RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2008; **22**: 694–700
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.3413

Fractionation and metabolic turnover of carbon and nitrogen stable isotopes in black fly larvae

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Received 2 November 2007; Revised 18 December 2007; Accepted 21 December 2007

Diet-tissue fractionation factors and metabolic turnover rates of $\delta^{15}N$ and $\delta^{13}C$ were assessed in laboratory-reared black fly (*Simulium vittatum* IS-7) larvae fed isotopically distinct diets. Five treatments consisted of using food with different $\delta^{15}N$ signatures throughout the experiments (19–26 days), a sixth shifted from a low to high $\delta^{15}N$ signature diet (*uptake*) on day 14, and the last shifted from a high to low $\delta^{15}N$ signature diet (*elimination*) on day 14. In the larvae, diet-tissue fractionation factors for $\delta^{13}C$, which were in steady state with food, ranged from −0.61 to 2.0, with a median of 1.87. The $\delta^{15}N$ diet-tissue fractionation factors were mostly negative, ranging from +2.85 to −24.96‰, with a single positive value from the *elimination* treatment in which larval $\delta^{15}N$ did not achieve steady state with the food. Diet-tissue fractionation factors also had a significant negative relationship ($r^2 = 0.98$) with $\delta^{15}N$ values in the food suggesting that nitrogen diet-tissue fractionation factors are ^{15}N concentration-dependent. The $\delta^{15}N$ of shed head capsules and feces were enriched in ^{15}N and could be mechanisms for elimination of ^{15}N by the larvae. For $\delta^{15}N$, metabolic turnover values based on the Hesslein model were highly consistent (0.40 to 0.43 $\delta^{15}N^{*}day^{-1}$) between uptake and elimination phases and across experiments and were an order of magnitude greater than growth rates. The rapid turnover of nitrogen in black fly larvae, which was orders of magnitude greater than measured in vertebrates, makes them an excellent indicator of short-term changes in nitrogen inputs to aquatic systems. Copyright © 2008 John Wiley & Sons, Ltd.

Stable isotope ratios of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) have become a useful tool for assessing the feeding ecology of organisms and the trophic structure of food webs.[1,2] These tracers provide an integrated estimate of feeding behavior over days to years, depending on the tissue and species analyzed,[3] as opposed to gut analysis which captures a 'snapshot' of what an organism has recently consumed. In addition, stable isotope analyses are attractive because accurate results can be achieved with small quantities of tissue, making them applicable to various sized organisms.

Although interpretation of data on stable isotope ratios appears to be straightforward, there are multiple caveats or assumptions that are typically overlooked, several of which have been recognized and addressed by Gannes *et al.*[4] and by Post,[5] but others have not.[6] For example, the diet-tissue fractionation of $\delta^{13}C$ and $\delta^{15}N$ has been investigated and shown to vary among species,[5] tissues,[7] amino acids,[8] prey quality,[9] and within species under varying environmental conditions (e.g. temperature[10]). Other assumptions, such as those related to the uptake and elimination kinetics of ^{15}N and ^{14}N in organisms after a diet switch, have not been as widely studied but have recently begun to be addressed.[11] Numerous calls for more laboratory studies to provide insight into the validity of the assumptions commonly employed in stable isotope ratio data interpretation have been made.[3,4,12,13]

Black fly larvae (Diptera: Simuliidae) can be dominant components of aquatic ecosystems.[14] Larvae are primarily filter-feeders, feeding on suspended organic matter in the range of 0.091 to 350 μm, but are also known to scrape deposited organic material on substrates to which they are attached (i.e. rocks, snags, trailing vegetation).[15] Simuliids are also one of the best known families of aquatic insects taxonomically and ecologically.[14] These attributes, along with the availability of a laboratory colony,[16] make simuliids an excellent model organism for studying stable isotopes in aquatic insects. A number of studies have utilized stable isotope ratios with black fly larvae,[17–19] creating a need to quantify stable isotope turnover in this family and in aquatic insects in general.

In this study, nutritionally similar but isotopically distinct diets were used to determine diet-tissue fractionation factors of $\delta^{15}N$ and $\delta^{13}C$ and to examine metabolic turnover during diet switches from a low to high $\delta^{15}N$ signature diet (*uptake*)

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and from a high to low $\delta^{15}N$ signature diet (*elimination*) in laboratory-reared *Simulium vittatum* Zetterstedt cytospecies IS-7 larvae. Based on studies in vertebrates,[11] we hypothesized that the values of metabolic turnover for $\delta^{15}N$ would differ between diet switches from high to low ^{15}N versus low to high ^{15}N. Based on rapid growth we also hypothesized that metabolic turnover in black fly larvae would be greater than in vertebrates and measurable using $\delta^{15}N$.

EXPERIMENTAL

Black flies

Simulium vittatum IS-7 eggs were obtained from the University of Georgia black fly colony,[16] which was established with eggs collected from Flaxmill Brook north of Cambridge, NY, USA in September 1981, and recently shown to be genetically similar to the wild type.[20] Black flies were reared as described by Gray and Noblet[16] with minor adjustments. Briefly, on day 0, the 30.5 cm × 102 cm runway of the rearing tank was covered with 24.5 cm × 25.5 cm strips of nylon screen (750 μm mesh; Sefar America, Kansas City, MO, USA) upon which cloth sheets containing approximately 10 000 eggs were pinned. Initially, the water temperature was between 25 and 27°C to allow for egg hatching and it was then allowed to slowly reach room temperature (20°C) by day 3.

Diets

Black fly larvae were fed one of three basic foods, *standard*, *worm-mixture* or *worm-only food*. The standard food, used to maintain the black fly colony, is a 50:50 mixture of soybean meal and Advanced Nutrition Rabbit Chow (Purina Mills, St. Louis, MO, USA). The worm-mixture food consisted of 2/3 standard food and 1/3 freeze-dried earthworms (*Eisenia foetida*), which were obtained from UNCO Industries Inc. (Racine, WI, USA). The worm-only food consisted of 100% freeze-dried earthworms. Foods were ground in a blender for 1 min and then pulverized into a fine powder using a 8000-D ball mill (SPEX Certiprep, Metuchen, NJ, USA).

Six different worm-mixture foods that varied in stable isotope signature were used in this study (Table 1). To create these isotopically distinct foods, the stable isotope signatures of earthworms were manipulated by rearing the worms in a soil that had varying levels of ^{15}N and ^{13}C.[11] Concentrations of ^{15}N and ^{13}C were manipulated by adding 5 g of ^{15}N-NH_4Cl (99.9% ^{15}N) and 10 g of 1-^{13}C-$CH_3CO_2Na3H_2O$ (99.8% ^{13}C) or by adding no labeled compounds to 1 L of potato and water slurry. This mixture was incubated for 7 days to allow for absorption of the heavy isotopes by resident bacteria and then thoroughly mixed into 40 L of loamy soil in a 100-L terrarium. Approximately 4000 earthworms were then added to the soil, and, after 8 days, the worms were removed, thoroughly washed, freeze-dried and pulverized into a powder for making diets.

Experimental methods

Five treatments were assessed, each differing by the food that was used, starting on days 7 and 14 (Table 1). For all treatments, on day 0, 4 L of standard food (2.0 g food/L) was poured directly into the reservoir of the rearing tank, and this was repeated on days 1 and 2. Additional food was not added until day 7 so that larvae could remove deposited food material on the screens; the standard food was used initially to ensure a common dietary baseline in the larvae among treatments. On day 7, treatment foods (~2.0 g of food/L; 7 L per day) were poured daily into an automated feeding system that pumped ~200 mL of the suspension into the rearing tank every 10 min (see Gray and Noblet[16]).

Three treatments used the same food, although the food type varied (Table 1) from day 7 until the end of the experiment (day 26). Larval samples were collected on days 10, 14, 18, 22 and 26. Two treatments involved a switch in the food on day 14, changing the food to one that was either depleted in ^{15}N (lower $\delta^{15}N$; *elimination treatment*) or enriched

Table 1. Stable isotope and C:N ratios of the food used in each treatment with corresponding diet-tissue fractionation values in black fly larvae. Stable isotope and C:N ratios are the mean ± 1 SE (n = 5 for treatments that used the same food throughout (collection on days 7, 10, 14, 18 and 22); n = 2 for food prior to the switch (collected on days 7 and 10) in the food switch treatments; n = 5 for food after the switch (collected on days 14, 15, 16, 18 and 22) in the food switch treatment). Diet-tissue fractionation values (n = 3, mean ± 1 SE) are from larvae collected on day 19 for the worm food and day 26 for the standard, mixed foods and food switch experiments

Treatment	Food $\delta^{13}C$ (‰)		Food $\delta^{15}N$ (‰)		Food C:N		Diet-tissue fractionation $\delta^{13}C$ (‰)	Diet-tissue fractionation $\delta^{15}N$ (‰)
Same food throughout								
Low worm food*	−24.27		16.34		4.4		1.90	−2.59
High worm food*	−20.95		39.96		5.0		−0.61	−13.53
Standard food	−23.9 ± 0.3		1.5 ± 1.0		12.7 ± 0.5		1.26 ± 0.01	−0.41 ± 0.04
Low mixed-food	−24.1 ± 0.1		2.6 ± 0.1		6.5 ± 0.1		1.95 ± 0.13	−0.01 ± 0.36
High mixed food	−23.6 ± 0.2		87.7 ± 3.0		6.7 ± 0.2		1.76 ± 0.19	−24.96 ± 0.22
Food switch	*Days 7–13*	*Days 14–26*	*Days 7–13*	*Days 14–26*	*Days 7–13*	*Days 14–26*		
High - Low	−24.6	−25.2 ± 0.1	32.1	1.5 ± 0.4	6.8	7.0 ± 0.1	1.87 ± 0.02	2.85 ± 0.14
Low - High	−24.3	−24.2 ± 0.1	2.3	31.7 ± 0.7	NA**	6.4 ± 0.2	1.88 ± 0.22	−9.95 ± 0.10

*Treatments were conducted in 30-L tanks under similar experimental conditions with fewer larvae to reduce the amount of food needed to conduct the full experiment. Thus the values represent the signatures of the larvae and food from only one repetition. These treatments were designed to help understand the assimilation of C and N from the worm portion of the food in the mixed food treatments.
**Food C:N values were lost for days 7–13.

DOI: 10.1002/rcm

in ^{15}N (high $\delta^{15}N$; *uptake treatment*) for the remainder of the experiment (till day 26) (Table 1). In these treatments, after sampling on day 14, the runway upon which the larvae were attached was removed from the rearing tank and immediately placed into a new rearing tank. A volume of 7 L of the new food (~2.0 g of food/L) was added to the reservoir of the new tank and an additional 7 L was added to the automated feeding system. The larvae were then fed 7 L of the new food suspension each day for the remainder of the experiment. To observe the change in stable isotope signatures after the food change, larval samples were taken on days 15 and 16 in addition to sampling on days 18, 22 and 26.

Sampling consisted of randomly removing three strips of the nylon screen from the lower three-quarters of the runway and scraping larvae from each screen. One larva representing the largest and smallest size class from each screen was placed into 70% ethanol for length and head-capsule measurement. The width of the head capsule was measured using an ocular micrometer in a Meiji dissecting scope (Meiji Techno America, San Jose, CA, USA) at 45× and larval instar was determined using the data of Hudson.[21] The length of the larvae (most anterior portion of the head capsule to the most posterior portion of the abdomen) was measured at 7×. Larval length was converted into biomass using the method of Benke *et al*.[22] The remaining larvae from each screen were transferred into 1.5-mL plastic centrifuge tubes, labeled and stored in the freezer at −20°C until stable isotope analysis.

Additional samples were collected on the last day of the experiment (day 26). The guts were removed from a subsample of 50 larvae prior to stable isotope analysis to assess the importance of gut contents on measured stable isotope ratios and develop a correction factor if necessary. Shed head capsules of molting larvae were collected from the rearing tank by placing a basket made of 250-μm mesh polypropylene screen at the bottom of the runway. Head capsules were washed to remove any extraneous material before being transferred into 1.5-mL centrifuge tubes and stored at −20°C until analysis.

Worm assimilation

To determine the extent to which the black fly larvae assimilated the worm portion of the mixed food compared with the standard food, two experimental treatments were conducted in smaller, 30-L rearing tanks using the two worm-only foods that differed in $\delta^{15}N$ signatures (Table 1). Approximately 1500 3-day-old larvae were transferred from the colony rearing tank onto the runway of the 30-L experimental tank containing 20 L of tap water (20°C). Larvae were fed 15 mL of a 40 g/L worm suspension daily and sampled as previously described except the sampling days were 7, 11, 15 and 19 and only one sample was obtained at each time point. Pupae began to appear on day 18; thus the majority of larvae collected on day 19 were near the end of their larval stage. Larvae were stored and dried before stable isotope analysis as previously described.

Stable isotope analysis

Prior to stable isotope ratio analysis, samples were dried in an oven (Precision Scientific, Chicago, IL, USA) for 72 h at 60°C and then desiccated for 24 h. Samples were pulverized and homogenized in the centrifuge tube with a stainless steel spatula for stable isotope analysis. Stable isotope ratios of nitrogen and carbon were determined on a DeltaPlus mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an elemental analyzer; the analytical precision was ±0.15‰. Stable isotope abundances are expressed in δ notation as the deviation from standards in parts per thousand (‰) according to the following equation:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000 \quad (1)$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$.

Modeling and statistical analysis

Mass-balance models have been most frequently used in diet-switch experiments to estimate proportional rates in stable isotope turnover due to metabolism and growth. The use of mass-balance models was initiated in Hesslein *et al*.[23] and can be written as:

$$X_{tissue(t)} = X_{diet} + (X_{tissue(0)} - X_{diet})e^{-(k+m)t} \quad (2)$$

where X_{diet} is the stable isotope ($\delta^{15}N$ or $\delta^{13}C$) signature of the larvae at equilibrium with a new diet, $X_{tissue(0)}$ is the initial stable isotope value of each larvae, $X_{tissue(t)}$ is the stable isotope value of the larvae at sampling time t, k is the growth rate per day (here set to 0), and m is the metabolic (non-growth) stable isotope turnover rate constant per day.

We fit Eqn. (2) to our data using the non-linear least-squares (nls) routine in the statistical package R.[24] Because larvae grew at a consistent rate throughout the experiment, we independently estimated the growth rate per day for each treatment group from linear regressions of average larval weights per day; these values were subsequently used in Eqn. (2) to estimate metabolic turnover and X_{diet}. The initial stable isotope value of each larva was set to equal the mean stable isotope value of larvae on day 10 of each treatment cycle and, because experiments were run close to steady state, we were able to achieve precise estimates of metabolic turnover and X_{diet} for each treatment. We quantified the uncertainty surrounding our parameter estimates using 95% confidence intervals from the *confint* method in the MASS library of R.[25]

From estimates of metabolic turnover the times to 50% (T_{50}) and 95% (T_{95}), dietary steady states with a new diet were calculated using the equation:

$$T = [\log(1) - \log(1 - \alpha)]/m \quad (3)$$

where α is the target percentage of steady state (e.g. 95%).

RESULTS

The only treatments where $\delta^{13}C$ or $\delta^{15}N$ signatures of the larvae with full guts were significantly different from larvae with guts removed was the high $\delta^{15}N$ signature food treatment. The mean $\delta^{15}N$ signatures ± standard error of the larvae that were fed on the high $\delta^{15}N$ food with guts present and sampled on day 26 was 66.71‰ ± 0.71 and those for larvae with guts removed were 62.69‰ ± 0.22 (paired t-test; $p < 0.05$). Thus, $\delta^{13}C$ and $\delta^{15}N$ signatures of larvae

DOI: 10.1002/rcm

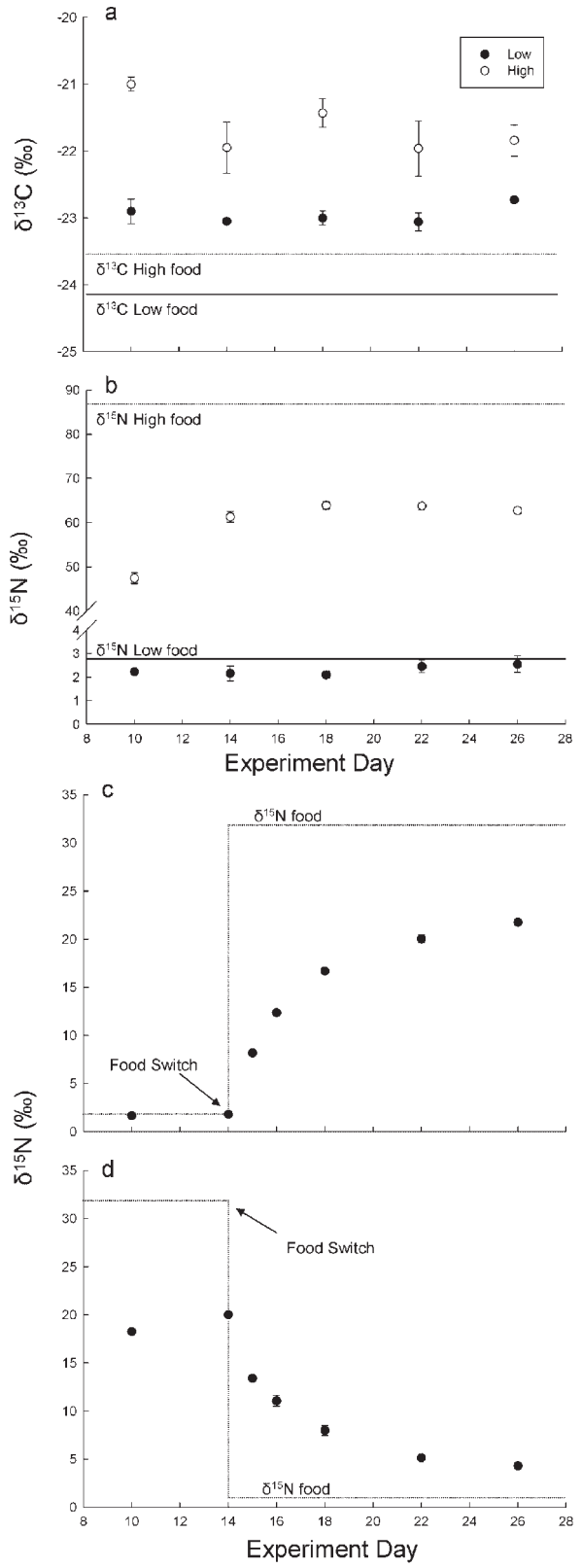

Figure 1. Stable isotope signatures in black fly larvae (each point is the mean ± 1 SE, n = 3) fed: (a, b) two foods with variable stable isotope signatures (low food: $\delta^{13}C = -24.1 \pm 0.12$ and $\delta^{15}N = 2.56 \pm 0.14$; high food: $\delta^{13}C = -23.6 \pm 0.22$ and $\delta^{15}N = 87.7 \pm 3.0$). (c) Larvae fed on a low $\delta^{15}N$ food and switched to a high $\delta^{15}N$ food after day 14. (d) Larvae fed on a high $\delta^{15}N$ food and switched to a low $\delta^{15}N$ food after day 14. Reference lines indicate either the $\delta^{13}C$ or $\delta^{15}N$ signatures of the food.

without guts removed were reported in all experiments except when the high worm food was used, in which case the $\delta^{15}N$ values at all collection times were adjusted by subtracting the difference between larvae with guts and those with guts removed on day 26.

Values of $\delta^{13}C$ in the black fly larvae were near steady state with their diet by the first collection day (day 10), and remained near steady state throughout the experiment, in all treatments (Fig. 1). Values of $\delta^{15}N$ in the larvae were near steady state with their food by the first collection day (day 10) in the three experiments that used foods with lower $\delta^{15}N$ signatures (~2‰) (Fig. 1). In the two experiments that used food with a higher $\delta^{15}N$ signature, steady state between the larvae and the food was reached on day 14 (Fig. 1).

Diet-tissue fractionation factors for $\delta^{13}C$ in black fly larvae were lower when food was 100% plant material, i.e., standard food (fractionation factor = 1.3), than when food was 1/3 worm and 2/3 plant material (fractionation factor = 1.8–2.0) (Table 1); although one of the worm-only treatments had a $\delta^{13}C$ diet-tissue fractionation factor of −0.61, fractionation factors for $\delta^{15}N$ were generally negative and were highly influenced by the magnitude of the $\delta^{15}N$ value in the food (Table 1), becoming more negative with increasing $\delta^{15}N$ values in the food (Fig. 2). The only experiment that generated a positive diet-tissue fractionation factor involved the switch from a food with a high $\delta^{15}N$ signature to a lower signature (*elimination*) which had a factor of +2.85 (Fig. 1).

Results from the mass-balance model were highly precise as larval $\delta^{15}N$-turnover was consistent among experimental treatments and the experiments were run to, or almost to, steady state (Fig. 3). While growth rates varied somewhat among treatments (0.035 to 0.049 $g^{*}day^{-1}$ from growth-curve estimates), metabolic turnover was consistent (0.33 to 0.41 $\delta^{15}N^{*}day^{-1}$) between the uptake and elimination phases of the experiment and was an order of magnitude greater than growth rates (Table 2). The lack of significant differences in

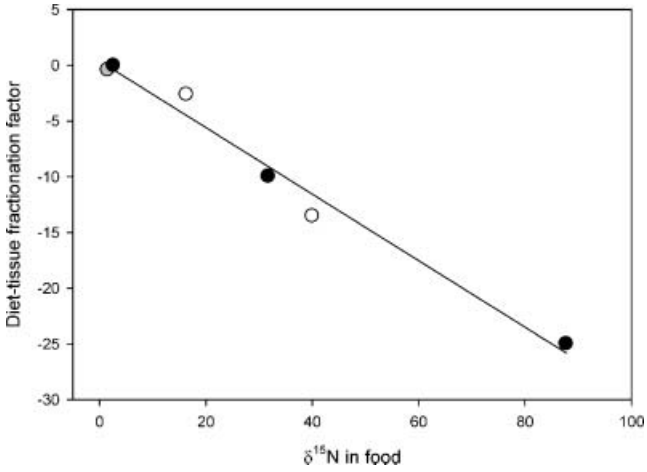

Figure 2. Relationship between diet-tissue fractionation factors (DTFFs) and $\delta^{15}N$ in the food for black fly larvae. Open circles are worm-only food treatments, closed circles are worm-mixture food treatments and the grey circle is the standard food treatment. Line represents linear regression ($r^2 = 0.98$; $p < 0.001$).

DOI: 10.1002/rcm

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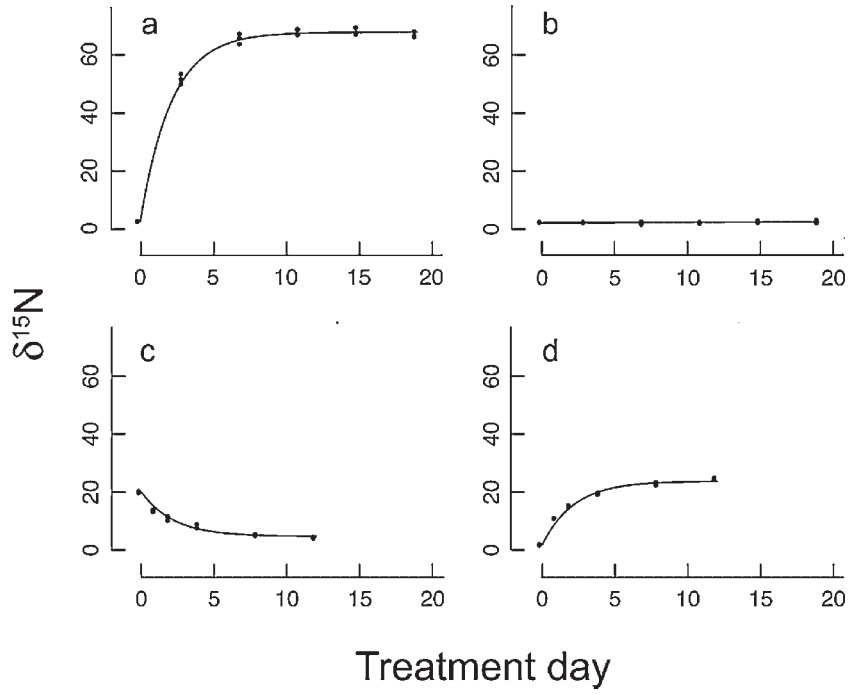

Figure 3. δ^{15}N trajectories in black fly larvae. Regression curves are least-squares fits for the model of Hesslein *et al.*[23] (see Table 3): (a) larvae fed high δ^{15}N for 19 days; (b) larvae fed low δ^{15}N for 19 days; (c) larvae fed high δ^{15}N for 7 days followed by a low δ^{15}N for 12 days with model on the final 12 days; and (d) larvae fed low δ^{15}N for 7 days followed by a high δ^{15}N for 12 days with model on the final 12 days. See Table 1 for δ^{15}N values of the food.

δ^{13}C values between foods prevented any effort to model δ^{13}C turnover.

DISCUSSION

Diet-tissue fractionation factors

Black fly larvae analyzed in this study had δ^{13}C diet-tissue fractionation factors higher than average (0.2‰) but within the normal range of literature values.[5,26] However, the δ^{15}N diet-tissue fractionation factors for black fly larvae were much lower than average (3.2‰), showing depletion in δ^{15}N compared with the food in six of the seven treatments. The exception was the treatment that switched the food of the larvae to one that was lower in δ^{15}N on day 14 (i.e. an elimination food switch), but δ^{15}N values in the larvae did not achieve steady state with the new food. In addition, the diet-tissue fractionation factors determined before the food switch in this experiment were depleted, consistent with the other treatments.

While studies that have reported δ^{15}N signatures for black fly larvae have not indicated depletion compared with their food source,[19,27–29] Lancaster and Waldron[30] suggest that simuliids might be depleted in δ^{15}N based on the signatures of *Ryacophila dorsalis* larvae which prey upon black fly larvae. Differences in larval diet-tissue fractionation among the aforementioned studies, including our own, could be related to the composition and quality of the diet.[9] However, we observed negative diet-tissue fractionation factors across a range of food types (Table 1). Therefore, food quality is not likely to have caused the observed patterns in nitrogen fractionation. A possible explanation for these negative δ^{15}N diet-tissue fractionation factors are elimination processes (i.e. molting and expelling feces) of the larvae. Head capsules are shed during each molt from one istar to the next (total of seven molts in *S. vittatum* IS-7). Head capsules

Table 2. Growth rates (k), tissue-specific estimates of metabolic turnover (m, 95% confidence intervals (CIs)) of δ^{15}N and estimated days to 50% (T_{50} with 95% CI) and 95% isotopic change (T_{95} with 95% CI) in black fly larvae. Estimates of m and X_{diet} (δ^{15}N signature of the larvae at equilibrium with a new diet) derived from least-squares regressions (nls) from the mass-balance model of Hesslein *et al.*[23] p-values are calculated t-tests of the parameter estimate being zero

Treatment	k (day^{-1})	$\hat{m}$(d^{-1})	$\hat{X}_{diet}$	T_{50} (days)	T_{95} (days)	p-value
Same food throughout						
High mixed food	0.035	0.411 (0.380–0.447)	63.7 (62.8–64.5)	1.4 (1.3–1.5)	6.6 (6.1–7.1)	<0.0001
Food switch						
High - Low	0.049	0.404 (0.339–0.482)	4.7 (3.9–5.4)	1.7 (1.4–1.9)	7.4 (6.2–8.8)	<0.0001
Low - High	0.038	0.332 (0.305–0.362)	21.5 (21.0–22.0)	1.5 (1.4–1.6)	7.7 (7.2–8.4)	<0.0001

DOI: 10.1002/rcm

collected in this study were enriched in $\delta^{15}N$ (1.2 and 7.1‰ higher than the larvae in the low and high mixed food treatments, respectively). Larvae are constantly feeding and releasing fecal pellets to make room for newly ingested food. One sample of fecal pellets from the larvae in this study showed that feces were highly enriched in $\delta^{15}N$ (143‰ higher than the larvae in the high worm food treatment). An additional factor might be the high metabolism (discussed below) and growth rate of the larvae, which may necessitate greater feeding and lower assimilation of food. Another possibility is that the larvae assimilated the worm portion of the diet differently from the other components (soybean meal and rabbit chow), which could confound the diet-tissue fractionation factors. Since the worm component was the only portion of the diet that was enriched for the high mixed food, if it were poorly assimilated the larvae would not achieve a $\delta^{15}N$ signature similar to that of the food. However, additional treatments with the worm-only food produced results consistent with those for larvae that had mixed foods. This is not surprising given that animal material is typically assimilated with greater efficiency than plant material.[31–33] In view of these results, there is a need for mechanistic studies of stable isotope dynamics in aquatic insects, and other organisms, to understand how assimilation affects isotope fractionation.

Critically, the degree to which the larvae were depleted in $\delta^{15}N$ was positively related to the $\delta^{15}N$ levels in the food (i.e. diet-tissue fractionation factors were more negative with increasing $\delta^{15}N$ in the food), showing clear concentration dependence in fractionation factors among experiments. To our knowledge, this relationship between $\delta^{15}N$ concentration of food and diet-tissue fractionation factors has not been previously demonstrated but has serious ramifications for field studies because diet-tissue fractionation factors are used to predict the trophic position of an organism and ultimately the food web structure. If diet-tissue fractionation factors differ with $\delta^{15}N$ values in food, there needs to be a consideration for scaling these fractionation factors to food source $\delta^{15}N$ values. This concept could be particularly important when interpreting data from ^{15}N-enrichment studies, for example in stream studies that use $^{15}NH_4$ to get a better understanding of nutrient dynamics or food resources.[29,34–38] In such studies, $\delta^{15}N$ can vary widely (by as much as 100‰) within sites over time and among sites on a downstream gradient from the source.[36] Likewise, comparison of data among studies which use different concentrations of $^{15}NH_4$ for enrichment could be problematic. Clearly, this warrants further laboratory experiments with foods varying in $\delta^{15}N$ concentrations and animals of differing trophic levels. Values of $\delta^{13}C$ did not differ sufficiently in the foods to address this issue with this isotope but they too need to be assessed.

Metabolic turnover of nitrogen

Nitrogen turnover rates in black fly larvae were orders of magnitude greater than those reported in cold-blooded vertebrate species (see MacNeil *et al.*,[11] Bosley *et al.*,[39] Hesslien *et al.*,[23] and Logan *et al.*[40]) and ten times the growth rates of the black fly larvae. These previous studies have concluded that growth rate dominates the rate of $\delta^{15}N$ change in cold-blooded vertebrates after a change in diet, although they focused on young, fast-growing animals, and metabolism might become more significant in vertebrates as growth rates decline. The growth rates of the black fly larvae in this study were high compared with these studies on vertebrates and, thus, a slow growth rate does not account for the significance of the metabolic turnover found in this study.

In comparison with other aquatic invertebrates, black fly larvae appear to have one of the highest $\delta^{15}N$-turnover rates documented. In a study by Hamilton *et al.*,[38] $\delta^{15}N$-turnover rates were determined for several aquatic macroinvertebrates. Simuliids were shown to have the highest turnover rates at 0.26/day while elmids had the lowest turnover rates at 0.033/day. Metabolic rates in aquatic insects have shown to be influenced by temperature, availability of food, and stress.[41–43] Thus, although metabolic turnover was high in the black fly larvae in this study compared with the study of Hamilton *et al.*,[38] metabolic rates and turnover would probably have been even higher with warmer water temperatures and greater food delivery.

Metabolic turnover rates of $\delta^{15}N$ did not differ between an uptake versus an elimination diet switch event, which is in contrast to results from similar studies in fish[11] and snakes (A.T. Fisk, unpublished data). Unlike in the experiments reported here, N stable isotope turnover rates in these studies were based on non-steady state conditions where only the initial and most dramatic changes in animal tissues $\delta^{15}N$ were observed. Further, both of these studies assumed that the steady-state $\delta^{15}N$ fractionation of sampled tissues over the experimental diets would be of the order of 2–4‰. If the concentration dependence we observed is a consistent phenomenon among consumers, the assumed steady-state values used by MacNeil *et al.*[11] would have biased metabolic turnover estimates in proportion to the differences among $\delta^{15}N$ signatures of the tissues at the start of each diet switch period.

The rapid metabolic turnover of $\delta^{15}N$ resulted in a time to the 50% steady state with a new diet of less than 2 days and a time to the 95% steady state of approximately 7 days. These short times mean that $\delta^{15}N$ signatures in black fly larvae, and probably in other fast-growing aquatic insect larvae, represent recent nitrogen stable isotope signatures in particulate organic matter in aquatic systems. Larvae that undergo a diet switch, or live in a stream that experiences a pulse of high or low $\delta^{15}N$ (e.g. from a waste discharge), would reflect this change very quickly. Thus, these organisms have great potential as monitors of recent nitrogen input, and, if collected frequently and then compared with longer-lived, slower metabolic organisms (e.g. mussels), they can provide information on temporal variation in nitrogenous inputs to aquatic systems.

Acknowledgements

Funding for this work was provided by the University of Georgia Research Foundation grant to Jay Overmyer. M. Aaron MacNeil was supported by an NSERC doctoral studentship. We thank Tom Maddox (UGA) for stable isotope analysis and David Rouse (UGA) for help with food and sample preparation.

DOI: 10.1002/rcm

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DOI: 10.1002/rcm