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Warmer waters increase the larval sea lamprey's (*Petromyzon marinus*) tolerance to the lampricide 3-trifluoromethyl-4-nitrophenol (TFM)

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ABSTRACT

Invasive sea lampreys (*Petromyzon marinus*) in the Great Lakes are controlled by applying the pesticide (lampricide) 3-trifluoromethyl-4-nitrophenol (TFM) to waters infested with larval lamprey. However, treatment effectiveness can be undermined by "residual" larval sea lamprey that survive TFM exposure, and subsequently complete metamorphosis into parasitic juvenile sea lamprey that prey on culturally and economically important fishes. We investigated how season and temperature influenced the TFM tolerance of larval sea lamprey. Acute toxicity tests on lamprey collected from the Au Sable River, Michigan, revealed that the 12-h LC_{50} and $LC_{99.9}$ were 2.0- to 2.5-fold greater in late spring and summer, than in early spring and fall. Subsequent toxicity tests indicated that greater TFM tolerance in summer was due to warmer temperatures, based on an almost 2-fold greater 12-h LC_{50} and $LC_{99.9}$ in warm (24 °C) compared to cool (6 °C) water. Variations in energy stores (glycogen, lipid, protein) or condition did not appear to affect TFM sensitivity. We conclude that higher water temperature is the primary factor driving the larval sea lamprey's greater tolerance to TFM during the summer, possibly due to an increase in their capacity to detoxify TFM. Considering seasonal variations in temperature may be prudent when selecting and treating sea lamprey infested streams with TFM to minimize treatment residuals. In the longer term, increases in average and peak water temperatures due to climate change could result in greater TFM requirements and costs due to the greater tolerance of larval sea lamprey to TFM at warmer temperatures.

Introduction

The piscicide, 3-trifluoromethyl-4-nitrophenol (TFM), has been used to control invasive sea lamprey (*Petromyzon marinus*) populations in the Great Lakes since the early 1960s (Applegate et al., 1961; Siefkes, 2017; Wilkie et al., 2019). Applied at regular intervals to nursery streams and rivers infested with larval sea lamprey, TFM specifically targets animals in their burrows, where they live as relatively sedentary, suspension feeders (Moore and Beamish, 1973; Sutton and Bowen, 1994). Current treatment protocols are based on the minimal lethal concentration (MLC) of TFM, which is defined as the amount of TFM needed to kill 99.9% of larval sea lamprey over 9 h (Bills et al., 2003). In practice, the concentrations used in treatments range from 1.2 to 1.5 times the MLC to ensure that treatment residuals (i.e., sea lamprey that survive TFM exposure) are minimal (McDonald and Kolar, 2007).

TFM exerts its toxicity by uncoupling mitochondrial oxidative phosphorylation, interfering with ATP production in both target and non-target fishes (Niblett and Ballantyne, 1976; Birceanu et al., 2011; Wilkie et al., 2019). By inducing a mismatch between energy supply and demand in the body, TFM forces the fish to rely on anaerobic metabolic pathways for survival. Once energy reserves, such as glycogen and high energy phosphagens (e.g., phosphocreatine) become depleted, ATP supply cannot keep up with ATP demand, and death soon follows (Wilkie et al., 2007; Birceanu et al., 2009, 2014; Clifford et al., 2012). While the mechanism of TFM action is similar among different aquatic organisms (Viant et al., 2001; Wilkie et al., 2007; Birceanu et al., 2011, 2014), the specificity of TFM is thought to be due to the lower capacity of larval sea lamprey to detoxify TFM compared to non-target fishes (see Wilkie et al., 2019 for recent review). Non-target fishes including rainbow trout (*Oncorhynchus mykiss*), ictalurid catfishes, and bluegill (*Lepomis macrochirus*) use phase II biotransformation to detoxify and excrete TFM, to which a glucuronic acid or sulphate functional group reacts with TFM to generate TFM-glucuronide or TFM-sulphate (Lech and Statham, 1975; Kane et al., 1994; Bussy et al., 2018a, 2018b), which are more water soluble and easier to excrete via the gastrointestinal or urinary tract (see Clarke et al., 1991 for review).

Residual larval sea lamprey, that survive lampricide treatments, and non-target mortality are ongoing challenges of the sea lamprey control program (Boogaard et al., 2003; McDonald and Kolar, 2007; Wilkie et al., 2019). Abiotic factors such as water pH and alkalinity can affect the acute toxicity of TFM to sea lamprey and non-target organisms by altering TFM bioavailability (Bills et al., 2003; O'Connor et al., 2017; Hlina et al., 2017). Less is known about how other factors, both abiotic and

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biotic, might affect the TFM sensitivity of larval sea lamprey. Scholefield et al. (2008) reported that the TFM sensitivity of larval sea lamprey could be >2-fold higher in the spring compared to late summer, suggesting that season and/or temperature have a marked influence on TFM toxicity independent of changes in water chemistry. However, the underlying physiological basis for these observations was not determined. The overarching goal of the present study was to determine which biotic and/or abiotic factors might explain seasonal variations in the sensitivity of larval sea lamprey to TFM.

Biotic factors such as life stage and body mass are known to affect TFM sensitivity in sea lamprey. Henry et al. (2015) demonstrated that TFM sensitivity was highest in sexually mature adults compared to the earlier larval life stages. Tessier et al. (2018) found that rates of TFM uptake were inversely proportional to body size in lamprey, and that larger larval sea lamprey survived for longer periods when exposed to the MLC of TFM, suggesting that larger larvae are a potential source of treatment residuals. Abiotic factors, such as differences in stream discharge and water chemistry, as well as pH and alkalinity, also influence TFM treatment success (Bills et al., 2003; McDonald and Kolar, 2007; Wilkie et al., 2019). Because TFM toxicity decreases with increasing water pH and alkalinity, TFM application rates are based on standard tables relating the MLC to water pH and alkalinity (Bills et al., 2003), and sometimes streamside toxicity tests (McDonald and Kolar, 2007). Previous treatment history and water discharge rates are also considered (P. Sullivan, Sea Lamprey Control Centre, Fisheries and Ocean's Canada, pers. comm.). Another abiotic variable, that has received surprisingly little attention, is temperature, which is not considered to be as important as pH or alkalinity when TFM application regimens are determined (e.g. Bills et al., 2003).

Given that TFM depletes energy reserves such as glycogen, which are also known to fluctuate seasonally in the sea lamprey (O'Boyle and Beamish, 1977), one goal of this study was to investigate if variation in whole body and tissue energy reserves influenced TFM sensitivity in sea lamprey. To this end, larval sea lamprey were collected in different seasons in 2013 (spring, early and late summer, fall) to relate variations in energy stores to seasonal differences in the TFM sensitivity of sea lamprey. Experiments were conducted at temperatures that corresponded to those at the time of capture, to most accurately reflect each season. A second set of follow-up experiments were then conducted the following year, using lamprey collected from the same river but at the same time of year (July 2014), to determine how differences in water temperature affected TFM sensitivity.

Material and methods

Collection site, experimental animals, holding, and temperature acclimation

Larval sea lamprey were collected from the Au Sable River, Michigan, United States, using pulsed-DC backpack electrofishing (ABP-2 Electrofisher, Electrofishing Systems, LLC, Madison, WI, USA) by United States Fish and Wildlife Service (USFWS) personnel in late April (experiments conducted in May), June, August and October 2013 (Series I – Effects of season on TFM tolerance) and in June 2014 (Series II – Effects of temperature on TFM tolerance). The Au Sable River is a tributary of Lake Huron and runs approximately 200 km through the northern Lower Peninsula of Michigan. The river is treated with TFM on a three-year cycle by USFWS personnel and underwent TFM treatment following our last collection of sea lamprey in 2014 (A. Jubar, USFWS, Ludington, MI pers. comm.). Following collection, the larval sea lamprey were transported to the U.S. Geological Survey, Hammond Bay Biological Station (HBBS; Millersburg, MI) in coolers containing aerated river water. Upon arrival at HBBS, animals were transferred to 30 L glass holding tanks (N = ~100 per aquaria), continuously receiving aerated Lake Huron water (pH 7.8 ± 0.4; alkalinity 85 mg $CaCO_3$ L^{-1}; hardness = 150 mg L^{-1} as $CaCO_3$; dissolved oxygen ≥80% saturation), with a 4–5 cm deep layer of sand lining the bottom of the tank to provide the larval sea lamprey with burrowing substrate. For Series I experiments, care was taken to ensure that holding temperatures at HBBS were maintained near those of the Au Sable River at the time of capture (±2 °C). This was done to ensure that different thermal exposure regimes did not result in metabolic adjustments that could confound data interpretation. In addition, the animals were held for no longer than one week before experiments were conducted to ensure that the physiology of the animals was as similar as possible to that at their time of capture. We were concerned that holding the animals for longer, combined with a lack of feeding or an artificial diet of yeast (e.g. Holmes and Youson, 1994), would have influenced body condition and energy stores prior to collecting tissues or measuring acute toxicity. Indeed, prolonged fasting can lead to reductions in tissue glycogen stores in fishes (Soengas et al., 2006; Polakof et al., 2012), a key analytical variable in our analysis.

To determine how temperature acclimation alone influenced TFM toxicity (Series II – Effects of temperature on TFM tolerance), larval sea lamprey collected from a different reach of the Au Sable were collected in June of the following year (2014), and transferred to larger, temperature controlled holding tanks (200 L) (N = ~250 animals per tank), filled with sand as described above. One group of sea lamprey were kept at the ambient temperature of the river at the time of collection (~12 °C), the second and third group were acclimated to nominal temperatures of either 6 °C or 24 °C by slowly cooling or warming the water by 1 °C per day, until the target temperatures were reached. The animals then remained at the nominal temperature for an additional 7–10 d, without feeding, prior to the collection of tissue samples and acute toxicity test experiments. Previous studies in our lab have indicated that such short-term fasts have little effect on tissue energy stores in larval sea lamprey due to their low metabolic rates (Wilkie et al., 2001). All animal holding conditions and experimental methods were approved by the Wilfrid Laurier University Animal Care Committee and followed Canadian Council of Animal Care (CCAC) guidelines.

Experimental protocols

Effects of season and temperature on the toxicity of TFM to larval sea lamprey

To determine how season (Series I - 2013) and temperature (Series II – 2014) affected the tolerance of larval sea lamprey to TFM, acute toxicity tests were performed over 12 h. The animals were then acclimated to the water temperature at which they were collected or the experimental temperature(s) for 7 to 10 d before performing acute toxicity tests. Each acute toxicity test was preceded by a preliminary range-finder toxicity test to determine the appropriate TFM concentrations to be used during the acute toxicity tests. Each range-finder test used nine glass aquaria (18 L) filled with aerated Lake Huron water (16 L) to which the appropriate amounts of TFM were added. The subsequent acute toxicity tests were conducted in triplicate, at six TFM concentrations ($N = 3$, plus one control; 19 aquaria in total) using a similar set-up, but larger aquaria (30 L).

Twelve hours prior to the range-finder or acute toxicity tests, each aquarium was filled with Lake Huron water and placed in a Living Stream (108″L x 24″W x 22″D, 190 gal; Frigid Units Inc., Toledo, OH) partially filled with re-circulating water maintained at the appropriate temperature using either a chiller or immersion heater (3/4 hp Delta Star chiller or 180 W direct immersion heater, manufacturer: Aqua Logic Incorporated, San Diego, CA). The aquaria, containing no animals, were dosed with sufficient amounts of field grade TFM (35% active ingredient dissolved in isopropanol, Clariant SFC GMBH WERK, Griesheim, Germany) to yield the appropriate target concentrations in each aquarium [0 (control), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 and 7.0 mg L^{-1}]. The range of TFM concentrations selected for the acute toxicity tests were based on the information generated in the range-finder tests, and varied according to season and temperature. For both sets of tests, water

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samples were collected after adding TFM and the next morning prior to adding animals to the aquaria, followed by immediate, spectrophotometric measurement of the water TFM concentration. Because measured TFM concentrations did not deviate by >10% from target concentrations, there was no need to make further adjustments to TFM concentrations in the tanks.

Larvae were removed from their holding tanks 12 h before tests were to be conducted, and left to acclimate in similar aquaria, containing the same water at the appropriate temperature, but containing no TFM. The day of testing, the larvae were randomly distributed to each test aquarium (N ~10 per aquaria in the range-finder tests; N ~15 for toxicity tests), and temperature, pH and dissolved oxygen were recorded immediately after the animals were added (0 h) and at 12 h of exposure. Survival was monitored hourly from 0 to 12 h; when animals appeared dead (immobile, no visible ventilation), survival was tested by gently pinching the caudal fin with tweezers. Unresponsive (dead) animals were immediately removed from the tanks, at which time body length and mass were measured. Surviving lamprey were euthanized with an overdose of tricaine methanesulfonate (1.5 g L^{-1} buffered with 3.0 g L^{-1} of $NaHCO_3$; MS-222; Syndel Labs, Nanaimo, BC, Canada). The toxicity tests followed ASTM International (formerly American Society and Testing Materials, 2007) guidelines, with toxicity expressed as the concentrations of TFM that were lethal to 50% and 99.9% of the animals tested over 12 h (12-h LC_{50} or 12-h $LC_{99.9}$).

Variation in the proximate body composition of sea lamprey with season or temperature

To determine how energy stores and proximate body composition of larval sea lamprey changed with season or temperature acclimation and how this could impact TFM toxicity, tissues were collected from the appropriate groups of larvae not exposed to TFM. The night before sampling, lamprey (N = 48) were distributed into twelve containers (750 mL) receiving aerated lake water that was maintained at the appropriate temperature. Each container contained 2 g of diffuse aquarium cotton to provide burrowing substrate (e.g. Wilkie et al., 2001). Temperature, pH, and dissolved oxygen were recorded immediately after the animals were added (0 h) and at 12 h. After 12 h, the lamprey were euthanized, one container at a time by cutting off water flow to the container, and adding a slurry of buffered tricaine methanesulfonate sufficient to anaesthetize the animals (0.5 g L^{-1} buffered with 1.0 g L^{-1} of $NaHCO_3$), before transferring them one at a time to a container with a lethal dose (1.5 g L^{-1} buffered with 3.0 g L^{-1} of $NaHCO_3$) of the anaesthetic. Lamprey were weighed, body length measured, and then dissected for collection of brain and liver. The brain, liver and remaining carcass were snap frozen in liquid nitrogen and stored at −80 °C until transported to WLU on dry ice. Upon arrival, tissues and carcasses were stored at −80 °C until analyzed.

Analytical techniques

Chemicals and reagents

Unless noted, all chemicals and reagents were purchased from the Sigma-Aldrich Chemical Co., St. Louis, Missouri, or BioShop Life Science Products, Burlington, Ontario.

Quantification of TFM concentrations in water

Quantification of TFM in water samples during range-finder and acute toxicity tests were made by measuring the absorbance of freshly collected water samples (within 1 h) against freshly prepared TFM standards (0, 0.5, 1.0, 2.0, 3.0, 5.0 and 7.0 mg L^{-1}) at a wavelength of 395 nm using a Genesys 6 spectrophotometer (Thermo Electron Corporation, MA, USA) using Standard Operating Procedures provided by the Sea Lamprey Control Centre (IOP: 012.4), Fisheries and Oceans Canada, Sault Ste. Marie, ON.

Glycogen analysis in lamprey carcass, brain and liver

Larval sea lamprey carcasses (N = 10–15 per experiment) were processed for glycogen analysis as described in Birceanu et al. (2009) and Henry et al. (2015). Briefly, the carcass was homogenized by mortar and pestle under liquid N_2, and approximately 100 mg of the powder was transferred into a 1.5 or 2.0 mL microcentrifuge tube containing four volumes of ice cold 8% PCA and 1 mmol L^{-1} EDTA solution, vortexed, placed on ice for 10 min and centrifuged for 5 min at 4 °C and 10,000*g*. The supernatant (400 μL) was then neutralized with 3 M K_2CO_3, vortexed, and the supernatant frozen in liquid nitrogen. To determine glycogen concentration, the supernatant was separated into two aliquots: the first was used to measure background glucose, and the second received an equal volume of 2 M sodium acetate buffer (adjusted to pH 4.5–4.6 with glacial acetic acid) and was incubated with 40 units (U) of amyloglucosidase solution to hydrolyze the glycogen into glucose (glucosyl units) for 2 h in a water bath at 37 °C. After incubation, 70% PCA was added to terminate the reaction, followed by neutralization using 3 M K_2CO_3. The free glucose in the background glucose aliquot and the amyloglucosidase treated aliquot was quantified using the LiquiColor® enzymatic method (Reference No. 1070–125, StanBio Laboratory, TX, USA) in 96 microwell plates at 500 nm (Epoch 2 Microplate Spectrophotometer, BioTek Instruments, Winooski, VT) after 10 min incubation at 37 °C. Brain and liver glycogen (N = 10–12 per experiment) were similarly processed, but within a chilled 1.5 mL centrifuge tube, to which ice-cold PCA was added followed by homogenization on ice using a small hand-held motorized plastic pellet pestle (Gerresheimer Kimble Kontes LLC, Düsseldorf, Germany).

Lipid analysis

Lipid content in the carcass was determined gravimetrically (N = 8–11 per experiment), using the chloroform:methanol extraction method (Lauff and Wood, 1996), after grinding the carcasses to a fine powder under liquid N_2, to which approximately 100 mg of tissue was added to 10 mL of chloroform:methanol (2:1) in a 20 mL glass scintillation vial and left to incubate for 12 h at 4 °C. After incubation, 2.6 mL of 0.9% NaCl solution was added and the samples were again left to incubate for 12 h at 4 °C. Next, a 5 mL syringe fitted with a 25 G needle was used to collect the 4-mL chloroform phase into a pre-weighed glass culture tube and chloroform was evaporated to dryness under a stream of nitrogen gas. The culture tubes were then transferred to a desiccator for 1 h to ensure that any residual chloroform had evaporated and were then re-weighed to determine the mass of lipid in the tube.

Protein quantification

Larval sea lamprey carcasses (N = 8–11 per experiment) were homogenized as described above, and four parts 50 mM tris (hydroxymethyl) aminomethane buffer (pH 7.4) were added to a 1.5 mL microcentrifuge tube. The slurry was further broken down with a hand-held homogenizer (PowerGen model 125 Homogenizer, Fisher Scientific, Mississauga, ON), vortexed and put on ice. The samples were then diluted 50 times for protein analysis, which was quantified spectrophotometrically using the bicinchoninic acid (BCA) assay (Smith et al., 1985), with bovine serum albumin for standards. Samples were incubated at 37 °C for 30 min and the absorbance was determined at 562 nm. Livers (N = 11–12 per experiment) were analyzed using the same method except that they were homogenized in five parts TRIS (hydroxymethyl) aminomethane buffer (50 mM, pH 7.4) containing protease inhibitor (1 mg, Sigma). Sample preparation and analysis were done as previously described.

Water content and dry ash analysis

Percent water content and dry ash in larval sea lamprey carcasses (N = 9–10 per experiment) were determined gravimetrically using standard methods. First, percent tissue water was determined in the carcasses (whole body minus brain and liver) of lamprey not exposed to TFM by grinding the carcass to a fine powder under liquid N_2.

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Approximately 50 mg of ground carcass was then placed in pre-weighed crucibles and dried to constant mass in a muffle furnace (Type 48000 Furnace, Barnstead International, Dubuque, Iowa) at 60 °C, over 48 h to determine % water content. The amount of dry ash was determined by combusting the dried tissue at 750 °C for 4 h, and expressed as the amount of ash per unit wet mass (g wet mass^{-1}).

Calculations and statistics

Hepatosomatic index (HSI) and condition factor (CF)

The HSI was based on the wet liver mass divided by body mass according to the following formula:

$$\text{HSI} = \text{liver mass (mg)}/\text{body mass (mg)} \times 100 \quad (1)$$

CF was calculated according to Holmes and Youson (1994) using the equation below:

$$\text{CF} = ([\text{mass (g)}]/\text{length (mm)}]^3) \times 10^6 \quad (2)$$

Log-Probit analysis was used to determine the 12-h LC_{50} and 12-h $LC_{99.9}$ values, plus the 95% confidence intervals (CI) in both Series I and Series II experiments using the R package 'ecotox', v1.3.3, available on CRAN (Hlina, 2017). Values were considered significantly different if the respective 95% CI's did not overlap (Finney, 1971). Differences in larval lamprey length, mass, proximate carcass composition (glucose and glycogen, lipid, protein, water and dry ash), brain and liver glycogen, liver protein and hepatosomatic index (HSI) were tested using analysis of variance (ANOVA) followed by Tukey post-hoc tests. In instances, where the assumptions of normality and homogeneity of variance were not met, non-parametric (Kruskal-Wallis) ANOVA, followed by Dunn's Test of Multiple Comparisons Using Rank Sums or Games Howel test were used. Statistical analyses were conducted using R (version 3.1.3), R Studio (version 3.2.3). Statistical significance was assessed at the $\alpha = 0.05$ level.

Results

Effects of season and temperature on body condition

Condition factor varied with season in the larval sea lamprey collected from the Au Sable River, MI, in 2013, averaging 1.70 ± 0.02 in May, increasing slightly to 1.88 ± 0.06 in June and then falling in August and October to values of 1.74 ± 0.02 and 1.63 ± 0.02, respectively (Table 1). The differences were mainly due to significant variation in the mass of the animals, which were significantly higher in May and October, than during summer months (Table 1). The mean lengths of the animals varied slightly, ranging from a high of 69.2 ± 1.1 mm in May to a low of 59.6 ± 0.7 mm in August (Table 1).

Unlike the previous year, the larval sea lamprey collected from the Au Sable River in 2014 were all collected within a few days of one another, and then acclimated to nominal temperatures of 6 °C (measured $T = 6.4 \pm 0.1$ °C), 12 °C (measured $T = 12.4 \pm 0.2$ °C;) or 24 °C (measured $T = 24.0 \pm 0.1$ °C) at the HBBS. Acclimation temperature did influence CF, but only slightly, significantly increasing from a value of 1.59 ± 0.02 at 6 °C to 1.66 ± 0.02 at 24 °C. At 12 °C, the CF was intermediate between the low and higher temperatures at 1.63 ± 0.06 (Table 2). The very small differences in CF, were reflected by an absence of any significant differences in body mass or length.

Effects of season and temperature on the acute toxicity of TFM

Series I: seasonal variation in TFM sensitivity

Not surprisingly, water temperature was lowest at 5.7 ± 0.1 °C in May, increasing to 21 ± 0.3 °C in June and peaking at 23.7 ± 0.1 °C in June, before decreasing in October to 11.6 ± 0.3 °C (Table 3). However, water pH also varied with season (Series I), increasing from a low of 7.70 ± 0.02 in May to 7.98 ± 0.03, stabilizing at pH 8.27 ± 0.01 through August and October (Table 3).

The dose-response curves depicting TFM sensitivity shifted far to the right between the spring and mid-to-late summer, indicating that TFM tolerance was greatest during the summer, before decreasing in the autumn (Fig. 1A). Differences in TFM sensitivity were quantified by calculating the respective TFM 12-h LC_{50}'s and the MLC's (12-h $LC_{99.9}$) for the lamprey. During the spring, the 12-h LC_{50} was 1.22 mg L^{-1} (CI = 1.17–1.27 mg L^{-1}) when water temperature was coolest, increasing more than two-fold in June to a value of 2.52 mg L^{-1} (CI = 2.34–2.72 mg L^{-1}), and peaking in August at 3.15 mg L^{-1} (CI = 3.04–3.26 mg L^{-1}), when the water was warmest ($T = 23.7 \pm 0.1$ °C). This was followed by an approximately 50% reduction in the 12-h LC_{50} to 1.64 mg L^{-1} (CI = 1.59–1.69 mg L^{-1}) in October, at which time the test water had cooled by almost 12 °C (Fig. 1B).

Similar, but more variable trends were observed when the acute toxicity data were expressed as the MLC of TFM, which increased more than two-fold from 1.92 mg L^{-1} (CI = 1.74–2.26 mg L^{-1}) in May to 4.04 mg L^{-1} (CI = 3.41–6.77 mg L^{-1}) in June, peaking in August to 4.56 mg L^{-1} (CI = 4.22–5.17 mg L^{-1}) before falling to 2.19 mg L^{-1} (CI = 2.06–2.41 mg L^{-1}) in October (Fig. 1B).

Series II – effects of temperature on TFM sensitivity

The nominal (target) water temperatures of 6, 12 and 24 °C closely matched measured water temperatures which averaged 6.4 ± 0.1 °C, 12.4 ± 0.2 °C and 24.0 ± 0.1 °C, respectively (Table 3). Water pH also varied, with respective values averaging 7.86 ± 0.02, 8.02 ± 0.01 and 8.22 ± 0.01 at 6, 12 and 24 °C (Table 3).

TFM tolerance was almost two-fold greater at 24 °C than at 6 °C, as depicted by marked rightward shifts in the TFM dose-response curves with increasing temperature (Fig. 2A). These observations were reflected by significant differences in the 12-h LC_{50}, which was 1.84 mg L^{-1} (CI = 1.79–1.89 mg L^{-1}) at 6 °C, increasing to 2.32 mg L^{-1} (CI = 2.24–2.39 mg L^{-1}) at 12 °C, and then to 3.40 mg L^{-1} (CI = 3.17–3.55 mg L^{-1}) at 24 °C (Fig. 2B). Similarly, the 12-h $LC_{99.9}$ increased with temperature from 2.56 mg L^{-1} (CI = 2.40–2.53 mg L^{-1}) at 6 °C, to 2.85 mg L^{-1} (CI = 2.71–3.31) mg L^{-1} at 12 °C, and then to 4.78 mg L^{-1} (CI = 4.31–6.32 mg L^{-1}) at 24 °C (Fig. 2B).

Table 1
Condition factor (CF), mass and length of larval sea lamprey collected from the Au Sable River, Michigan in the spring, summer, and autumn of 2013. Temperature of river water indicated in parentheses. Data presented as mean ± 1 SEM. Data sharing a common letter are not significantly different from on another ($P < 0.05$). CF = (mass (g)/length (mm))^3 × 1. (Holmes and Youson, 1994).

Month of collection	CF	Mass (g)	Length (mm)	N
May (6 °C)	1.70 ± 0.02^{a,b}	0.62 ± 0.03^{a}	69.2 ± 1.1^{a}	290
June (20 °C)	1.88 ± 0.06^{c}	0.44 ± 0.02^{b}	61.1 ± 1.2^{b}	291
August (23 °C)	1.74 ± 0.02^{b}	0.39 ± 0.01^{b}	59.6 ± 0.7^{b}	313
October (12 °C)	1.63 ± 0.02^{a}	0.56 ± 0.03^{a}	68.6 ± 1.1^{a}	247

Table 2
Condition factor (CF), mass and length of larval sea lamprey collected from the Au Sable River, Michigan in June 2014 and acclimated to temperatures of 6, 12 or 24 °C for 7–-10 days. Data presented as mean ± 1 SEM. Data sharing a common letter are not significantly different from one another ($P < 0.05$). CF = (mass (g)/length (mm))^3 × 10^6 (Holmes and Youson, 1994).

Acclimation temperature	CF	Mass (g)	Length (mm)	N
6 °C	1.59 ± 0.02^{a}	0.69 ± 0.03^{a}	74.0 ± 1.6^{a}	315
12 °C	1.63 ± 0.06^{a,b}	0.61 ± 0.03^{a}	70.0 ± 1.2^{a}	315
24 °C	1.66 ± 0.02^{b}	0.52 ± 0.01^{a}	65.6 ± 1.1^{a}	314

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Table 3
Mean water temperatures and pHs measured during 12-h acute toxicity tests of larval sea lamprey captured at different times of the year (Series I) or acclimated to nominal temperatures of 6, 12 or 24 °C (Series II). Data presented as mean ± 1 standard error of the mean.

Experiment	Measured temperature	pH	N
Series I – effect of season on TFM tolerance			
May	5.7 ± 0.1 °C	7.70 ± 0.02	38
June	21.0 ± 0.3 °C	7.98 ± 0.03	38
August	23.7 ± 0.1 °C	8.27 ± 0.01	38
October	11.6 ± 0.3 °C	8.27 ± 0.01	38
Series II – effect of temperature on TFM tolerance			
Nominal T = 6 °C	6.4 ± 0.1 °C	7.86 ± 0.02	42
Nominal T = 12 °C	12.4 ± 0.2 °C	8.02 ± 0.01	42
Nominal T = 24 °C	24.0 ± 0.1 °C	8.22 ± 0.01	42

Effects of season and temperature on the proximate body composition of sea lamprey

Glucose and glycogen

Season had no effect on the concentrations of carcass (whole body minus brain and liver) glucose in larval sea lamprey, which averaged 7.2 ± 0.2 μmol g^{-1} ww, 7.3 ± 0.1 μmol g^{-1} ww, 7.2 ± 0.1 μmol g^{-1} ww and 7.3 ± 0.1 μmol g^{-1} ww in May, June, August and October, respectively (data not shown). There were however, differences in glycogen, which averaged 24.2 ± 5.4 μmol g^{-1} ww in May, 21.1 ± 4.4 μmol g^{-1} ww and 19.5 ± 3.5 μmol g^{-1} in June and August, before significantly decreasing to 11.3 ± 2.8 μmol g^{-1} ww in October (Fig. 3A). Mean brain glycogen exhibited more pronounced reductions, averaging 131.2 ± 20.0 μmol g^{-1} ww and 128.0 ± 8.8 μmol g^{-1} ww in May and June, respectively. By August, however, brain glycogen had significantly decreased to 83.3 ± 7.4 μmol g^{-1} ww, where it remained through October (85.4 ± 9.5 μmol g^{-1} ww; Fig. 3A). The opposite trend was observed in the liver, which was lowest in May, at 6.4 ± 1.2 μmol g^{-1} ww, and then doubled in June to 12.8 ± 1.3 μmol g^{-1} ww, followed by a slight drop to 9.6 ± 0.9 μmol g^{-1} ww in August, before peaking at 15.5 ± 1.5 μmol g^{-1} ww in October (Fig. 3A).

Acclimation to warmer waters led to slight reductions in mean carcass glucose concentration, which averaged 8.8 ± 1.3 μmol g^{-1} ww, 7.4 ± 0.3 μmol g^{-1} ww, and 6.9 ± 0.1 μmol g^{-1} ww in larval lamprey at 6, 12 and 24 °C, respectively (data not shown). Similarly, carcass glycogen was significantly lower at 24 °C, where it averaged 6.0 ± 1.2 μmol g^{-1} ww, compared to values of 9.0 ± 1.5 and 10.4 ± 2.1 μmol g^{-1} ww at 6 °C and 12 °C, respectively (Fig. 3B).

Mean brain glycogen was inversely proportional to acclimation temperature, averaging 173.9 ± 24.1 μmol g^{-1} ww at 6 °C, 147.6 ± 24.1

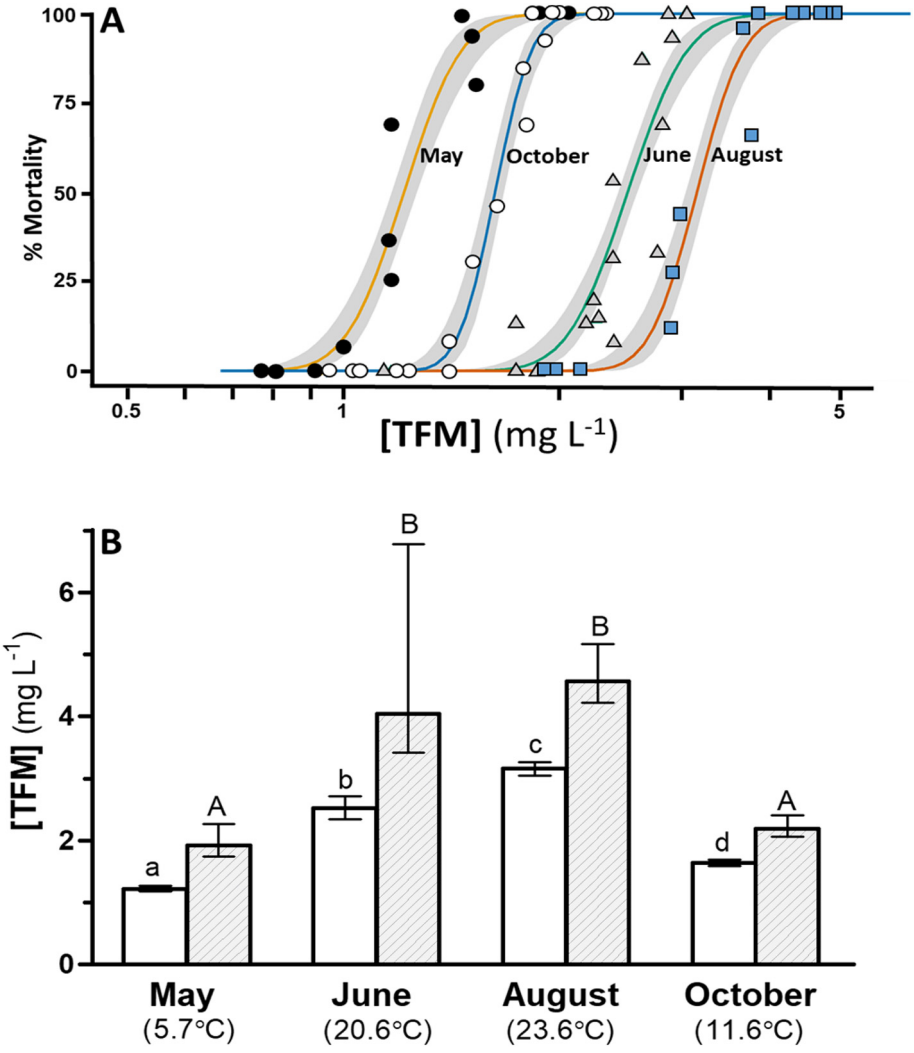

Fig. 1. Effects of season on the acute toxicity of TFM to larval sea lamprey. (A) Dose-response curves depicting changes in the toxicity of 3-trifluoromethyl-4-nitrophenol (TFM) to larval sea lamprey collected in May, June, August and October 2013 from the Au Sable River, MI, and (B) the corresponding 12-h LC_{50} and minimum lethal concentration (12-h $LC_{99.9}$; MLC) of TFM to the same animals. Tests were conducted at the water temperature in which the larval lamprey were collected (in brackets). Data presented as the 12-h LC_{50} (hatched bars) or the MLC (solid bars) ± 95% confidence interval (CI). Values in which the CIs do not overlap are significantly different from one another. N = 246–360 larval sea lamprey per test.

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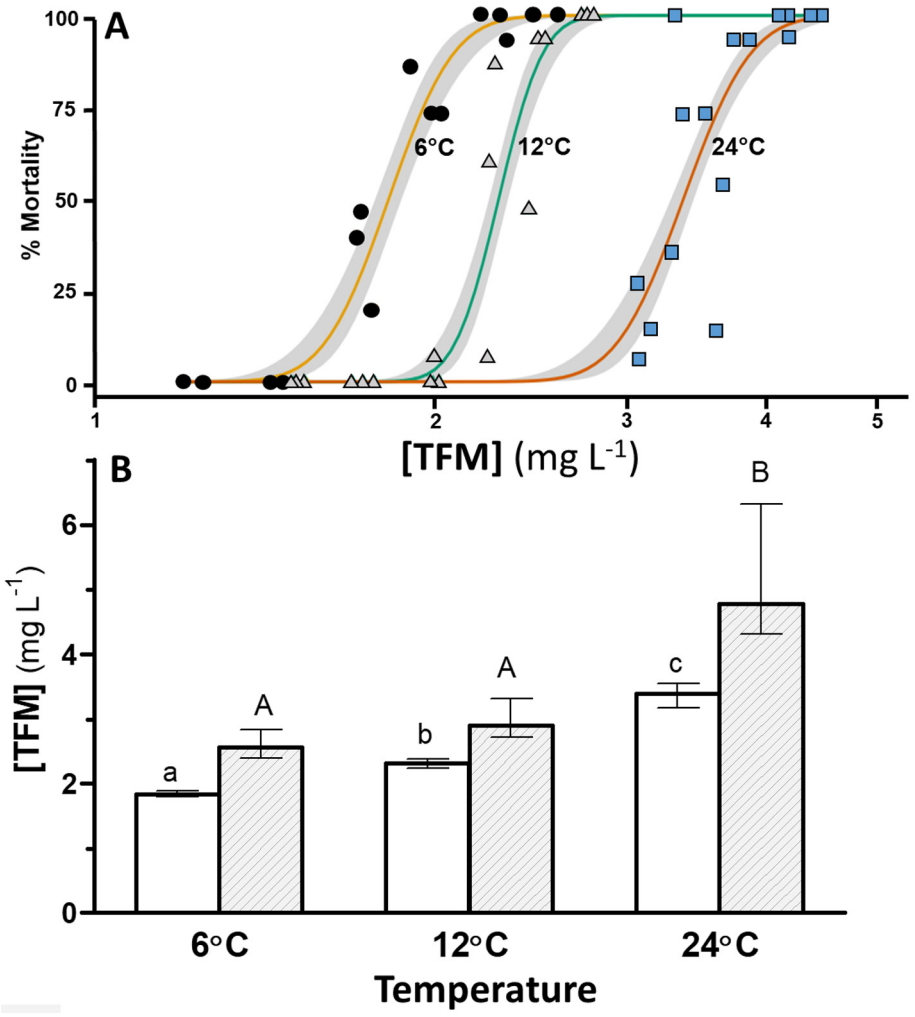

Fig. 2. Effects of temperature on the acute toxicity of TFM to larval sea lamprey. (A) Dose-response curves depicting changes in the toxicity of 3-trifluoromethyl-4-nitrophenol (TFM) with temperature to larval sea lamprey collected in July 2014 from the Au Sable River, MI, and (B) the corresponding 12-h LC_{50} and minimum lethal concentration (12-h $LC_{99.9}$; MLC) of TFM to the same animals. Data presented as the 12-h LC_{50} (hatched bars) or the MLC (solid bars) ± 95% confidence interval (CI). Values in which the CIs do not overlap are significantly different from one another. $N = 314–315$ larval sea lamprey per temperature.

μmol g^{-1} ww at 12 °C, and 58.0 ± 5.0 μmol g^{-1} ww at 24 °C (Fig. 3B). There were no significant effects of temperature on mean liver glycogen, which averaged 11.2 ± 1.0 μmol g^{-1} ww, 11.1 ± 0.8 μmol g^{-1} ww and 11.6 ± 1.0 μmol g^{-1} ww at 6, 12 and 24 °C, respectively (Fig. 3B).

Whole-body lipid

Season had a pronounced effect on mean carcass lipid in larval sea lamprey, which decreased in a stepwise fashion between May, when carcass lipid averaged 160 ± 29 mg g^{-1} ww, decreased to 115 ± 16 and 114 ± 18 mg g^{-1} ww in the summer (June and August), followed by a further decline in October to 47 ± 11 mg g^{-1} ww (Fig. 4A). However, acclimation temperature had no significant effect on carcass lipid, which averaged 109 ± 9, 102 ± 19, and 99 ± 17 mg g^{-1} ww, at 6, 12, and 24 °C, respectively (Fig. 4B).

Whole-body and liver protein

Mean carcass protein concentrations did not change with season in larval sea lamprey, averaging 85.7 ± 7.7 mg g^{-1} ww in May, 63.5 ± 4.9 mg g^{-1} ww and 51.8 ± 5.5 mg g^{-1} ww in the summer, and 72.4 ± 4.5 g mg^{-1} ww in the fall (Fig. 5A). Temperature acclimation also had no significant effect on mean carcass protein, which averaged 75.5 ± 12.4, 59.7 ± 3.4, and 60.9 ± 12.6 mg g^{-1} ww at 6, 12, and 24 °C, respectively (Fig. 5B).

Liver protein concentrations differed with season but not temperature. Mean liver protein averaged 127.0 ± 7.2 mg g^{-1} ww in May and was reduced by approximately 25% to 95.5 ± 4.9 mg mg^{-1} ww and 88.6 ± 8.1 mg g^{-1} ww in the summer. However, liver protein rebounded to a concentration of 113.3 ± 5.1 mg g^{-1} ww in the fall (Fig. 5A). Mean liver protein concentrations in the temperature experiments averaged 106.3 ± 5.0, 102.4 ± 3.4, and 108.4 ± 8.7 mg g^{-1} ww at 6, 12 and 24 °C, respectively (Fig. 5B).

Water content and dry ash

The mean water content of larval sea lamprey carcass in May was 77.7 ± 1.8%, but dropped significantly in the summer to 72.7 ± 1.3% and 74.4 ± 1.1% in the summer (June, August, respectively), before significantly increasing to 81.4 ± 0.7% in October (Fig. 6A). Acclimation to different temperatures had no significant effect on the carcass water content of larval lamprey, which averaged 77.0 ± 1.0%, 77.9 ± 0.5%, and 77.6 ± 0.9% at 6, 12 and 24 °C, respectively (Fig. 6B).

Carcass dry ash also varied seasonally, averaging 0.85 ± 0.02% in May, and then slightly decreasing to 0.79 ± 0.02% and 0.80 ± 0.02% in June and August. By October, mean dry ash was lowest, at 0.69 ± 0.04% (Fig. 6A). Temperature acclimation resulted in greater variation in mean dry ash of larval sea lamprey. However, there were no significant differences among the animals acclimated to 6, 12 and 24 °C,

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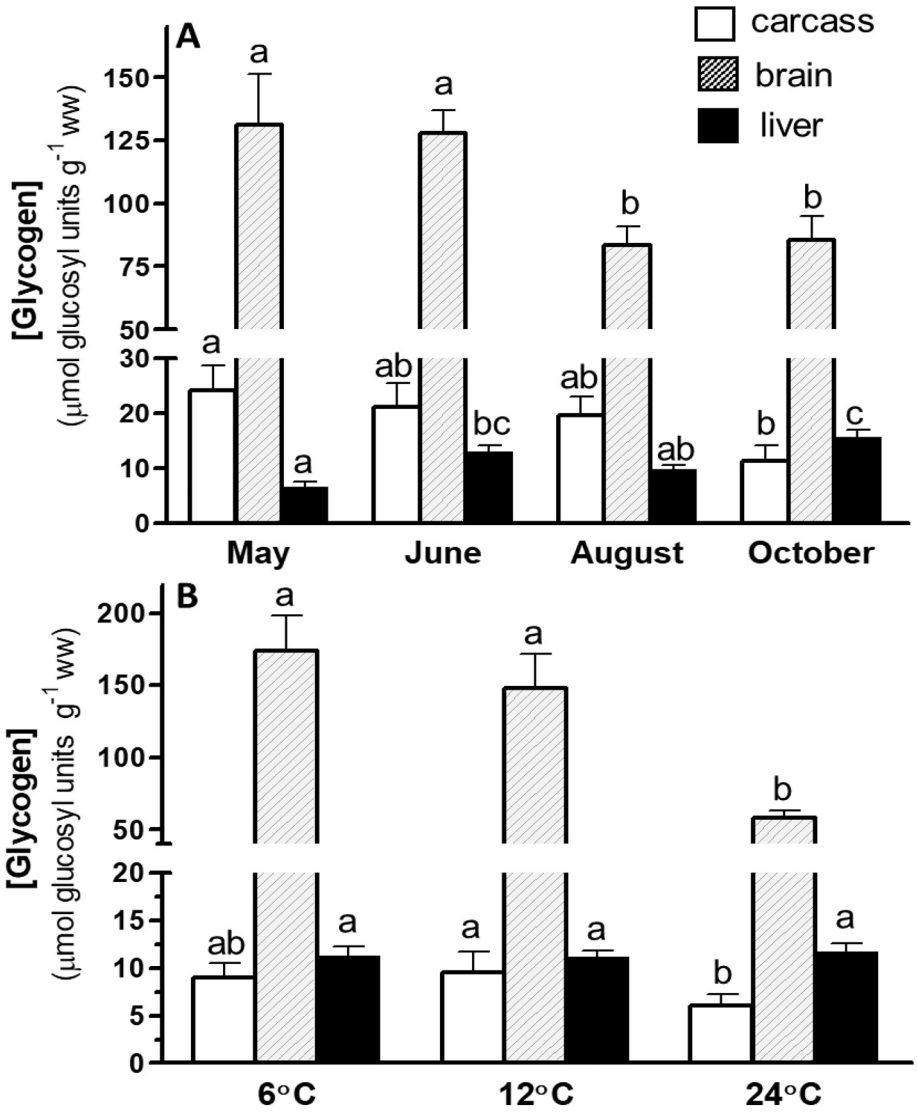

Fig. 3. Effects of season and temperature on glycogen stores. Changes in carcass (open bars), brain (hatched bars) and liver (solid bars) glycogen concentrations of larval lamprey collected from the Au Sable River, MI in (A) May, June, August or October 2013, or (B) acclimated to nominal temperatures of 6 °C, 12 °C or 24 °C after capture in July of 2014. Data presented as the mean + 1 SEM, $N = 11–12$ per sample period. For a given tissue, bars sharing the same letters were not statistically significant from one another.

which averaged 0.84 ± 0.01%, 0.79 ± 0.02% and 0.96 ± 0.09%, respectively (Fig. 6B).

Hepatosomatic index analysis

Mean hepatosomatic index (HSI) changed with season in larval sea lamprey, averaging 1.21 ± 0.06 in May, before significantly decreasing to 1.00 ± 0.05 and 0.79 ± 0.07 in the summer (June and August), and then increasing to 1.09 ± 0.05 in October. Temperature also had a pronounced effect on mean HSI in larval sea lamprey, which averaged 1.34 ± 0.04 at 6 °C, decreased significantly to 1.09 ± 0.07, and then decreased again to 0.99 ± 0.04 (Table 4).

Discussion

Effects of season and temperature on the acute toxicity of TFM

Previous studies have demonstrated that the TFM sensitivity of larval sea lamprey varies with season (Applegate et al., 1961; Scholefield et al., 2008), but the underlying mechanisms were poorly understood. The present study demonstrates that the greater tolerance of sea lamprey to TFM in the summer is due primarily to corresponding increases in water temperature. Applegate et al. (1961) first reported that the biological activity of TFM varies seasonally, with the maximum toxic effects of TFM occurring during the late fall, winter and early spring, and then declining through spring and summer. Using sea lamprey collected from streams in Michigan, Scholefield et al. (2008) demonstrated that TFM toxicity was greatest in the spring (May to June) compared to late summer (July to August), when the 9-h LC_{50} and MLC ($LC_{99.9}$) were up to 1.5 to 2-fold greater in a given stream. They also reported that spring 9-h MLC test values were similar to those predicted by pH–alkalinity charts (104% to 117% of the chart values), which are used to calculate TFM application amounts based on water pH and alkalinity measurements (Bills et al., 2003). In contrast, the corresponding 9-h MLC test values measured in the summer were 32–170% higher than those predicted by the charts. They also noted that the discrepancies from the charts in the summer were unrelated to differences in alkalinity or pH, each of which affect the bioavailability of non-ionized TFM, which is the main determinant of TFM accumulation in lamprey (Hunn and Allen, 1974; Hlina et al., 2017).

The findings of the present study strongly suggest that the greater tolerance of sea lamprey to TFM in the summer was mainly due to warmer water temperatures, rather than differences in energy reserves. This conclusion is supported by the strong trend between temperature and the acute toxicity of TFM (12-h LC_{50}, 12-h MLC). At first glance,

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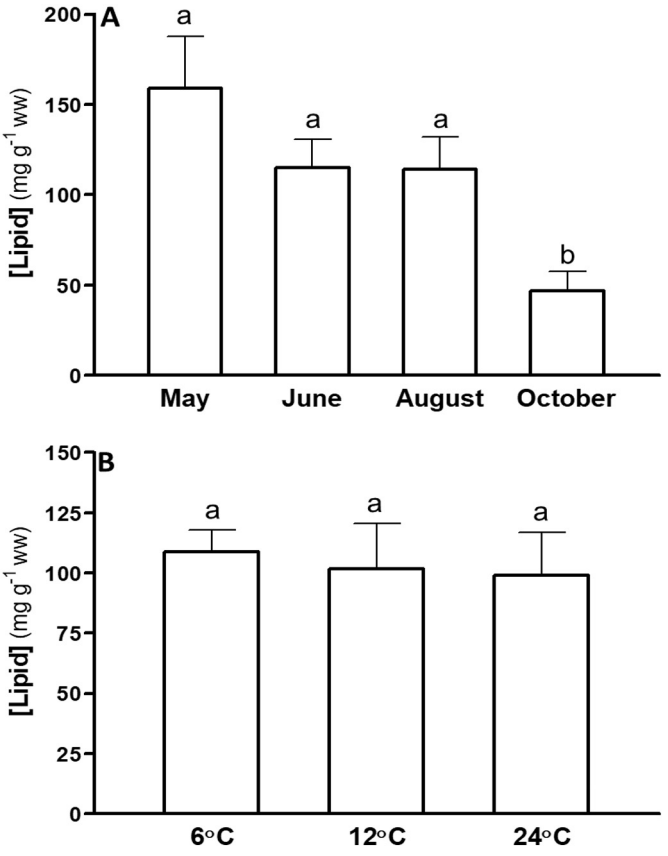

Fig. 4. Effects of season and temperature on lipid stores. Influence of (A) season and (B) acclimation temperature on carcass lipid concentrations in larval sea lamprey captured from the Au Sable River, Michigan. Data presented as the mean + 1 SEM, N = 8–11 for each month, and N = 8 at each temperature. Bars sharing the same letters denote values that were not statistically significant from one another.

this observation seems counter-intuitive because rates of TFM uptake and accumulation increase in proportion to water temperature (B.L. Hlina. unpub. data). However, toxicity depends upon the relative balance between toxicant accumulation and detoxification/elimination. We propose that while increasing temperatures result in increased rates of TFM accumulation, the capacity of larval lamprey to detoxify TFM increases to a greater degree, resulting in greater TFM tolerance. We defend this hypothesis based on two lines of evidence. First, the larval sea lampreys' capacity to detoxify TFM may be more complicated and efficient than previously thought (Bussy et al., 2018a, 2018b). Second, the toxicity of similar phenolic compounds has also been demonstrated to decrease with increasing temperature.

While the overall capacity of sea lamprey to detoxify TFM is lower than most non-target fishes, earlier studies clearly demonstrated that they do have a limited capacity to detoxify TFM (Lech, 1974; Lech and Statham, 1975; Kane et al., 1994). These studies demonstrated that in non-target fishes such as rainbow trout, the detoxification of TFM relies on Phase II metabolism by which TFM undergoes glucuronidation by combining a glucuronic acid functional group with TFM, making it more water soluble and easier to excrete via the intestine or renal routes (Lech, 1974; Kane et al., 1994). Although sea lamprey express the genes for UDP-glucuronyl transferase (B.L. Hlina, unpub/data), which mediates glucuronidation, corresponding enzyme activities are significantly lower than in most non-target fishes (Kane et al., 1994). For many years, glucuronidation was considered the primary TFM detoxification pathway, but very recently Bussy et al. (2018a, 2018b) demonstrated that TFM detoxification is more complex than previously thought, and that other phase II processes, including sulfation, and phase I processes such as reductive and oxidative metabolism, may also be quantitatively important. We therefore suggest that at higher acclimation temperatures, the combined activities of these enzymes may be sufficient to increase the sea lamprey's capacity to detoxify TFM by Phase II and possibly Phase I detoxification pathways. Because the reduced amino metabolite of TFM is also more abundant than the sulfated and glucuronide conjugates in sea lamprey (Bussy et al., 2018b), it may also play a more important role in TFM detoxification. Future studies, addressing the role of these pathways and metabolites in TFM detoxification, or toxicity, and how their production is influenced by increased temperature would be very informative.

A complication of the above interpretation is that TFM uptake, like other toxicants, would also be expected to increase with temperature as the lamprey's metabolic and respiratory demands increase, resulting in increased ventilatory flow across the gills and greater delivery of TFM-laden water to the gills. For many toxicants, this results in greater accumulation and presumably greater toxicity (see Ficke et al., 2008 for a review). However, toxicity is equal to the difference between TFM accumulation and detoxification/elimination. Thus, if increases in the rate of TFM detoxification were more sensitive than rates of uptake to increasing temperatures, then TFM tolerance would be expected to increase with temperature. Indeed, it is notable that the toxicity of two other similar phenolic compounds, and also known to uncouple oxidative phosphorylation, 4-nitrophenol and 2,4-nitrophenol, decreased in rainbow trout (*Oncorhynchus mykiss*) with increases in water temperature (Howe et al., 1994). Howe et al. (1994) also reasoned that an increased capacity to detoxify these compounds in warmer waters explained the greater survival of rainbow trout. Phenol toxicity to silver perch (*Bidyanus bidyanus*), rainbow trout (*Oncorhynchus mykiss*),

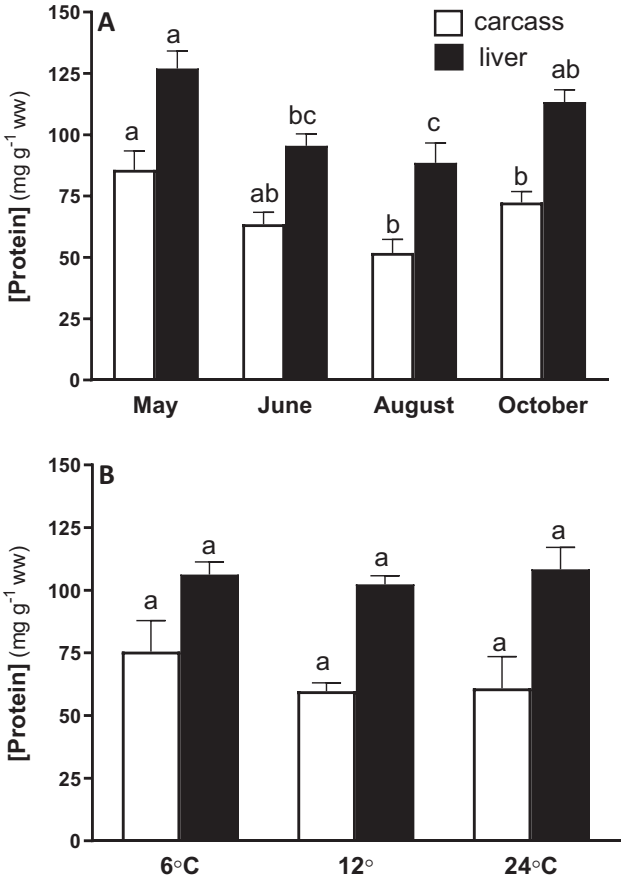

Fig. 5. Effects of season and temperature on protein stores. Changes in carcass (open bars), and liver (solid bars) protein concentration with (A) season and (B) acclimation temperature in larval lamprey collected from the Au Sable River, MI. Data presented as the mean + 1 SEM, N = 10–12 per sample period. For a given tissue, bars sharing the same letters denote values that were not statistically significant from one another.

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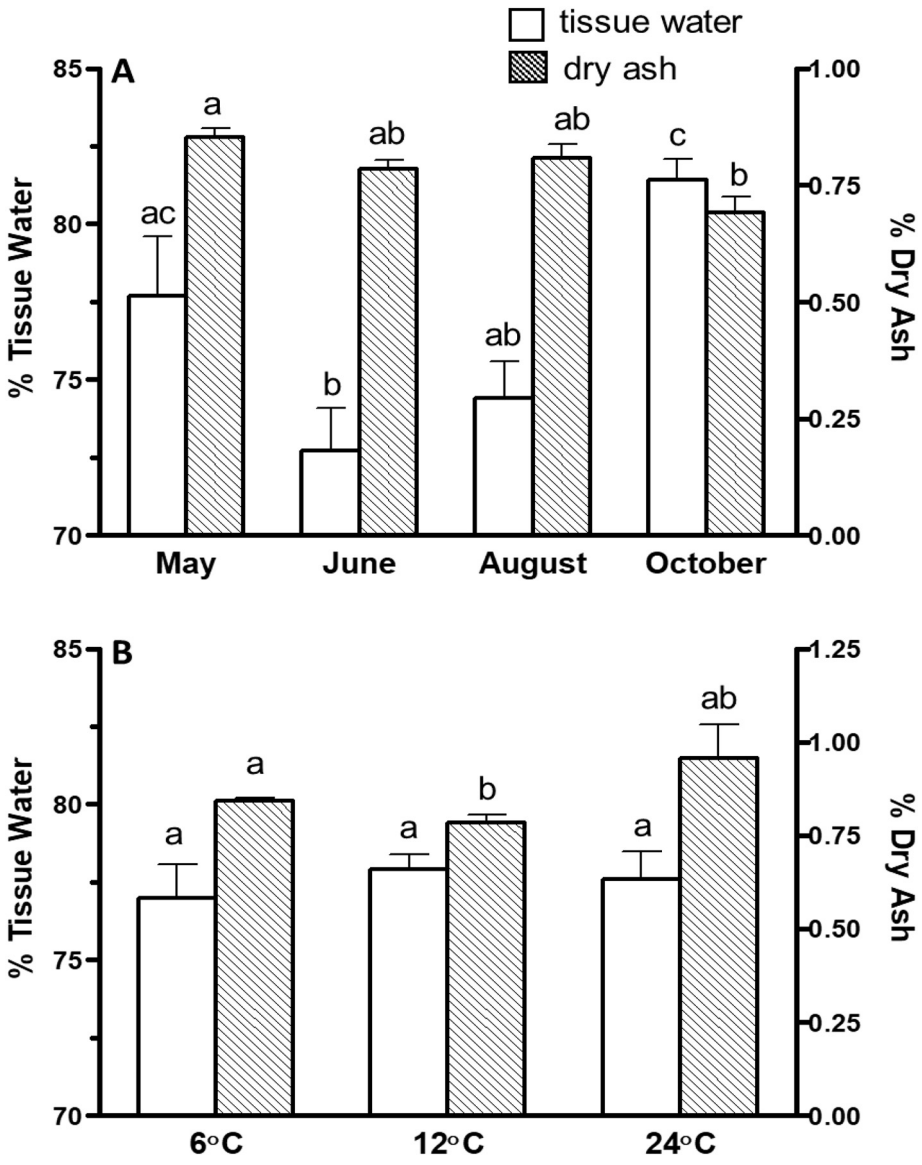

Fig. 6. Effects of season and temperature on water content and dry ash. Changes in carcass water content (open bars) and dry ash (hatched) in larval lamprey collected from the Au Sable River, MI in (A) May, June, August or October 2013, or (B) acclimated to nominal temperatures of 6 °C, 12 °C or 24 °C after capture in July of 2014. Data presented as the mean + 1 SEM, N = 8–11 carcasses per sample period. Bars sharing the same letter denote values that are not statistically different from one another.

rainbowfish (*Melanotaenia duboulayi*) and western carp gudgeon (*Hypseleotris klunzingeri*) also decreased with increases in temperature that were well within the thermal tolerance ranges of these fishes (Patra et al., 2015). However, toxicity increased as water temperatures approached the thermal tolerance thresholds of each fish due to the combined effects of toxicant exposure and thermal stress. It should be noted that the maximum temperatures to which the sea lamprey in the present study were exposed, 24 °C, was well below their upper critical thermal tolerance limits of 29.5–31 °C (Potter and Beamish, 1975) and not far outside their preferred thermal niche of 17.8–21.8 °C (Holmes and Lin, 1994).

Why would TFM uptake be influenced less by temperature than TFM detoxification? The answer is probably related to TFM's mode of uptake, which depends on passive diffusion of non-ionized TFM across the gills (Hunn and Allen, 1974; Hlina et al., 2017). With a pKa of 6.07, parent TFM is a weak acid that contains an ionizable hydroxyl group, existing as either non-ionized TFM (TFM-OH) or ionized (TFM-O$^-$), with latter dominating as water pH increases in the circum-neutral range characteristic of most rivers and streams in the Great Lakes basin (Hunn and Allen, 1974; Hubert, 2003; Hlina et al., 2017). Because TFM uptake occurs by passive diffusion, down favorable TFM-OH gradients rather than transporter-mediated, as is the case with ionized toxicants such as metals, its uptake is influenced more by internal concentrations in the blood. As TFM-OH increases in the blood, this would have the effect

Table 4
Hepatosomatic index (HSI) of larval sea lamprey captured at different times of the year in 2013 (Series I) or acclimated to nominal temperatures of 6, 12 or 24 °C (Series II). Data presented as mean ± 1 standard error. Data sharing the same letters are not statistically different from one another.

Experiment	HSI	N
Series I – effect of season on TFM tolerance		
May (5.6 °C)	1.21 ± 0.06^{a}	12
June (20.6 °C)	1.00 ± 0.05^{b}	12
August (23.7 °C)	0.79 ± 0.07^{c}	12
October (11.7 °C)	1.09 ± 0.05^{ab}	12
Series II – effect of temperature on TFM tolerance		
6 °C	1.34 ± 0.04^{a}	10
12 °C	1.09 ± 0.07^{b}	11
24 °C	0.99 ± 0.04^{b}	12

of reducing inwardly directed TFM-OH gradients and therefore TFM uptake. While higher temperatures would lead to greater TFM uptake rates in the early stages of exposure, internal TFM-OH concentrations that reduce or even eliminate the inwardly directed TFM gradient would be reached more quickly and uptake would slow. Ionized toxicants, such as metals, would not necessarily behave in the same manner because uptake would involve either primary or secondary active transport, and could take place against reduced or outwardly directed electrochemical gradients. TFM detoxification, on the other hand, would not be similarly affected because it is metabolized enzymatically, and its actions dictated by substrate concentration and its affinity for various enzymes of detoxification (i.e. Michaelis-Menten kinetics). In addition to seasonal variation, larval sea lamprey could be subjected to acute changes in temperature over a few hours or days, depending on the season, water flow, canopy cover or changing weather conditions. In the present study we chose to acclimate the lamprey and conduct acute toxicity tests at one of three temperatures to avoid introducing confounding variables into our study. As other poikilothermic animals, however, the metabolic rate of lamprey would be expected to simultaneously change with water temperature, resulting in accompanying changes in oxygen demands and ventilation rate, and in the rates of biochemical reactions, including those involved in TFM detoxification. For this reason, we suspect that similar changes in TFM sensitivity would accompany acute changes in temperature, which could further complicate TFM application routines, but this question requires further study.

The findings of the present study contrasts with early work by Dawson et al. (1975), who reported no clear relationship between temperature and TFM toxicity in larval sea lamprey exposed to TFM at 7, 12, 17 and 22 °C in static toxicity tests. It is worth noting however, that they did not report how long the animals had been in captivity or their condition, which we controlled for in the present study by using freshly caught animals (within 2–3 weeks of capture) followed by gradual acclimation to the test temperatures. These earlier studies (Dawson et al., 1975) did not report how long the sea lamprey were acclimated to each temperature tested, nor to the different water pH and water hardness conditions examined. Acute exposure to such conditions could have resulted in additional physiological disturbances, which could have obscured the animal's responses to TFM. They also used a different formulation of TFM, 35.7% TFM dissolved in demethylformamide (DMF), as opposed to the current formulation which is dissolved in isopropanol.

It is notable that TFM toxicity in rainbow trout increased with temperature (Marking and Olson, 1975), exposed to TFM under similar static conditions as the larval lamprey in Dawson et al. (1975). However, it is premature to make comparisons to the present findings because rainbow trout have a markedly different physiology than larval sea lamprey including much higher metabolic rates, a greater capacity to detoxify TFM and markedly different organization of the gills (e.g. Kane et al., 1994; Rovainen, 1996; Tessier et al., 2018). A goal of future studies should be to understand more about how trout and other non-target fishes respond to TFM at different temperatures compared to sea lampreys.

Given its effects on TFM speciation and uptake, differences in water pH could have contributed to the variation in TFM toxicity observed with season or temperature. However, the differences in toxicity we observed were more pronounced than those expected based on differences in pH alone. Along with prior treatment history, the application rates of TFM to sea lamprey infested streams are often determined from charts relating the expected 12-h MLC (12-h $LC_{99.9}$) of TFM to measurements of water pH and alkalinity (Bills et al., 2003). The MLC measurements reported in the present study were in fact approximately 0.5 mg L^{-1} higher than the chart value in May, and 1.9 and 1.4 mg L^{-1} higher in June and August, respectively (Table 5). Interestingly, the 12-h MLC was 1 mg L^{-1} lower in October, when water pH remained high (pH ~8.3), but temperature was much lower at 11.6 °C compared to 23.7 °C in August (Table 5). A clearer pattern emerged when we compared the observed and predicted MLC values in sea lamprey acclimated to different temperatures, with the difference increasing from 0.5 mg L^{-1} at 6 °C to 2.0 mg L^{-1} at the 24 °C (Table 6). These findings suggest that the risk of underestimating the MLC is greater at warmer temperatures. These observations lend further support to our argument that temperature strongly influences the TFM sensitivity of larval sea lamprey, in addition to water pH.

Rather than the MLC, i.e. the amount of TFM needed to eradicate 99.9% of larval sea lamprey during a treatment (the $LC_{99.9}$), TFM is typically applied at 1.2–1.5 times the MLC (McDonald and Kolar, 2007). This insurance margin minimizes the risk of residual sea lampreys that survive treatment by temporarily avoiding the TFM treatment block by seeking refuge near groundwater inputs or entering tributaries, escaping into side-channels, or remaining burrowed longer. This insurance margin may also offset differences in sea lamprey tolerance to TFM due to season and temperature. For instance, when the predicted MLCs (Table 5) are multiplied by a factor of 1.4, the doses of TFM that would be used in the field fall within or above the confidence intervals (CI) of the observed MLC in May, August and October, but not in June (Table 5). This approach also results in TFM concentrations that fall above or within the CI of the observed MLC for lamprey acclimated to 6 °C and 12 °C, but below the CI at 24 °C (Table 6). In other words, applying TFM at 1.2–1.5 times the MLC compensates for season and temperature variations in TFM sensitivity, but not in all cases, with the greatest risk of residual sea lampreys occurring in summer when temperatures are warmest. Sea lamprey control agents have noted similar trends in the field, reporting that less TFM is required to achieve total mortality in the spring and fall, compared to the summer (B. Scotland and B. Morrison, Sea Lamprey Control Centre, Fisheries and Oceans Canada. Pers. Comm.).

Effects of season and temperature on proximate body composition

Exposure to TFM leads to a reduction of glycogen reserves in the brain and liver of sea lamprey and non-target fishes (Birceanu et al., 2009, 2014; Clifford et al., 2012; Henry et al., 2015) due to lower ATP production rates arising from TFM interference with mitochondrial oxidative phosphorylation (Birceanu et al., 2011). As a result, the animals rely on anaerobic energy reserves such as glycogen and phosphocreatine to make up for the shortfall in ATP supply, which likely culminates in death when these reserves fail to meet ATP demands (Birceanu et al., 2009; Clifford et al., 2012). If glycogen reserves were lower in the spring

Table 5
Differences in observed and predicted minimum lethal concentration (12-h MLC = 12-h $LC_{99.9}$) of TFM to larval sea lamprey at different times of the year and water pH. Observed MLC ($MLC_{Observed}$; expressed in mg L^{-1} plus upper and lower confidence intervals) also presented in Fig. 1. Predicted MLC ($MLC_{Predicted}$) based on pH and alkalinity values obtained from charts depicting expected 12-h MLC of TFM to larval sea lamprey (Bills et al., 2003). Measured alkalinity of Lake Huron water at Hammond Bay Biological Station assumed to average 90 mg $CaCO_3$ L^{-1}. Predicted MLC multiplied by 1.4 times. pH values not sharing common letters are significantly different from one another ($P < 0.05$).

Month	pH	$MLC_{Observed}$	$MLC_{Predicted}$	1.4 x $MLC_{Predicted}$	$MLC_{Observed}$ – $MLC_{Predicted}$
May (5.6 °C)	7.70 ± 0.02[a]	1.92 (1.74 to 2.26)	1.4	1.96	0.52 (0.34 to 0.86)
June (20.6 °C)	7.98 ± 0.03[b]	4.04 (3.41 to 6.78)	2.1	2.94	1.94 (1.31 to 4.68)
August (23.7 °C)	8.27 ± 0.01[c]	4.56 (4.22 to 5.17)	3.2	4.48	1.36 (1.02 to 1.97)
October (11.7 °C)	8.27 ± 0.01[c]	2.19 (2.06 to 2.41)	3.2	4.48	−1.01 (−1.14 to −0.79)

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Table 6
Differences in observed and predicted Minimum Lethal Concentration (12-h MLC = 12-h $LC_{99.9}$) of TFM to larval sea lamprey acclimated to different water temperatures and water pH. Observed MLC data ($MLC_{Observed}$; expressed in mg L^{-1} plus upper and lower confidence intervals) also presented in Fig. 2. Refer to Table 5 for additional details.

Acclimation temp. (°C)	pH	$MLC_{Observed}$	$MLC_{Predicted}$	1.4 x $MLC_{Predicted}$	$MLC_{Observed}$ – $MLC_{Predicted}$
6 °C	7.86 ± 0.02[a]	2.56 (2.40 to 2.53)	1.9	2.66	0.52 (0.34 to 0.86)
12 °C	8.02 ± 0.01[b]	2.85 (2.71 to 3.31)	2.1	2.94	0.74 (0.61 to 1.21)
24 °C	8.22 ± 0.01[c]	4.78 (4.31 to 6.32)	2.8	3.92	1.98 (1.51 to 3.52)

due to decreased nutrient supply during the winter months, we predicted that larval lamprey would be more vulnerable to TFM. This hypothesis was not supported, however. In fact, glycogen reserves were highest in brain and carcass in the spring-early summer, before dropping through late summer and fall, suggesting that seasonal differences in these energy stores had little impact on TFM tolerance.

Unlike other vertebrates, the brain and meningeal tissue of the sea lamprey has very high glycogen reserves (e.g. Rovainen, 1970; Foster et al., 1993; Clifford et al., 2012). Indeed, glycogen levels in lamprey brain and meningeal tissue are at least four times those found in other vertebrates (Rovainen et al., 1971; Foster et al., 1993). These brain glycogen stores provide the lamprey with a large reservoir of glucose, an essential fuel for the central nervous system of chordates (Polakof et al., 2012). Glucose supply to the brain is via glycogenolysis, which yields glucose-6-phosphate, which in turn is converted to glucose via the enzyme glucose-6-phosphatase (Rovainen et al., 1971; Polakof et al., 2012). In other vertebrates, the liver fulfills this role, which in the lamprey appears to be less important (O'Boyle and Beamish, 1977; Polakof et al., 2012). Because brain glycogen can drop by >50% (Clifford et al., 2012) following TFM exposure, it was predicted that lower initial brain glycogen concentrations would make sea lamprey more susceptible to toxicity. However, brain glycogen was maintained through the winter, which argues against this prediction in these groups of lamprey.

Liver glycogen, on the other hand, was lower in the spring, which might be because it was needed to sustain blood glucose through the winter. Indeed, carcass glucose stores were remarkably stable throughout the year. Liver glycogen stores had more than doubled by the fall, but the liver glycogen storage patterns were not suggestive of any role in TFM tolerance. Nor were the patterns in carcass glycogen reserves, which steadily decreased, indicative of any role in tolerance. Because the bulk of the whole body is muscle, most of the glycogen likely reflects intramuscular stores, which would be essential for fueling burst swimming or burrowing (Wilkie et al., 2001). Because metabolic rate and activity levels were higher in the warmer, summer months, it is possible that steady state glycogen stores were lower for this reason. Similarly, O'Boyle and Beamish (1977) reported comparable declines in muscle glycogen through the late spring and summer in non-metamorphosing sea lamprey. The reductions in glycogen stores in the carcass were not likely a consequence of changes in tissue water content, which could have resulted in lower wet weight glycogen concentrations. Instead, carcass water actually decreased between early spring and late summer, before increasing markedly in the fall, which is also consistent with previous observations made in non-metamorphosing sea lamprey (Lowe et al., 1973). Also arguing against any interaction between tissue glycogen stores and TFM tolerance were the decreases in brain glycogen concentration though the spring and summer, when the 12-h LC_{50} and 12 h $LC_{99.9}$ were highest. Although glycogen concentration, particularly in the brain, is affected by TFM exposure, the present findings suggest that glycogen stores are not a reliable predictor of TFM tolerance in sea lamprey.

Season had a pronounced effect on whole body lipid in larval sea lamprey but temperature did not. Lipid concentrations were highest in May, and continuously declined through the summer and fall. These observations were similar to earlier work by Lowe et al. (1973), who made similar observations in larval sea lamprey studied over a one-year period. Current studies suggest that higher lipid stores in the early spring were probably due to the onset of feeding following the spring thaw, followed by increased lipid consumption with warming waters. Indeed, this was noted by Kao et al. (2010) who showed that larval lamprey acclimated to 24 °C had total lipid amounts in the liver and kidneys that were more 30% lower than in larvae acclimated to 13 °C. Relatively lower lipid contents in the liver and kidneys of 24 °C-acclimated lamprey primarily resulted from a reduction in stored lipid reserve, triacylglycerol, but not structural lipid or phospholipid (Kao et al., 2010). Nevertheless, it may be worth additional investigation to study whether lipid stores additional amounts of TFM in larger pre-metamorphic lamprey that have much higher lipid reserves (Lowe et al., 1973; O'Boyle and Beamish, 1977; Kao et al., 2010), and thus contribute to increased TFM tolerance.

Lowe et al. (1973) previously reported that whole-body protein does not vary seasonally in larval lamprey, as shown in the present study. The protein composition (5.1–8.5% ww) measured was slightly lower than reported by Lowe et al. (1973), which averaged (about 10.5 to 12.5% of the wet weight). These differences could be explained by a range of factors, including that, in the present study, lamprey generally had higher lipid reserves than those studied by Lowe et al. (1973), and the animals were generally smaller as well.

Seasonal variations in liver protein were evident, but temperature had no effect on liver protein. The reduced liver protein in the summer (June and August) coincided with a lower HSI at this time which could be attributed to increased metabolic demands associated with living at warmer temperatures. There was also some glycogen accretion in the liver during the summer, which could have reduced the relative proportion of protein in the liver. The percent protein content in lamprey also increases with metamorphosis due to lipid consumption during this non-trophic life phase (Lowe et al., 1973); but the lamprey in this study were not approaching metamorphosis so the observed trend cannot be attributed to pre-metamorphosis or metamorphosis. Although low whole-body and liver protein coincides with higher TFM tolerance in the late summer (August), it is unlikely to be a preferred source of ATP production during TFM exposure because the oxidation of amino acids arising from proteolysis mainly relies on oxidative phosphorylation to generate ATP.

The ranges in water content values observed (72–81%) are consistent with earlier work on larval sea lamprey (Lowe et al., 1973). In general, season seemed to have more effect on larval sea lamprey water content than temperature, although no trends were observed between water content or dry ash and TFM sensitivity. Body mass throughout the present experiment was similar in all groups, and the condition factor was >1.5, suggesting that the animals were all in good health. The slightly higher CF in the spring larvae coincided with a high carcass water content is consistent with their emergence from a period of slow growth during the winter months (Busacker et al., 1990). That lipid, glycogen and protein were highest at this time suggests that even in the spring, the animal's nutrient reserves were not compromised. Virtually nothing is known about fuel use during the overwintering period in sea lamprey, but the present findings imply that they are highly adept at conserving energy during this time. Similarly, because acclimation to different temperatures had little effect on CF, body mass and energy reserves, it demonstrates that the animals subjected to higher temperatures readily coped.

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Conclusions

In conclusion, the tolerance of sea lamprey to TFM is lowest in spring; and then markedly increases through summer when water temperatures and food availability are greatest, before dropping in the fall. The hypothesis that TFM tolerance was related to greater energy stores was not supported, however, because lipid and glycogen reserves were in fact lowest during late summer, when TFM tolerance was highest. Rather, there was a strong relationship between water temperature and TFM tolerance which led to two-fold increases in the 12-h LC_{50}. Thus, the likelihood of residual lamprey following control treatments is likely greater at warmer temperatures which could compromise lampricide effectiveness during the warm summer months. Sea lamprey control efforts in the Great Lakes could be further complicated by future increases in water temperature due to climate change, which would increase the amounts of TFM required for lampricide applications and further increase the risk of residual sea lamprey. Dependent on the CO_2 emission scenarios provided by the Intergovernmental Panel on Climate Change, mean air temperatures in the basin are projected to increase between 1.5 and 3 °C under a low CO_2 emission scenario or up to 7 °C under a high emission scenario by 2100 (Angel and Kunkel, 2010) which would result in higher average and peak water temperatures and longer ice-free periods (Bartolais et al., 2015). Thus, it may be prudent to incorporate season and water temperature into current models that are used to evaluate the amounts of TFM required for lampricide applications; and, where practical, modify TFM treatment schedules by treating large streams or rivers earlier or later in the year when temperatures are cooler and sea lamprey most sensitive to TFM.

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