holmes and Lin (1994) extrapolated SMR values ranging between 1 and 2.7 μmol $O_2$ $g^{-1}h^{-1}$. Potter and Rogers (1972) also reported resting metabolic rates of 1.6–1.7 μmol $O_2$ $g^{-1}h^{-1}$ for burrowed larvae. Sea lamprey larvae are thought to have a relatively sedentary filter-feeding lifestyle. Hence, it is not surprising that larvae have a low metabolic rate compared to free-swimming fish of similar size (Lewis, 1980).

Achieving starvation in filter-feeding animals can be a complicated process, as the withdrawal of new food does not guarantee that the animals have stopped feeding on already-suspended matter. As such, while food was withheld 2 days prior to the experiments, we cannot guarantee that the animals had fully stopped digesting materials before the oxygen measurements began, as the sediment in their housing contains detritus, their primary food source (Sutton and Bowen, 1994; Yap and Bowen, 2003), upon which they could still feed. However, sea lamprey larvae differ from other detritivorous fish in that they have an unspecialized, simple digestive tract that breaks down food at a slower pace (Sutton and Bowen, 1994; Yap and Bowen, 2003). As such, we would not expect that any remnants that the animal could have ingested in the 48h prior to the experiments would have been able to cause any significant increases in metabolic rate.

### 4.3. Rapid return to SMR following TFM exposure

The $\dot{M}_{O_2}$ of the surviving animals rapidly declined following the switch from TFM to clean (TFM-free) water, reaching SMR-level measurements within 4h (Fig. 4). This steep drop likely correlates with the clearance of TFM from the internal organs and tissues such as the muscle and liver. The $t_{1/2}$ for $\dot{M}_{O_2}$ to return to SMR following TFM exposure was approximately 1.8 h (mean value) and was found to have no significant relationship with TFM concentration, which is characteristic of biological half-life estimates (Endrenyi, 1998). Hence, the gradual drop in $\dot{M}_{O_2}$ was likely due to residual TFM that continued to stimulate mitochondria $\dot{M}_{O_2}$ during the TFM washout phase. This rapid restoration of $\dot{M}_{O_2}$ to

near SMR levels contrasts results of Hlina et al. (2017), who reported that lamprey injected with radio-labelled TFM ($^{14}$C-TFM) took approximately 24h to clear 95% of an injected dose (86 nmol $g^{-1}$ body mass), though it is not known at which point in this process $\dot{M}_{O_2}$ would have returned to resting levels. We also do not know what the peak concentrations of TFM were in the larvae in the present study, which could profoundly impact the total time required to clear TFM from the body. Further, previous work from our lab demonstrated that larval sea lamprey exposed to TFM for 12h and then transferred to clean (TFM-free) water, eliminated TFM from the body with a half-life of 9.7 h (M. Le Claire and M.P. Wilkie, unpublished findings), greatly exceeding the half-life measured for $\dot{M}_{O_2}$ here. In other words, $\dot{M}_{O_2}$ returns to SMR even while the sea lamprey likely still retains some TFM in the body. This could be because residual TFM concentrations are no longer sufficiently high to interfere with oxidative phosphorylation.

In addition to TFM's effects on mitochondrial $\dot{M}_{O_2}$ during the depuration period, it is also likely that at least part of the post-exposure $\dot{M}_{O_2}$ was due to the need to restore metabolic homeostasis in the sea lamprey. The time it took for $\dot{M}_{O_2}$ to return to SMR (2–4h) was similar to that taken for larval sea lamprey to restore energy stores including ATP, PCr, and glycogen, and to metabolize lactate, following short-term exposure (4h) to the 12h LC99.9 of TFM (~7.6 mg $L^{-1}$; Clifford et al., 2012). This concentration of TFM matches exposure concentrations sea lamprey would experience during lampricide treatments based on the pH and alkalinity of the test water used (Bills et al, 2003). It is therefore tempting to speculate that the sustained elevation of $\dot{M}_{O_2}$ after TFM removal was at least partially attributable to excess post-TFM exposure oxygen consumption, which would be analogous to the excess post-exercise oxygen consumption (EPOC) observed in many fish species following exhaustive exercise (Scarabello et al., 1991a, 1991b; Zhang et al., 2018), including larval sea lamprey (Wilkie et al., 2001).

EPOC refers to the additional $O_2$ that must be consumed to restore metabolic homeostasis following intense exercise in vertebrates (Scarabello et al., 1991a, 1991b; Zhang et al., 2018). It is notable that EPOC in exhaustively exercised larval sea lamprey is also intense and brief, with $\dot{M}_{O_2}$ increasing more than 3- to 4-fold following exhaustive chasing and lasting only 2–4h. Wilkie et al. (2001) found an impressively rapid recovery of muscle PCr and glycogen stores (< 0.5h) following exercise, which had been reduced by more than 50%, and lactate was restored to resting levels over a similar time course. Based on the present and earlier observations, larval sea lamprey have a high capacity to restore metabolic homeostasis via increases in $\dot{M}_{O_2}$. Further experiments combining respirometry, measurement of TFM clearance rates, and/or terminal sampling for the determination of tissue TFM and energy store concentrations could help further resolve how these variables relate to each other.

### 4.4. *Apparent night-time increase in* $\dot{M}_{O_2}$

A notable finding was that tested sea lamprey larvae showed increased night-time $\dot{M}_{O_2}$ prior to TFM exposure (Fig. 1A), suggesting an apparent pattern in night-time activity. Although this hypothesis needs to be more rigorously tested, this interesting observation is in line with the known tendency for larval lampreys to move predominantly at night (Dawson et al., 2015; Potter, 1980). This likely represents an innate tendency that allows them to avoid predation during daylight hours, a phenomenon common across the animal kingdom (e.g., Iwasa, 1982; Moore et al., 1995).

## 5. Conclusion

The present study demonstrates that TFM exposure results in dose-dependent increases in the $\dot{M}_{O_2}$ of larval sea lamprey. The dose-dependent manner in which $\dot{M}_{O_2}$ increases at the whole animal level likely reflects the dose-dependent increases in state 4 (leak respiration) $\dot{M}_{O_2}$ reported in isolated mitochondria exposed to TFM (Birceanu et al., 2011; Borowiec et al., 2022). The present observations also provide further evidence that TFM is an uncoupler of oxidative phosphorylation.

Our results showcase the possibility of using measurements of whole animal $\dot{M}_{O_2}$ in TFM dose–response trials to determine TFM sensitivity. This is particularly relevant given that the toxicity of TFM varies with key abiotic factors, particularly pH, alkalinity, and temperature (e.g., Bills et al., 2003; Muhametsafina et al., 2019; Schueller et al., 2024), therefore requiring testing under various conditions reflecting the environment in which the species of interest is found. There are several non-target species that share streams with larval sea lamprey, and therefore are likely to be exposed to TFM (Boogaard et al., 2003; Neave et al., 2021; Wilkie et al., 2019). Examples include young-of-the-year lake sturgeon (*Acipenser fulvescens*), some species of catfishes (family Ictaluridae), mudpuppy (*Necturus maculosus*), and native lamprey species (e.g., northern brook lamprey *Ichthyomyzon fossor*; silver lamprey *I. unicuspis*). The development of a non-lethal approach to determine TFM sensitivity could be particularly relevant for at-risk species, for which lethal toxicity testing would raise concerns from an ethical and societal perspective. If the strong relationship between TFM concentration and $\dot{M}_{O_2}$ found here also proves true for non-target species, respirometry could be used to test the TFM sensitivity of species of conservation concern, including invertebrates. This respirometry-based method could also be adapted for stream-side application (e.g., inside a trailer), providing sea lamprey control agents with an "early-warning system" to prevent accidental, non-target mortality prior to treatments in the field. Ultimately, this approach would reduce the need for lethal experimentation (i.e., traditional toxicity tests or bioassays), which is undesirable when working on protected, at-risk species.

## 6. Author contribution statement

The project was conceived by HF and MPW. HF designed and built the respirometers, along with the software used to control the devices. Experiments were conducted by LDS under the guidance of HF, as part of an undergraduate honours thesis research project. All authors contributed to the interpretation of the data, and the writing and review of this manuscript.

## 7. Data availability

The respirometry data collected for this study and the respective R analysis scripts are available as a Zenodo repository: https://doi.org/10.5281/zenodo.13371444.

## Declaration of competing interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jglr.2025.102536.

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