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Isotopic enrichment without change in diet: an ontogenetic shift in $\delta^{15}N$ during insect metamorphosis

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Summary

1. Insects are frequently sampled in food web studies and are assumed to follow the pattern of enrichment in ^{15}N across trophic levels observed for other organisms. However, few studies have examined ^{15}N discrimination of metamorphosing insects.

2. We measured the $\delta^{15}N$ of larvae, pupae and adults of four Lepidoptera (*Bombyx mori*, *Galleria mellonella*, *Manduca sexta* and *Vanessa cardui*), one Diptera (*Sarcophaga* sp.) and one Coleoptera (*Tenebrio molitor*) fed on artificial diets.

3. The tissues of larvae were enriched in ^{15}N relative to their diet and this enrichment was explained by the production of ^{15}N-depleted frass. Surprisingly, the tissues of adults were enriched in ^{15}N relative to larvae in all but one species (*T. molitor*). Because, we measured the tissues of adults immediately after they emerged from pupal cases, the enrichment was not due to a change in diet. Rather, it was due to the excretion of ^{15}N depleted metabolic waste ('meconium') that resulted from protein metabolism during metamorphosis.

4. To our knowledge, the ^{15}N enrichment that seems to accompany metamorphosis in holometabolous insects had not been previously recognized. This enrichment must be accounted for in ecological studies that rely on stable isotopes to identify both the trophic position and diet of insects.

Key-words: nitrogen, development, larvae, stable isotope, trophic position

Introduction

The analysis of naturally occurring stable isotope ratios of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) has rapidly become one of the most widely used tools in animal ecology. Stable isotopes are used to infer an animal's diet, trophic position and hence its role in an ecological community (Post 2002; Martinez del Rio & Wolf 2005). Because consumers are enriched in ^{15}N relative to their diet (DeNiro & Epstein 1981; Peterson & Fry 1987), measurements of nitrogen stable isotope ratios ($\delta^{15}N$) are widely used to assign trophic position. Generally, ecologists use 3·4‰ as the mean trophic enrichment in $\delta^{15}N$ or $\Delta^{15}N$($\Delta^{15}N = \delta^{15}N_{diet} - \delta^{15}N_{consumer}$) in trophic studies (DeNiro & Epstein 1981; Post 2002).

Although there is significant evidence to support trophic enrichment, $\Delta^{15}N$ values vary and range from –3‰ to 6‰ (Post 2002; McCutchan *et al.* 2003; Vanderklift & Ponsard 2003). In response to evidence of variation in $\Delta^{15}N$, recent studies are incorporating more precise species-specific estimates of $\Delta^{15}N$ into food web studies (e.g. Doi *et al.* 2006). Several mechanisms have been hypothesized to explain variation in $\Delta^{15}N$. These include food quality (Adams & Sterner 2000; Robbins, Felicetti & Sponheimer 2005), growth rate (Martinez del Rio & Wolf 2005), form of nitrogenous waste (Vanderklift & Ponsard 2003), fasting (Ben-David *et al.* 1999), nutrient routing (Kelly 2000) and functional feeding group (Vander Zanden & Rasmussen 2001). Overall, the physiological mechanisms for ^{15}N enrichment are poorly understood and it is likely that the discrimination factor for ^{15}N in consumers is a function of multiple processes. It is widely accepted that isotopic discrimination of ^{15}N occurs during amino acid synthesis, whereby amine groups containing ^{15}N are retained relative to those containing ^{14}N (Gaebler, Vitti & Vukmirov 1966). The net result is that ^{15}N is preferentially retained in consumers' tissues while ^{14}N is excreted as waste (Minagawa & Wada 1984; Gannes, O'Brien & Martinez del Rio 1997).

For holometabolous insects, the remodelling of larval to adult tissues during pupation involves complex metabolic processes that lead to the production of a significant amount of 'waste' nitrogen (called meconium). Holometabolous insects dispose of this nitrogenous waste immediately after emergence from the pupal case (Chapman 1998). There is evidence from previous studies that $\delta^{15}N$ enrichment occurs during insect development, particularly during metamorphosis

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Table 1. Nitrogen stable isotope (‰) values and percent nitrogen, carbon, molar C : N ratios (by dry mass) and sources of artificial diets in this study

Species	$\delta^{15}N$	%N	%C	C : N	Source
Bombyx mori	1·9	4·4	43·6	11·5	Educational Science
Galleria mellonella	3·4	1·1	42·5	46·5	Berkshire Biological
Manduca sexta	3·6	4·9	36·7	8·7	University of Arizona Center for Insect Science
Vanessa cardui	0·6	2·5	45·1	21·7	Educational Science
Sarcophaga haemorrhoidalis					Wards Natural Science
Tenebrio molitor	3·6	3·5	45·6	15·4	University of Wyoming Zoology and Physiology

(Mihuc & Toetz 1994; McCutchan *et al.* 2003; Patt *et al.* 2003; Doi *et al.* 2007). We investigated whether protein catabolism causes enrichment in ^{15}N between larvae and adults during metamorphosis.

Methods

INSECT REARING

We reared the following six species of holometabolous insects from larvae to emergence from pupae: Lepidoptera: *Bombyx mori* (L.) (silkworm moth,), *Galleria mellonella* (L.) (wax moth,), *Manduca sexta* L. (tobacco hornworm) and *Vanessa cardui* (Linn.) (painted lady butterfly), Diptera: *Sarcophaga* sp. (flesh fly) and Coleoptera: *Tenebrio molitor* L. (mealworm). Insects and their species-specific artificial diets were obtained from commercial and educational sources and fed throughout larval development (Table 1). The carbon and nitrogen elemental composition, molar C : N ratios, and $\delta^{15}N$ values of the artificial diets are presented in Table 1. We separated larvae from each species into groups of five individuals and housed them in clear plastic cylindrical containers (10·5 cm diameter × 7·5 cm height) within an environmental chamber (25 ± 1 °C), 50% (± 10%) relative humidity and a 12 : 12 h L : D photocycle. Food was provided *ad libitum* and frass was removed daily. Two days after pupation, we placed individual pupae in 10·2 × 2·5 cm clear plastic cylinders until collection date or eclosion of adults. Larvae and pupae of *Sarcophaga* sp. were sampled immediately upon delivery, therefore diet and frass were not available for analysis.

SAMPLE COLLECTION AND PROCESSING

We analysed the isotopic composition of 8–10 larvae, pupae and adults of each species. Insects were euthanized by freezing and the gut contents of larvae were removed before processing. We collected samples of diet and frass from late-instar larvae. Pupae samples were frozen at the midpoint of pupation for each species. Adults, meconium and exuviae (pupal case) were collected immediately after eclosion and stored frozen at –30 °C. Meconium samples in pupal cylinders were thawed and rinsed with *c.* 15 mL of distilled water, filtered through 25 μm filter paper into a 50 mL glass vial, and dried at 50 °C. Dried meconium was removed from the sides of each vial and homogenized. All samples were freeze-dried, homogenized and stored in a desiccator until weighed for stable isotope analysis. Powdered samples were loaded (*c.* 1·0 mg) into pre-cleaned tin capsules for isotopic and elemental analysis of nitrogen.

Nitrogen isotope and carbon and nitrogen elemental analyses were analysed using a Thermo Finnigan Delta plus XP (Waltham, MA, USA) continuous flow inlet stable isotope ratio mass spectrometer at the University of Wyoming Stable Isotope Facility. Peptone ($\delta^{15}N$ = 5·60‰, AIR, USGS40 8542) and glycine ($\delta^{15}N$ = 0·73‰, AIR, IAEAN2) internal laboratory standards were used for $\delta^{15}N$ and sample precisions were ±0·2‰. Stable isotope ratios were expressed using standard delta notation (δ) in parts per mil (‰) as:

$$\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000$$

where R_{sample} and $R_{standard}$ are the ratios of ^{15}N : ^{14}N of the sample and standard, respectively. Samples were referenced against the international standard, atmospheric nitrogen (AIR) for $\delta^{15}N$.

STATISTICS

We tested whether $\Delta^{15}N_{larvae}$ ($\Delta^{15}N_{larvae} = \delta^{15}N_{larvae} - \delta^{15}N_{diet}$) values were significantly different from 0 with one-sample *t*-tests, and compared $\Delta^{15}N_{larvae}$ and $\Delta^{15}N_{adult}$ ($\Delta^{15}N_{adult} = \delta^{15}N_{adult} - \delta^{15}N_{diet}$) using one-way analysis of variance (ANOVA). We compared the $\delta^{15}N$ of larvae and exuviae of each species with one-way ANOVA. For the larvae and adults of several species, we were able to measure the $\delta^{15}N$ of both frass and meconium for individuals within species; we compared these values using paired *t*-tests. All tests were performed using SPSS 12·0·1 (SPSS for Windows; SPSS Inc., Chicago, IL, USA).

Results

In all species the larvae had more positive $\delta^{15}N$ values than their diets (Fig. 1). $\Delta^{15}N_{larvae}$ ranged from 1·3‰ to 3·9‰ and was significantly different from 0 in all species (Table 2). The $\Delta^{15}N_{adult}$ values were also significantly greater than 0 for all species and ranged from 2·4‰ to 5·0‰. $\Delta^{15}N_{adult}$ was significantly higher than $\Delta^{15}N_{larvae}$ in five out of the six insect species sampled (Table 2). The exception was *T. molitor*. In this species, $\Delta^{15}N_{adult}$ did not differ from $\Delta^{15}N_{larvae}$. Frass $\delta^{15}N$ was significantly more negative (i.e. depleted in ^{15}N) than larvae in all species analysed and was lighter than the diet in three of the four species for which we had data (Table 3 and Fig. 1). Meconium was also depleted in ^{15}N relative to adult tissues (Table 3). Exuviae was depleted in ^{15}N relative to larvae in *B. mori*, *G. mellonella*, *V. cardui* and *S. haemorrhoidalis*. In contrast, the exuviae of *M. sexta* and *T. molitor* was enriched in ^{15}N (i.e. $\delta^{15}N$ was more positive) relative to larvae.

Discussion

Our study demonstrates that metamorphosis can affect the $\Delta^{15}N$ of larval vs. adult stages of insects. Although a handful

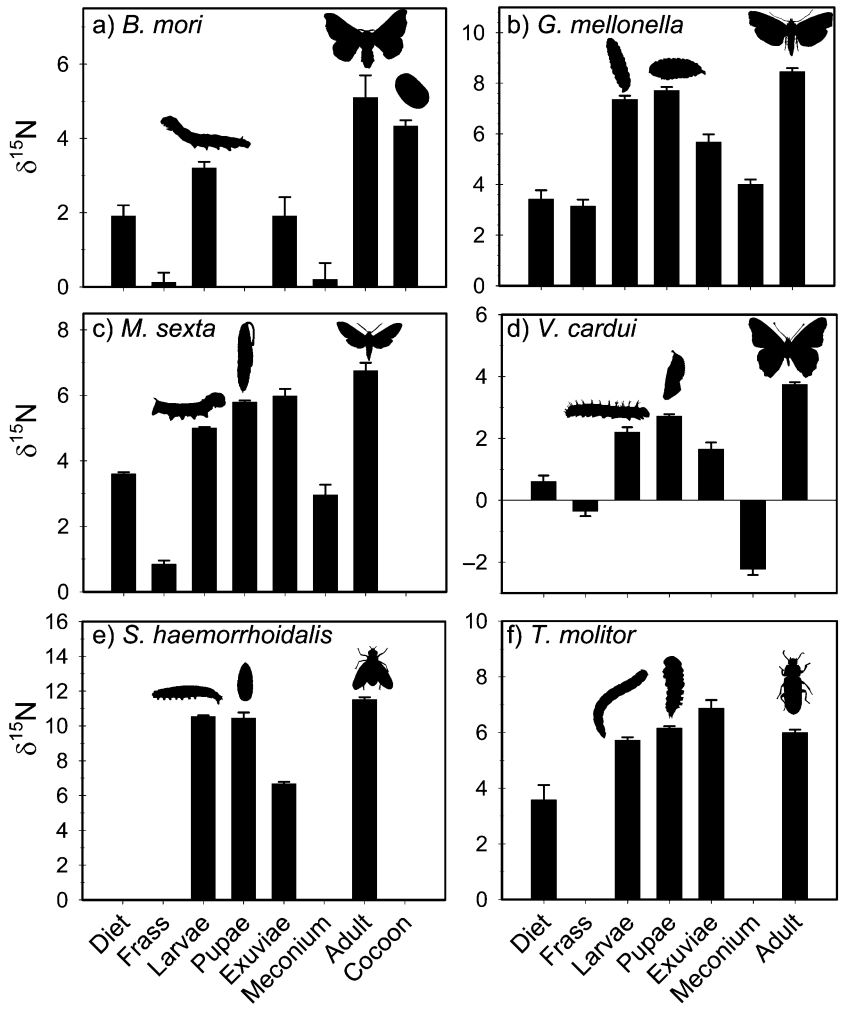

Fig. 1. Insect larvae are significantly enriched in ^{15}N relative to their diets. In addition, adults of the study species were enriched in ^{15}N relative to larvae with the exception of *T. molitor*. The enrichment in ^{15}N in the tissues of insects is probably due to the production of ^{15}N-depleted nitrogen waste products (frass and meconium). Bars represent mean ± SE.

Table 2. $\Delta^{15}N$ (‰) of larval and adult stages for each insect species

Species	$\Delta^{15}N_{Larvae}$	$\Delta^{15}N_{Adult}$	$F_{x,y}$	F	P
Bombyx mori	1·3 ± 0·4*	3·2 ± 1·3*	$F_{1,8}$	9·36	0·02
Galleria mellonella	3·9 ± 0·5**	5·0 ± 0·4**	$F_{1,19}$	30·5	<0·001
Manduca sexta	1·4 ± 0·1**	3·2 ± 0·7**	$F_{1,16}$	68·1	<0·001
Vanessa cardui	1·6 ± 0·5**	3·1 ± 0·2**	$F_{1,18}$	79·2	<0·001
Tenebrio molitor	2·2 ± 0·3**	2·4 ± 0·3**	$F_{1,18}$	3·57	0·07

Significance of *t*-test ($\Delta\delta^{15}N > 0$) shown as $^*P < 0{\cdot}001$ and $^{**}P < 0{\cdot}01$; *F* statistics and *P*-values for one-way ANOVA comparisons of $\Delta^{15}N_{larvae}$ and $\Delta^{15}N_{adult}$ ($\Delta^{15}N_{larvae} = \delta^{15}N_{larvae} - \delta^{15}N_{diet}$; $\Delta^{15}N_{adult} = \delta^{15}N_{adult} - \delta^{15}N_{diet}$).

of studies have observed $\delta^{15}N$ enrichment after metamorphosis for Lepidoptera (McCutchan *et al.* 2003), Diptera and Trichoptera (Mihuc & Toetz 1994; Doi *et al.* 2007) and Neuroptera (Patt *et al.* 2003), our data suggest a potential mechanism of $\delta^{15}N$ enrichment. Variation in discrimination of ^{15}N among holometabolous insects likely reflects the metabolic processes involved in the formation of adult tissues and the production of nitrogenous waste during metamorphosis.

The relative amount of internal reconstruction and tissue recycling during metamorphosis is determined by the similarity of larval and adult forms. Compared to the other insect Orders in this study, Coleoptera larvae resemble adults more and undergo less tissue reconstruction (Chapman 1998). This difference may explain the similarity in $\Delta^{15}N$ of larval and adult stages of *Tenebrio*. Since we only observed one species of Coleoptera, we cannot judge if this pattern holds across other species in this Order. However, in a study of three species of beetles, $\delta^{15}N$ values showed little difference between larvae and adults (Scrimgeour *et al.* 1995). Interestingly, $\delta^{15}N$ values of adult beetles in the same study were enriched by *c.* 3‰–4‰ compared to larvae after overwintering as pupae. Nitrogen metabolism during diapause may be an additional source of ^{15}N discrimination for species that do not undergo large turnover of N during metamorphosis. Kohzu *et al.* (2004) found that adults of the aquatic beetle, *Helodes* sp. were enriched in $\delta^{15}N$ by *c.* 5‰ compared to larvae; however,

Table 3. $\delta^{15}N$ values (mean ± SD) differed among larvae and frass in all species tested. Adult tissues were enriched in ^{15}N relative to meconium in all species tested. Exuviae differed in $\delta^{15}N$ from larval tissues, but the direction of the difference was not consistent across species

Species	Larvae	Frass	*P**	Adult	Meconium	*P**	Larvae	Exuviae	*P**
Bombyx Mori	3·2 ± 0·4	0·1 ± 0·6	0·001	5·1 ± 1·3	0·2 ± 1·0	0·001	3·2 ± 0·4	1·9 ± 1·1	0·04
Galleria mellonella	7·4 ± 0·5	3·1 ± 0·8	0·001	8·5 ± 0·4	4·0 ± 0·6	0·001	7·4 ± 0·5	5·7 ± 1·0	0·001
Manduca sexta	5·0 ± 0·1	0·9 ± 0·4	0·001	6·8 ± 0·7	3·0 ± 0·9	0·001	5·0 ± 0·1	6·0 ± 0·6	0·001
Vanessa cardui	2·2 ± 0·5	−0·4 ± 0·5	0·001	3·7 ± 0·2	−2·2 ± 0·6	0·001	2·2 ± 0·5	1·7 ± 0·7	0·05
Sarcophaga haemorrhoidalis	10·6 ± 0·2	na		11·3 ± 0·4	na		10·6 ± 0·2	6·7 ± 0·3	0·001
Tenebrio molitor	5·7 ± 0·3	na		6·0 ± 0·3	na		5·7 ± 0·3	6·9 ± 0·9	0·001

Letters denote significant difference between groups, $P < 0·05$; na, not available. *Results for paired *t*-test analyses.

because the study period spanned 3 years of field collections, it is difficult to determine whether enrichment was caused by metamorphosis, N metabolism during diapause, or adult diet.

Our data are consistent with the idea that ^{15}N enrichment is a consequence of the excretion of ^{15}N-depleted waste products. Both frass and meconium were depleted in ^{15}N. Meconia excreted from newly emerged adults were depleted by 3·8‰–5·9‰ compared to adults and were composed of 16·8‰–22·1% N. Recent studies have hypothesized that the form of N waste products can lead to differences in trophic enrichment (Vanderklift & Ponsard 2003). In general, terrestrial insects largely excrete uric acid, however, ammonia, allantoin and allantoic acid are also found in varying amounts in excreta of the insect Orders in this study (Chapman 1998). While not identified for the species in this study, the form of nitrogenous excreta may have been an important factor in kinetic discrimination against ^{15}N during metamorphosis (Vanderklift & Ponsard 2003).

We observed surprising variation in differences in $\delta^{15}N$ of exuviae compared to larvae (Table 3). If exuvia is largely composed of chitin, and chitin is depleted in $\delta^{15}N$ relative to both diet and proteins (Minagawa & Wada 1984; Schimmelmann & DeNiro 1986), exuviae is expected to be depleted in $\delta^{15}N$ compared to larvae. Once the ^{15}N-depleted exuvia is formed, the remaining pool of N in the pupa would be enriched, contributing to overall enrichment of adult tissues; however, we observed this pattern in only four of the six species sampled. Both *M. sexta* and *T. molitor* had exuviae that were enriched in ^{15}N relative to diet and larval values. The molar C : N ratio of pure chitin is 6·9, however, when chitin is cross-linked with proteins it has a lower C : N ratio (Schimmelmann & DeNiro 1986). C : N of exuviae ranged from 3·0 to 5·8 across species in this study. *Manduca sexta* had the lowest exuviae C : N (*c.* 3·0), but this pattern did not hold for *T. molitor*, which had a C : N of 5·4. Cocoons of *B. mori*, made of silk proteins and secretions, were enriched compared to larval values, probably reflecting ^{15}N-enriched silk proteins. Variation in $\delta^{15}N$ for exuviae in this study illustrates that exuviae should not be used as indicators of larvae or diet until differences between exuviae and larvae are studied for a species.

In conclusion, enrichment during metamorphosis must be accounted for in ecological studies that rely on stable isotopes and that aim to identify the trophic position and diet of insects. Ecological studies that include either insects of different life stages or sampling of different tissue types (i.e. exuviae vs. actual larvae) must recognize that life stages may differ in tissue-to-diet isotopic ^{15}N discrimination. In insects, tissue-diet ^{15}N discrimination appears to be explained by the production of ^{15}N-depleted waste products.

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