Using stable isotopes and C:N ratios to examine the life-history strategies and nutritional sources of larval lampreys

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Natural abundance stable-isotope analysis (δ¹³C and δ¹⁵N) and C:N ratios were used to study the ammocoete phase of two common non-parasitic lamprey species (least brook lamprey Lampetra aepyptera and American brook lamprey Lethenteron appendix) in two tributaries of the Ohio River (U.S.A.). The C:N ratios suggest that each species employs different lipid accumulation strategies to support its metamorphosis and recruitment into an adult animal. Ammocoete δ¹³C values generally increased with increasing C:N values. In contrast to δ¹³C, ammocoete δ¹⁵N values were weakly related to the total length (Lₜ) in L. aepyptera, but positively correlated to both Lₜ and C:N ratios in L. appendix. In L. appendix, C:N also correlated positively with Lₜ, and presumably age. A Bayesian mixing model using δ¹³C and δ¹⁵N was used to estimate nutritional subsidies of different potential food resources to ammocoetes at each site. The models suggested that although nutritional subsidies to ammocoetes varied as a function of site, ammocoetes were generally reliant on large contributions (42–62% at three sites) from aquatic plants. Contributions from aquatic sediment organic matter were also important at all sites (32–63%) for ammocoetes, with terrestrially derived plant materials contributing smaller amounts (4-33%). These findings provide important insights into the feeding ecology and nutrition of two species of lampreys. They also suggest that similar and other quantitative approaches are required to (1) fully understand how the observed stable-isotope ratios are established in ammocoetes and (2) better assess ammocoete nutritional subsidies in different natal streams.

Key words: ammocoetes; Lampetra aepyptera; Lethenteron appendix; MixSIR.

INTRODUCTION

Lampreys (Order: Petromyzontiformes) are an ancient group of jawless fishes with a complex life history. As a result, many populations of lampreys are currently threatened by anthropogenic activities that have degraded habitat for all life stages and impeded the migration of juveniles and adults (Renaud, 1997; Close et al., 2002; Mesa & Copeland, 2009). Larval lampreys (i.e. ammocoetes) may be particularly vulnerable to human activities, in part because of their extended residence time filter feeding in low-flow sandy or silty sections of the stream bed (Potter, 1980; Potter et al., 1986; Mundahl

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et al., 2005). Although considerable interest has developed in lamprey conservation (Renaud, 1997; Close et al., 2002), numerous questions about ammocoete biology and ecology remain, including the nutritional sources that support ammocoetes as they develop. The importance of the ammocoete stage to lamprey life cycles and the ability of humans to influence lamprey populations are high during the larval stage. A more quantitative understanding of the food sources supporting ammocoete growth and recruitment is still needed.

Prior dietary studies of ammocoetes have focused on gut-content analysis and have concluded that algae are important for nutrition (Sutton & Bowen, 1994; Yap & Bowen, 2003; Mundahl et al., 2005). Ammocoete gut content is primarily composed of amorphous detritus (Sutton & Bowen, 1994); however, this is difficult to categorize as to its ultimate sources, and as such the original starting material contributing to ammocoete growth remains debatable. Natural abundance isotopes represent a potential approach for assessing the types of organic matter (OM) supporting ammocoete somatic growth and energetic maintenance. Naturally occurring isotopes of C and N in organic materials may be used to trace these elements from their potential food sources to organisms, food webs and even ecosystems, provided the relative abundance of each isotope can be measured in both the relevant potential food sources and the target organisms (Peterson & Fry, 1987; Post, 2002; Cole et al., 2011). In biological systems, the lighter isotope of an element is processed at the enzymatic level to a slightly greater extent than the heavier isotope due to mass-dependent effects, resulting in isotopic fractionation (Hoefs, 2004). Therefore, fractionation results in more of the lighter isotope of a given element being lost (i.e. depleted) during respiration, and a greater relative retention (i.e. enrichment) of the heavier isotope within biomass. For example, $\delta^{15}N$ fractionates at c. 3‰ for each trophic level whereas $\delta^{13}C$ fractionates c. 1‰ per trophic level, although there is variation around these means (Peterson & Fry, 1987). Natural abundance stable isotopes may therefore provide insight to both the trophic level and the food sources of an organism or population.

Because biological systems cause fractionation during the use of various elements, the stable-isotope ratios in organisms can be different and tracked through consumers that depend on these organisms. For instance, stable-isotope ratios of terrestrial plants usually have a $\delta^{13}C$ of c. $-28‰$ in C$_3$ plants and c. $-13‰$ in C$_4$ plants (Peterson & Fry, 1987). In contrast, the $\delta^{15}N$ values are more variable, but are usually c. 0‰ in both groups of plants (Peterson & Fry, 1987). Although a consumer’s dependence on C$_3$ or C$_4$ plants could not be distinguished with $\delta^{15}N$, because both sources are identical, the use of $\delta^{13}C$ could distinguish the percentage contribution from each food source to the animal.

Prior work on lampreys with stable isotopes has provided important insights into their feeding ecology, but has primarily targeted parasitic juveniles and adults (Adams et al., 2008; Harvey et al., 2008; Inger et al., 2010). For example, work in the Laurentian Great Lakes suggested that juvenile sea lamprey Petromyzon marinus L. 1758 feeding was more locally influenced because dispersal was more limited than initially thought (Harvey et al., 2008). Work on anadromous populations of P. marinus has shown that the biomass of spent adults provides marine-derived carbon (C) and nutrients to local streams after spawning (Weaver et al., 2015). Inger et al. (2010) also used stable isotopes to determine whether non-native fish species were supporting native lamprey populations, and if lamprey populations were migratory. These authors found that a freshwater parasitic population fed primarily on brown trout Salmo trutta L. 1758 and
common bream *Abramis brama* (L. 1758) within the local water body instead of migrating to the ocean. Isotopes have also suggested that differences in the size of adult lamprey are probably because of differences in feeding strategies and growth of juveniles (Adams *et al.*, 2008). It is similarly predicted that the time to maturation in ammocoetes of different species and populations of lampreys is also controlled by differences in larval food resources, but ammocoete diets have not been the target of stable-isotope studies, although this life stage is critical for recruitment and maintenance of lamprey populations.

Stable-isotope analysis simultaneously provides estimates of the C:N ratio of the sample. The C:N ratios of aquatic animals (including many fish species) have been used as a proxy for estimating lipid content in a broad range of consumer organisms (Post *et al.*, 2007). Lipid content is important in controlling ammocoete metamorphosis (Moore & Potter, 1976; O’Boyle & Beamish, 1977), although not the only factor (e.g. temperature and photoperiod) (Manzon *et al.*, 2015). Lampreys use one of two strategies to accumulate lipids to complete metamorphosis (Hardisty, 2011). In one strategy, lipid levels are maintained at low levels until an ammocoete approaches its maximum size, at which point it ceases significant growth in length and rapidly accumulates lipids. If the animal fails to reach a lipid threshold before the end of the growing season, it maintains its lipid levels and will attempt to increase them the following year (O’Boyle & Beamish, 1977) (the all-or-none strategy). The other documented strategy has animals accumulating lipids annually without depleting them to initial levels. As a result, the following year’s initial lipid level is always higher than the previous year. Lipid accumulation continues until lipid levels reach levels high enough for metamorphosis to occur (Moore & Potter, 1976) (the rising-tide strategy; Hardisty, 2011). Whether these strategies are specific to a given species is currently unclear, and more work is required to determine how variable these life histories are in lamprey populations.

In this study, ammocoete tissues and their potential nutritional sources were examined using natural abundance stable isotope (δ\(^{13}\)C and δ\(^{15}\)N), and C:N analyses to better understand the feeding and nutritional ecology of ammocoetes. Stable isotopes were used to identify and quantify the nutritional resources supporting the critical ammocoete stage of two species of non-parasitic lamprey: least brook lamprey *Lampetra aepyptera* (Abott 1860) and American brook lamprey *Lethenteron appendix* (DeKay 1842), common throughout their ranges in the U.S.A. (Page & Burr, 1991). The C:N ratios of ammocoete tissue were also used to examine potential accumulation strategies of different biochemical classes of their biomass.

### MATERIALS AND METHODS

*Lampetra aepyptera* ammocoetes were collected from the Clear Fork River in the Muskingum catchment of north-central Ohio, U.S.A., whereas *L. appendix* ammocoetes were collected from the Mad River in the Great Miami catchment of western Ohio (Fig. 1). Both the Clear Fork and Mad Rivers are within the Mississippi River basin. For the Clear Fork, sampling was conducted on both third (Clear Fork River 1) and first (Clear Fork River 2) order stretches of the river. The Clear Fork catchment consists of 47% agricultural land use, 41% forest cover and 12% urban development (Jin *et al.*, 2013). Sampling on the Mad River was conducted on both third (Mad River 1) and second (Mad River 2) order reaches. The Mad River catchment is dominated by agricultural land use (81%), whereas the remaining land is primarily forest (18%) (Jin *et al.*, 2013). Both rivers were sampled in July and November 2010.
STABLE-ISOTOPE ANALYSIS OF AMMOCOETES

AMMOCOETE COLLECTION

Ammocoetes (six to 13 individuals per site at each sampling time) were collected using a backpack electrofisher (model ETS, Electrofishing LLC; www.etselectrofishing.com) following electrofishing procedures described by Moser et al. (2007) and the manufacturer’s recommendations. Upon emergence from their burrows, ammocoetes were immobilized, netted, rinsed of sediment and wrapped individually in pre-baked (500°C for 4 h) sheets of aluminium foil before being sealed in plastic bags and placed on dry ice. Upon return to the laboratory, ammocoetes were frozen at −20°C until processing.

COLLECTION OF POTENTIAL AMMOCOETE FOOD SOURCES

The top 8 cm of stream sediment cores were collected from undisturbed locations in stream areas in which ammocoetes were also collected. Cores were collected using pre-cleaned (10% HCl) 60 ml plastic syringes and then frozen on dry ice. Upon return to the laboratory, the cores were stored at −20°C until processing and analysis.

Terrestrial soil samples were excavated within c. 10 m of the stream to a depth of c. 30 cm using a clean spade. Collected soils were wrapped in pre-baked aluminium foil and frozen in plastic bags on dry ice in the field. Samples were stored at −20°C until processing.

Samples of the dominant species of terrestrial (e.g. Acer spp., Rosa multiflora and Urticaceae spp.) and aquatic vegetation (e.g. Ludwigia spp. and Vallisneria americana) were collected by hand-picking leaf material and rinsed in stream water to remove non-plant material. Aquatic vegetation was limited to submerged plants and algal mats; emergent and floating vegetation were not observed in the study areas. Samples were stored in individual plastic bags and frozen on dry ice until return to the laboratory and stored at −20°C, after which they were processed.

STABLE-ISOTOPE ANALYSES

Ammocoetes were thawed before their total length (LT) was measured to the nearest mm and their wet mass (M) measured to the nearest 0·01 g. Muscle tissue from each ammocoete (n = 72)
was then removed following isotopic clean protocols. The gut was kept intact while dissecting
the animal to prevent any gut contents from contaminating the samples. The muscle tissue was
dried at 60°C for 48 h, homogenized by grinding and stored in pre-baked glass scintillation vials
placed in a clean, airtight polycarbonate desiccator (<10% relative humidity) until isotopic anal-
ysis. Aquatic and terrestrial plant tissues were treated identically to ammocoete muscle tissue.
The condition factor (K) was calculated for each fish as $K = 10^6ML_T^{-3}$, where $M$ is in g and $L_T$
is in mm.

Thawed stream sediment cores were sectioned by cutting each core into 1 cm increments with
a clean metal razor blade, except for the portion closest to the water–sediment interface, which
was divided into two 0.5 cm segments. Each segment was dried at 60°C for 24 h and then
homogenized using a clean mortar and pestle. Homogenized samples were fumed with con-
centrated stock HCl for 24–48 h in a clean glass desiccator to remove inorganic C. Following
acid-fuming, sediments were placed under vacuum for 24–48 h to remove residual acid fumes
and then dried for 24 h at 60°C. Terrestrial soil samples were processed in the same manner.

Sub-samples of each sample type were packed in tin capsules for analysis of $\delta^{13}C$ and $\delta^{15}N$ at
the University of California, Davis, to be run on a PDZ Europa ANCA-GSL elemental analyser
(EA) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (IRMS; Sercon Ltd; www.sercongroup.com), or at the Ohio State University’s Stable Isotope Biogeochemistry Lab-
oratory on a Costech EA with continuous flow by ConFlo III interfaced to a Finnigan Delta Plus
IV IRMS (Thermo Scientific; thermoscientific.com). The s.d. for replicate analyses of standards
using both instruments was $\leq 0.2‰$ for $\delta^{13}C$ and $\leq 0.3‰$ for $\delta^{15}N$.

STATISTICAL ANALYSIS

ANCOVA of the $\delta^{13}C$ values of ammocoetes was performed and included species, dates the
interactions of species and site (species was tested as a nested factor since only one species was
found at each site) and the interaction of species, date and site, as well as the covariates
$L_T$ and C:N. In addition, a separate ANCOVA of ammocoete $\delta^{15}N$, which did not include the covariate
of $L_T$ but included the covariate C:N ratios, and the effects of site, date and species’ interaction
with site, and the interaction of species, date and site, was performed. As aquatic sediment OM
was analysed from different depths that could be isotopically unique, depth was included as a
factor, as well as site or date, and $\delta^{13}C$ and $\delta^{15}N$ were analysed using a MANOVA. Sample size
was too small to include site and date simultaneously in the MANOVA as no d.f. was available
for the error term.

ISOTOPE MASS BALANCE MODEL

The Bayesian stable-isotope mixing model MixSIR (Moore & Semmens, 2008; Solomon
et al., 2011) was used to estimate contributions of potential nutritional sources to ammocoetes.
MixSIR incorporates uncertainty in consumer and source isotopic values and trophic fraction-
atation in its estimates of source contribution to better express the predicted contributions and
uncertainties in food source contributions (Moore & Semmens, 2008; Kim et al., 2011). Ammoco-
etes and their potential nutritional resources were modelled separately for each site. The
isotopic means and s.d. of potential sources used in the model were drawn only from sam-
ple collected at the site, except for aquatic plants at Clear Fork River 2. At Clear Fork River 2,
only one aquatic plant sample was present, and therefore no s.d. could be calculated. As a
result, the s.d. used in the model for aquatic plants at Clear Fork River 2 was the greatest of all
the potential nutritional sources at that site.

The contributions of the following potential nutritional resources were included in the models:
(1) aquatic plants, (2) aquatic sediment OM and (3) terrestrial plants (Table I). While aquatic
sediments contain terrestrial and aquatic plant-derived materials, these materials may undergo
significant post-depositional diagenetic modification and ageing (Fogel & Tuross, 1999; Hoefs,
2004) that alters the availability and reactivity of the OM to consumer organisms. Therefore,
aquatic sediment OM was used as an independent source in all models. Terrestrial soils were
poorly differentiated isotopically from other sources at all sites, except at Mad River 1. At Mad
River 1, where terrestrial soils were isotopically most different from other sources, they were
still similar to aquatic sediment OM and were not included explicitly in the model. Aquatic
Table I. Mean δ^{13}C and δ^{15}N values [‰, mean ± s.d. (n)] of potential nutritional sources for *Lampetra aepyptera* ammocoetes from the Clear Fork River sites (CFR1 and CFR2) and *Lethenteron appendix* in the Mad River sites (MR1 and MR2) used in food source modelling in this study

<table>
<thead>
<tr>
<th>Source</th>
<th>δ^{13}C</th>
<th>δ^{15}N</th>
<th>δ^{13}C</th>
<th>δ^{15}N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFR1</td>
<td>CFR2</td>
<td>MR1</td>
<td>MR2</td>
</tr>
<tr>
<td>Aquatic plants</td>
<td>-28.5 ± 3.0 (4)</td>
<td>13.8 ± 7.6 (4)</td>
<td>-30.9 (1)</td>
<td>7.4 (1)</td>
</tr>
<tr>
<td>Aquatic sediment organic matter</td>
<td>-24.3 ± 2.0 (8)</td>
<td>4.3 ± 0.8 (8)</td>
<td>-27.5 ± 0.6 (15)</td>
<td>3.5 ± 1.1 (15)</td>
</tr>
<tr>
<td>Terrestrial plants</td>
<td>-30.4 ± 1.3 (9)</td>
<td>4.2 ± 3.0 (9)</td>
<td>-30.6 ± 1.7 (10)</td>
<td>2.1 ± 1.7 (10)</td>
</tr>
<tr>
<td>Terrestrial soils</td>
<td>-29.0 ± 1.7 (3)</td>
<td>2.0 ± 2.0 (3)</td>
<td>-28.1 ± 1.5 (3)</td>
<td>3.9 ± 1.6 (3)</td>
</tr>
<tr>
<td>Aquatic plants</td>
<td>-33.4 ± 1.8 (6)</td>
<td>10.8 ± 1.1 (6)</td>
<td>-30.1 ± 2.8 (4)</td>
<td>6.4 ± 0.8 (4)</td>
</tr>
<tr>
<td>Aquatic sediment organic matter</td>
<td>-18.1 ± 3.9 (8)</td>
<td>4.2 ± 1.1 (8)</td>
<td>-24.1 ± 3.6 (8)</td>
<td>3.6 ± 0.8 (8)</td>
</tr>
<tr>
<td>Terrestrial plants</td>
<td>-31.2 ± 1.0 (9)</td>
<td>2.8 ± 2.4 (9)</td>
<td>-30.1 ± 1.5 (10)</td>
<td>3.3 ± 3.1 (10)</td>
</tr>
<tr>
<td>Terrestrial soils</td>
<td>-23.3 ± 3.8 (3)</td>
<td>4.0 ± 1.3 (3)</td>
<td>-23.3 ± 2.4 (3)</td>
<td>3.6 ± 2.1 (3)</td>
</tr>
</tbody>
</table>

sediment OM showed no differences between depths in either the MANOVA that examined dates (depth d.f. = 8, residual d.f. = 22, Pillai = 0.24, *P* > 0.05) or sites (depth d.f. = 8, residual d.f. = 11, Pillai = 0.99, *P* > 0.05) and were therefore pooled by site for each model.

For the purposes of modelling, it was assumed that all lamprey isotopic values were derived from the potential nutritional sources that were measured. For modelling purposes, ammocoetes were considered primary consumers, as found in previous studies (Mallatt, 1982; Sutton & Bowen, 1994). Trophic level isotopic fractionation was estimated from previously published controlled feeding studies on Pacific lamprey *Entosphenus tridentatus* (Richardson 1836) (Uh et al., 2014) and followed published trophic fraction values of different consumer organisms from the literature of 0.4 ± 6.0‰ for δ^{13}C and 1.2 ± 1.5‰ for δ^{15}N (Post, 2002). All models were run with 1 × 10^6 iterations, which resulted in an importance ratio of <0.001 and >1000 posterior draws in all cases, following the recommended guidelines for determining if the model output has estimated true posterior distributions (Moore & Semmens, 2008).

**RESULTS**

**AMMOCOETE CHARACTERISTICS**

A total of 72 ammocoetes were collected in this study, 14 *L. aepyptera* at Clear Fork 1, 19 *L. aepyptera* at Clear Fork 2, 19 *L. appendix* at Mad River 1 and 20 *L. appendix* at Mad River 2. *Lampetra aepyptera* collected in this study had a mean ± s.d. *L*_T of 7.9 ± 3.8 cm [Fig. 2(a)] and ranged from 1.8 to 14.6 cm. *Lethenteron appendix* had a mean ± s.d. *L*_T of 14.5 ± 2.7 cm and ranged from 8.0 to 18.9 cm [Fig. 2(b)]. The C:N values for *L. aepyptera* were right-skewed with a mode at 4:5 [Fig. 3(a)]. The C:N of *L. appendix* had three distinct peaks centred around 4:7, 6-5 and 8:4 [Fig. 3(b)]. The C:N of *L. aepyptera* was positively correlated with *L*_T at both Clear Fork River 1 (*r^2 = 0.29, *P* < 0.01) [Fig. 4(a)] and at Clear Fork River 2 (*r^2 = 0.35, *P* < 0.01). For the Mad River, *L. appendix* *L*_T was positively correlated to C:N at Mad River 2 (*r^2 = 0.24, *P* < 0.05) [Fig. 4(b)], but not at Mad River 1 (*r^2 = 0.05, *P* > 0.05).

The δ^{13}C values of *L. aepyptera* ammocoetes were positively correlated with *L*_T at both Clear Fork River 1 (*r^2 = 0.35, *P* < 0.05) [Fig. 4(c)] and Clear Fork River 2...
Fig. 2. Total length ($L_T$) distributions of (a) Lampetra aepyptera from the Clear Fork River and (b) Lethenteron appendix from the Mad River. [■], samples collected in July; [□], samples collected in November. NB some July and November samples are overlaid.

($r^2 = 0.48, P < 0.001$). The relationship between $\delta^{13}C$ and $L_T$ for L. appendix was not significant at Mad River 1 ($r^2 = 0.04, P > 0.05$), but at Mad River 2, the relationship was positively related to $L_T$ ($r^2 = 0.27, P < 0.05$) [Fig. 4(d)]. There were no relationships at any site between $\delta^{15}N$ and $L_T$ in either species ($P > 0.05$ at all sites) (Table SI, Supporting Information). The relationship between $K$ and C:N ratios was not significant for L. aepyptera at Clear Fork River 1 ($r^2 = 0.01, P > 0.05$) or 2 ($r^2 = 0.001, P > 0.05$), but was significant ($r^2 = 0.13, P < 0.05$) with a weakly positive slope [mean with 95% c.i. = 0.04 (0.01–0.08)] for L. appendix at Mad River when both sites were combined (Table SI, Supporting Information). The mean ± s.d. $K = 2.4 ± 0.2 (n = 14)$ and $2.7 ± 0.5 (n = 19)$ for L. aepyptera at Clear Fork River 1 and Clear Fork River 2, respectively. The average $K$ for L. appendix was lower at both Mad River 1 (mean ± s.d. = 1.6 ± 0.2, $n = 19$) and Mad River 2 (mean ± s.d. = 1.8 ± 0.2, $n = 20$) than at either Clear Fork site.

**SPECIES COMPARISONS**

Lampetra aepyptera and L. appendix ammocoetes had significantly different mean $\delta^{13}C$ values (ANCOVA, $F_{1,63} = 19.71, P < 0.001$), and date means were also significantly different ($F_{1,63} = 47.74, P < 0.001$). The interaction of species and site was significant ($F_{2,63} = 23.25, P < 0.001$), but the interaction of species, date and site was not ($F_{3,64} = 2.59, P > 0.05$). Both $L_T$ ($F_{1,63} = 35.59, P < 0.001$) [Fig. 4(b), (d)] and C:N ($F_{1,63} = 34.85, 63, P < 0.001$) [Fig. 5(a), (c)] were significant covariates.

An ANCOVA of $\delta^{15}N$ showed that species ($F_{1,64} = 35.03, P < 0.001$) and date ($F_{1,64} = 14.39, P < 0.001$) were both significant, as was the interaction of species and site ($F_{2,64} = 20.15, P < 0.001$) was significant. The interaction of species, date and site
Fig. 3. C:N ratio distributions of (a) Lampetra aepyptera from the Clear Fork River and (b) Lethenteron appendix from the Mad River.

was not significant \( (F_{3,64} = 1.67, P > 0.05) \), whereas the covariate (C:N) did have a significant effect on \( \delta^{15}N \) \( (F_{3,64} = 12.81, P < 0.001) \) [Fig. 5(b), (d)].

**ISOTOPIC MIXING MODELS**

Estimates of the contributions of different potential nutritional resources to ammocoetes at Clear Fork River 2, Mad River 1 and Mad River 2 were similar. Median contributions for aquatic plants at these sites ranged from 42 to 62%, whereas aquatic sediment OM ranged from 32 to 37% (Fig. 6). At Clear Fork River 1, aquatic plants contributed little to ammocoete nutrition (3%), but aquatic sediment OM remained important (63%). Terrestrial plant contributions were variable between sites, but had a mean ± s.d. contribution of 18 ± 15% (Fig. 6).

**DISCUSSION**

**AMMOCOETE C:N DISTRIBUTIONS**

Although neither total nor specific lipids were measured directly in this study, the C:N ratios of ammocoete muscle tissue may provide insight into the lipid accumulation strategies of *L. aepyptera* and *L. appendix*. The majority of *L. aepyptera* ammocoetes had relatively low C:N ratios (≤5.0), but the largest animals had C:N ratios ≥7.0 [Fig. 3(a)], suggesting that the studied populations may follow an all-or-none strategy. Elevated C:N ratios were not limited to the largest animals as ammocoetes may metamorphose over a wide range of sizes (Purvis, 1970; Potter, 1980). The present
Fig. 4. (a, b) *Lampetra aepyptera* and (c, d) *Lethenteron appendix* ammocoetes C:N ratios and total length ($L_T$) for the (a) Clear Fork River (●, Clear Fork River 1 ($y = 0.56 + 0.63x; r^2 = 0.29, P < 0.05$); ○, Clear Fork River 2 ($y = 3.80 + 0.14x; r^2 = 0.35, P < 0.01$)) and (c) Mad River (■, Mad River 1; □, Mad River 2 ($y = 3.10 + 0.27x; r^2 = 0.24, P < 0.05$)) study sites, and ammocoete $\delta^{13}C$ values $L_T$ at the (b) Clear Fork River (●, Clear Fork River 1 ($y = -31.00 + 0.53x; r^2 = 0.35, P < 0.05$); ○, Clear Fork River 2 ($y = -28.00 + 0.36x; r^2 = 0.48, P < 0.01$)) and (d) Mad River (■, Mad River 1; □, Mad River 2 ($y = -29.00 + 0.25x; r^2 = 0.26; P < 0.05$)) study sites. Regression lines are colour-coded to match the corresponding data.

data [Fig. 4(a)] suggest that the minimum size for metamorphosis in *L. aepyptera* in this study is c. 10 cm, which is within the reported values for metamorphosing and adult *L. aepyptera* in a previous study (Docker & Beamish, 1991).

In contrast to *L. aepyptera*, the C:N distribution of *L. appendix* ammocoetes in this study suggests that the studied population utilizes a rising-tide approach of lipid storage [Fig. 3(b)]. The three C:N maxima may result from sequential years of increasing lipid content during animal growth. In addition, the C:N peak at c. 8.5 for *L. appendix* is broader than the other two peaks, suggesting that multiple year classes may begin to overlap during what has been called the ‘arrested growth’ stage (Hardisty & Potter,
1971). Older individuals that fail to reach the lipid levels required for metamorphosis may still be able to maintain relatively high lipid levels, whereas rapidly growing younger *L. appendix* ammocoetes overtake older ammocoetes and exaggerate the C:N peak at c. 8 [Fig. 3(b)].

The minimum mean C:N ratio for *L. appendix* (4:1) was not different from the minimum C:N for *L. aepyptera* (4:1); however, it is unlikely YOY *L. appendix* were captured because the smallest animal captured was 8·0 cm. The weak relationship between the C:N ratio, which appears useful in determining age and $L_T$, which is frequently used to establish ages in fishes for *L. appendix* [Fig. 4(c)], also suggests that $L_T$ is a poor proxy for ammocoete age (Kelso & Todd, 1993; Quintella et al.,...
Fig. 6. Median nutritional subsidies to all size classes of ammocoetes for (a) Lampetra aepyptera in the Clear Fork River sites. ■ Clear Fork River 1; □ Clear Fork River 2 sites. (b) Lethenteron appendix at the Mad River sites. ■ Mad River 1 ammocoetes; □ Mad River 2 ammocoetes. All values were calculated by the Bayesian model MixSIR (see Materials and methods for details). Lower and upper error bars correspond to 5 and 95% posterior proportional contributions, respectively.

2003). Entosphenus tridentatus ammocoetes were observed to have relatively low mean C:N ratios (mean = 5.0) in two published studies (Limm & Power, 2011; Uh et al., 2014), which were similar to the lowest measured values in this study (4.1) and had a limited range (4.6–5.1). These values for E. tridentatus also suggest an all-or-none lipid accumulation strategy similar to L. aepyptera in this study, but require confirmation over a broader range of streams and seasons. The present finding that $K$ was not correlated with C:N ratios suggests that condition factors in lampreys may not always be useful for detecting lipid accumulation. Future work to confirm that C:N values track lipid content in ammocoetes should examine both lipid-extracted tissues (and specific lipid abundances) and non-extracted tissues in multiple species and populations in order to better establish the relationship between lipid concentration and C:N curves that are specific for ammocoetes (Logan et al., 2008).

**ISOTOPIC CORRELATIONS**

The $\delta^{13}$C values of both L. aepyptera and L. appendix ammocoetes increased with $L_T$ [Fig. 4(b), (d)]. The increase in $\delta^{13}$C values may result from of a shift in the animals’ food and nutritional resources during growth, or from one or more physiological mechanisms that alter ammocoete isotopic ratios through time. Consumer $\delta^{13}$C values have
been found to be influenced by the lipid content of a sample (DeNiro & Epstein, 1977; Post et al., 2007), and ammocoete muscle tissue has been found to have relatively high lipid content that is both size and age dependent (up to 18% by wet mass) (Moore & Potter, 1976; O’Boyle & Beamish, 1977). Interestingly, when C:N is assumed to correlate with age [as in L. appendix; Fig. 4(c), (d)], $\delta^{13}C$ values increased as a function of C:N ratio by c. 5‰, in spite of the probable increasing lipid content. Lipids may still have reduced the $\delta^{13}C$ values of ammocoete body tissue, but its effect appeared weaker than the mechanism driving the $\delta^{13}C$ values in a positive direction. Lipid correction of $\delta^{13}C$ values has been found not to be applicable to all species and tissue types (Kiljunen et al., 2006). Ammocoetes may produce greater relative amounts of biosynthesized intramuscular $^{13}C$-enriched lipids (Stott et al., 1997), or may assimilate specific $^{13}C$-enriched lipid compounds of plant materials (i.e. such as sterols and fatty acids) (Canuel et al., 1997). Either or both of these mechanisms may help explain the relationship between ammocoete $\delta^{13}C$ and C:N observed in this study.

Other studies have measured the stable-isotope signatures of ammocoetes and have reported a broad range of $\delta^{13}C$ values (mean range: $-18.9$ to $-28.1$‰) (Van Riel et al., 2006; Shirakawa et al., 2009; Evans & Limburg, 2015), and this large range may be
a characteristic of ammocoetes. The $\delta^{13}C$ values of ammocoete muscle tissue in other studies also routinely approached $-20\%_e$, even without lipid extraction (Van Riel et al., 2006; Shirakawa et al., 2009; Limm & Power, 2011; A. Hollett, unpubl. data). It is clear that further work on ammocoetes lipid content and composition, as a function of size, age and growth rate is required to more fully understand the unusual relationship between their C:N and $\delta^{13}C$ values.

The range in $\delta^{15}N$ values of *L. aepyptera* ammocoetes [Fig. 5(b)] suggests that N sources to the Clear Fork River *L. aepyptera* are highly variable. The Clear Fork River catchment is evenly divided between agriculture (47%) and forested (41%) land uses, with smaller levels of urban development (12%). Each of these land-use types and their dominant forms of nutrient N may influence the $\delta^{15}N$ values of terrestrial and aquatic OM (Lake et al., 2001; Anderson & Cabana, 2005). Most natural sources of vegetation have a $\delta^{15}N$ close to 0‰ (Peterson & Fry, 1987), whereas human waste is often $>8\%_e$ (McClelland et al., 1997). Agricultural fertilizers are known to have $\delta^{15}N$ values of +3 to +12‰ which is consistent with the measured ammocoete $\delta^{15}N$ values at both the Clear Fork and Mad Rivers [i.e. after accounting for enrichment from trophic fractionation; Fig. 5(b)] (Chang et al., 2002). The $\delta^{15}N$ value of ammocoetes at both Mad River sites was 7.6 ± 0.5‰ (mean ± s.d., n = 39). The $\delta^{15}N$ of *L. appendix* ammocoetes at both sites in the Mad River suggests that N sources to the Mad River are more similar along the study reach than at the Clear Fork. The Mad River catchment is primarily agricultural lands (81%), with smaller amounts of forested land use (18%), and the remainder being developed (c. 1%).

At both Mad River sites, *L. appendix* $\delta^{15}N$ values were negatively correlated with C:N [Fig. 5(d)], but lipids are known to have low N content and are not expected to influence $\delta^{15}N$ values (DeNiro & Epstein, 1977). If *L. appendix* ammocoetes, in general, have one or more underlying physiological mechanisms altering $\delta^{15}N$, then the animals’ $\delta^{15}N$ may also vary over the course of the larval life stage. The $\delta^{15}N$ values of ammocoetes in other studies have been found to be relatively stable, and in cases where animal $\delta^{15}N$ values could be compared with $\delta^{15}N$ values of potential food sources or other consumers, ammocoetes appeared to be first-order consumers (Bilby et al., 1996; Marty et al., 2009; Shirakawa et al., 2009; Limm & Power, 2011; Evans & Limburg, 2015).

**ISOTOPIC MIXING MODEL ESTIMATES**

Isotope–isotope plots of ammocoetes and their potential nutritional sources suggest that Clear Fork River and Mad River animals derived their nutrition from multiple sources of OM at all sampling sites (Fig. 7). The present models assume that ammocoete isotopic signatures are derived from the measured potential nutritional sources and indicate that aquatic plants were usually important (range: 3–62%; Fig. 6). Ammocoetes were also subsidized significantly by aquatic sediment OM (range: 32–63%) (Fig. 6). Only at Clear Fork River 1, aquatic plants did not contribute a substantial portion of ammocoete biomass [range: 0.3–9.4‰; Fig. 6(a)]. This may be the result of elevated $\delta^{15}N$ values observed in aquatic plants at this site [Fig. 7(a)]. If aquatic plants had been consumed by ammocoetes even in minute quantities, the $\delta^{15}N$ signature of animals at Clear Fork 1 should have been greatly elevated, but mean ± s.d. *L. aepyptera* ammocoete $\delta^{15}N$ values were 5.3 ± 1.4 (n = 14), which was similar to *L.
aepyptera ammocoete δ¹⁵N values at Clear Fork River 2 of 7.4 ± 1.0 (n = 19). The variability of aquatic plant δ¹⁵N values at Clear Fork River 1, however, makes it difficult to accurately assess the true contribution of aquatic plants to ammocoetes at this site.

At all sites, the contribution from aquatic plants and sediments composed the primary sources of nutrition for ammocoetes (66–95%; Fig. 6). A previous study of *L. appendix* ammocoetes in Minnesota (U.S.A.) using gut-content analysis demonstrated that undifferentiated detritus made up >85% of the ingested material by volume (Mundahl et al., 2005). The present models further suggest that algae, although visually rare in ammocoete guts (Sutton & Bowen, 1994), are nutritionally significant, suggesting that ammocoetes may selectively digest the relatively rarer algal material at higher efficiencies than terrestrial plant materials and soil and sediment OM. Ammocoete growth is therefore probably controlled, at least in part, by land use and stream productivity, as previous studies have suggested (Morkert et al., 1998; Griffiths et al., 2001). Finally, the importance of algae to ammocoete diet and nutrition supports the contention that seasonal differences in both allochthonous and autochthonous primary production (Morkert et al., 1998; Griffiths et al., 2001), whereas not explicitly examined in this study, may be an important determinant of ammocoete nutrition (Yap & Bowen, 2003; Mundahl et al., 2005). Although terrestrial detrital OM dominates the OM in temperate streams (Cole & Caraco, 2001), its importance is more limited to ammocoetes (4–33%; Fig. 6). While terrestrial OM is common in aquatic systems, its quality is thought to be lower than autochthonous OM and may be more difficult for consumers to digest (Guenet et al., 2010). Ammocoetes have little in the way of specialized gut morphology to digest detritus and rely instead on long residence times within the gut to extract nutrients (Sutton & Bowen, 1994), probably making such OM less digestible than more labile forms such as aquatic plant OM.

This study provides compelling evidence that *L. aepyptera* and *L. appendix* have different lipid accumulation strategies. The isotopic and C:N relationships observed for ammocoetes here are atypical of those of most aquatic consumers. Long-term controlled feeding studies, similar measurements of other species of lamprey and more detailed isotopic and elemental measurements of whole and lipid-extracted tissues are necessary to more fully understand these unique aspects of ammocoete nutrition and feeding ecology.

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**Supporting Information**

Supporting Information may be found in the online version of this paper:

**Table S1.** Correlations of measured variables in this study and the equation describing each relationship (if the *P* < 0.05), *r*² and *P* values

**Fig. S1.** Ammocoete condition factor (*K*) v. C:N ratio for (a) the Clear Fork River and (b) the Mad River study sites. •, Clear Fork River 1; ○, Clear Fork River 2; ■, Mad...
River 1; Mad River 2. The correlation indicate for the Mad River is for both sites combined.

References


**Electronic Reference**