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PROFILES OF BIOCHEMICAL TRACERS IN UNIONID MUSSELS ACROSS A BROAD GEOGRAPHICAL RANGE

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ABSTRACT As large, long-lived filter feeders with high clearance rates, unionid mussels are capable of altering nutrient cycling in freshwater food webs. Because the effects of mussel communities on ecosystem processes result largely from their feeding behavior, we need to understand how they sample and process particulate matter. Our objective was to determine whether measuring a suite of biochemical markers in unionid mussels, from different populations of 2 species (*Amblema plicata*, *Actinonaias ligamentina*) across a broad geographical range (4 North American rivers), would help identify their primary dietary sources. Stable isotope data were able to differentiate mussels among rivers, and sometimes species. Data on $\delta^{15}\text{N}$ revealed that mussels fed across trophic levels but that diet varied with habitat and perhaps species. Although mussels contained the same types of fatty acids regardless of river or species, we nonetheless observed considerable variation in essential fatty acid content across rivers. Essential fatty acid profiles were dominated consistently by arachidonic acid. Source-specific fatty acid biomarkers suggested that bacterial and detrital resources might be as important as algae in many habitats. In summary, biochemical metrics differed more among rivers than between species. This suggests that mussels are either considerably adaptable in their dietary requirements or that they feed nonselectively. Understanding nutritional profiles in mussels will help restore unionid communities, which may lead to the reestablishment of their critical role as nutrient recyclers in freshwater food webs.

KEY WORDS: unionid mussels, biochemical tracers, stable isotopes, fatty acids, freshwater food webs

INTRODUCTION

Unionid mussels are a guild of relatively sedentary, suspension-feeding bivalves that play central roles in freshwater ecosystems particularly when their biomass is large relative to water volume and where hydrological residence time is long (Vaughn 2010). Mussels transfer nutrients and energy from the water column to the sediments through their filtering activity, and they excrete nutrients that stimulate primary and secondary production (e.g., Vaughn et al. 2008). Even moderate densities of mussels may influence ecosystem processes. For example, in the Kiamichi River, Oklahoma, mussels (densities up to 64/m²) influenced the distribution and abundance of other benthic organisms including periphyton and invertebrates (Spoonner & Vaughn 2006). Similarly, in the South Fork Eel River, California, *Margaritifera falcata* (Gould 1850) (density, ~1/m²) played a significant role in local food webs by increasing fine particulate matter of sediments, which subsequently increased the abundance and biomass of other macroinvertebrates (Howard & Cuffey 2006).

Unionid populations are declining rapidly throughout North America, presumably as a result of overharvesting, widespread habitat destruction, pollution, land-use change, and exotic species introductions (Strayer et al. 2004). To augment declining populations, resource managers use captive propaga-

tion as the primary means to conserve and replenish mussel populations. However, captive propagation has had variable success, most likely because we have inadequate information on the nutritional requirements of wild mussels. The lack of data on diets of mussels in the wild is limiting conservation efforts because we cannot develop captive diets for adults or juveniles nor can we assess the nutritional suitability of food resources in habitats targeted for mussel relocation efforts.

Direct observation of feeding is impractical in unionids, so other techniques have to be used to examine diet. Stomach content analysis is unreliable because it does not distinguish between what is ingested and what is assimilated (Vaughn & Hakenkamp 2001, Strayer et al. 2004). Biochemical tracers (e.g., stable isotopes and fatty acids (FAs)) may provide less biased, longer term dietary information than gut content analysis (Kelly & Scheibling 2012). Stable isotopes of N and C are often measured in aquatic biota because signatures of these 2 isotopes are thought to reflect trophic position and carbon (energy) flow, respectively, in aquatic and terrestrial food webs (Thorp et al. 1998, Finlay 2001). Nitrogen stable isotope ratios are generally used to infer trophic position via differential fractionation of the heavier ¹⁵N isotope in consumer diets as they move up the food chain (usually 3–4‰ per trophic level (Post 2002)). Furthermore, primary consumers, like mussels, are often used to obtain baseline isotopic values in aquatic food webs (e.g., Cabana & Rasmussen 1996, Howard et al. 2005). Carbon stable isotope ratios are used to identify food sources. For example, pelagic organisms are generally less enriched in ¹³C as a result of reduced fractionation associated

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with phytoplankton production, whereas benthic organisms undergo greater C fractionation and therefore often have more enriched ^{13}C signatures (France 1995). Sulfur isotopes are frequently measured in marine systems to distinguish animals with a sulfur-based nutrition (e.g., consuming sulfur-oxidizing bacteria) from those that consume phytoplankton-derived organic matter (Vetter & Fry 1998). Given the potential importance of bacteria in unionid diets (discussed later), this isotope may yield additional data on food sources. However, isotope ratios often fail to yield definitive results because potential sources often outnumber isotopes (Phillips & Gregg 2003) and because extraneous factors (e.g., current velocity, dietary C:N ratios) may influence fractionation (Finlay 2001). For example, $\delta^{13}\text{C}$ data from invertebrates and fish in the Mississippi River did not provide clear separation of C sources (DeLong et al. 2001, Herwig et al. 2004). Similarly, stable isotope analysis alone has been unable to identify unequivocally food sources for mussels (Nichols & Garling 2000, Christian et al. 2004). These ambiguities highlight the need for multiple lines of evidence to understand the dietary needs of mussels (Strayer 2008).

A technique that may add additional sensitivity to stable isotope analysis for tracking unionid diets is FA analysis. Quantitative FA analysis has emerged as an increasingly reliable method to measure food sources (Iverson 2009) and food quality (Wacker & von Elert 2004) in a variety of biota. These analyses are based on the distinctive profiles of the more than 70 unique FAs that can be quantified by gas chromatography and often provide better separation of food sources than stable isotope ratios. The placement of a double bond in the n-3 (also called omega-3) or n-6 (omega-6) position during the synthesis of polyunsaturated FA (PUFA) is almost exclusively the domain of plants, fungi, and bacteria. Most animals cannot synthesize 18C PUFA, but they can convert 18C PUFA into long-chain PUFAs such as eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic acids (DHA; 22:6n-3); however this latter ability is limited and restricted in most animals, including bivalves (Chu & Greaves 1991). Polyunsaturated FAs are important in many biological processes, including cell membrane physiology and as precursors to hormones (Brett & Müller-Navarra 1997, Arts & Kohler 2009). For example, the presence of certain dietary FAs has been shown to enhance growth in zooplankton (Müller-Navarra et al. 2000, Wacker & von Elert 2001) and fish (Copeman et al. 2002). In marine bivalves, EPA and DHA have been demonstrated to be essential for growth and survival (Berntsson et al. 1997).

Fatty acids have also been used as trophic markers in aquatic food webs (e.g., Goedkoop et al. 1998, Dalsgaard et al. 2003). Three characteristics of FAs make them useful tracers of diets and food web structure (Iverson 2009). First, organisms are able to biosynthesize, modify chain length, and introduce double bonds in FAs, but they are subject to biochemical limitations during these processes. That certain FAs cannot be produced *de novo* is a valuable tool for assessing an organism's physiological requirements and tracking its food sources. Second, unlike other dietary nutrients (e.g., proteins and carbohydrates), FAs are released from ingested lipid molecules during digestion where they are often incorporated unchanged into tissues. Third, fat is often stored in reservoirs (e.g., adipose and/or muscle tissues) for long periods, thus providing a more integrative perspective on diet compared with conventional gut content analyses.

There are few data on biochemical tracers in unionid mussels (but see McGoldrick et al. (2009) and Mezek et al. (2011)). Previous bivalve studies have largely been in marine systems, and were often undertaken for aquaculture-related purposes. In this exploratory effort, we used long-lived, filter-feeding unionid mussels as samplers and processors of particulate matter in rivers. Our objective was to determine whether measuring a suite of biochemical markers in unionid mussels, from different populations of species across a broad geographical range, would help identify their primary dietary sources. Our study provides information on dietary elements common to each species across a range of habitats and assesses the sensitivity of various biochemical techniques for diet analysis.

MATERIALS AND METHODS

Biochemical tracers were measured from 2 widely distributed mussel species, *Amblema plicata* (three-ridge, Say 1817) and *Actinonaias ligamentina* (mucket, Lamarck 1819). We chose these 2 coexisting species because they dominate community biomass and associated ecosystem services differentially in many eastern North American rivers (Spooner & Vaughn 2008). We sampled mussels from 4 North American rivers. These rivers were chosen because they had high-quality mussel beds, which we defined as dense, species-rich assemblages that contained a range of age classes, including young individuals of several species and few attached zebra mussels (*Dreissena polymorpha*, Pallas 1771). The Little River was sampled near Idabel, Oklahoma; the Cass River was sampled near Vassar, Michigan; the Upper Mississippi River was sampled near La Crosse, Wisconsin; and the Sydenham River was sampled near Florence, Ontario (Table 1).

We did not measure potential food sources for 2 reasons. First, prior stable isotope studies have been unable to identify food sources accurately (e.g., Nichols & Garling 2000, Christian et al. 2004) so it is unclear what food should be sampled. Second, new techniques to separate live food from dead food have been developed (e.g., Hamilton et al. 2005) that might give better resolution to stable isotope data, but the quantity of food required often limits this method's application and sensitivity.

About 10 *Amblema plicata* and 10 *Actinonaias ligamentina* were sampled in August 2006 from each river (except for *A. ligamentina* in the Upper Mississippi River). Mussels were

TABLE 1.

Physical and biological features of 4 North American river systems where unionid mussels were sampled for biochemical markers in August 2006.

River, State or Province	Primary land use	Mean discharge (m ³ /s)	Mussel density (site, n/m ²)	No. of mussel species (site)
Little River, OK	Forest*	183	43.0	14
Cass River, MI	Agriculture	15	NA	NA
Upper Mississippi River, WI	Agriculture	3,576†	8.8	15
Sydenham River, Ontario	Agriculture	12	11.3	21

* Although there is a forested riparian zone, much of the surrounding lands are agricultural. † Excludes the Missouri River. NA, data not available.

placed in coolers containing river-water-soaked burlap cloth and transported to the laboratory. In the laboratory, mussels were weighed (wet), and shell dimensions (length, width, height) were determined. A piece of foot tissue (mean, 5.1 g wet weight) was removed, frozen at -80°C , and subsequently freeze-dried and homogenized prior to measurement of biochemical tracers. We used foot tissue because it has been shown to integrate long-term nutrient sources (Raikow & Hamilton 2001) and can be sampled noninvasively. Biochemical tracers were chosen to represent different biological processes: general body condition (glycogen), nutritional condition (FA), trophic position ($\delta^{15}\text{N}$), food sources ($\delta^{13}\text{C}$, $\delta^{34}\text{S}$, FA), and elemental composition (molar C:N ratio). Glycogen was measured using the methods outlined in Newton et al. (2001), and tissue C and N levels were derived from stable isotope analyses.

Stable isotope samples ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$) were run in continuous-flow mode using a Thermo-Finnigan Deltaplus Advantage gas isotope-ratio mass spectrometer (Bremen, Germany) interfaced with a Costech Analytical ECS4010 elemental analyzer (Valencia, CA). Helium flow rate was 10–130 mL/min for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, and 120 mL/min for $\delta^{34}\text{S}$. Oxygen flow rate was 80 mL/min for all isotopes. For $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, a 3-m gas chromatograph column was used (55°C) for peak separation, in combination with 1 quartz (combustion) tube filled with chromium oxide and silvered cobaltous/cobaltic oxide ($1,020^{\circ}\text{C}$) and 1 quartz (reduction) tube filled with reduced copper (650°C). For $\delta^{34}\text{S}$, a CHNS 1-m gas chromatograph column was set at 90°C for peak separation, in combination with 1 quartz tube filled with tungsten oxide and pure copper wire ($1,020^{\circ}\text{C}$) and a second quartz tube filled with quartz turnings (800°C). Data were normalized using 4 (CH6, CH7, N1, and N2 from the International Atomic Energy Agency for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) or 6 (S1, S2, S3, SO5, and SO6 from the International Atomic Energy Agency, and NBS 127 from the National Bureau of Standards for $\delta^{34}\text{S}$) external standards. Peach leaves (National Institute of Standards and Technology 1547 for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and bovine liver (National Institute of Standards and Technology 1577b for $\delta^{34}\text{S}$) were the working standards. Isotope data were expressed relative to Vienna Pee Dee Belemnite for $\delta^{13}\text{C}$, relative to air for $\delta^{15}\text{N}$, and relative to Canyon Diablo Triolite for $\delta^{34}\text{S}$. Stable isotope analyses were done by the Colorado Plateau Stable Isotope Laboratory (Flagstaff, AZ).

Forty-one FAs were quantified using a 3-step procedure comprising gravimetric extraction, derivatization, and quantification on a gas chromatograph following the methods of Zellmer et al. (2004). Fatty acid methyl esters (FAME) were identified using a 37-component FAME standard (Supleco no. 47885-U) by comparing peak retention times between samples and standards. The FAME standard was run as a 4-point standard curve with each set of 20 samples. The standard curve was used to quantify the amount of each FA in a sample. An internal standard, 5 α -cholestane (Sigma no. C-8003), was added to the tissue before extraction to estimate percent recovery. Additional single FA standards were used to expand the range of quantifiable FAME to include other important FAs (e.g., docosapentaenoic acid [DPA], 22:5n-3c) not included in the 37-component FAME standard. Fatty acid data were reported as mass fractions (i.e., as micrograms of FAME per milligram dry mass of tissue).

In addition to individual FAs, we also classified FAs into 8 groups, including saturated FAs (SAFAs), monounsaturated

FAs (MUFAs), PUFAs, n-3 FAs, n-6 FAs, bacterial FAs, algal FAs, and essential FAs (EFAs). We defined bacterial FAs as the sum of pentadecanoic acid (15:0), pentadecanoic acid (iso, 15:0i), pentadecanoic acid (anteiso, 15:0ai), and heptadecanoic acid (17:0); and algal FAs as the sum of linoleic (LIN, 18:2n-6), α -linolenic acid (ALA, 18:3n-3), arachidonic acid (ARA, 20:4n-6), EPA, and DHA. Essential FAs are those FAs that cannot be synthesized by organisms at rates sufficient to meet basic biochemical requirements and thus must be obtained through the diet (Kainz et al. 2004). We defined EFAs as LIN, ALA, ARA, EPA, and DHA.

Quality assurance and quality control measures were estimated with all biochemical tracers. Precision was $\pm 0.1\%$ or better for $\delta^{13}\text{C}$, $\pm 0.2\%$ or better for $\delta^{15}\text{N}$, and $\pm 0.3\%$ or better for $\delta^{34}\text{S}$. The relative SD of working standards was 0.2% for $\delta^{13}\text{C}$ and 10.0% for $\delta^{15}\text{N}$ ($n = 17$), and 4.2% for $\delta^{34}\text{S}$ ($n = 14$). The mean percent difference between 84 duplicate samples for total lipid analysis was 2%. Average methylation efficiencies based on tests with nonmethylated cis-11,14-eicosadienoic acid (20:2), EPA, and DHA was close to 1.0. Seven blind tissue samples were analyzed in triplicate for stable isotopes, FAs, and glycogen. Relative SDs averaged 0.2 for $\delta^{13}\text{C}$, 0.8 for $\delta^{15}\text{N}$, 9.3 for $\delta^{34}\text{S}$, 6.1 for total FAs, and 19.9 for glycogen.

Given that we only sampled 1 mussel population in each river, these data are largely descriptive and our inferences are relative to the specific mussel bed in each river. We pooled all the biochemical tracer data and used nonmetric multidimensional scaling as a data exploration technique to identify groupings of river reaches and species (using Primer-E software (Clarke & Warwick 2001)). Fatty acid data were fourth-root transformed and stable isotope, glycogen, and C:N ratios were normalized prior to analysis. All biochemical analyses were based on Euclidian distance matrices. We used a 2-way crossed analysis of similarities (ANOSIM) to test for significant differences in biochemical tracers between species or among rivers. Analysis of similarities compares the similarity of values within groups with the similarity of values among groups. Analysis of similarity is analogous to ANOVA except it uses Monte Carlo simulation to determine significance rather than probability inferences from an assumed statistical distribution. In ANOSIM, a global R is computed such that 2 or more groups containing the same values have $R = 0$ (i.e., similarity within groups equals the similarity among groups), whereas groups of values that have nothing in common have $R = 1$. The significance of the result is also calculated and $P > R$ was considered significant if ≤ 0.05 . If the ANOSIM was significant, similarity percentages were estimated to identify those variables that contribute to the dissimilarity between species or among rivers.

RESULTS

Mussels were significantly larger from the Cass River than from the other 3 rivers. Mean shell length was 137.4 ± 2.9 mm (SE), 109.3 ± 2.4 mm, 108.5 ± 8.7 mm, and 92.4 ± 2.2 mm for *Amblema plicata* in the Cass River, Little River, Sydenham River, and Upper Mississippi River, respectively, and 143.4 ± 4.9 mm, 115.9 ± 8.7 mm and 112.2 ± 1.8 mm for *Actinonaias ligamentina* in the Cass River, Sydenham River, and Little River, respectively. Glycogen concentrations in mussels were highly variable among rivers but similar between species (range, 53–79 mg/g dry weight in *A. plicata*, 53–75 mg/g dry weight in

TABLE 2.

Mean (± 1 SE) stable isotope values (measured in parts per thousand), glycogen (measured in milligrams per gram dry weight), percent C, percent N, percent S, and molar C:N ratios in *Amblyma plicata* and *Actinonaias ligamentina* mussels from 4 rivers in North America.

Variable	Little River, OK	Cass River, MI	Sydenham River, ON	Upper Mississippi River, WI
<i>Amblyma plicata</i>				
$\delta^{13}\text{C}$	-30.19 ± 0.20	-31.38 ± 0.08	-32.64 ± 0.08	-28.49 ± 0.05
$\delta^{15}\text{N}$	16.36 ± 0.18	13.39 ± 0.09	13.56 ± 0.14	12.14 ± 0.11
$\delta^{34}\text{S}$	5.57 ± 0.15	-5.33 ± 0.20	-3.43 ± 0.24	-7.24 ± 0.15
Glycogen	64.89 ± 9.39	78.80 ± 7.74	73.20 ± 8.23	53.16 ± 4.40
C (%)	45.27 ± 3.35	45.95 ± 1.06	46.98 ± 0.57	47.29 ± 0.28
N (%)	11.75 ± 1.94	11.76 ± 1.42	12.40 ± 0.77	13.11 ± 0.45
S (%)	0.68 ± 0.10	0.71 ± 0.07	0.80 ± 0.04	0.88 ± 0.09
C:N	4.57 ± 0.17	4.61 ± 0.18	4.44 ± 0.08	4.21 ± 0.04
<i>Actinonaias ligamentina</i>				
$\delta^{13}\text{C}$	-31.10 ± 0.11	-31.63 ± 0.11	-32.88 ± 0.09	—
$\delta^{15}\text{N}$	14.39 ± 0.16	13.50 ± 0.14	13.54 ± 0.25	—
$\delta^{34}\text{S}$	5.71 ± 0.06	-5.62 ± 0.14	-3.83 ± 0.14	—
Glycogen	55.37 ± 5.09	74.96 ± 6.98	53.17 ± 8.21	—
C (%)	44.59 ± 1.76	45.60 ± 1.25	46.61 ± 0.94	—
N (%)	12.15 ± 0.93	11.92 ± 1.84	12.92 ± 0.59	—
S (%)	0.72 ± 0.04	0.75 ± 0.04	0.78 ± 0.02	—
C:N	4.29 ± 0.06	4.58 ± 0.27	4.22 ± 0.05	—

—, not sampled.

A. ligamentina; Table 2). Molar C:N ratios ranged from 4.2–4.6 in both *A. plicata* and *A. ligamentina* (Table 2).

Stable isotopes in mussels varied substantially among rivers (Fig. 1). Overall, mussels were quite depleted in ^{13}C , as indicated by their $\delta^{13}\text{C}$ values, which ranged from -28‰ to -33‰ in *Amblyma plicata* and from -31‰ to -33‰ in *Actinonaias ligamentina* (Table 2). Stable N data varied from 12.1–16.4 ‰ , with the highest $\delta^{15}\text{N}$ values in both species from Oklahoma and the lowest $\delta^{15}\text{N}$ values in *A. plicata* in Wisconsin (Table 2). Stable S isotope was also highly variable among rivers; $\delta^{34}\text{S}$ was negative in both species from Michigan, Ontario, and Wisconsin (range, -3‰ to -7‰) and was positive in both species from Oklahoma ($\sim 5\text{‰}$, Table 2). Across species and rivers, mussels averaged 46% C, 12% N, and 0.8% S (Table 2).

Thirty-two of the 41 FAs (chain length, ≥ 14 C) were found in both species and all rivers (Table 3). We did not observe any FAs that were unique to either of the 2 species examined. Five FAs for which we assayed were not found in any mussels (17:1, 18:2n-6t, 21:0, 23:0, and 24:0). Myristoleic (14:1n-5) was found in both species in Oklahoma, Ontario, and Wisconsin; cis-13, 16-docosadienoic acid (22:2) was found in *Actinonaias ligamentina* in Ontario and Michigan and in *Amblyma plicata* in Ontario, Oklahoma, and Wisconsin; nervonic acid (24:1n-9) was found in both species from Ontario and in *A. plicata* in Wisconsin; and omega-6 docosapentaenoic acid (22:5n-6) was only found in mussels from Oklahoma. Mean total lipid (DW basis) was low and ranged from 4.7–5.7% in *A. plicata* and from 4.5–6.2% in *A. ligamentina* (Fig. 2). The dominant SAFAs, MUFAs, and PUFAs were consistent between species and across rivers (palmitic acid, 16:0; oleic acid, 18:1n-9; and ARA, respectively). The dominant n-3 FA in *A. ligamentina* (all rivers) and *A. plicata* in Wisconsin, Michigan, and Ontario was DPA (22:5n-3). In Oklahoma, the dominant n-3 FA in *A. plicata* was ALA (18:3n-3). The dominant n-6 FA in both species and in all rivers was ARA.

Contents of EFAs were variable among rivers. For example, EPA contents in *Amblyma plicata* from Oklahoma were $\sim 50\%$ less than EPA contents in *A. plicata* from the other 3 rivers (Fig. 2). Similarly, ARA contents in *Actinonaias ligamentina* from Michigan were 25–40% less than ARA contents in *A. ligamentina* from the other 2 rivers (Fig. 2). Regardless of species, EFAs were dominated by ARA. The ARA:EPA ratio was consistent between species, but this ratio was about 2-fold higher in mussels from both species in Oklahoma than in mussels from

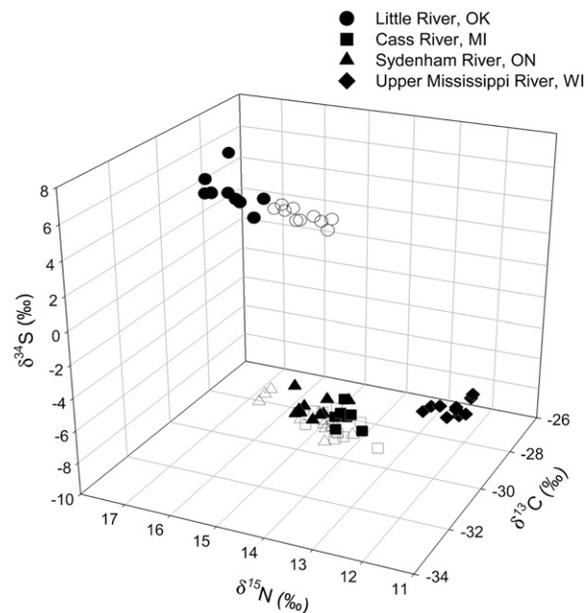


Figure 1. Stable isotopes of $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ in *Amblyma plicata* (solid symbols) and *Actinonaias ligamentina* (open symbols) mussels from 4 North American Rivers.

TABLE 3.

Contents of 36 fatty acids (measured in micrograms per milligram dry weight ± 1 SD) in 2 mussel species from 4 North American Rivers.

Fatty acid	<i>Actinonaias ligamentina</i>			<i>Amblema plicata</i>			
	Sydenham River, ON	Cass River, MI	Little River, OK	Sydenham River, ON	Cass River, MI	Little River, OK	Upper Mississippi River, WI
C14:0	0.071 \pm 0.041	0.032 \pm 0.025	0.044 \pm 0.020	0.059 \pm 0.031	0.039 \pm 0.035	0.046 \pm 0.031	0.046 \pm 0.032
C15:0ai	0.005 \pm 0.004	0.003 \pm 0.002	0.007 \pm 0.003	0.004 \pm 0.002	0.001 \pm 0.003	0.003 \pm 0.003	0.002 \pm 0.004
C15ai	0.153 \pm 0.020	0.086 \pm 0.025	0.145 \pm 0.031	0.178 \pm 0.044	0.125 \pm 0.049	0.109 \pm 0.067	0.174 \pm 0.041
C14:1n5	0.013 \pm 0.040	0.000 \pm 0.000	0.048 \pm 0.047	0.005 \pm 0.016	0.000 \pm 0.000	0.026 \pm 0.048	0.001 \pm 0.004
C15:0	0.216 \pm 0.176	0.119 \pm 0.049	0.110 \pm 0.017	0.133 \pm 0.085	0.144 \pm 0.113	0.087 \pm 0.027	0.144 \pm 0.094
C16:0i	0.022 \pm 0.005	0.021 \pm 0.007	0.021 \pm 0.005	0.016 \pm 0.005	0.011 \pm 0.004	0.012 \pm 0.003	0.014 \pm 0.006
C15:1	0.257 \pm 0.038	0.149 \pm 0.044	0.072 \pm 0.067	0.198 \pm 0.030	0.110 \pm 0.051	0.068 \pm 0.083	0.179 \pm 0.035
C16:0	2.905 \pm 0.526	2.348 \pm 0.327	2.910 \pm 0.294	2.791 \pm 0.372	2.298 \pm 0.440	2.588 \pm 0.576	2.552 \pm 0.288
C16:1n7	0.473 \pm 0.085	0.558 \pm 0.171	0.266 \pm 0.077	0.665 \pm 0.199	0.780 \pm 0.037	0.366 \pm 0.116	1.002 \pm 0.120
C17:0	0.359 \pm 0.194	0.297 \pm 0.097	0.449 \pm 0.031	0.390 \pm 0.160	0.371 \pm 0.181	0.394 \pm 0.070	0.430 \pm 0.042
C16:2n4	0.279 \pm 0.054	0.170 \pm 0.046	0.287 \pm 0.049	0.346 \pm 0.052	0.249 \pm 0.096	0.227 \pm 0.110	0.381 \pm 0.063
C16:3n4	0.018 \pm 0.008	0.008 \pm 0.006	0.018 \pm 0.006	0.023 \pm 0.008	0.012 \pm 0.010	0.016 \pm 0.010	0.017 \pm 0.005
C18:0	1.676 \pm 0.457	1.078 \pm 0.232	1.418 \pm 0.113	1.611 \pm 0.408	1.140 \pm 0.387	1.234 \pm 0.246	1.521 \pm 0.292
C18:1n9t	0.182 \pm 0.124	0.072 \pm 0.047	0.074 \pm 0.057	0.195 \pm 0.176	0.099 \pm 0.063	0.086 \pm 0.070	0.129 \pm 0.091
C18:1n9c	1.514 \pm 0.150	1.154 \pm 0.174	1.232 \pm 0.156	1.655 \pm 0.237	1.392 \pm 0.140	1.137 \pm 0.234	1.433 \pm 0.130
C18:1n7	0.212 \pm 0.077	0.157 \pm 0.038	0.169 \pm 0.028	0.287 \pm 0.056	0.242 \pm 0.027	0.306 \pm 0.354	0.228 \pm 0.025
C18:2n6c	0.717 \pm 0.116	0.499 \pm 0.077	0.751 \pm 0.092	0.833 \pm 0.166	0.518 \pm 0.101	0.821 \pm 0.171	0.687 \pm 0.075
C20:0	0.046 \pm 0.008	0.039 \pm 0.009	0.075 \pm 0.012	0.037 \pm 0.005	0.028 \pm 0.015	0.053 \pm 0.010	0.048 \pm 0.011
C18:3n6	0.019 \pm 0.006	0.019 \pm 0.006	0.029 \pm 0.015	0.019 \pm 0.005	0.020 \pm 0.010	0.030 \pm 0.015	0.028 \pm 0.005
C20:1n9	0.173 \pm 0.043	0.127 \pm 0.035	0.219 \pm 0.026	0.230 \pm 0.053	0.162 \pm 0.021	0.222 \pm 0.053	0.167 \pm 0.028
C20:1n7	0.033 \pm 0.007	0.057 \pm 0.086	0.044 \pm 0.004	0.044 \pm 0.010	0.042 \pm 0.009	0.043 \pm 0.013	0.068 \pm 0.009
C18:3n3	0.423 \pm 0.098	0.274 \pm 0.087	0.476 \pm 0.085	0.474 \pm 0.172	0.244 \pm 0.052	0.548 \pm 0.239	0.270 \pm 0.045
C20:2	0.053 \pm 0.022	0.035 \pm 0.017	0.085 \pm 0.019	0.074 \pm 0.030	0.044 \pm 0.014	0.069 \pm 0.029	0.054 \pm 0.013
C22:0	0.024 \pm 0.012	0.018 \pm 0.018	0.024 \pm 0.005	0.014 \pm 0.010	0.009 \pm 0.010	0.015 \pm 0.014	0.022 \pm 0.011
C20:3n9	0.019 \pm 0.009	0.007 \pm 0.005	0.032 \pm 0.020	0.030 \pm 0.022	0.008 \pm 0.007	0.024 \pm 0.020	0.009 \pm 0.004
C20:3n6	0.020 \pm 0.007	0.017 \pm 0.004	0.034 \pm 0.004	0.021 \pm 0.005	0.017 \pm 0.005	0.031 \pm 0.010	0.030 \pm 0.006
C22:1n9	0.014 \pm 0.008	0.006 \pm 0.006	0.012 \pm 0.002	0.017 \pm 0.007	0.006 \pm 0.005	0.012 \pm 0.003	0.014 \pm 0.004
C20:3n3	0.020 \pm 0.053	0.002 \pm 0.004	0.071 \pm 0.092	0.031 \pm 0.019	0.018 \pm 0.004	0.087 \pm 0.103	0.011 \pm 0.009
C20:4n6	1.819 \pm 0.214	1.379 \pm 0.176	2.300 \pm 0.224	1.559 \pm 0.147	1.181 \pm 0.225	1.511 \pm 0.327	1.617 \pm 0.157
C22:2	0.002 \pm 0.007	0.003 \pm 0.006	0.000 \pm 0.000	0.007 \pm 0.008	0.000 \pm 0.000	0.002 \pm 0.007	0.002 \pm 0.005
C20:5n3	0.731 \pm 0.174	0.719 \pm 0.165	0.431 \pm 0.054	0.607 \pm 0.163	0.594 \pm 0.174	0.310 \pm 0.114	0.670 \pm 0.076
C24:1n9	0.002 \pm 0.003	0.000 \pm 0.000	0.000 \pm 0.000	0.003 \pm 0.007	0.000 \pm 0.000	0.000 \pm 0.000	0.003 \pm 0.007
C22:4n6	0.454 \pm 0.118	0.427 \pm 0.054	0.650 \pm 0.091	0.358 \pm 0.037	0.273 \pm 0.052	0.408 \pm 0.086	0.511 \pm 0.085
C22:5n6	0.000 \pm 0.000	0.000 \pm 0.000	0.029 \pm 0.065	0.000 \pm 0.000	0.000 \pm 0.000	0.031 \pm 0.071	0.000 \pm 0.000
C22:5n3c	1.040 \pm 0.238	0.824 \pm 0.133	0.529 \pm 0.114	0.815 \pm 0.265	0.574 \pm 0.150	0.377 \pm 0.145	0.831 \pm 0.091
C22:6n3	0.249 \pm 0.076	0.190 \pm 0.050	0.128 \pm 0.027	0.284 \pm 0.098	0.199 \pm 0.061	0.138 \pm 0.052	0.215 \pm 0.026

the other rivers. Thus, the ARA:EPA ratio was 4.9, 2.0, 2.6, and 2.4 in *A. plicata* from Oklahoma, Michigan, Ontario, and Wisconsin, respectively, and was 5.3, 2.0, and 2.5 in *A. ligamentina* in Oklahoma, Michigan, and Ontario, respectively.

The percent of total FAs that were algal derived ranged from 25–31% across species and rivers (Fig. 2). The percent of total FAs that were bacterially derived ranged from 5.1–5.8% in *Amblema plicata* and from 4.7–5.4% in *Actinonaias ligamentina* (Fig. 2). The mean ratio of bacterial:algal FAs ranged from 0.18–0.23 in *A. plicata* and from 0.17–0.19 in *A. ligamentina*.

Results from nonmetric multidimensional scaling indicated that the biochemical tracers varied significantly among rivers ($P = 0.001$, $R = 0.78$) and between species ($P = 0.001$, $R = 0.24$; Fig. 3). River-specific contrasts indicated that there were differences in biochemical tracers in mussel tissue among all 4 rivers ($P_s = 0.001$, $R_s \geq 0.44$; Table 4). The species-specific contrast was driven largely by species differences in the Little River, Oklahoma ($P = 0.001$, $R = 0.40$) and the Sydenham River,

Ontario ($P = 0.03$, $R = 0.14$). There were marginal differences between species in the Cass River, Michigan ($P = 0.06$, $R = 0.15$). Stable isotopes and glycogen were responsible for much of the observed separation among rivers (Table 4). For example, stable isotopes contributed substantially (~60%) to the observed differences among rivers in all river-specific contrasts. Similarly, glycogen was responsible for ~28% of the observed differences in 5 of the 6 river-specific contrasts. Three variable combinations accounted for 54–78% of the separation between species or among rivers, but the precise variables involved differed among species- and river-specific contrasts.

DISCUSSION

Stable Isotope Analyses

Based solely on stable isotope signatures, prior studies suggest that mussels appear to feed on similar resources even

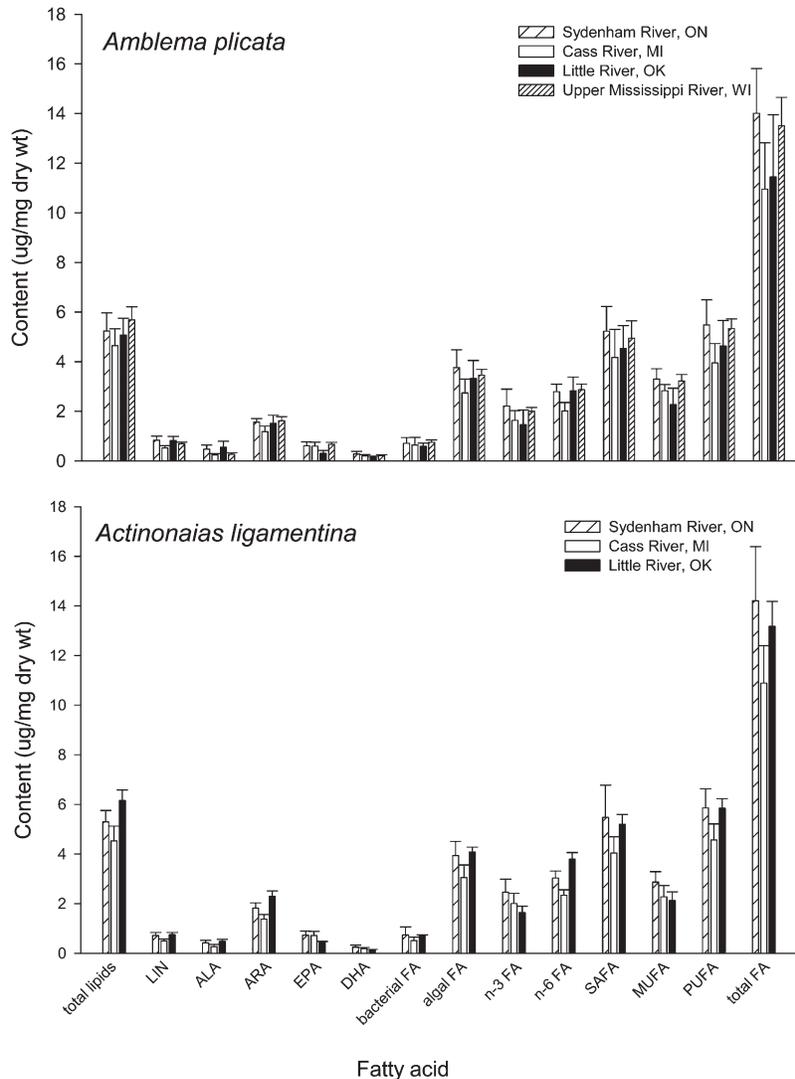


Figure 2. Mean percent total lipids and fatty acid (FA) content (measured in micrograms per milligram dry weight ± 1 SD) in *Amblema plicata* and *Actinonaias ligamentina* mussels from 4 rivers in North America. Bacterial FAs are the sum of 15:0, 15:0i, 15:0ai, and 17:0. Algal FAs are the sum of 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, and 22:6n-3. MUFA, monounsaturated FA; PUFA, polyunsaturated FA; SAFA, saturated FA.

in different ecosystems and seasons (Nichols & Garling 2000, Christian et al. 2004, Howard et al. 2005). However, given what we know about mussels and potential dietary sources in the wild, it is unlikely that individuals of several species across small streams to large rivers are feeding on the same resources (Hamilton et al. 2004, Delong 2010). This suggests that either stable isotopes are not sensitive enough to identify dietary sources, that previous studies were unable to identify and sample potential food sources, or that food source signatures change in a way that we do not yet understand. In particular, prior studies have been largely unable to separate detrital, fungal, and microbial food sources within biofilm matrices (Cross et al. 2005).

In our study, stable isotope signatures were able to separate mussels among rivers and, to a lesser extent, between species. Similar to prior studies (Nichols & Garling 2000, Christian et al. 2004), our data suggest that mussels are depleted in ^{13}C compared with the food sources that are usually thought of as mussel food (i.e., phytoplankton). This suggests that either

additional highly ^{13}C -depleted foods (e.g., methanotrophic bacteria) are being consumed by mussels or that mussels selectively assimilate ^{13}C -depleted fractions from a heterogeneous FPOM pool (Strayer 2008), but see discussion on bacterial fractionation later).

Elevated $\delta^{15}\text{N}$ values can indicate assimilation of microbially enriched materials (e.g., Goedkoop et al. 2006); thus, the more enriched $\delta^{15}\text{N}$ values seen in mussels from the Little River suggest that these mussels might be more efficient at assimilating microbial biomass than mussels in the other rivers or that bacterial loads are higher in this river. However, microbial assimilation of inorganic N may influence the $\delta^{15}\text{N}$ content of microbes, thereby complicating interpretation of $\delta^{15}\text{N}$ as a trophic tracer (Caraco et al. 1998). Differences in $\delta^{15}\text{N}$ values of mussels across rivers may reflect differential use of inorganic N by microbes or different ^{15}N content in inorganic N (e.g., from different amounts of fertilizers), as well as in the food web or in mussel diets. Other studies have demonstrated higher $\delta^{15}\text{N}$ values in consumers from agricultural or urban watersheds

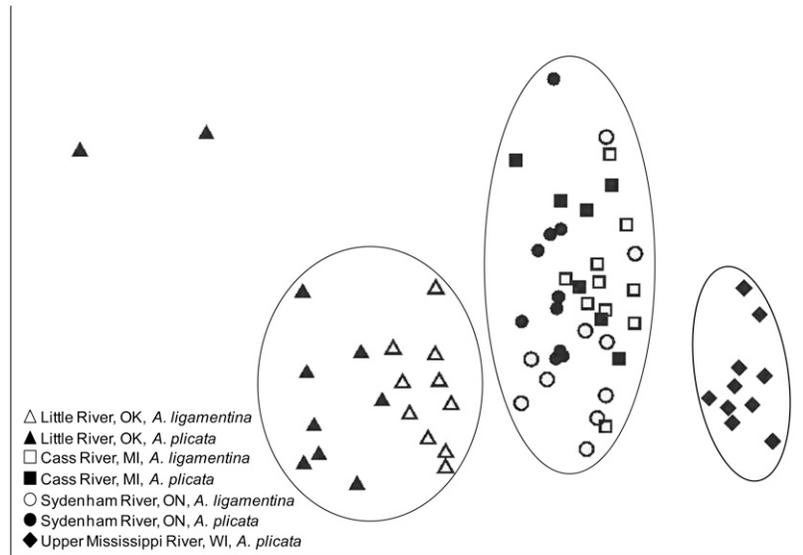


Figure 3. Nonmetric multidimensional scaling plot of biochemical tracers in *Amblema plicata* and *Actinonaias ligamentina* mussels from 4 North American Rivers. 2D stress, 0.15. Ellipses were drawn by hand to illustrate river differences.

(e.g., Vander Zanden et al. 2005); thus, greater $\delta^{15}\text{N}$ values in the Little River may reflect anthropogenic inputs from the watershed.

A compilation of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ data across 17 mussel species in 14 rivers suggests that dietary components vary by system more than by species (Fig. 4). Previous $\delta^{15}\text{N}$ data (e.g., Vander Zanden & Rasmussen 1999) suggest that mussels in some oligotrophic habitats, such as subalpine lakes, are consumers of primary production. However, mussels in more productive habitats appear to feed across multiple trophic levels and to eat primary producers and various consumer groups (Nichols & Garling 2000). Data regarding $\delta^{13}\text{C}$ suggest that mussels feed heavily on bacteria and phytoplankton in small temperate streams (Nichols & Garling 2000, Raikow & Hamilton 2001, Christian et al. 2004), whereas they appear to feed largely on phytoplankton in large productive rivers such as the Mississippi (Thorp et al. 1998). However, our lack of knowledge regarding the potential contribution of highly depleted methanotrophs or the selective assimilation of materials from heterogeneous pools makes it unclear what mussels assimilate.

Much of the separation in dietary components in our study was driven by large differences in $\delta^{34}\text{S}$ values among rivers. In particular, mussels from the Little River, Oklahoma, were enriched with ^{34}S compared with the other 3 rivers. A paper mill ~20 km upstream from our site in the Little River is the likely source of S. Variation in $\delta^{34}\text{S}$ can indicate loadings of $\delta^{34}\text{S}$ -enriched sodium sulfate used in the digestion of pulp (Dube et al. 2006). Wayland and Hobson (2001) also reported a consistent pattern of $\delta^{34}\text{S}$ enrichment in food chain components from upstream to downstream in 2 rivers receiving effluent from pulp and paper mills.

Ecological Stoichiometry

Stoichiometry (i.e., ratios of C, N, and P) is the study of an organism’s balance between the availability of multiple limiting

elements within the environment and its physiological requirements for metabolism. If nutrient availability is imbalanced with respect to diet, consumers should retain as much of the limiting nutrient as possible, while excreting the nutrients that are in excess (Sterner & Elser 2002). Measuring these ratios is useful to understand how different species assimilate C and N differentially to obtain what they need from their food. In the current study, the molar C:N ratio varied little between species or among rivers (range, 4.2–4.6). These values are similar to those reported in other mussel species in other systems. For example, Christian et al. (2008) reported a mean C:N ratio of

TABLE 4.

Results of nonmetric multidimensional scaling analysis of biochemical tracers in 2 mussel species (*Actinonaias ligamentina*, *Al*, and *Amblema plicata*, *Ap*) in 4 North American Rivers (Little River, OK; Cass River, MI; Sydenham River, ON; and Upper Mississippi River, WI).

Contrast	Global R	Variable 1		Variable 2		Variable 3		Sum (%)
		%	Variable	%	Variable	%	variable	
Ap-Al	0.24	27.7	Glycogen	15.7	C:N	14.6	$\delta^{15}\text{N}$	58.0
OK-MI	0.86	30.7	$\delta^{34}\text{S}$	16.2	$\delta^{15}\text{N}$	13.7	Glycogen	60.6
OK-ON	0.82	21.3	$\delta^{34}\text{S}$	18.1	$\delta^{15}\text{N}$	14.6	$\delta^{13}\text{C}$	54.0
OK-WI	0.95	39.4	$\delta^{15}\text{N}$	23.8	$\delta^{34}\text{S}$	7.1	Glycogen	70.3
MI-ON	0.44	32.2	Glycogen	11.3	$\delta^{13}\text{C}$	10.2	C:N	53.7
MI-WI	0.91	35.1	$\delta^{13}\text{C}$	18.2	Glycogen	14.1	C:N	67.4
ON-WI	0.95	55.4	$\delta^{13}\text{C}$	13.9	Glycogen	9.0	$\delta^{15}\text{N}$	78.3

A global R value equal to 0 suggests that the similarity within groups equals the similarity among groups, whereas groups of values that have nothing in common have an R value equal to 1. Variables contributing to the separation between species or among rivers are also reported (e.g., glycogen concentration is responsible for 28% of the separation between species). $P > R$ for all contrasts was 0.001.

FAs were unable to explain differences in mussel assemblages between species or among rivers. Patterns in n-3 FAs, n-6 FAs, SAFAs, MUFAs, PUFAs, and total FA contents suggest that the *quantity* of food available to mussels was generally lowest in Michigan and Oklahoma, and highest in Ontario, regardless of species. Similarly, EPA and DHA contents suggest that the *quality* of available food was generally poorest in mussels from Oklahoma and highest in mussels from Ontario.

The high content of ARA, relative to DHA and EPA, in freshwater mussels was notable. In the current study, ARA contents ranged from 11–18% of the total FA pool in mussels, which compares favorably to values of ~25% in *Lampsilis siliquoidea* and *Lampsilis cardium* in Lake St. Clair (M. Arts, unpubl. data). Similarly high concentrations of ARA have been found in other freshwater bivalves (Pollero et al. 1981, Hagar & Dietz 1986). These data are in contrast to marine bivalves and other freshwater invertebrates that usually have much lower concentrations of ARA, relative to EPA or DHA. For example, a survey of FAs in 58 genera of aquatic insects reported ARA concentrations that ranged from only 0.4–7% of the total FAs (Hanson et al. 1985, Ahlgren et al. 2009). Similarly, n-6 FAs (including ARA) were a minor component of the FA pool in nonbivalve macroinvertebrate consumers in a third-order stream (Torres-Ruiz et al. 2007).

It is unclear why freshwater mussels contain high contents of ARA. Mussels could get ARA through 2 processes. First, they could obtain ARA from their diet, but because ARA concentrations generally increase as a function of body size (e.g., Kainz et al. 2004), algae contain low ARA concentrations. Also, although some marine bacteria (Nichols et al. 1997) can synthesize ARA, freshwater bacteria are not known to produce ARA. Second, mussels may be able to produce ARA by elongation and desaturation of LIN (18:2n-6), an FA that is found in many green algae and cyanobacteria species (Ahlgren et al. 1992). However, there is still debate on how effective this bioconversion process may be. Some researchers suggest that it is a common, albeit inefficient, process in animals (Cook & McMaster 2002), and others have concluded that although animals can desaturate and elongate shorter chain PUFAs to highly unsaturated fatty acids the process is often insufficient to meet the organism's needs (e.g., Gonzalez-Baro & Pollero 1988). Interestingly, Hagar and Dietz (1986) found that ARA concentrations in freshwater mussels do not vary seasonally, suggesting that ARA is actively regulated at high levels, perhaps as a result of its function as a precursor for prostaglandin synthesis. Kainz et al. (2010) also documented high retention of ARA during fasting in the benthic amphipod *Diporeia* spp.

Much of the interest in ARA stems from its known functions in physiological processes. For example, ARA is required for cortisol formation—a compound that allows fish to mitigate stress (Koven et al. 2001). However, given that our samples came from diverse, dense, and reproducing mussel beds, we assume that high levels of ARA are not the result of overly stressed mussels. Unfortunately, we know little about intra- and interspecific interactions in mussels, so it is difficult to speculate whether there is resource facilitation and/or competition occurring in dense mussel beds (Vaughn et al. 2007, Spooner & Vaughn 2009). In marine bivalves, ARA has been associated with the development of gametogenesis and initiation of egg release (Soudant et al. 1996). The reproductive periods of

Amblema plicata and *Actinonaias ligamentina* are roughly May to July and August to May, respectively. Although *A. ligamentina* may have been undergoing gametogenesis at the time of sampling (and perhaps *A. plicata* in Oklahoma), the high levels of ARA seen in *A. plicata* and other species suggest that reproduction alone cannot explain the elevated levels of ARA in freshwater mussels. In other mussel species, high retention of ARA throughout the year was hypothesized to result from its importance in prostaglandin synthesis (Hagar & Dietz 1986). Mussels are much longer lived than the other freshwater invertebrates that have been studied; perhaps high levels of ARA in mussels are related to their long life span, such that mussels could conceivably accumulate a much higher proportion of ARA than is normally present in their diet. It is unclear at this time why freshwater mussels have high levels of ARA, but it may involve its importance in maintaining physiological processes. It is clear, however, that the high concentrations of this essential FA in mussels constitute a rich source of ARA for their consumers.

Fatty acids are useful as source-specific biomarkers because it is often possible to quantify algal, bacterial, and allochthonous-derived FA compounds (Kainz & Fisk 2009). The n-3:n-6 FA ratio has been used as a putative marker of the relative amount of terrestrial (<1) or aquatic (>1) food sources (Desvillettes et al. 1994). Our low n-3:n-6 ratio (range, 0.4–0.8) suggests a diet rich in n-6 FAs (likely driven by the high ARA content). In a study of macroinvertebrates in a small stream, Torres-Ruiz et al. (2007) reported n-3:n-6 ratios greater than 1 in all consumers. Similarly, Wacker and von Elert (2004) observed n-3:n-6 ratios of 2.8–5.8 in *Dreissena polymorpha*. In a review of n-3:n-6 ratios in freshwater zoobenthos, deposit-feeding species often had lower ratios (i.e., <1) than organisms with other feeding modes (Ahlgren et al. 2009). Perhaps this marker is sensitive to the feeding ecology of an organism that may limit the utility of this ratio in unionid mussels at this time.

In summary, dietary tracers in unionid mussels varied more among rivers than between species. This suggests that mussels might be considerably adaptable in their dietary requirements. We suggest that what may be most important in these rivers is that there is enough food (be it algae or bacteria) rather than the particular type of food, although this hypothesis needs to be tested in other systems, species, tissues, and over time. Nutritional balance and food requirements vary seasonally, and unionid mussels must satisfy needs for maintenance metabolism, reproduction, and somatic growth. These results can also be used to understand the cycling of nutrients in river systems and how unionid mussels might mitigate this process. Efforts to restore and conserve unionid populations will require more in-depth information on food–resource linkages among mussel species and between the mussel community and its surrounding ecosystem. Restoration of dense and diverse mussel assemblages would help reestablish their role in river food webs (i.e., nutrient cycling) to the benefit of other biota.

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