Journal of Molluscan Studies

The Malacological Society of London

Journal of Molluscan Studies (2013) **79**: 163–167. doi:10.1093/mollus/eyt008

SHIFTS IN STABLE-ISOTOPE SIGNATURES CONFIRM PARASITIC RELATIONSHIP OF FRESHWATER MUSSEL GLOCHIDIA ATTACHED TO HOST FISH

MARK W. FRITTS[1,3], ANDREA K. FRITTS[1], SCOTT A. CARLETON[2] AND ROBERT B. BRINGOLF[1]

[1]*Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA 30602, USA;*
[2]*U.S. Geological Survey, New Mexico Cooperative Fish and Wildlife Research Unit, New Mexico State University, Las Cruces, NM 88003, USA; and*
[3]*Present address: Illinois Natural History Survey, Illinois River Biological Station, Havana, IL 62644, USA*

Correspondence: R.B. Bringolf; e-mail: bringo@uga.edu

(Received 29 August 2012; accepted 12 February 2013)

ABSTRACT

The parasitic nature of the association between glochidia of unionoidean bivalves and their host fish (i.e. the role of fish hosts in providing nutritional resources to the developing glochidia) is still uncertain. While previous work has provided descriptions of development of glochidia on fish hosts, earlier studies have not explicitly documented the flow of nutrition from the host fish to the juvenile mussel. Therefore, our objective was to use stable isotope analysis to quantitatively document nutrient flow between fish and glochidia. Glochidia were collected from nine adult *Lampsilis cardium* and used to inoculate *Micropterus salmoides* ($n = 27$; three fish per maternal mussel) that produced juvenile mussels for the experiment. Adult mussel tissue samples, glochidia, transformed juvenile mussels and fish gill tissues were analysed for δ^{15}N and δ^{13}C isotope ratios. We used a linear mixing model to estimate the fraction of juvenile mussel tissue derived from the host fish's tissue during attachment. Our analyses indicate a distinct shift in both C and N isotopic ratios from the glochidial stage to the juvenile stage during mussel attachment and development. Linear mixing model analysis indicated that 57.4% of the δ^{15}N in juvenile tissues were obtained from the host fish. This work provides novel evidence that larval unionoideans are true parasites that derive nutrition from host fish during their metamorphosis into the juvenile stage.

INTRODUCTION

Freshwater mussels (Unionoidea) are characterized by complex life cycles composed of multiple, distinct life stages, including a parasitic larval stage referred to as a glochidium. After fertilization, the embryonic mussels are retained in specialized maternal gill structures, marsupia, until their development is complete and they are ready to be released as glochidia (Wächtler, Dreher-Mansur, & Richter, 2001). Following their release from the marsupium, glochidia must attach to a suitable host (usually a fish) to facilitate their metamorphosis into the juvenile mussel stage (Arey, 1921; Coker *et al.*, 1921). A detailed explanation of glochidial metamorphosis has been given by Chumnanpuen *et al.* (2011). The evolution of larval attachment in Unionoidea is understood to have begun as a phoretic relationship in which larvae attached to host fish gained the selective advantage of dispersal to new habitats (Watters, 2001). However, the parasitic nature of larval freshwater mussels and, specifically, the role that the fish hosts play in providing nutritional resources to the developing glochidia, is still uncertain (Kat, 1984; Barnhart, Haag, & Roston, 2008).

In recent years, the global decline of freshwater mussel populations has spurred new efforts to understand the complex life cycles of these species. However, glochidial metamorphosis to the juvenile stage has been studied less than other periods of development. Fisher & Dimock (2002a, b) provided descriptions of the changing morphology and biochemical composition of developing *Utterbackia imbecillis*. The mantle cells of larval *U. imbecillis* resemble the digestive cells of other adult bivalves (Owen, 1970, 1973; Pal, 1972; Robledo & Cajaraville, 1996; Fisher & Dimock, 2002a) and are therefore likely to be capable of deriving nutrition from host fish tissues. However, unlike those of other unionoideans, the glochidia of *U. imbecillis* are reported to be capable of direct development without attachment to a host (Lefevre & Curtis, 1911) and may exhibit morphological and physiological idiosyncrasies not found in the larvae of other species. Although glochidia of some taxa (e.g. Margaritiferidae) are known to grow substantially during

encystment, to date no other studies have investigated the topic of nutrient transfer between mussel larvae and host fish in a more typical unionoidean species that is only capable of metamorphosis while attached to a suitable host.

Stable-isotope analysis (SIA) is a method increasingly used to document parasitic relationships in aquatic organisms (Doucett, Giberson & Power, 1999; Pinnegar, Campbell & Polunin, 2001; Deudero, Pinnegar & Polunin, 2002; Fry, 2008; Doi *et al*., 2010). Stable isotopes of nitrogen (δ^{15}N) and carbon (δ^{13}C) are commonly used for assessing trophic relationships in ecological studies (Polunin & Pinnegar, 2002). Differences among δ^{13}C values observed in carbon-fixing organisms can be used to evaluate the source of primary productivity supporting species at higher trophic levels in food webs (France, 1995). Normally, the δ^{13}C values of consumers are 1–2‰ higher than those of their diet (DeNiro & Epstein, 1978; Post, 2002). Values of δ^{15}N typically increase by 2–5‰ with each corresponding increase in trophic position, thereby providing an indication of the trophic level of organisms in a community (DeNiro & Epstein, 1981; Minagawa & Wada, 1984; Vander Zanden, Cabana, & Rasmussen, 1997; Vander Zanden *et al*., 1999; Post, 2002). In previous studies, the increase, or enrichment, of isotopes in consumer tissues compared with that of its diet is attributed to the increased retention of the heavier isotopes during specific biochemical processes (e.g. protein amination and deamination for δ^{15}N and respiration for δ^{13}C (DeNiro & Epstein, 1978, 1981; Macko *et al*., 1986)). However, these trends in δ^{13}C and δ^{15}N fractionation have not been consistent across studies of common aquatic parasites (Boag *et al*., 1998; Pinnegar *et al*., 2001; Deudero *et al*., 2002; Doi *et al*., 2010). Pinnegar *et al*. (2001) reported that four taxa of fish parasites (Cestoda, Nematoda, Isopoda and Copepoda) were consistently lower in δ^{15}N with respect to host fish tissue. Description of patterns of isotopic fractionation between additional host organisms and their parasites could offer new insights in studies of isotopic ecology, especially the fundamental biochemical processes that govern the distribution of isotopes in ecosystems.

Because previous studies have not explicitly documented the flow of nutrition to developing glochidia, our objective was to use stable isotope analysis to quantitatively describe the flow of nutrients between fish hosts and mussel glochidia.

MATERIAL AND METHODS

Tissue collection and production of juveniles

Adult *Lampsilis cardium* (Rafinesque, 1820) were collected from Pool 8 of the Mississippi River near La Crosse, Wisconsin, USA, in October 2011. Brooding female mussels were placed in river water in aerated coolers and transported to the United States Geological Survey Upper Midwest Environmental Science Center where they were held in aerated well water (13–15°C) and fed a continuous supply of a combination of concentrated microalgae diets (Reed Mariculture Instant Algae Nanno 3600 and Reed Mariculture Instant Algae Shellfish Diet 1800). The mussels were shipped to the Aquatic Science Laboratory at the University of Georgia on 28 November 2011; tissue collection and the inoculation of host fish with glochidia occurred 2 d after arrival.

Nine female mussels were used for this experiment. Glochidia were extracted from each mussel and subsamples were retained for stable isotope analysis; tissue biopsies were also extracted from the maternal donor's foot and gill. Glochidia from each female mussel were used to inoculate three *Micropterus salmoides* (Lacépède, 1802) ($n = 27$; three fish per maternal mussel). The host fish were inoculated by immersion in a suspension of 4000 glochidia per litre of water. Fish were placed in the inoculation bath for 15 min, while the glochidia were kept in suspension with the use of a large rubber-bulb pipette and vigorous aeration. Upon completion of the inoculation, fish were removed from the bath and placed into individual tanks for monitoring. Host fish were inoculated with glochidia on 30 November 2011 and monitored daily for release of juveniles for a four-week period. Fish were housed in a modified recirculating aquaculture system (AHAB®; Aquatic Habitats Inc., Apopka, FL, USA), with the outflow from each tank equipped with a 153-μm mesh filter cup to recover juvenile mussels released from the fish. Metamorphosed juveniles were collected and preserved for stable-isotope analysis. Once the metamorphosis period was complete and microscopic analysis ensured that no encysted glochidia remained on the fish gill tissue, the host fish were euthanized and their gills removed for analysis. As a control, three additional naïve fish were reared in the same AHAB system and gill tissues were preserved for analysis. Replicates of juveniles and fish gill tissue for each respective mussel donor ($n = 9$) were pooled at this time. Water temperature, dissolved oxygen and pH in each fish holding system were measured with a Hydrolab Quanta (Hach Hydromet, Loveland, CO, USA) and recorded daily. Ammonia concentrations were monitored weekly using a LaMotte colorimeter (LaMotte Co., Chestertown, MD, USA). All water chemistry parameters were maintained within suitable levels for aquatic organisms throughout the duration of the study (temperature = 21–22°C, dissolved oxygen = 7.5–8.2 mg/l, pH = 6.9–7.6, total ammonia ≤ 0.1 mg/l.

Stable-isotope analysis

Prior to stable-isotope analysis, all adult mussel tissue samples, glochidia, transformed juvenile mussels and fish gill tissue were freeze-dried for >48 h in a Labconco 4.5 freeze-dryer (Labconco Co., Kansas City, MO, USA). The dried samples were pulverized with a handheld glass stirring rod. A ~1 μg sub-sample of each sample was weighed and sealed in a tin capsule for analysis of ^{15}N/^{14}N and ^{13}C/^{12}C ratios by a Thermo Finnigan DeltaPlus mass-spectrometer (Thermo Finnigan, San Jose, CA, USA) at the Odum School of Ecology, University of Georgia.

By convention, C and N isotope ratios are expressed in delta notation (δ), the deviation from standards in parts per thousand (‰), according to the following equation:

$$\delta X = \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}}\right) - 1\right] \times 10^3, \quad (1)$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio ^{13}C/^{12}C or ^{15}N/^{14}N. Values were normalized to the international isotope standards, PeeDee Belemnite (PDB) carbonate for CO_2 and atmospheric nitrogen (AIR) for N_2. Analytical error reported by the Analytical Chemistry Laboratory, Odum School of Ecology, University of Georgia, was ±0.2‰ for δ^{13}C and ±0.15‰ for δ^{15}N.

The observed values of both δ^{13}C and δ^{15}N were non-normally distributed. We therefore used a Kruskal-Wallis test to determine whether there were any differences among maternal tissues, glochidia, juvenile mussels, fish gill tissue and fish feed. We then used a Mann–Whitney U-test ($\alpha < 0.05$ as significance criterion) to facilitate pairwise comparisons between individual groups.

We calculated the isotopic difference between an animal's tissue and their diet as:

$$\Delta X = \delta X_{\text{Consumer}} - \delta X_{\text{Diet}}, \quad (2)$$

where X is ^{13}C or ^{15}N.

We used a linear mixing model to estimate the fraction of juvenile mussel tissue derived from the host fish's tissue during attachment (Carleton, Wolf & Martinez del Rio, 2004).

$$\delta X_{\text{juvenile}} = (P)(\delta X_{\text{Glochidia}}) + (1 - P)(\delta X_{\text{fish}}), \quad (3)$$

where X is ^{15}N. This model estimates the fraction (P) contributed from the glochidia, with an isotope composition equal to $\delta X_{\text{Glochidia}}$, relative to the fraction ($1-P$) contributed by the host fish, with an isotope composition equal to δX_{Fish} so that:

$$P = \frac{\delta X_{\text{Juvenile}} - \delta X_{\text{Fish}}}{\delta X_{\text{Glochidia}} - \delta X_{\text{Fish}}}, \quad (4)$$

where X is ^{15}N. Because shell carbonate was not removed from glochidia or juvenile mussel samples, we were unable to separate the influence of shell C on the ^{13}C value of larval and juvenile mussel tissue from host tissue. Therefore, mixing model analysis was not applied to carbon isotope values derived from glochidia and juvenile tissue samples.

RESULTS

The first juvenile mussels were recovered 12 days after inoculation. Peak collection of juveniles occurred 18 days after inoculation and the last of the juveniles were collected 26 days post-inoculation. All host fish survived the period of juvenile mussel metamorphosis. Results of the Kruskal–Wallis test for both δ^{13}C and δ^{15}N indicated significant differences among the four tissues evaluated ($\chi^2_4 = 33.7464$, $P < 0.0001$) (Table 1). Pairwise comparisons of individual sample groups showed a statistically significant difference ($P < 0.05$) in δ^{13}C and δ^{15}N among all samples except maternal foot and gill tissues ($P = 0.59$ in both comparisons). Maternal foot and gill tissues were combined into a single group (termed 'maternal donor tissues') in subsequent analyses. A separate analysis determined that there was no significant difference between isotopic values of naïve host fish and those fish inoculated with glochidia ($P = 0.48$).

Carbon-isotope ratios were lowest in maternal donor tissues (expressed $\delta X \pm 1$ SD ‰; -29.5 ± 0.50‰). The glochidia extracted from brooding female mussels were higher in δ^{13}C compared with maternal tissues (-25.1 ± 1.46‰). Following metamorphosis on the host fish's gills, we observed a statistically significant change ($n = 9$; $z = -3.5321$; $P = 0.0004$) in δ^{13}C in juvenile mussels (-19.6 ± 0.36‰) from that of the original glochidia. These transformed juvenile mussels more closely resembled δ^{13}C in host-fish gill tissues (-20.1 ± 0.23‰) (Fig. 1).

The δ^{15}N values of glochidia (13.06 ± 0.59‰) were higher in comparison to maternal donor tissues (12.1 ± 0.48‰).

Table 1. δ^{13}C and δ^{15}N values (mean ± SD) of maternal mussel (*Lampsilis cardium*) tissues, glochidia, juvenile mussels, fish gill tissue and fish feed.

Tissue	*n*	δ^{15}N ± 1 SD	δ^{13}C ± 1 SD
Maternal donor	9	12.12 ± 0.48	−29.49 ± 0.50
Glochidia	9	13.06 ± 0.59	−25.09 ± 1.46
Juvenile mussels	9	10.69 ± 0.59	−19.56 ± 0.36
Host fish gill tissues	9	8.93 ± 0.14	−20.05 ± 0.23
Naïve fish gill tissues	3	9.11 ± 0.34	−19.79 ± 0.30
Fish feed	2	6.00 ± 0.08	−21.22 ± 0.06

Kruskal-Wallis test results for both δ^{13}C and δ^{15}N indicated that there were significant differences among the four tissues ($X^2_4 = 33.7464$, $P < 0.0001$) and pairwise comparisons of individual sample groups showed a statistically significant difference ($P < 0.05$) in δ^{13}C and δ^{15}N among all samples.

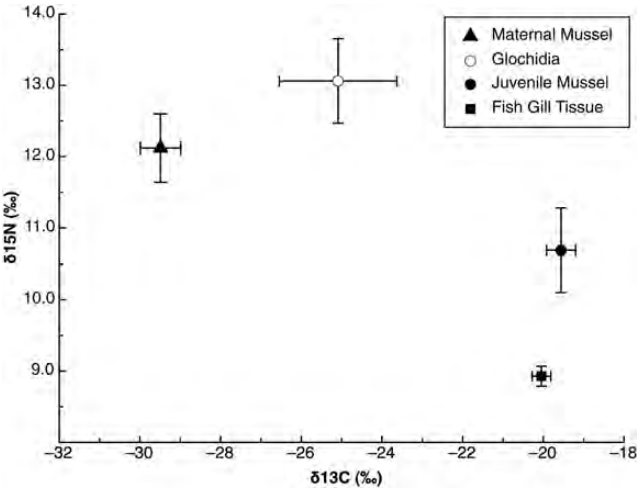

Figure 1. δ^{13}C and δ^{15}N values (mean ± SD) of maternal mussel (*Lampsilis cardium*) tissues (filled triangle), glochidia (open circle), juvenile mussels (closed circle) and fish gill tissue (closed square). Maternal mussel values are the average of isotopic values observed for gill and foot tissue.

Following metamorphosis, juvenile mussels exhibited a significant shift in δ^{15}N (10.7 ± 0.59‰) from glochidia ($n = 9$; $z = -2.6063$; $P = 0.008$) and were higher relative to fish gill tissues (8.93 ± 0.14‰) (Fig. 1). Linear mixing model analysis indicated that 57.4% ± 4.1 of the δ^{15}N in juvenile tissues was obtained from the host fish.

DISCUSSION

A distinct shift occurred in C and N isotopic ratios from the glochidial stage to the juvenile stage during development of *Lampsilis cardium*. This work strengthens the evidence that glochidia are true parasites that derive nutrition from a host fish during their metamorphosis into the juvenile stage. We have also demonstrated the utility of stable-isotope analyses to assess nutritional sources during the unionid life cycle.

The δ^{13}C values of the maternal mussel tissues (*c.* −29‰) are indicative of a diet composed primarily of suspended algae (C_3 plant, O'Leary, 1988) and bacteria (Nichols & Garling, 2000). However, the higher δ^{13}C values of glochidia (*c.* −25‰) may represent the influence of a number of different geochemical and physiological factors. Previous studies have indicated that glochidia derive nutrition via some combination of lecithotrophy (consumption of egg cytoplasm) and matrotrophy (direct exchange of nutrition via interlamellar septa) (Wood, 1974; Schwartz & Dimock, 2001). The morphology of unionid gills appears to facilitate the transfer of nutrition to glochidia (Schwartz & Dimock, 2001); Silverman, Steffens & Dietz (1985) and Silverman, Kays, & Dietz (1987) demonstrated that 90% of the total calcium in the developed glochidial valve was derived from maternal gill tissues. Observed carbon-isotope ratios in glochidia could also be attributed to the presence of carbonates in the valves of the developing glochidia. Fry & Allen (2003) documented enriched δ^{13}C signatures in *Dreissena polymorpha* shells (*c.* −9‰) and additional studies suggest that aquatic invertebrates usually produce carbonates that resemble ambient dissolved inorganic carbon (DIC) pools (McConnaughey *et al.* 1997, Dettman, Reische & Lohmann, 1999; McConnaughey & Gillikin, 2008). The removal of inorganic carbon from samples of aquatic invertebrates via acidification is a common practice in many stable-isotope studies as a means of selectively analysing dietary

(organic) carbon (Jacob *et al*., 2005). However, the removal of inorganic carbon from glochidia and juvenile samples in this study was not feasible given the very small masses (<0.5 μg) collected for analysis. Future studies should investigate the relative ^{13}C contribution of maternally derived nutritional sources (lecithotrophy and matrotrophy) and ambient DIC pools. Furthermore, future research should be designed to investigate the relative contribution of matrotrophy to the development of mussel species with both short-term and long-term brooding periods.

The relative $\delta^{15}N$ enrichment documented in maternal and glochidia tissues likely reflects the influence of industrial and municipal effluents and agricultural runoffs deposited into the upper Mississippi River (Fry & Allen, 2003). The $\delta^{15}N$ observed between maternal tissues and glochidia, and between fish tissues and juvenile mussels, is roughly +1 and +1.75‰, respectively, and is within accepted ^{15}N fractionation patterns representative of the isotopic shift (Δ15N) reported between trophic levels (Post, 2002). Martinez del Rio *et al*. (2009) suggests that $\Delta^{15}N$ should be lower in animals that are actively growing because of the increased retention of dietary proteins. Glochidia are actively developing both within the female marsupium and after attachment to a host fish (Schwartz & Dimock, 2001; Fisher & Dimock, 2002b). Furthermore, Kreeger, Hawkins & Bayne (1996) demonstrated that adult marine mussels (*Mytilus edulis*) were nutritionally limited in amino N and that most dietary protein was completely metabolized to meet anabolic demands. Given these findings, it is conceivable that the relatively low $\Delta^{15}N$ in glochidia and juvenile mussels is attributable to their high growth rates and increased retention of dietary protein. The relative variability of $\Delta^{15}N$ between hosts and parasites observed in previous studies may be attributable to a range of different assimilation rates of dietary N in individual parasite species and highlights the need for future experiments to investigate variations of $\Delta^{15}N$ among a wider range of biota.

Two testable hypotheses provide explanations for the enriched $\delta^{15}N$ signature of juvenile mussels relative to the gill tissues of the host fish (Fig. 1). First, the enriched value could be driven by the rate at which developing glochidia grow new tissues and turnover maternally inherited tissues. Tissue turnover varies widely between tissues within an organism, across taxa, and differs according to whether replacement occurs solely through catabolic tissue replacement or through a combination of new tissue growth and catabolism (Dalerum & Angerbjörn, 2005; Carleton & Martinez del Rio, 2010). Despite the fact that glochidia are rapidly growing, adding new tissues derived from the fish host, and replacing maternally inherited tissues through catabolic tissue turnover, results from the isotopic analysis and the linear mixing model indicate that glochidia are not attached to the fish for long enough to allow for complete turnover of maternal $\delta^{15}N$ (Fig. 1). Second, because the isotopic shift in our study is from an enriched to a more depleted value (maternal to host tissue), it is difficult to ascertain if the enriched $\delta^{15}N$ value of juvenile mussels relative to their host is representative of the isotopic shift observed between trophic levels (Post, 2002). Indeed, the difference observed in this study ($\Delta^{15}N = 1.76 \pm 0.60$) between the juvenile mussel and the fish host is within the realm of reported values for trophic isotope shifts (Post, 2002). Since the mussel is essentially feeding on its host's tissue, it is plausible that the value we measured represents a trophic level shift in $\delta^{15}N$. It would be relatively easy to resolve these two competing hypotheses by designing a study where the shift from glochidia to juvenile mussel is from a depleted to an enriched isotopic value instead of an enriched to depleted as we have done in this study. Regardless of which hypothesis is ultimately supported, our $\delta^{15}N$ results clearly demonstrate that developing glochidia derive nutrients from their host fish.

Beyond the morphological work of previous researchers documenting the digestion of host fish tissue (Fisher & Dimock, 2002a), this experiment has provided the first successful documentation of nutritional exchange between host fish and the parasitic larvae of a freshwater mussel species. However, the significance of these findings is limited by the fact that only one mussel species was used for these analyses. Future studies should use additional unionoidean species to determine whether observed isotopic trends are consistent among species that exhibit different glochidial morphologies and growth patterns. There is a need for future studies to assess the dynamics of isotopic incorporation in specific tissues of both adult and larval freshwater mussels. Future research should also investigate the effects of diet and food quality on fractionation patterns in individual tissues in adult mussels. Additional efforts should be devoted to determining the relative contribution of dietary carbon and assimilated DIC on the δC^{13} values of molluscan tissues. Controlled studies of isotopic incorporation will facilitate the use of stable-isotope analyses to investigate the diets of freshwater mussels, especially regarding the selective digestion or evacuation of individual food sources.

ACKNOWLEDGEMENTS

We are grateful for the assistance of T. Newton and A. Ganser for assistance in obtaining mussels, Owens & Williams Fish Hatchery for supplying largemouth bass, and T. Maddox for isotopic analyses. Funding was provided by the Warnell School of Forestry and Natural Resources at the University of Georgia.

REFERENCES

AREY, L.B. 1921. An experimental study on glochidia and the factors underlying encapsulation. *Journal of Experimental Zoology*, **33**: 463–499.

BARNHART, M.C., HAAG, W.R. & ROSTON, W.N. 2008. Adaptations to host infection and larval parasitism in Unionoida. *Journal of the North American Benthological Society*, **27**: 370–394.

BOAG, B., NEILSON, R., ROBINSON, D., SCRIMGEOUR, C.M. & HANDLEY, L.L. 1998. Wild rabbit host and some parasites show trophic level relationships for δ13C and δ15N: a first report. *Isotopes in Environmental Health Studies*, **34**: 81–85.

CARLETON, S.A. & MARTINEZ DEL RIO, C. 2010. Growth and catabolism in isotopic incorporation: a new formulation and experimental data. *Functional Ecology*, **24**: 805–812.

CARLETON, S.A., WOLF, B.O. & MARTINEZ DEL RIO, C. 2004. Keeling plots for hummingbirds: a method to estimate carbon isotope ratios of respired CO_2 in small vertebrates. *Oecologia*, **141**: 1–6.

CHUMNANPUEN, P., KOVITVADHI, U., CHATCHAVALVANICH, K., THONGPAN, A. & KOVITVADHI, S. 2011. Morphological development of glochidia in artificial media through early juvenile of freshwater pearl mussel, *Hyriopsis (Hyriopsis) bialatus* Simpson, 1900. *Invertebrate Reproduction and Development*, **55**: 40–52.

COKER, R.E., SHIRA, A.F., CLARK, H.W. & HOWARD, A.D. 1921. Natural history and propagation of fresh-water mussels. *Document No. 893. Bulletin of the US Bureau of Fisheries*, **37**: 77–181.

DALERUM, F. & ANGERBJÖRN, A. 2005. Resolving temporal variation in vertebrate diets using naturally occurring stable isotopes. *Oecologia*, **144**: 647–658.

DENIRO, M.J. & EPSTEIN, S. 1978. Influence of diet on the distribution of carbon isotopes in animals. *Geochimica et Cosmochimica Acta*, **42**: 495–506.

DENIRO, M.J. & EPSTEIN, S. 1981. Influence of diet on the distribution of nitrogen isotopes in animals. *Geochimica et Cosmochimica Acta*, **45**: 341–351.

DETTMAN, D.L., REISCHE, A.K. & LOHMANN, K.C. 1999. Controls on the stable isotope composition of seasonal growth bands in aragonitic fresh-water bivalves (Unionidae). *Geochimica et Cosmochemica Acta*, **63**: 1049–1057.

DEUDERO, S., PINNEGAR, J.K. & POLUNIN, N.V.C. 2002. Insights into fish host–parasite trophic relationships revealed by stable isotope analyses. *Diseases of Aquatic Organisms*, **52**: 77–86.

DOI, H., YURLOVA, N.I., VODYANITSKAYA, S.N., KANAYA, G., SHIKANO, S. & KIKUCHI, E. 2010. Estimating isotope fractionation between cercariae and host snail with the use of isotope measurement designed for very small organisms. *Journal of Parasitology*, **96**: 314–317.

DOUCETT, R.R., GIBERSON, D.J. & POWER, G. 1999. Parasitic association of *Nanocladius* (Diptera: Chironomidae) and *Pteronarcys biloba* (Plecoptera: Pteronarcyidae): insights from stable-isotope analysis. *Journal of the North American Benthological Society*, **18**: 514–523.

FISHER, G.R. & DIMOCK, R.V. 2002a. Ultrastructure of the mushroom body: digestion during metamorphosis of *Utterbackia imbecillus* (Bivalvia:Unionidae). *Invertebrate Biology*, **121**: 126–135.

FISHER, G.R. & DIMOCK, R.V. 2002b. Morphological and molecular changes during metamorphosis in *Utterbackia imbecillus* (Bivalvia: Unionidae). *Journal of Molluscan Studies*, **68**: 159–164.

FRANCE, R.L. 1995. Differentiation between littoral and pelagic food webs in lakes using stable carbon isotopes. *Limnology and Oceanography*, **40**: 1310–1313.

FRY, B.J. 2008. *Stable isotope ecology*. Edn 3. Springer, New York.

FRY, B.J. & ALLEN, Y.C. 2003. Stable isotopes in zebra mussels as bioindicators of river-watershed linkages. *River Research and Applications*, **19**: 683–696.

JACOB, U., MINTENBECK, K., BREY, T., KNUST, R. & BEYER, K. 2005. Stable isotope food web studies: a case for standardized sample treatment. *Marine Ecology Progress Series*, **287**: 251–253.

KAT, P.W. 1984. Parasitism and the Unionacea (Bivalvia). *Biological Reviews*, **59**: 189–207.

KREEGER, D.A., HAWKINS, A.J.S. & BAYNE, B.L. 1996. Use of dual-labeled microcapsules to discern the physiological fates of assimilated carbohydrate, protein carbon, and protein nitrogen in suspension-feeding organisms. *Limnology and Oceanography*, **41**: 208–215.

LEFEVRE, G. & CURTIS, W.C. 1911. Metamorphosis without parasitism in the Unionidae. *Science*, **33**: 863–865.

MACKO, S.A., ESTEP, M.F., ENGEL, M.H. & HARE, P.E. 1986. Kinetic fractionation of stable nitrogen isotopes during amino acid transamination. *Geochimica et Cosmochimica Acta*, **50**: 2143–2146.

MARTINEZ DEL RIO, C., WOLF, N., CARLETON, S.A. & GANNES, L.Z. 2009. Isotope ecology ten years after a call for more laboratory experiments. *Biological Reviews*, **84**: 91–111.

McCONNAUGHEY, T.A., BURDETT, J., WHELAN, J.F. & PAULL, C.K. 1997. Carbon isotopes in biological carbonates: respiration and photosynthesis. *Geochimica et Cosmochimica Acta*, **61**: 611–622.

McCONNAUGHEY, T.A. & GILLIKIN, D.P. 2008. Carbon isotopes in mollusk shell carbonates. *Geo-Marine Letters*, **28**: 287–299.

MINAGAWA, M. & WADA, E. 1984. Stepwise enrichment of 15N along food chains: further evidence and the relation between δ15N and animal age. *Geochimica et Cosmochimica Acta*, **48**: 1135–1140.

NICHOLS, S. & GARLING, D. 2000. Food-web dynamics and trophic-level interactions in a multispecies community of freshwater unionids. *Canadian Journal of Zoology*, **78**: 871–882.

O'LEARY, M.H. 1988. Carbon isotopes in photosynthesis. *BioScience*, **38**: 328–336.

OWEN, G. 1970. The fine structure of the digestive tubules of the marine bivalve *Cardium edule*. *Philosophical Transanctions of the Royal Society of London, B. Biological Sciences*, **258**: 245–260.

OWEN, G. 1973. The fine structure and histochemistry of the digestive diverticula of the protobranchiate bivalve *Nucula sulcata*. *Proceedings of the Royal Society of London, B. Biological Sciences*, **183**: 249–264.

PAL, S.G. 1972. The fine structure of the digestive tubules of *Mya arenaria* L. II. Digestive cell. *Proceedings of the Malacological Society of London*, **40**: 161–170.

PINNEGAR, J.K., CAMPBELL, N. & POLUNIN, N.V.C. 2001. Unusual stable isotope fractionation patterns observed for fish host-parasite trophic relationships. *Journal of Fish Biology*, **59**: 494–503.

POLUNIN, N.V.C. & PINNEGAR, J.K. 2002. Trophic ecology and the structure of foodwebs. In: *The handbook of fish biology and fisheries. Vol. 1* (P.J.B. Hart & J.D. Reynolds, eds), pp. 301–320. Blackwell Science, Oxford.

POST, D.M. 2002. Using stable isotopes to estimate trophic position: models, methods, and assumptions. *Ecology*, **83**: 703–718.

ROBLEDO, Y. & CAJARAVILLE, M.P. 1996. Ultrastructural and cytochemical study of the Golgi complex of molluscan (*Mytilus galloprovincialis*) digestive cells. *Cell & Tissue Research*, **284**: 449–458.

SCHWARTZ, M.L. & DIMOCK, R.V. 2001. Ultrastructural evidence for nutritional exchange between brooding unionid mussels and their glochidia larvae. *Invertebrate Biology*, **120**: 227–236.

SILVERMAN, H., KAYS, W.T. & DIETZ, T.H. 1987. Maternal calcium contribution to glochidial shells in freshwater mussels (Eulamellibranchia: Unionidae). *Journal of Experimental Zoology*, **242**: 137–146.

SILVERMAN, H., STEFFENS, W.L. & DIETZ, T.H. 1985. Calcium from extracellular concretions in the gills of freshwater unionid mussels is mobilized during reproduction. *Journal of Experimental Zoology*, **236**: 137–147.

VANDER ZANDEN, M.J., CABANA, G. & RASMUSSEN, J.B. 1997. Comparing trophic positions of freshwater fish calculated using stable nitrogen isotope ratios (^{15}N) and literature dietary data. *Canadian Journal of Fisheries and Aquatic Sciences*, **54**: 1142–1158.

VANDER ZANDEN, M.J., SHUTER, B.J., LESTER, N. & RASMUSSEN, J.B. 1999. Patterns of food chain length in lakes: A stable isotope study. *American Naturalist*, **154**: 406–416.

WÄCHTLER, K., DREHER-MANSUR, M.C. & RICHTER, T. 2001. Larval types and early postlarval biology in naiads (Unionoida). In: *Ecology and evolution of the freshwater mussels Unionoida* (G. Bauer & K. Wächtler, eds), pp. 93–125. *Ecological Studies* 145. Springer, Berlin, Germany.

WATTERS, G.T. 2001. The evolution of the Unionacea in North America, and its implications for the worldwide fauna. In: *Ecology and evolution of the freshwater mussels Unionoida* (G. Bauer & K. Wächtler, eds), pp. 281–310. *Ecological Studies* 145. Springer, Berlin, Germany.

WOOD, E.M. 1974. Development and morphology of the glochidium larvae of *Anodonta cygnea* (Mollusca: Bivalvia). *Journal of Zoology*, **173**: 1–13.