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Changes in carbon and nitrogen stable isotopes of chironomid larvae during growth, starvation and metamorphosis

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We conducted experiments to determine isotope changes in the deposit-feeding chironomid larvae *Chironomus acerbiphilus* during feeding, starvation and metamorphosis. Isotope changes in chironomid larvae occurred mainly during growth and rarely afterward. This finding indicates that chironomid isotope turnover mainly occurs in conjunction with growth and suggests that chironomid larvae only break down newly assimilated food for energy during periods of no growth. Chironomid $\delta^{13}C$ values significantly increased throughout the starvation experiment, indicating that chironomids preferentially break down components with lower $\delta^{13}C$ content during starvation. We found significant changes in chironomid isotope ratios (^{15}N enrichment) during pupation. This evidence suggests that the physiological condition of animals (such as during an active growth phase or pre- or post-molting) is important to their stable isotope ratios. Copyright © 2007 John Wiley & Sons, Ltd.

The natural abundances of carbon and nitrogen stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) are an important tool in studies of food webs and biogeochemical processing in ecosystems. Stable isotope techniques are increasingly used to define trophic levels and diet of consumers. This is because the manner in which the stable isotopic composition of a consumer's tissues reflects its diet is fairly predictable.[1–3]

To date, many isotope feeding experiments have yielded the information that the time of the stable isotopic composition of a consumer's tissues reflected its diet.[4–7] Ecological applications of stable isotope techniques will be enhanced by experimental validation of the assumptions that are made when they are used in a field context.[8]

In aquatic ecosystems, deposit-feeding animals form important links between sediment organic matter and carnivorous fish, and chironomid larvae form important links of lake food webs. However, although there are many reports of changes in isotope levels in animals during their growth,[9–11] very few studies in benthic macroinvertebrates such as chironomid larvae have investigated differences in changes in isotope levels in animals between periods of growth and no feeding (starvation).

To estimate the changes in isotope levels in benthic macroinvertebrates, we conducted feeding experiments using deposit-feeding chironomid larvae (*Chironomus acerbiphilus* Tokunaga; Diptera: Chironomidae), and diets with different carbon and nitrogen isotope levels. We measured and compared isotope changes in the chironomid larvae during their growth (feeding) periods and after growth had stopped (starvation).

Many invertebrates metamorphose from larval to adult stages. During metamorphosis, the internal concentrations of stable isotopes may change rapidly. The nitrogen isotope enrichment of the arthropod larvae stages was found to be significantly lower than that of adults using meta-analysis, supporting a role for metamorphosis effects.[12] Thus, stable isotopes are probably enriched as an animal changes from larva to adult, even if the adult isotope levels reflect those of the larval diet. Moreover, the adults of aquatic invertebrates provide a link between the aquatic and terrestrial food webs, since many adults are fed by terrestrial animals such as birds.[13] Thus, to use stable isotopes as natural tracers in food-web analyses of aquatic and terrestrial ecosystems, the extent of isotope enrichment during metamorphosis must be known.

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In this study, we determined the degree of isotope enrichment in *C. acerbiphilus* during metamorphosis from the final larval stage to the adult stage by measuring the isotope values of larvae and adults hatched in the laboratory.

EXPERIMENTAL

Experiment 1: Changes in isotope levels in feeding *C. acerbiphilus*

Three types of diets were used for the feeding experiments: boiled corn (a C_4 plant), tetramin fish food(Tetra-Japan Co., Tokyo, Japan), and sediments from Lake Katanuma, Japan. The sediments were collected directly from the lake bottom at a depth of 0.5 m using an Ekman-Birge sediment sampler. The C and N contents of the diets were measured using an elemental analyzer (NA-2500, CE Instruments, Milan, Italy). The corn, tetramin and sediment values were $49.5 \pm 0.5\%$ C and $2.0 \pm 0.1\%$ N, $42.3 \pm 0.7\%$ C and $1.1 \pm 0.4\%$ N, $12.2 \pm 1.0\%$ C and $0.8 \pm 0.1\%$ N, respectively (mean $\pm$ 1SE, $n = 4$).

Third-instar *C. acerbiphilus* specimens were also collected from the lake bottom at a depth of 0.5 m using an Ekman-Birge sediment sampler in October 2002. This was the stable season of the larval density based on the circulation periods of the lake. The larvae were allowed to molt to the fourth instar for 1 day in Petri dishes (2.3 cm in diameter) containing filtered lake water (2 cm deep) at 20°C. All individuals that had molted after 1 day were collected and one chironomid larvae reared in a new Petri dish under the same conditions, except that they were fed one of the three experimental diets (each diet was provided to five replicates).

During the experiment, the chironomids were transferred to new Petri dishes and were provided with fresh food every 3 days and feces was removed. Each diet provided enough food for growth of the larvae, similar to our previous experiment[14] that used the same temperature, light and Petri dish conditions as in this experiment. In a pilot experiment, fourth-instar chironomid larvae pupated 11 to 15 days after molting. Thus, in the feeding experiment, we collected five fourth-instar larvae immediately after molting (on day 0) and at 3, 6, 9 and 12 days after molting for isotope analyses. The larvae were placed into filtered lake water for 24 h to clear their guts.[14] They were then freeze-dried and weighed using an electric microbalance (HR-60, A&D Co., Tokyo, Japan) before their isotope ratios were measured. The relative growth of the larvae was calculated by (dry weight of day x) $\times$ (dry weight of day 0)$^{-1} \times 100$ (%) (x is experiment days from day 3 to day 12).

Experiment 2: Changes in isotope levels in starving *C. acerbiphilus*

A second subset of fourth-instar *C. acerbiphilus*, collected from Lake Katanuma in October 2002, was used in experiment 2. The experimental conditions and procedures were the same as for experiment 1, except that the chironomids were not provided with food. After the experiment, the larvae were not placed into filtered lake water to clear their guts as in experiment 1. They were then freeze-dried and weighed using an HR-60 electric microbalance before their isotope ratios were measured, and the growth rate of the larvae calculated by the above equation.

Experiment 3: Changes in isotope levels in *C. acerbiphilus* between the fourth instar and emergence

Fourth-instar *C. acerbiphilus* specimens, collected from Lake Katanuma in October 2002, were used in experiment 3. Approximately 10 per 60 individuals were not fed and were allowed to emerge as adults in Petri dishes (2.3 cm in diameter) containing filtered lake water (2 cm deep) at 20°C. These 6 male and 4 female adults that hatched in the period from 0 to 3 days, and the 10 collected chironomid larvae (3 days starvation in the same conditions), were used for isotope analysis.

Analysis of stable isotope ratios

All samples were preserved in a freezer at −20°C until the stable isotope ratios were analyzed. The carbon and nitrogen isotope ratios of each sample (1 larva for the analysis) were measured with an isotope ratio mass spectrometer (DELTA plus, Finnigan Mat, Bremen, Germany) connected to an NA-2500 elemental analyzer. The results are reported using delta notation: $\delta^{13}C$ or $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$ (‰), where R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (for $\delta^{13}C$ or $\delta^{15}N$, respectively). PDB (Pee Dee Belemnite) and atmospheric nitrogen were used as the standards for $\delta^{13}C$ and $\delta^{15}N$, respectively.

Calculating isotope turnover

We calculated the isotope turnover, at which the isotopes in each diet were converted into *C. acerbiphilus* larval biomass, using two isotope mixing models modified from our previous study.[14] The two models are as follows:

$$\delta^{13}C_c = (1 - f)\delta^{13}C_i + f(\delta^{13}C_d + \Delta^{13}C) \quad (1)$$

$$\delta^{15}N_c = (1 - f)\delta^{15}N_i + f(\delta^{15}N_d + \Delta^{15}N) \quad (2)$$

where subscripts c, i and d refer to the chironomid larvae after feeding on corn or tetramin, the chironomids on day 0, and diets, respectively, and f is the isotope turnover in chironomids fed on a diet. We used mean isotope values of chironomid larvae for the model, since we could not measure the same individual before and after feeding. The $\Delta^{13}C$ and $\Delta^{15}N$ terms are the carbon and nitrogen isotope fractionation, respectively, for chironomid larvae. We used 0.3‰ and 5.4‰ for $\Delta^{13}C$ and $\Delta^{15}N$, respectively; these values were obtained in previous feeding experiments that used pure samples of benthic diatoms and phytoplankton collected from Lake Katanuma.[14] The $\Delta^{13}C$ and $\Delta^{15}N$ of the chironomid larvae fed on benthic diatoms (C/N = 5.7), phytoplankton (C/N = 7.1) and sediment from 0.5 m depth (C/N = 15.6) were not significantly different from those in the previous experiment,[14] indicating that there is no stoichiometric impact on the $\Delta^{13}C$ and $\Delta^{15}N$ of larvae such as *Daphnia*.[15] Moreover, the corn, tetramin and sediment had from 0.8 to 2.0% N; thus the N contents of the diets did not have a major effect on the $\Delta^{15}N$ of chironomid larvae.[11]

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Statistical analysis

We preformed one-way analysis of variance (ANOVA) to test the differences in the isotope values and dry weights of the chironomid larvae in the experiments. When the ANOVA results showed that the variations were significant ($p < 0.05$), we also preformed a multiple comparison Tukey test to compare the values of each treatment. All statistical analyses were performed in Microsoft Excel 2004 for Mac.

RESULTS

Experiment 1: Isotope levels in fed larvae

The dry weights of chironomid larvae fed on each diet increased rapidly and significantly from 0 to 6 days (multiple comparison Tukey test: $p < 0.05$, $n = 5$), from 0.16 mg on day 0 to as high as 0.6 mg on day 6 (Fig. 1). After 6 days, the weights tend to increase gradually, although not significantly different among the days (Tukey test: $p > 0.05$, $n = 5$). From day 0 to day 6, the relative growths of chironomids that were fed corn, tetramin and sediment were 262.5, 335.3 and 337.5%, respectively (Table 1). From 6 to 12 days, growth rates changed little, ranging from 262.5 to 337.5%, 325.0 to 362.5% and 337.5 to 406.3% for corn, tetramin and sediment, respectively. The growth of the chironomid species was greater than those of shrimp and squid (11–23 days[4]) and krill (6–8 weeks[5]) in the previous turnover experiment studies.

The $\delta^{13}C$ value was significantly greater for the corn diet (-12.0 ± 0.2‰, mean ± 1 SE) than for tetramin (-22.5 ± 0.2‰) or sediment (-22.3 ± 0.3‰; Fig. 2; Tukey test: $p < 0.05$, $n = 5$). The $\delta^{13}C$ value was also significantly greater for the corn than for the chironomid larvae on day 0 (Tukey test: $p < 0.05$, $n = 5$). The $\delta^{13}C$ values for the tetramin and sediment diets were similar and did not significantly differ from those of the chironomid larvae on day 0 (Tukey test: $p > 0.05$, $n = 5$).

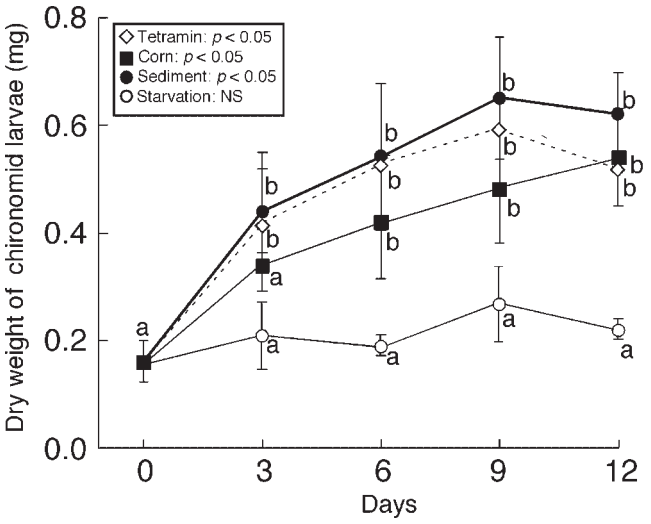

Figure 1. Dry weights of *C. acerbiphilus* larvae fed different diets or no diet (starvation). Different letters indicate significant differences in weight between sampling days for each treatment. The *p* values describe the significance of the treatment effect (Tukey test: $n = 5$). Data are means ± 1 SE ($n = 5$). NS = non-significant.

Table 1. The growth (from day 0 to day x; %) and carbon and nitrogen isotope turnover (%) of fourth-instar chironomid larvae after molting (days) in feeding experiments. NS = non-significant between the isotope ratios of the chironomid larvae at day 0 to day × (3–12)

		Growth	Turnover	
Treatments	days	(%)	C (%)	N (%)
Corn	3	212.5	NS	49.4
	6	262.5	48.8	65.6
	9	300.0	55.0	60.0
	12	337.5	64.4	56.7
Tetramin	3	250.0	NS	47.3
	6	335.3	NS	53.3
	9	362.5	NS	62.7
	12	325.0	NS	55.2
Sediment	3	275.0	NS	NS
	6	337.5	NS	NS
	9	406.3	NS	NS
	12	387.5	NS	NS

Changes in $\delta^{13}C$ in the chironomid larvae varied among the three diets (Fig. 2). The $\delta^{13}C$ values of the chironomid larvae fed on corn significantly increased during the first 6 days (from -20.7 ± 0.2‰ to -16.1 ± 0.2‰; Tukey test: $p < 0.05$, $n = 5$) and thereafter tended to increase gradually but non-significantly (Tukey test: $p > 0.05$, $n = 5$). The $\delta^{13}C$ values of the chironomid larvae fed on tetramin and sediment showed no significant change between each sampling day, approaching the $\delta^{13}C$ values of those diets.

Because the $\delta^{13}C$ value of the corn differed from the $\delta^{13}C$ values of *C. acerbiphilus* larvae on day 0, we were able to calculate the carbon turnover from the changes in $\delta^{13}C$ of corn-fed chironomids (Table 1). The carbon turnover of the chironomids fed on corn was 48.8% at 6 days, indicating that 48.8% of the carbon in chironomid tissue was metabolized. However, after 6 days, growth slowed and the carbon isotope ratios of the chironomids did not change significantly. The mean growth rate and the carbon turnover rate of the corn-fed chironomids did not differ significantly (paired t test: $p > 0.05$, $n = 3$; Table 1). Thus, these rates changed similarly throughout the experiment.

The $\delta^{15}N$ values of the three diets differed significantly (Tukey test: $p < 0.05$, $n = 5$; Fig. 3). The $\delta^{15}N$ values of the tetramin, corn and sediment were 6.1 ± 0.3‰, 1.8 ± 0.2‰ and -2.5 ± 0.2‰ (mean ± 1 SE, $n = 5$), respectively.

The $\delta^{15}N$ values of the chironomid larvae fed on corn significantly increased from 1.8 ± 0.2‰ to 4.1 ± 0.1‰ over the first 6 days (Tukey test: $p < 0.05$, $n = 5$, Fig. 3) and then ceased to change. The $\delta^{15}N$ values of the tetramin-fed larvae significantly increased from 1.8 ± 0.2‰ to 6.6 ± 0.3‰ over the first 9 days (Tukey test: $p < 0.05$, $n = 5$) and decreased slightly thereafter. In contrast, the $\delta^{15}N$ values of the larvae fed on sediment did not change during the experiment (Tukey test: $p > 0.05$, $n = 5$).

The nitrogen isotope turnover of chironomids that were fed corn and tetramin were 65.6% and 53.3%, respectively, at 6 days (Table 1); the rates did not change significantly thereafter (Tukey test: $p > 0.05$, $n = 5$). The mean growth rate

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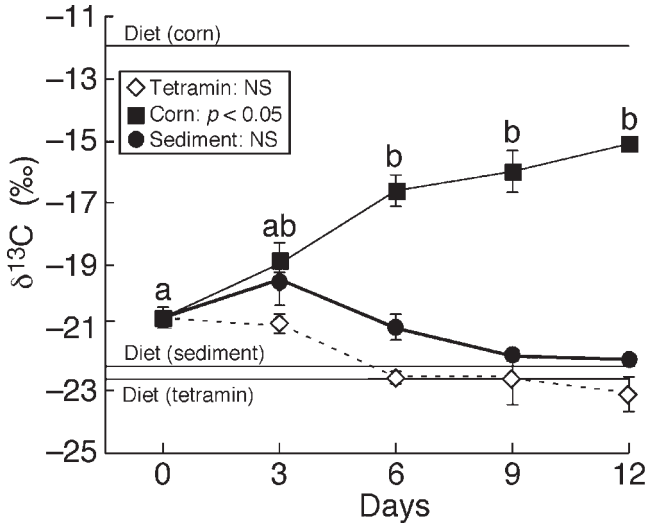

Figure 2. Values of δ^{13}C of *C. acerbiphilus* larvae fed each diet. Different letters indicate significant differences in δ^{13}C between sampling days for each treatment. The *p* values describe the significance of the treatment effect (Tukey test: $n = 5$). Data are means ± 1 SE (n = 5). NS = non-significant.

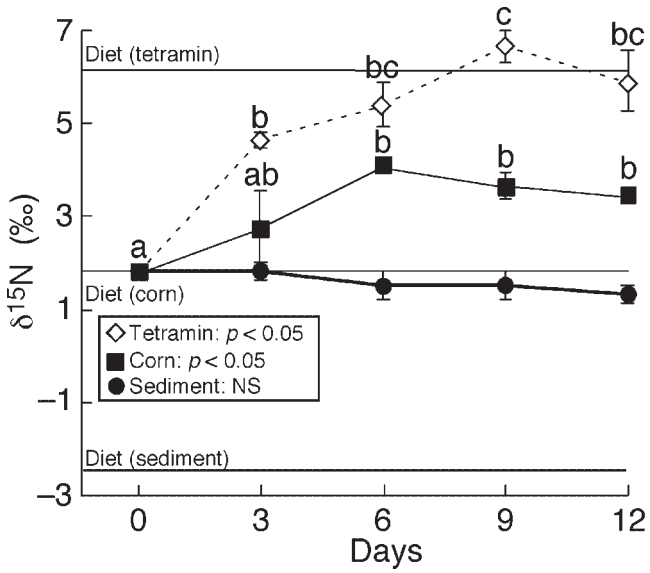

Figure 3. Values of δ^{15}N of *C. acerbiphilus* larvae fed each diet. Different letters indicate significant differences in δ^{15}N between sampling days for each treatment. The *p* values describe the significance of the treatment effect (Tukey test: $n = 5$). Data are means ± 1 SE ($n = 5$). NS = non-significant.

and the nitrogen isotope turnover of chironomids fed corn and tetramin did not differ significantly (paired t-test: $p > 0.05$, $n = 3$; Table 1), indicating that these rates changed similarly throughout the experiment. In addition, from 6 to 12 days, the nitrogen turnover in corn-fed chironomids (56.7–65.6%) did not significantly differ from the corresponding carbon turnover rates (48.8–64.4%) (paired t-test: $p > 0.05$, $n = 3$; Table 1).

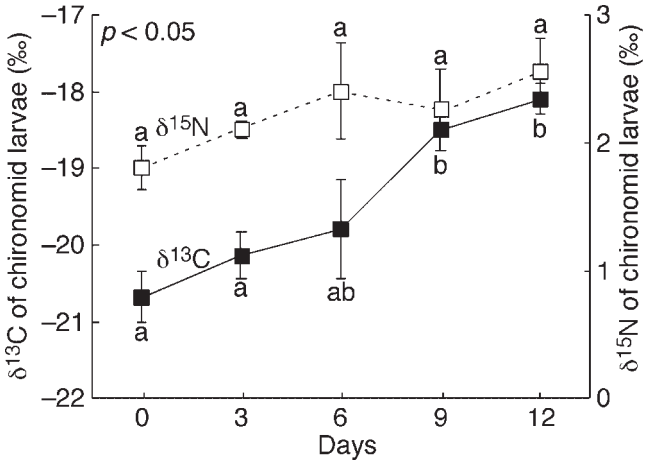

Figure 4. Values of δ^{13}C and δ^{15}N of *C. acerbiphilus* larvae during starvation. Different letters indicate significant differences in isotope values between sampling days for each treatment. The *p* values describe the significance of the treatment effect (Tukey test: $n = 5$). Data are means ± 1 SE ($n = 5$).

Experiment 2: Isotope changes in starved larvae

The dry weight of chironomid larvae did not change significantly during the starvation experiment (Fig. 1), but δ^{13}C values tended to increase (Fig. 4). The δ^{13}C values at 9 and 12 days (−18.5 to −18.1‰) were significantly higher than those at 0 and 3 days (−20.7 to −20.1‰; Tukey test: $p < 0.05$, $n = 5$; Fig. 4). The δ^{15}N values also increased slightly during starvation, but they did not differ significantly between 0 and 12 days.

Experiment 3: Isotope changes between fourth-instar larvae and adults

We measured and compared the isotope values of adult chironomids that emerged in the laboratory 0 to 3 days after they were collected from Lake Katanuma as fourth-instar larvae and chironomids collected in the fourth instar. The δ^{13}C values of adults tended to be lower than those of fourth-instar larvae in the laboratory. The δ^{15}N values of the adults were significantly greater (by 0.7−1.6‰) than those of the larvae (t-test, $p < 0.01$, $n = 10$; Fig. 5).

DISCUSSION

Isotope turnover in feeding

We measured changes in isotope levels in chironomid larvae and calculated growth (%) for up to 12 days after molting. The growths and the carbon and nitrogen isotope turnover calculated by using isotope data were similar on each sampling day. This similarity suggests that isotope changes were mainly due to assimilation into new body tissues. During periods of little weight gain (between 6 and 12 days), the isotope turnover rates were slightly changed. These results indicate that isotope turnover in chironomid larvae mainly occurred along with growth (0–6 days), and thus that larvae may only break down their food for energy during periods of little or no growth.

Our results showed that, from 6 to 12 days, the nitrogen and carbon isotope turnover in corn-fed chironomid larvae

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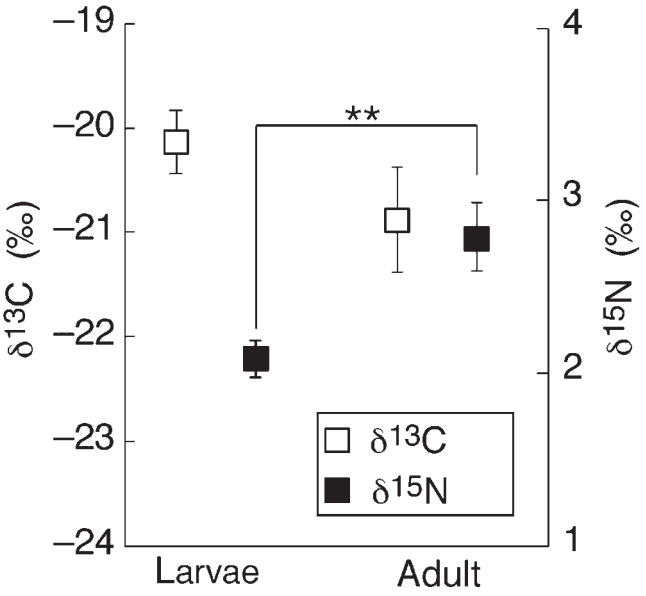

Figure 5. Values of $\delta^{13}C$ and $\delta^{15}N$ of *C. acerbiphilus* adults and fourth-instar larvae. Different letters indicate significant differences in isotope values between the larvae and the adults. ** indicates the significance of the treatment effect (t-test, $p < 0.01$, $n = 10$). Data are means ± 1 SE ($n = 10$).

(56.7–65.6% and 48.8–64.4%, respectively) did not differ significantly, suggesting that chironomids may convert carbon and nitrogen in the same pattern. Moreover, nitrogen isotope turnover did not differ significantly between chironomids that were fed tetramin and those fed corn. This similarity suggests that chironomids convert isotopes at the same rate–about 60%–during the fourth instar regardless of diet.

In other studies, the time from the initial to 50% turnover of the aquatic invertebrates varied, e.g. 4 days (rotifer[16]) and 10 days (shrimp[4]). In this study, however, we found a time of 3–6 days from the initial to 50% turnover with higher growth rate than in those other invertebrates. Thus, the isotope turnover times of the aquatic invertebrates were largely dependent on their growth rate.

The $\delta^{13}C$ and $\delta^{15}N$ primary producers in aquatic ecosystems varied temporally due to their growth conditions, algal mat thickness, and the supply of dissolved inorganic carbon and nitrogen.[17,18] If the potential food sources for the benthic food webs have various and temporal $\delta^{13}C$ and $\delta^{15}N$ values, the chironomid larvae may reflect these temporal isotope values of the potential food sources, because of their rapid isotope turnover.[10,11] Thus, the chironomid larvae may be useful when considering the detailed temporal variations of the potential food sources for benthic food webs.

Isotope turnover in starvation

In the starvation experiment, chironomid $\delta^{13}C$ values significantly increased (by 2.6‰) from 0 to 12 days. The larvae may obtain energy for maintenance from their somatic components during starvation, although their body weights did not significantly decrease. The chironomids probably metabolized somatic components with relatively lower $\delta^{13}C$ values and released more ^{12}C as CO_2 during starvation. The $\delta^{13}C$ values of fatty acids are lower (by 1–2‰) than those of somatic components,[19] suggesting that the metabolized component in this study was probably fatty acids.

The chironomid $\delta^{15}N$ values were not significantly changed from 0 to 12 days, indicating that during starvation the $\delta^{15}N$ values were slightly increased. Values of $\delta^{15}N$ reported for starving birds and snails were slightly higher than for their fed counterparts.[20,21] The slight increase in $\delta^{15}N$ of starved chironomid larvae may be expected as a result of the excretion of isotopically light ammonium.[22] Thus, the differences in carbon and nitrogen isotope ratios between periods of starvation and feeding appear to be similar regardless of diet or species. Moreover, the chironomid larvae hibernate in the winter season, and the stable isotope of the larvae are predicted to increase in the hibernation condition of the larvae. The stable isotope values of the starving animals should be increased, since mainly light isotopes are used in catabolism, and, in starvation, these are not replaced.[8,20] However, there were not significant isotope changes in the starved animals for krill and mysids.[5,6] Thus, the isotope changes in the starved animals were dependent on the species.

Isotope turnover in metamorphosis

In experiment 3, the $\delta^{15}N$ values for the adults hatched in the laboratory were significantly higher than for larvae, indicating nitrogen isotope enrichment during pupation. Development of the new exoskeleton occurs during the pupation stages, while the materials of the exuviae were those that had been synthesized during one cycle before the last one. In fact, $\delta^{13}C$ and $\delta^{15}N$ were higher and lower, respectively, in mysid exuviae than in mysid muscle.[6] Exuviae of chironomid larvae, released at molting and pupation, may also have relatively higher $\delta^{13}C$ and lower $\delta^{15}N$ values reflecting the previous values of the larvae, and this would explain the values in emergent adults. Thus, the isotopic changes in the metamorphosis of the invertebrates should be considered when estimating the migration of the adults and the food-web analysis between aquatic and terrestrial food webs. However, the isotope signatures of adult chironomids may simply reflect those of the larval diets, because the differences in the isotope values were small between larvae and adults compared with those among the diets. It may thus be possible to estimate the migration of the chironomid larvae using the stable isotope technique as in previous fish studies.[23,24]

The results of our feeding experiment showed that isotope fractionation and changes in isotope levels mainly occurred during chironomid growth (periods of weight increase). However, our results also suggest that significant changes in carbon and nitrogen isotope ratios occur even during periods of starvation. Enrichment of heavy isotopes (^{13}C and ^{15}N) was observed in starving chironomid larvae, when weight loss was not significant. Like many insects, chironomids undergo holometabolous development, which includes a non-feeding, quiescent period, i.e. the pupal stage. We found evidence for significant changes in chironomid isotope ratios (^{15}N enrichment) during pupation. This suggests that the physiological condition of animals (such as during an active growth phase or pre- or post-molting) is important to their stable isotope ratios.

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