Research paper

Influence of body size, metabolic rate and life history stage on the uptake and excretion of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) by invasive sea lampreys (*Petromyzon marinus*)

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ABSTRACT

Invasive sea lamprey (*Petromyzon marinus*) are controlled in the Great Lakes using the lampricide 3-tri-fluoromethyl-4-nitrophenol (TFM), which is applied to streams infested with larval lamprey. However, lamprey that survive treatments (residuals) remain a challenge because they may subsequently undergo metamorphosis into parasitic juvenile animals that migrate downstream to the Great Lakes, where they feed on important sport and commercial fishes. The goal of this study was to determine if body size and life stage could potentially influence sea lamprey tolerance to TFM by influencing patterns of TFM uptake and elimination. Because mass specific rates of oxygen consumption ($\dot{M}O_2$) are lower in larger compared to smaller lamprey, we predicted that TFM uptake would be negatively correlated to body size, suggesting that large larvae would be more tolerant to TFM exposure. Accordingly, TFM uptake and $\dot{M}O_2$ were measured in larvae ranging in size from 0.2–4.2 g using radio-labelled TFM (^{14}C-TFM) and static respirometry. Both were inversely proportional to wet mass (*M*), and could be described using the allometric power relationship: $Y = aM^b$, in which $\dot{M}O_2 = 1.86 M^{0.53}$ and *TFM Uptake* $= 7.24 M^{0.34}$. We also predicted that body size would extend to rates of TFM elimination, which was measured following the administration of ^{14}C-TFM (via intraperitoneal injection). However, there were no differences in the half-lives of elimination of TFM ($T_{1/2}$-TFM). There were also no differences in $\dot{M}O_2$ or TFM uptake amongst size-matched larval, metamorphosing (stages 6–7), or post-metamorphic (juvenile) sea lamprey. However, the $T_{1/2}$-TFM was significantly lower in larval than post-metamorphic lamprey (juvenile), indicating the larval lamprey cleared TFM more efficiently than juvenile lamprey. We conclude that larger larval sea lamprey are more likely to survive TFM treatments suggesting that body size might be an important variable to consider when treating streams with TFM to control these invasive species.

1. Introduction

Sea lamprey (*Petromyzon marinus*) spend the first 2–7 years of life burrowed in the substrate of streams as filter-feeding larvae (often called ammocoetes) before metamorphosing into juvenile lamprey. In this life stage, sea lamprey ingest large amounts of blood from teleost fishes while attached to their hosts using a newly formed oral disc and rasping tongue (Beamish and Potter, 1975; Manzon and Youson, 2015). However, during the 12–20 month parasitic juvenile stage, a single sea lamprey may kill up to 20 kg of fish (Swink, 2003). The invasion of the Great Lakes by sea lamprey (*Petromyzon marinus*) in the early 20th century decimated commercial, recreational and culturally significant fisheries (Lawrie, 1970; Smith and Tibbles, 1980). The American and Canadian governments jointly responded to the crisis, forming the Great Lakes Fishery Commission, which implemented a wide range of measures to control sea lamprey populations including barriers and traps to block the migration of adult spawning sea lamprey, sterile male release and chemical control methods using lampricides (Applegate et al., 1961; Smith and Tibbles, 1980; Bergstedt and Twohey, 2007; McLaughlin et al., 2007; McDonald and Kolar, 2007).

Two lampricides, 3-trifluoromethyl-4-nitrophenol (TFM) and ni-closamide, are used to control larval sea lamprey but TFM is used in the majority of treatments, and is the focus of the present study. Typically applied for 12 h at 1.5X the 9 h-MLC (minimum lethal concentration) every 3–4 years to streams and rivers containing the larvae, a single application of TFM targets multiple generations at once, which can vary widely in body-size (McDonald and Kolar, 2007). Indeed, larval size is used to predict the likelihood of larvae entering metamorphosis, a key criteria used help select streams for lampricide treatment (Christie et al., 2003; McDonald and Kolar, 2007).

It is not clear, however, how body size influences sea lamprey sensitivity to TFM. As in other animals, mass specific rates of oxygen consumption ($\dot{M}O_2$) and gill ventilation are inversely related to mass and scale allometrically in lampreys (Hill and Potter, 1970; Lewis and Potter, 1977; Galloway et al., 1987; Wilkie et al., 2001). As an organism's size increases, its mass specific metabolic rate generally increases allometrically as described by the equation $Y = aM^b$, where Y is respiration rate (or other physiological variable of interest), M is body mass, and a is a scaling coefficient that varies between different taxa (Glazier, 2005). The scaling (mass) exponent, b, is generally fixed near 0.75 amongst different groups of animals (Gillooly et al., 2001; Glazier, 2005), but Killen et al. (2010) have pointed-out that there may be substantive variation due to differences in the metabolic capacity and lifestyle of different animals. The main objective of this study was to test the hypothesis rates of TFM uptake, like $\dot{M}O_2$, scale allometrically with body mass in sea lamprey. If such a relationship exists, it would suggest that larger larvae would have correspondingly lower mass specific rates of TFM uptake. Such animals would be more likely to survive TFM treatments, providing a pool of "residual" lamprey that could complete metamorphosis into juveniles and subsequently migrate into the Great Lakes to feed and subsequently kill large numbers of commercial, game and culturally significant fishes. Accordingly, we measured rates of TFM uptake using radio-labeled TFM (^{14}C-TFM), along with measurements of routine $\dot{M}O_2$, in different size groups of larval lamprey.

Rates of TFM excretion were similarly compared to larval mass following intraperitoneal (IP) injection of ^{14}C-TFM. We predicted that higher rates of gill ventilation and greater surface area: volume ratios in small larval sea lampreys (Lewis, 1980) would allow them to clear TFM more quickly from their bodies after re-introduction into clean (TFM-free) water. To test this possibility, rates of TFM clearance by different sizes of larval and juvenile sea lampreys were measured during depuration in clean water following the administration of ^{14}C-TFM by intraperitoneal injection.

In addition to body size, sea lamprey sensitivity to TFM could also be affected by life stage (Henry et al., 2015). Following metamorphosis, the feeding apparatus, gills and internal organs are completely restructured as the animals enter the parasitic, blood-feeding juvenile phase of their life cycle (Youson and Potter, 1979; Wilkie, 2011). The $\dot{M}O_2$ of other lamprey species has also been reported to increase during and following metamorphosis (Lewis and Potter, 1977; Galloway et al., 1987). Thus, a related goal was to use respirometry and radio-labeled TFM to test the hypothesis that post-metamorphic juveniles had higher $\dot{M}O_2$ and rates of TFM uptake than similarly sized larvae.

2. Methods and materials

2.1. Sea lamprey collection and holding

Larval sea lampreys (*Petromyzon marinus*) were collected using backpack, pulsed DC electrofishing (ABP-2 Electrofisher, Electrofishing Systems, LLC, Madison, WI, USA) from streams draining into Lake Michigan by United States Fish and Wildlife Service personnel (Luddington, MI) in the spring of 2014. The lamprey were held at the Hammond Bay Biological Station (Millersburg, MI) in Lake Huron water (flow rate 1 L min^{-1}; temperature 14–16 °C) for 1–2 months, until transported to Wilfrid Laurier University (WLU) where they were held in 110 L tanks, continuously receiving aerated well-water (pH = 7.8–8.1; Alkalinity = 205 mg as CaCO$_3$ L^{-1}; [Na$^+$] = 1.2 mmol L^{-1}; [Ca^{2+}] = 3.8 mmol L^{-1}; Conductivity = 903 μS; T = 14–16 °C) at a flow rate of 1.0 − 2.0 L min^{-1}. The bottom of each tank was lined with sand (4 − 5 cm) to provide the animals with burrowing substrate, and they were fed a slurry of baker's yeast once per week (1.0 g yeast per sea lamprey; Holmes and Youson, 1994; Wilkie et al., 1999).

Metamorphosing (stages 6–7 combined) and juvenile (post-metamorphic) lamprey were a subset of animals that underwent metamorphosis at WLU or were provided courtesy of Dr. Barb Zielinski (Department of Biology, University of Windsor, Windsor, Ontario). The animals from WLU were not fed prior to experiments, but the animals from U. Windsor had ingested the blood of rainbow trout (*Oncorhynchus mykiss*) for 2–3 weeks prior to their shipment to WLU. Animals from WLU that were in stages 6–7 of metamorphosis, along with juveniles, were used to determine $\dot{M}O_2$ and TFM uptake. The U. Windsor animals were only used for TFM clearance experiments, after a two week non-feeding period. No burrowing substrate was provided for juvenile lamprey, which do not burrow.

All experiments were approved by the Wilfrid Laurier Animal Care Committee, and followed the guidelines and principles of the Canadian Council of Animal Care (CCAC).

2.2. Experimental set-up and respirometry chambers

Experiments were completed in a system comprised of a 40 L head tank, to which well-water was pumped from a lower reservoir, and vigorously aerated to maintain dissolved O$_2$ at greater than 90% saturation. Water flowed from the head tank via flow-splitters into individual 100 mL respirometry chambers (~10 mL min^{-1}), which were positioned in an underlying PVC tray (1 m × 2 m × 0.1 m). All chambers were immersed in a water bath, which was continually fed well water to maintain temperatures at approximately 12 °C. A removable standpipe in the tray was used to raise water levels so that chambers could be completely submerged during $\dot{M}O_2$ measurements (see below).

Each glass chamber was darkened (with black electrical tape) to minimize stress to these negatively phototactic animals (Binder et al., 2013). A plastic mesh insert, elevated 4 cm off the chamber bottom using a perforated polypropylene tube [1 cm inner diameter (ID)], supported the animal, and the tube enclosed a small (5 mm) magnetic stir bar to mix the water during $\dot{M}O_2$ determinations. The chambers were covered by a plastic lid, through which a water inflow tube and two polyethylene air-lines were inserted [Intramedic Clay Adams, 0.58 mm diameter (PE50)] to oxygenate and gently mix the water, except during periods when $\dot{M}O_2$ was determined. When $\dot{M}O_2$ was measured, the plastic lid was replaced with a black rubber stopper (size 10), through which two 21-gauge needles were inserted. Each needle was attached to externally mounted 3-way stopcocks, which served as water sampling ports, Within the chamber, each needle was connected to a length of polyethylene tubing (PE50) that extended to 1–2 cm above the bottom.

2.3. Experimental protocols

2.3.1. Effects of body size and life history stage on oxygen consumption ($\dot{M}O_2$)

A minimum of 12 h prior to experiments, individual larvae (N = 56; length = 61–146 mm; mass 0.2–4.2 g) were transferred to the respirometry chambers, to which 1 g of diffuse aquarium cotton was added to minimize spontaneous movement of these burrow-dwelling animals (thigmokinesis; Wilkie et al., 1999). The chambers were then fitted with the plastic lid and airlines, aerated water flow to each chamber initiated (~10 mL min^{-1}), and the animals left overnight. The next day, water flow to each chamber was cut-off and each chamber was completely filled with oxygenated water (to overflowing). The rubber stopper-sampling port apparatus was then carefully inserted into the chamber opening, taking care not to trap any bubbles, and the water level raised to completely submerge the entire chamber under water to prevent atmospheric O$_2$ from entering the chamber during $\dot{M}O_2$ determinations (Cech and Brauner, 2011).

Magnetic stir plates positioned beneath the tray were then turned on to activate the stir-bars in each chamber to initiate gentle but thorough mixing of the water during the subsequent $\dot{M}O_2$ measurement period.

To ensure adequate mixing of the chamber, electrical tape was removed from one container and dye was injected into it via the sampling port. The example container contained 1 g of diffuse cotton, no animal, and had the electrical tape removed. It was observed that homogenous mixing of the dye throughout the chamber occurred after approximately 5 min. To further test the scope of our measurements, a subset of larvae were acclimated for 12 h to the chambers and their resting $\dot{M}O_2$ was measured. They were then taken from the chambers and exercised, and $\dot{M}O_2$ increased by six fold. They were allowed to recover, and $\dot{M}O_2$ was found to decrease, giving us more confidence in the measurements.

Water samples (2 × 1 mL) were withdrawn using gas tight Hamilton syringes (2 × 1 mL) at 0, 0.5 and 1.0 h. The PO_2 in the chambers decreased linearly during this period (mean R^2 = 0.95), but in the few cases where this was not the case, the data were not used. The first sample (1 mL) at each sample point was discarded to eliminate dead-space water in the siphon tube, followed by measurement of water PO_2 using the second 1 mL sample. Two consecutive measures of water PO_2 were made (2 × 500 μL) using a Fibox 3 oxygen meter (PreSens Regensburg, Germany; Software PST3v602 version 5.32) using an acrylic flow-through cell coupled to a fiber optic oxygen probe (Integr Planar Oxygen Sensor FTC-PSt3). The PO_2 in each respirometer was never allowed to decrease below 100 Torr to prevent hypoxia (Cech and Brauner, 2011). Torr was then converted to μmol L^{-1} O_2 for the $\dot{M}O_2$ calculations based on the saturation constant and partial pressure relationship of Henry's Law (Boutilier et al., 1984).

An identical protocol was used to determine how life history stage influenced $\dot{M}O_2$. In these experiments, conducted from October–November 2015, the $\dot{M}O_2$ of larvae (N = 20; 2.7 ± 0.2) were compared to animals in stages 6–7 (N = 10; 2.7 ± 0.2), and juvenile lampreys (N = 15; 2.7 ± 0.2). Because lamprey emerge from their burrow following metamorphosis, cotton was removed from the chambers holding stage 6–7 and juvenile animals. Care was also taken to size-match the lamprey between the groups, to ensure that variation due to size differences were not introduced.

2.3.2. Effects of body size and life history stage on TFM uptake

Following $\dot{M}O_2$ determination (1 h) on the different size groups of larval lamprey or life stages (larval, late-stage metamorphosis, post-metamorphic), the rubber stopper-sampling port apparatus and the cotton (for larvae) was removed from each container and replaced with the container lid, followed by the re-establishment of water flow and aeration. After 30 min, water flow was again cut-off, and the water volume in the container adjusted to exactly 100 mL (without correction for body mass; see calculations below), and 0.5 μCi of ^{14}C-TFM mixed with cold TFM (Mean specific activity = 229 ± 7 CPM nmol^{-1} TFM) was then added to each chamber to yield a total TFM exposure concentration of 4.6 mg L^{-1} TFM (actual concentration 4.8 ± 0.05 mg L^{-1}), which is equivalent to the 12-h LC_{50} of TFM in larval sea lampreys in WLU well water (Birceanu et al., 2009). Water samples (10 mL) were then collected at 0, 1 and 3 h using the external sampling ports attached to the lid, and stored at 4 °C until processed for determination of ^{14}C-TFM radioactivity and total "cold" (non-radioactive) concentration of TFM. After 3 h, lamprey were euthanized with an overdose of tricaine methanesulfonate anaesthetic (1.5 g L^{-1}; Syndel Canada, Nanaimo, BC) buffered with 3 g L^{-1} sodium bicarbonate (Birceanu et al., 2009). The animals were rinsed for 30 s in a 50 mg L^{-1} solution of cold (non-radioactive) TFM to dislodge any surface bound ^{14}C-TFM, followed by a final rinse with de-ionized water. The animal carcasses were then blotted dry with a paper towel, weighed and transferred to 50 mL polypropylene test-tubes (Falcon tubes) to which 8–12 mL 1 N HNO₃ was added (dilution factors by mass accounted for in calculations). Carcasses were digested for 24 h at 60 °C, vortexed every 8–12 h and then centrifuged for 5 min at 1200g using an IEC Medilite clinical centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) prior to preparing the supernatant for measurements of ^{14}C-TFM radioactivity (Section 2.4.2).

2.3.3. Relationship between body size and life stage on rates of TFM clearance

The TFM elimination rates were measured in different sizes of larval (N = 52; mass = 0.3–2.4 g) lamprey administered ^{14}C-TFM labeled TFM solution (Mean Specific Activity = 99 CPM nmol^{-1}) by intraperitoneal injection (IP), and then measuring the appearance of ^{14}C-TFM radioactivity appearing in nominally TFM-free (clean) water over 24 h. Approximately 60 min before commencing experiments, radio-labelled TFM was administered to each animal at a dose of 100 nmol TFM g^{-1} wet weight, which represents the approximate total concentration of TFM that is reached in larval sea lampreys experiencing mortality during TFM exposure (Le Claire 2014). Immediately following TFM administration, the sea lamprey were transferred to the same respirometry chambers described above, containing 100 mL of TFM-free water, and left for 1 h to allow the TFM to become evenly distributed in the tissues prior to collecting water samples. Water samples (10 mL) were then collected at different time intervals (0–1 h, 1–2 h, 2–4 h, 4–6 h, 8–12 h, 22–24 h), and stored at 4 °C until processed for measurement of cold and radioactive TFM (Section 2.4). Mortality rates were approximately 10% after 24 h, as determined by applying a tail-pinch (using forceps) to animals that appeared un-responsive at the end of the experiment (24 h). Any lamprey that died were subsequently excluded from the data set. Fresh water was added to the containers between 4 h and 12 h, when the volume in each container was re-adjusted to 100 mL. Temperature (12.3 ± 0.4 °C) and pH (8.0 ± 0.03) was monitored to ensure similar conditions between replicates. An identical protocol was used to compare TFM clearance rates of the larvae to juveniles (N = 12).

2.4. Analytical methods

2.4.1. Non-radioactive TFM concentration

The concentration of total ("non-radioactive") TFM in water samples were determined, without modification, using a NovaSpec II spectrophotometer (Pharmacia Biotech, Cambridge, England, UK) set to a wavelength of 395 nm (Fisheries and Oceans Canada Standard Operating Procedure IOP: 012.4; Birceanu et al., 2009). Precision standards (0, 4, 8, 12 mg L^{-1} TFM) were provided courtesy of the Sea Lamprey Control Centre, Fisheries and Oceans Canada (DFO) (Sault Ste. Marie, Ontario).

2.4.2. TFM radioactivity

After the carcasses of larval, stage 6–7, and juvenile lamprey were digested (Section 2.3.2), exactly 2 mL (in duplicate) of the resulting supernatant was added to 4 mL of UltimaGold organic scintillation cocktail (PerkinElmer, Waltham, MA, USA), vigorously mixed, and left overnight to minimize chemiluminescence prior to measuring beta radioactivity (quantified as counts per minute, CPM) the next day using a Beckman-Coulter, LC 6500 Multipurpose Scintillation Counter (Beckman-Coulter, Fullerton, CA, USA). HiSafe Optiphase aqueous scintillation cocktail (4 mL; PerkinElmer, ibid) was added to water samples (2 mL) in duplicate, which were also left overnight prior to beta radioactivity quantification using the same instrument. Quench curves, generated by adding known amounts of ^{14}C-TFM to non-radioactive tissue digests (2 mL) and to water samples (2 mL) indicated that quench correction was not needed.

2.5. Calculations and statistical analysis

2.5.1. $\dot{M}O_2$ and TFM uptake

The following equation, modified from Cech (1990), was used to calculate $\dot{M}O_2$ (μmol g^{-1} h^{-1}):

$$\dot{M}O_2 = \frac{(C_{O2(i)} - C_{O2(f)})*V}{(M)(\Delta T)} \tag{1}$$

Where $C_{O2(i)}$ is the concentration (μmol L^{-1}) of O_2 in the water at the

beginning and $C_{O2(f)}$ is the concentration at the end of the measuring period ($\mu mol\,L^{-1}$); V is the volume of the respirometer (L), M is the mass of the sea lamprey (g), and ΔT is the duration of the measurement period (h).

The rate of TFM uptake ($nmol\,g^{-1}\,h^{-1}$) was calculated according to Hlina et al. (2017) using the following equation:

$$TFM\ Uptake = \frac{whole\ body\ CPM}{MSA*\Delta T} \quad (2)$$

Where whole body CPM is the beta radioactivity accumulated by the animal measured in counts per minute g^{-1} wet mass, MSA is the mean specific activity of ^{14}C-TFM in water samples ($CPM\,nmol^{-1}$ TFM), and ΔT is exposure time (h).

2.5.2. TFM clearance

The clearance rates of TFM were calculated in two steps, based on the appearance of ^{14}C-TFM beta radiation in water samples collected from the holding containers during the post-injection (described above) depuration period in TFM-free water using the following series of calculations. First, ^{14}C-TFM excretion rate, expressed in counts per minute g^{-1} body mass,was calculated as described below:

$$J^{out}{}_{14_{C-TFM}} = \frac{(CPM_i - CPM_f)*V}{M*\Delta T} \quad (3)$$

Where CPM_i and CPM_f are the respective beta radioactivity of the water samples at the beginning and end of each sample period measured in counts per minute mL^{-1}, respectively, and M, V and ΔT are as previously stated.

Second, the total TFM efflux (clearance) rate was calculated by dividing $J^{14}{}_{C-TFM}$ (Eq. (3)) by the MSA of ^{14}C -TFM:

$$TFM\ efflux(clearance)\ rate = \frac{J^{out}{}_{14_{C-TFM}}}{MSA} \quad (4)$$

Where MSA is the mean specific activity of the ^{14}C-TFM injected into the sea lamprey in $CPM\,nmol^{-1}$ TFM (e.g. Evans, 1967; Wilkie et al., 2006).

The TFM clearance rates for each animal were then used to calculate the elimination half-life for TFM ($T_{1/2}$-TFM), to more accurately ascertain if TFM elimination varied according to body size. Calculations for elimination half-life are more robust measures of the capacity of animals to unload drug or toxicant loads because the measures take into account variation in the amounts of substance administered and sampling time. Calculations were based on the total amount of TFM remaining in the carcass after 24 h of depuration and then using the preceding TFM efflux rate of each animal to back-calculate the amount of TFM remaining in the body of the lamprey at each given water sample period (24 h, 22 h, 12 h, 8 h, 6 h, 4 h, 2 h, 1 h, 0 h) as described by the following equation:

$$[TFM]_n = ([TFM]_{n-1} + (TFM\ efflux\ rate)_n\ X\ \Delta T) \quad (5)$$

Where $[TFM]_{(n)}$ is the amount of TFM remaining in the body ($nmol\,g^{-1}$ body mass) at a given time period (ΔT, measured in h) $[TFM]_{n-1}$ is the amount of TFM remaining in the body at the end of preceding sample period ($nmol\,g^{-1}$ body mass), (TFM efflux rate)$_n$ is the measured TFM efflux rate during the corresponding flux period ($nmol\,g^{-1}\,h^{-1}$) and TFM uptake was assumed to be negligible.

The amount of TFM calculated to be in the body ($nmol\,g^{-1}$) at each interval vs time was analyzed using log-linear plots, and the slope of the line (m) used to calculate the rate constant, K:

$$K = m*(-2.303) \quad (5)$$

Which was then used to calculate the $T_{1/2}$-TFM (Voet et al., 2006; LeClaire, 2014):

$$T_{1/2} - TFM = \frac{0.639}{K} \quad (6)$$

2.5.3. Statistical analysis

All data was analyzed using the statistical program JMP (version 12.0.1; SAS Institute Inc. 2015; Cary, NC), with the exception of the hierarchical clustering analysis which was performed using the hclust() function in R (version 3.3.2; R Core Team 2016; Vienna, Austria). Where appropriate, data were presented as the mean $\pm$ 1 standard error of the mean (SEM). Statistical significance was evaluated using one-way or two-way ANOVA (α = 0.05), as appropriate after first testing for normality and homogeneity of variance using Shapiro-Wilk's test and Levene's test. Least squares linear regression analysis was used to analyze $\dot{M}O_2$ and TFM uptake data with body size on log transformed data, and allometric equations were derived from results of linear regression of the lines for log–log whole body $\dot{M}O_2$ or TFM uptake rate versus body size. Grubb's outlier analysis was used and detected two outliers from the $T_{1/2}$-TFM data, which were subsequently removed from the dataset.

3. Results

3.1. Effects of body size on oxygen consumption ($\dot{M}O_2$)

The routine $\dot{M}O_2$ of larval sea lamprey was inversely related to body size, decreasing exponentially with body mass (wet mass) from peak values of $7.2\,\mu mol\,g^{-1}\,h^{-1}$ in a 0.3 g animal, to a low of $0.5\,\mu mol\,g^{-1}\,h^{-1}$ in the largest larvae (Fig. 1A). Log-log plots of the whole body $\dot{M}O_2$ ($\mu mol\,h^{-1}$) vs body mass yielded a significant linear relationship (Fig. 1A – inset), described by the equation:

$$\log \dot{M}O_2 = 0.53\ \log M + 0.27 \quad (R^2 = 0.49;\ P < 0.0001) \quad (7)$$

Re-arrangement of the equation yielded the following allometric power relationship:

$$\dot{M}O_2 = 1.86\,M^{0.53} \quad (8)$$

3.2. TFM uptake

Similar to routine $\dot{M}O_2$, rates of TFM uptake were also inversely related to body mass, exponentially decreasing from a high of near $20\,\mu mol\,g^{-1}h^{-1}$ in the smallest larvae to approximately $1\,\mu molg^{-1}h^{-1}$ in the largest (4.2 g) animals (Fig. 1B). Log transformation of the whole body TFM uptake rates and body mass also yielded a statistically significant linear relationship (Fig. 1B – inset) described by:

$$\log TFM\ uptake\ rate = 0.34\log M + 0.86\ (R^2 = 0.46;\ P < 0.0001) \quad (9)$$

Re-arrangement of this Eq. (9) yielded the following allometric power relationship:

$$TFM\ uptake\ rate = 7.24\,M^{0.34} \quad (10)$$

There was also a strong linear relationship between the routine mass specific $\dot{M}O_2$ ($\mu mol\,g^{-1}\,h^{-1}$) and mass specific TFM uptake ($nmol\,g^{-1}\,h^{-1}$). Not surprisingly, larvae with higher routine $\dot{M}O_2$ also took up TFM at faster rates compared to those with low $\dot{M}O_2$ (Fig. 2; $R^2 = 0.39$; P < 0.001).

3.3. Effects of life history stage on oxygen consumption and TFM uptake

The $\dot{M}O_2$ of larval sea lamprey was nearly identical ($1.3 \pm 0.2\,\mu mol\,g^{-1}\,h^{-1}$) to rates measured in late metamorphosing (stage 6–7; $1.4 \pm 0.2\,\mu mol\,g^{-1}\,h^{-1}$) and juvenile animals ($1.2 \pm 0.1\,\mu mol\,g^{-1}\,h^{-1}$) (Fig. 3A; P = 0.84). Similarly, the rate of TFM uptake in larval lamprey ($4.3 \pm 0.3\,nmol\,g^{-1}h^{-1}$) was not statistically different from metamorphosing ($3.8 \pm 0.4\,nmol\,g^{-1}h^{-1}$) or the juveniles ($4.7 \pm 0.5\,nmol\,g^{-1}h^{-1}$) (Fig. 3B; P = 0.34).
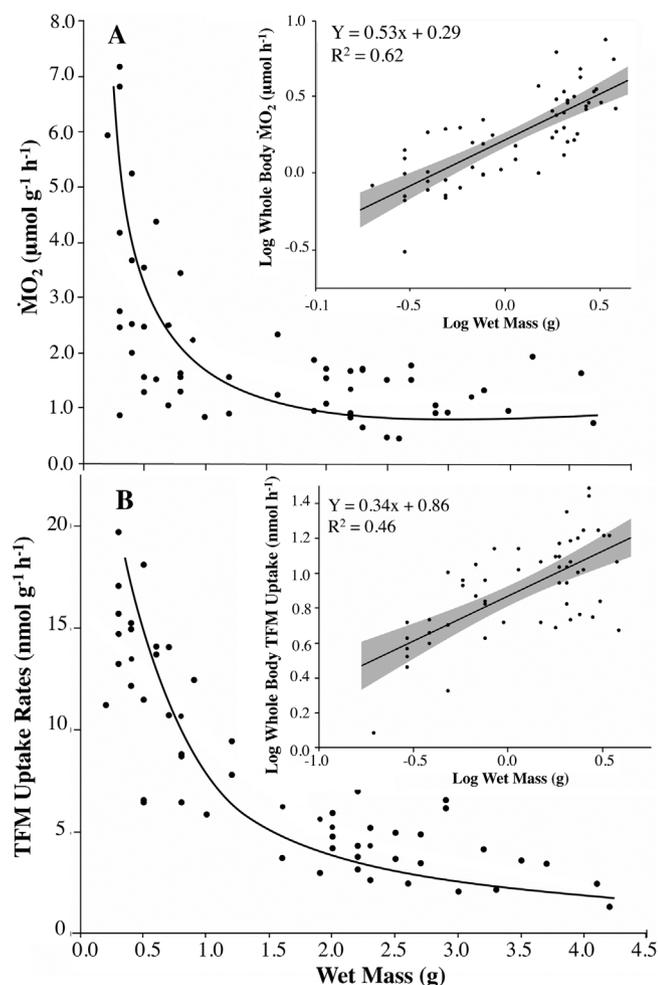
Fig. 1. Relationship between oxygen consumption ($\dot{M}O_2$) and TFM uptake rates with sea lamprey body mass with a line of best fit. **(A)** mass specific routine $\dot{M}O_2$ versus body mass (g) was quantified in individual larval sea lamprey (N = 56; 0.2 − 4.2 g) for 1 h using static respirometry in WLU well water, followed by **(B)** the measurement of mass specific TFM uptake rates using ^{14}C-labelled TFM (4.6 mg L^{-1}), to which sea lampreys were exposed for 3 h. Insets depict respective log–log relationships between whole body $\dot{M}O_2$ and TFM uptake vs log body mass from which the allometric power relationships Y = aM^b were derived (see text for details). Log-log relationships were determined using least squares linear regression.

3.4. Relationship between body size, life stage and post-TFM clearance

The amount of TFM administered (IP injection) to both larval and juveniles averaged 57 ± 15 nmol TFM g^{-1} (data not shown), reflecting the approximate amount of TFM that accumulates in the body following exposure to the 12h-LC$_{100}$ in the lab (Lech and Statham, 1975; LeClaire, 2014).

Overall, body size did have an affect on TFM clearance rates, with smaller animals initially (1–2 h) excreting TFM at significantly higher rates than larger animals (Fig. 4A). The highest rates of TFM clearance for all body size groups were observed in the first 0–2 h following ^{14}C-TFM administration (Fig. 4A). Thereafter, TFM clearance rates gradually declined in both groups before stabilizing between 8 and 24 h, when the majority of TFM had been cleared (Fig. 4A). There was also no relationship between body mass and the T$_{1/2}$-TFM of larval sea lamprey (Fig. 4B, P = 0.31), which averaged 7.0 ± 1.0 h. Although, the TFM clearance rates of larvae were not significantly different from those of size-matched juvenile lampreys (P = 0.28), the T$_{1/2}$-TFM was 30% lower in the larvae (P < 0.001), suggesting that sea lamprey have a greater capacity to eliminate TFM during this earlier life stage (Fig. 5).
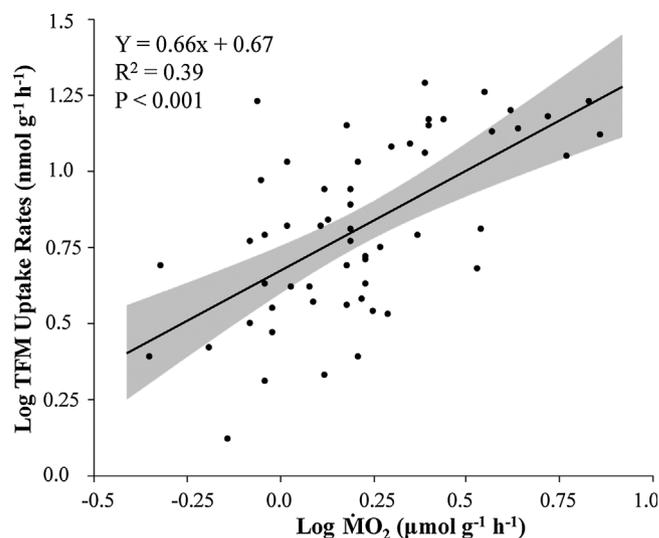

Fig. 2. Log-log relationship between mass specific routine $\dot{M}O_2$ and TFM uptake rate in larval sea lamprey. Routine $\dot{M}O_2$ was determined for 1 h in each larval sea lamprey (N = 56; 0.2–4.2 g), prior to exposure to radiolabeled TFM (^{14}C-TFM; 4.6 mg L^{-1} TFM) for 3 h. The line of best fit was determined using least squares linear regression, and data presented ± the 95% confidence interval (shaded zone), and statistical significance assessed using ANOVA.

4. Discussion

The application of TFM to streams containing larval lampreys every 2–5 years is a key component of the sea lamprey control program in the Great Lakes, one of the most successful aquatic invasive species control programs in the world (GLFC, 2011; Siefkes, 2017). However, the success of TFM applications is sometimes undermined by "residual" larval lamprey that are able to survive treatment (McDonald and Kolar, 2007). Yet, the underlying factors that lead to "residual" lamprey remain poorly defined. The present findings suggest that the much lower rates of TFM uptake by larger lamprey may explain why some animals are able to survive treatment. Our results further suggest that TFM uptake scales allometrically with body size, as does routine $\dot{M}O_2$ in larval lampreys (Hill and Potter, 1970; Lewis 1980; Wilkie et al., 2001). Thus, larger larvae with lower routine $\dot{M}O_2$ are much less susceptible to TFM than smaller animals. This also corresponds with the observations of personnel in the field, who report that larger larval lampreys are usually the last group of animals to succumb during TFM treatments (B. Morrison, DFO, Sea Lamprey Control Centre, pers. Comm.)

4.1. Oxygen consumption ($\dot{M}O_2$) and TFM uptake scale allometrically with body size

Like other vertebrates, the relationship between metabolic rate and body size can described using Kleiber's allometric scaling approaches, where metabolic rate ($\dot{M}O_2$) is a power function of body mass (Kleiber, 1947; Goolish, 1991). Such relationships are described by the well-known equation Y = aM^b, where Y is the dependent variable (e.g. $\dot{M}O_2$), M is the mass of the animal, b is the universal mass (aka. scaling) exponent which corrects for changes in $\dot{M}O_2$ with body mass, and a is an empirically derived proportionality constant generated from log–log plots of whole body $\dot{M}O_2$ vs. M (Kleiber, 1947; Peters, 1983; Glazier, 2013). Unlike proportional relationships, where the value of b is close to 1, b is usually near 0.75 in many mammal and poikilothermic animals, including lampreys (Kleiber 1947; Lewis, 1980; Hughs, 1984; Goolish, 1991; Hill and Potter, 1970; Wilkie et al., 2001; Nelson, 2016). In this study, however, $\dot{M}O_2$ = 1.86 M$^{0.53}$ yielded a mass scaling exponent of b = 0.53, which was much lower than expected. On the other hand, a = 1.86, which was near respective values reported in larval *Ichthyomyzon hubbsi* and *Lampetra planeri* (1.74 and 1.62; Lewis, 1980).
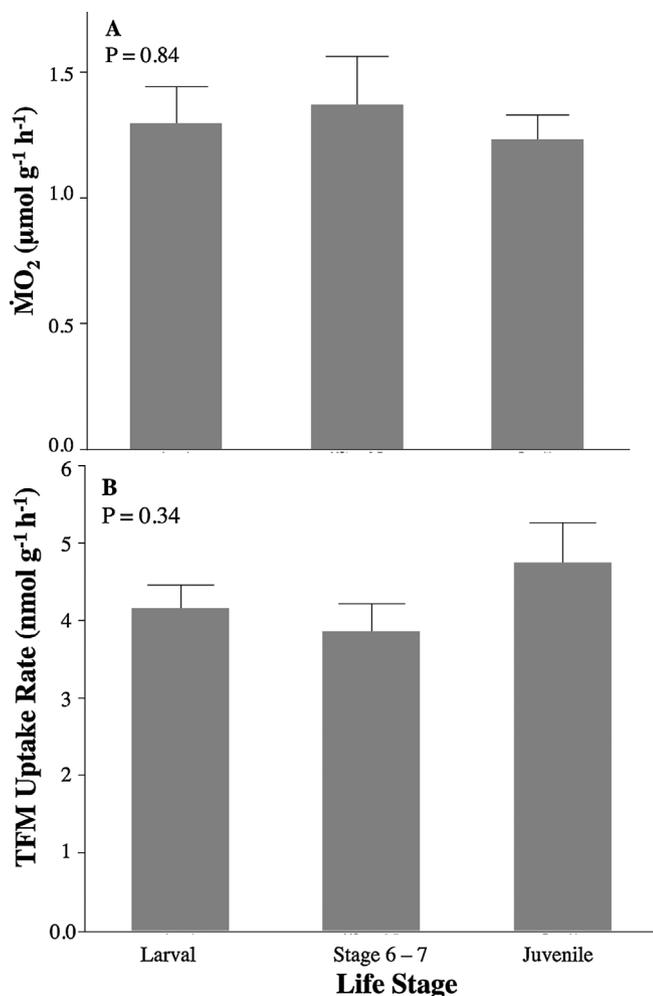
Fig. 3. Effects of life stage on $\dot{M}O_2$ and TFM uptake by sea lampreys. **(A)** Routine $\dot{M}O_2$ and **(B)** rates of TFM uptake were determined in larval (N = 19, mass = 2.7 ± 0.2 g), metamorphosing (stage 6–7; N = 10, mass = 2.7 ± 0.2 g), and juvenile (N = 15, mass = 2.7 ± 0.2 g) sea lamprey. No significant differences were observed amongst the different life stages for $\dot{M}O_2$ (P = 0.84) or rates of TFM uptake (P = 0.34). Data presented as the mean ± 1 SEM.

Fig. 4. Effects of body size on A) TFM clearance rates and B) elimination half-life of TFM ($T_{1/2}$-TFM) of larval sea lampreys. Clearance rates of TFM were determined by administering ^{14}C-TFM labeled TFM solution (Mean Specific Activity = 99 CPM nmol^{-1}) to 3 size groups of larval sea lamprey [2.0–2.9 g (N = 6); 1.0–1.9 g (N = 18); 0.2–0.9 g (N = 28)] by intraperitoneal injection (IP). Rates of TFM clearance were then calculated by measuring the appearance of ^{14}C-TFM radioactivity appearing in the water over 24 h. See method and materials for further details. Calculations of the respective $T_{1/2}$-TFM for each animal were based on the measured TFM clearance rate for each lamprey following IP administration of TFM (N = 52; mass = 0.2–2.9 g). See method and materials for further details.

Typically, low mass scaling exponents are observed in fishes with a high metabolic rate, for example thunniform swimmers such as tuna (Killen et al., 2010). In contrast, larval lampreys are burrow-dwelling, relatively sedentary animals with low metabolic rates (Hill and Potter, 1970; Lewis, 1980). If $\dot{M}O_2$ was overestimated in the larval lamprey, that would tend to lower the value of the mass exponent, but this seems unlikely in the present study because burrowing substrate (diffuse cotton) was provided to calm the animals (thigmokinesis; Wilkie et al., 1999, 2001) during $\dot{M}O_2$ measurements. Such precautions are crucial because rates of $\dot{M}O_2$ can be up to two-fold higher in unburrowed compared to burrowed larvae (Potter and Rogers, 1972; Wilkie et al., 2001).

In an earlier study on larval sea lamprey ranging in mass from 1.3 to 3.9 g, and collected from tributaries in Atlantic Canada, the mass exponent was equal to 0.83 (Wilkie et al., 2001), which is more in-line with the theoretical value of 0.75. In the present study, however, we used lampreys with a wider range of body masses (0.2–4.2 g) that were collected in the Great Lakes. Thus, the animals included in our study included very small young of the year animals and larger, older animals (1 + or older). Despite their similar appearance, the metabolic profile of lamprey is highly variable during the larval phase (O'Boyle and Beamish, 1977). The most notable changes take place in the latter stages of larval life, which is characterized by a period of arrested growth the year prior to metamorphosis, which is accompanied by
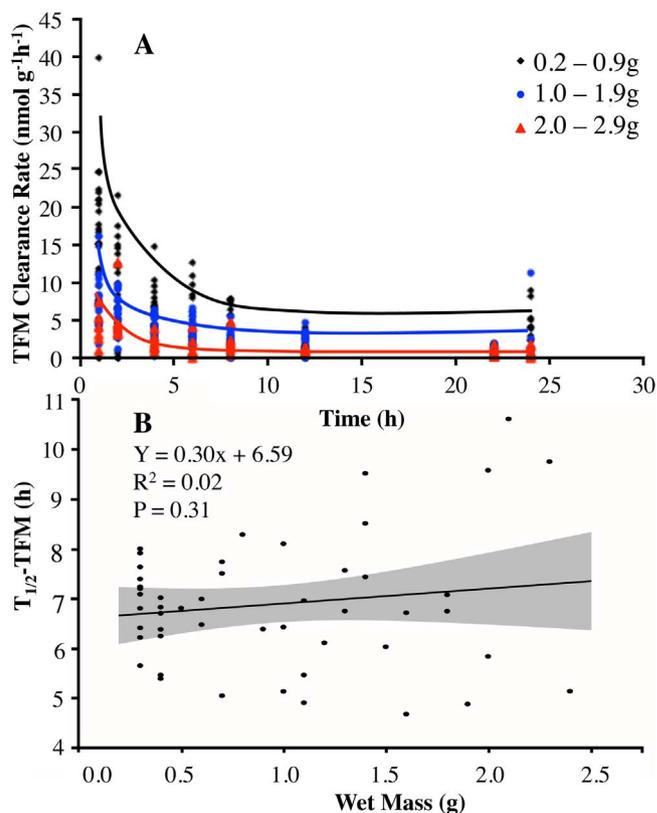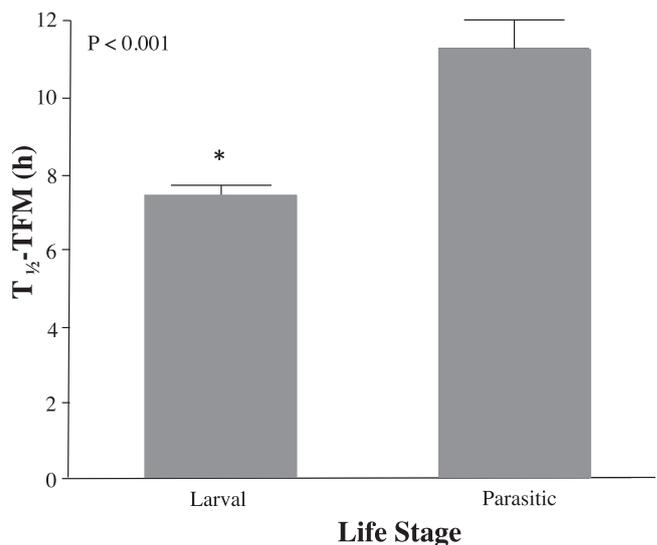

Fig. 5. Effects of life stage on the elimination half-life of TFM ($T_{1/2}$-TFM) of sea lampreys. Calculations of $T_{1/2}$-TFM presented as the mean ± 1 SEM for larval (N = 56; mass = 1.0 ± 0.1 g) and juvenile (N = 12; mass = 3.3 ± 0.2 g) sea lampreys following IP administration of TFM. Asterisk denotes significantly lower $T_{1/2}$-TFM of larval lampreys compared to juvenile lampreys (P < 0.001).
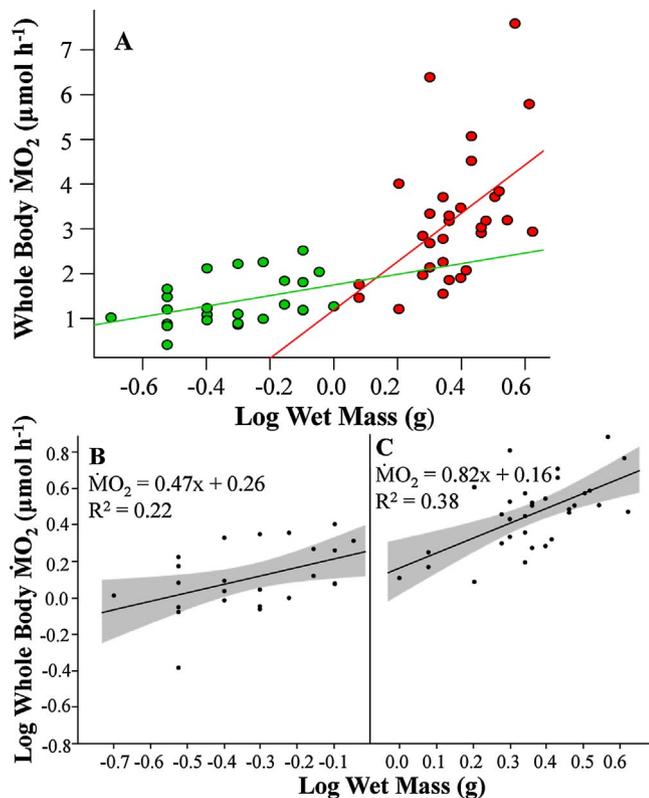
Fig. 6. Metabolic rate varies within body size for larval sea lamprey. Two groups of lamprey were detected within the population from analyzing the data with hierarchial clustering analysis (Ward error sum of squares). The two groups could be identified based on mass A) where larvae < 1 g (N = 24) had different allometric scaling relationships than those > 1 g (N = 32). The scaling relationships are depicted in B) for those < 1 g where $\log(\dot{M}O_2) = 0.47(\log x) + 0.26$ and in C) where the equation to describe the > 1 g larvae is $\log(\dot{M}O_2) = 0.82(\log x) + 0.16$.

major changes in the metabolic profile of the larvae (Lowe et al., 1973; O'Boyle and Beamish, 1977). Changes include the accumulation of large amounts of lipid, which provide the animals with the fuel needed to survive during the non-trophic period of metamorphosis (Lowe et al., 1973; O'Boyle and Beamish, 1977; Kao et al., 1997). Unfortunately, there are no measures of how such changes affect metabolic rate in larval lamprey, but it seems reasonable to predict that metabolic rate would be much lower during this period of arrested growth relative to earlier life stages.

Given the influence that developmentally-mediated changes in metabolic rate might have had on measures of the mass exponent of $\dot{M}O_2$ (Killen et al., 2010), hierarchical clustering analysis (Ward error sum of squares) was used to further analyze the $\dot{M}O_2$ vs body size data set. This analysis revealed that there were two distinct size groups (or "clusters"; Fig. 6A). In these two groups we observed different allometric relationships with $\dot{M}O_2$; in animals that were less than 1.0 g in mass, the relationship between body mass and $\dot{M}O_2$ was described by the equation $\dot{M}O_2 = 1.82\,M^{0.47}$ (Fig. 6B). In animals weighing more than 1 g (1.0–4.2 g), however, the corresponding power relationship was $\dot{M}O_2 = 1.45\,M^{0.82}$ (Fig. 6C), where the mass scaling exponent, $b = 0.82$, was much closer to the expected value of 0.75 and virtually identical to values previously measured in similarly sized larval sea lampreys ($b = 0.83$; Wilkie et al., 2001).

Because lamprey have a much lower capacity to biotransform TFM compared to other fishes, interpretation of TFM uptake data was not complicated by the simultaneous elimination of this compound (Lech, 1974; Lech and Statham, 1975; Kane et al., 1993). Like $\dot{M}O_2$, TFM uptake also increased allometrically, as described by the power relationship: TFM Uptake $= 7.24\,M^{0.34}$. In contrast to $\dot{M}O_2$, b was lower for TFM uptake, averaging 0.34, indicating that TFM uptake was

disproportionately higher in smaller larval sea lamprey than would be predicted based solely on $\dot{M}O_2$. The proportionality constant, a, for TFM uptake was also much higher than for $\dot{M}O_2$, at 7.24. The different allometric profiles of TFM uptake compared to $\dot{M}O_2$ may be due to differences in their modes of uptake. Whereas O_2 is taken up down partial pressure gradients generated across the gills, followed by its binding to hemoglobin (e.g. Randall, 1972; Wilson and Laurent, 2002), TFM uptake mainly takes place down diffusion gradients in its un-ionized (phenolic form; Hunn and Allen, 1974; Bills et al., 2003; Hlina et al., 2017) and possibly in its ionized form via unidentified transporters, possibly organic anion transporters (Hlina et al., 2017).

The significant correlation between TFM uptake and routine $\dot{M}O_2$ ($R^2 = 0.39$ P < 0.001; Fig. 2), suggests that the higher rates of gill ventilation needed to sustain oxygen delivery in smaller lampreys likely results in greater rates of TFM uptake than in larger animals. Such observations are in accord with predictions made by Brauner et al. (1994), who suggested that fishes with higher $\dot{M}O_2$ should have greater toxicant loads when exposed to organic xenobiotics. Their predictions were subsequently confirmed by Yang et al. (2000) who exposed different sizes and species of fishes to different hydrophobic (lipophilic) xenobiotics (1,2,4,5-tetrachlorobenzene; 3,4,5,6-tetrachloroguaiacol; 4,6-dichlorobenzediol) and noted a strong relationship between the rates of accumulation and $\dot{M}O_2$ ($R^2 = 0.942$). We noted a weaker correlation with TFM uptake and $\dot{M}O_2$ in the present study, however. This may be partially explained by the greater lipophilicity of the compounds studied by Yang et al. (2000), as defined by log octanol:water coefficient (log Kow's > 4.0) values that were generally much higher than that of TFM (log Kow = 2.77). However, differences in the mechanisms of action of the chemicals to which the animals were exposed also differed. Unlike TFM, which increases $\dot{M}O_2$ due to its uncoupling effects on mitochondrial oxidative phosphorylation (Birceanu et al., 2011), none of the organic chemicals examined by Yang et al. (2000) appear to affect $\dot{M}O_2$ in fishes (Brauner et al., 1994; Yang and Randall, 1997). By causing acute increases in $\dot{M}O_2$, exposure to TFM would likely result in a positive feedback loop resulting in greater rates of TFM uptake, decreasing the strength of the relationship between TFM uptake and routine $\dot{M}O_2$. Future experiments that simultaneously examine the relationship between TFM uptake and $\dot{M}O_2$ would therefore be informative.

4.2. Oxygen consumption ($\dot{M}O_2$) and TFM uptake are unchanged following metamorphosis

Although larval lampreys are relatively sedentary with significantly lower $\dot{M}O_2$ than comparably sized teleost fishes (Lewis, 1980; Wilkie et al., 2001), they are capable of substantially increasing their $\dot{M}O_2$ following brief bursts of activity, which might occur during intense swimming or even burrowing (Wilkie et al., 2001). Routine $\dot{M}O_2$ has also been reported to increase during metamorphosis in some lamprey species, including the non-parasitic European brook lamprey (*L. planeri*) and parasitic European river lamprey (*Lampetra fluviatilis*), to levels comparable with more active teleost fishes (Lewis and Potter, 1977; Lewis 1980). These changes are thought to be related to the transition from a sedentary, burrow-dwelling life style to a more active, free-swimming life stage following metamorphosis in parasitic species of lampreys such as the European river lamprey (Lewis, 1980). For this reason, we hypothesized that rates of TFM uptake would be greater in comparably sized post-metamorphic (juvenile) lampreys and larval lamprey. However, the rates of TFM uptake and $\dot{M}O_2$ were similar in larval and post-metamorphic sea lamprey.

As stated above, the presence of burrowing substrate in this and previous studies (Hill and Potter, 1970; Wilkie et al., 2001) makes it unlikely that $\dot{M}O_2$ was overestimated in larval lamprey. Further precautions, such as keeping the animals in the dark for the duration of $\dot{M}O_2$ measurements and making measurements at the same time each day, likely minimized variability. The absence of a distinct photoperiod may have also muted any diurnal (nightly) fluctuations in $\dot{M}O_2$ that are

known to occur in transforming and juvenile lampreys, but not in larval animals (Claridge and Potter 1975; Lewis and Potter, 1977). Interspecies differences between sea lamprey and the European river and brook lampreys cannot be ruled out either. Leach (1946) noted no difference between the $\dot{M}O_2$ of larval and post-metamorphic sea lamprey, but his experiments were done at higher temperatures and without burrowing substrate for the larval animals. However, it should be noted that in sea lamprey, metamorphosis results in a depletion of essential energy reserves as characterized by declines in lipid and glycogen stores (Lowe et al., 1973; O'Boyle and Beamish, 1977). In other words, the animals are in a state of prolonged starvation (3–4 months) that would make it necessary to conserve metabolic reserves until they begin feeding as juvenile parasites, which would preclude any pronounced increases in $\dot{M}O_2$ until such time. It is unknown if similar changes accompany metamorphosis in other species of lamprey, but this could be a fruitful area of inquiry.

The structure of the gills, presumed to be the main route of TFM uptake, also changes markedly during metamorphosis (Lewis and Potter, 1977; Lewis 1980; Rovainen, 1996). In larvae, gills are unidirectionally ventilated with water entering the pharynx via the oral hood before crossing the gills and exiting via the branchiopores. Following metamorphosis, gills are tidally ventilated and water is pumped out of the re-structured branchiopores (gill pouches) for irrigation of the gills during feeding or attachment to substrate (Lewis and Potter, 1976; Lewis, 1980; Rovainen, 1996). Despite such structural changes, the surface areas for gas exchange (secondary lamella) are similar in larval and post-metamorphic lampreys and do not differ relative to body size (Lewis and Potter, 1976; Lewis, 1980; Rovainen, 1996). In the absence of any change in functional gill surface area or $\dot{M}O_2$, it is perhaps not surprising that metamorphosis failed to result in differences in the rates of TFM uptake amongst larval, metamorphosing, or juvenile sea lamprey. However, further study including more detailed morphological and ultrastructural analysis of lamprey gill structure and function during metamorphosis, including measurements of branchial surface area are required.

4.3. Relationship between body size and life stage on rates of TFM clearance

In the early stages of depuration in TFM-free water, smaller larval sea lamprey excreted TFM at a faster rate compared to larger ones. The greater gill:body surface ratio may facilitate more passive diffusion of TFM from the body of a small lamprey. The branchial surface area and body surface area were not quantified in the present study, but should be addressed in future studies. The presence of dermal capillaries in the larvae, along with a relatively thin epidermis (Potter et al., 1996), may have contributed to some TFM loading and unloading. But, given the much greater relative surface area of the gills, along with high rates of convective flow of water across the gills, the skin likely plays a minor role in TFM elimination and uptake.

Excretion via the renal routes is another possibility, but this would require TFM to be bio-transformed via UDP-glucuronosyltransferase into its more water soluble glucuronide conjugate to be excreted via the urine or bile, as it is in coho salmon (*Oncorhynchus kisutch*) and largemouth bass (*Micropterus salmoides*) (Hunn and Allen, 1975; Schultz et al., 1979). Sea lamprey have a much lower capacity to detoxify and excrete TFM in this manner (Hunn and Allen, 1974; Lech and Statham, 1975; Kane et al., 1993), suggesting that TFM excretion is likely limited to passive diffusion of the un-ionized parent compound across the gills (Hunn and Allen, 1974; Hlina et al., 2017). Indeed, the half-life for TFM elimination by larval sea lamprey observed in the present study ($T_{1/2}$-TFM = 7 ± 1 h) was much high than values reported by Lech et al. (1973) in rainbow trout ($T_{1/2}$-TFM = 1.6 h), which are also known to conjugate TFM to TFM-glucuronide (Kane et al., 1993; Vue et al., 2002; Birceanu et al., 2014).

Despite a low capacity to detoxify TFM to TFM-glucuronide, 90% of the injected TFM was cleared by the lamprey within 24 h regardless of body size. The rapid reduction in internal TFM concentrations following depuration in TFM-free water may help explain why larval lampreys are able to rapidly restore internal energy stores following short-term TFM exposure. Clifford et al. (2012) demonstrated that larval sea lamprey fully restore internal glycogen stores and phosphocreatine within 2–4 h following short-term (4–6 h) exposure to the 12-h LC_{100} of TFM. Due to TFM's uncoupling effects on mitochondrial oxidative phosphorylation (Birceanu et al., 2011), TFM exposure results in greater reliance and reductions in these anaerobic energy reserves to make up for short-falls in ATP supply (Viant et al., 2001; Birceanu et al., 2009; Clifford et al., 2012; Henry et al., 2015). Thus, despite their relative inability to detoxify TFM, sea lamprey are resilient to short-term TFM exposure. In the early stages of post-TFM exposure, it also appears that smaller larvae can initially clear TFM at a faster rate than larger ones following their introduction into clean, TFM-free water.

After 24 h it was determined that the larval sea lamprey had excreted more TFM compared to the parasitic (juvenile) sea lamprey. Moreover, the 30% lower $T_{1/2}$-TFM of larval lamprey underscored their greater capacity to excrete TFM than the post-metamorphic animals. This greater capacity to clear TFM could be because larval lamprey irrigate the gills in a unidirectional manner (Rovainen, 1996), which would result in a larger, sustained outward blood-water gradient for TFM when bulk water concentrations of the lampricide are low. In contrast, the gradients across the tidally ventilated gills of the juveniles would be less because some TFM would likely remain in the gill pouches following expiration, resulting in lower blood-water TFM gradients and less efficient clearance of the lampricide.

5. Conclusions

In conclusion, large larval sea lamprey may be a more likely source of "residual" lampreys following TFM treatment than smaller animals. With a much higher mass specific $\dot{M}O_2$, smaller sea lampreys likely have greater ventilation rates, which leads to higher rates of TFM uptake. Because TFM uptake, like $\dot{M}O_2$, scales allometrically in larval sea lamprey, TFM uptake can be described using the power function: *TFM Uptake* $= 7.24 \, M^{0.34}$ to predict how much and how quickly TFM is accumulated by lampreys during lampricide treatments. By determining how body size dependent differences in routine $\dot{M}O_2$ of larval sea lamprey influenced their rates of TFM uptake and excretion, predictions can also be made about biotic factors that may affect a sea lamprey's ability to survive a typical TFM treatment. Differences in tolerance to TFM based on body size, life stage and $\dot{M}O_2$ may be important factors to consider when selecting streams for lampricide treatments, and for predicting the likelihood of some sea lamprey surviving TFM applications.

Finally, numerous factors are known to influence toxicant bioavailability, uptake, distribution and elimination by fishes. Different water pHs, hardness and ion content are known to alter the speciation and bioavailability of metals (Paquin et al., 2002), not to mention the bioavailability of organic compounds including phenols with ionizable functional groups such as TFM, which can influence their lipid solubility and diffusability across respiratory epithelia (Hunn and Allen, 1974; Erickson et al., 2006). However, only a few studies have addressed how body size can influence the kinetics of xenobiotic uptake, despite a wealth of studies demonstrating a clear allometric relationship between metabolic rate and body size amongst and between numerous aquatic species. Learning more about the allometric relationships between xenobiotics and body size in aquatic animals could therefore increase our ability to predict the toxicity of such compounds with greater accuracy.

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