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Intra-Body Variation and Ontogenetic Changes in the Isotopic Composition ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) of Beetles (Coleoptera)

S. M. Tsurikov[a], A. A. Goncharov[b], and A. V. Tiunov[b]

[a]*Faculty of Biology, Lomonosov Moscow State University, Moscow, 111991 Russia*
e-mail: smtsurikov@rambler.ru
[b]*Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, 119071 Russia*

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Abstract—Stable isotope analysis, which is widely used for studying trophic relationships of invertebrates, requires a correct estimation of trophic fractionation factors as well as the proper choice of reference tissues. Different organs and body parts of large insects may vary in the isotopic composition of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$), though detailed information is available for a limited set of species only. In the field-collected larvae and adults of *Oryctes nasicornis* (Scarabaeidae) and *Uloma rufa* (Tenebrionidae), the range of $\delta^{13}C$ and $\delta^{15}N$ values for different body parts and organs (muscles, legs, wings, elytra, fat body, gonads, etc.) was 4.8‰ and 2.4‰, respectively. We suggest that the muscle tissue or legs of large insects should be preferably selected for isotope analysis. The isotopic composition of nitrogen in beetles did not depend on the ontogenetic stage and was not affected by metamorphosis. In larvae and adults of *Tribolium confusum* (Tenebrionidae) reared in the laboratory culture, the trophic fractionation factors of carbon ($\Delta^{13}C$) and nitrogen ($\Delta^{15}N$) were within the expected values (from −1 to 1.5‰ and from 3 to 5‰, respectively), though depended on the diet. Changes in the $\delta^{13}C$ values during ontogenesis reflected changes in the lipid content and were strongly correlated with the C/N ratio.

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Isotope analysis is widely used for studying the structure of food webs and the trophic relations of organisms. The methods of this analysis, including the techniques for collection and processing of biological material, are being rapidly developed (Tarroux et al., 2010; Krab et al., 2012), but many aspects of practical importance remain insufficiently studied. The organic compounds composing the tissues of living organisms vary in their isotopic composition ($^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios). In particular, lipids and chitin contain less ^{13}C and ^{15}N as compared with proteins (Webb et al., 1998). Different tissues and parts of the insect bodies vary considerably in the mass content of muscle proteins, chitin, and lipids, which results in differences in their isotopic composition (Wehi and Hicks, 2010). Considerable variation of the carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic composition within the same organism was demonstrated in some vertebrates (Vanderklift and Ponsard, 2003; Caut et al., 2009), millipedes (Semenyuk and Tiunov, 2011), orthopterans (Wehi and Hicks, 2010), and aphids (Perkins et al., 2013). Correspondingly, in ecological studies of large invertebrates there is a problem of selecting the right body parts or organs for isotope analysis. This problem is difficult to solve due to the scarcity of available data on the isotopic composition of different insect tissues (DeNiro and Epstein, 1978, 1981; Gratton and Forbes, 2006; Tibbets et al., 2008). Some researchers analyzed certain body segments or tagmata (Tillberg et al., 2006; Traugott et al., 2007), internal organs (Perkins et al., 2013) or the exoskeleton (Tayasu et al., 2002); but in the majority of the published works, whole insects were used for analysis.

Holometabolous development, characteristic of some insect orders, involves complex biochemical transformations during metamorphosis (Schwanwitsch, 1949), which may lead to changes in the isotopic composition of the insect tissues. Indeed, an increase in the ^{15}N content in adults as compared to larvae was demonstrated in several species of insects (Doi et al., 2007; Tibbets et al., 2008). However, it remains almost unknown to which extent the isotopic composition of the young adults reflects that of the larvae.

The goal of this work was to compare the isotopic composition of different body parts of beetle larvae and adults and to follow the changes in the isotopic composition during the development of these holometabolous insects.

Table 1. The carbon and nitrogen isotopic composition ($\Delta^{13}C$ and $\Delta^{15}N$ values) and the C/N mass ratio of different body parts and organs of the larvae of *Oryctes nasicornis* ($n = 5$) and *Uloma rufa* ($n = 4$)

Body parts	*Oryctes nasicornis*			*Uloma rufa*		
	$\Delta^{13}C$, ‰	$\Delta^{15}N$, ‰	C/N	$\Delta^{13}C$, ‰	$\Delta^{15}N$, ‰	C/N
Head	0.6 ± 0.3 a	2.4 ± 0.5 ab	4.7 ± 0.1 a	3.1 ± 0.4 a	1.7 ± 0.1 b	4.3 ± 0.2 a
Legs	1.1 ± 0.3 a	2.5 ± 0.8 ab	4.4 ± 0.1 a	–	–	–
Integuments	0.1 ± 0.5 a	0.6 ± 0.3 a	4.4 ± 0.1 a	2.4 ± 0.3 a	0.7 ± 0.3 a	4.7 ± 0.2 a
Muscles	0.5 ± 0.8 a	3.0 ± 0.2 b	5.5 ± 0.8 a	4.0 ± 0.1 a	2.8 ± 0.1 c	3.9 ± 0.1 a
Gut wall	–0.2 ± 0.2 a	2.4 ± 0.6 ab	5.6 ± 0.4 a	2.7 ± 0.5 a	2.3 ± 0.2 bc	7.2 ± 1.9 a
Fat body	–3.1 ± 0.7 b	3.0 ± 0.3 b	21.4 ± 3.4 b	–	–	–

Tissues that are significantly different ($p < 0.05$, Tukey's HSD test) within each column are marked with different letters.

Table 2. The carbon and nitrogen isotopic composition ($\Delta^{13}C$ and $\Delta^{15}N$ values) and the C/N mass ratio of different body parts and organs of the adults of *Oryctes nasicornis* ($n = 5$) and *Uloma rufa* ($n = 4$)

Body parts	*Oryctes nasicornis*			*Uloma rufa*		
	$\Delta^{13}C$, ‰	$\Delta^{15}N$, ‰	C/N	$\Delta^{13}C$, ‰	$\Delta^{15}N$, ‰	C/N
Head	–0.8 ± 0.4 ab	2.6 ± 0.2 ab	5.5 ± 0.3 a	2.8 ± 0.1 a	2.2 ± 0.2 b	4.6 ± 0.2 a
Legs	1.0 ± 0.2 cb	3.1 ± 0.2 b	4.1 ± 0.1 a	2.7 ± 0.2 a	2.2 ± 0.1 b	4.4 ± 0.1 a
Integuments	0.6 ± 0.2 cb	2.7 ± 0.2 ab	4.3 ± 0.1 a	–	–	–
Wings	1.8 ± 0.4 c	4.0 ± 0.3 b	3.9 ± 0.1 a	2.8 ± 0.2 a	2.6 ± 0.1 ab	4.7 ± 0.3 a
Elytra	–0.5 ± 0.1 b	2.1 ± 0.1 a	5.1 ± 0.1 a	2.8 ± 0.1 a	1.6 ± 0.1 b	4.5 ± 0.1 a
Muscles	0.6 ± 0.7 cb	3.0 ± 0.2 ab	4.7 ± 0.7 a	3.3 ± 0.3 a	3.0 ± 0.1 a	4.1 ± 0.1 a
Gonads	0.1 ± 0.4 cb	2.8 ± 0.2 ab	5.2 ± 0.3 a	3.1 ± 0.4 a	3.3 ± 0.2 a	4.4 ± 0.3 a
Malpighian tubules	–0.2 ± 0.7 cb	2.2 ± 0.2 a	5.3 ± 0.7 a	–	–	–
Gut wall	0.6 ± 0.3 cb	2.2 ± 0.5 a	4.4 ± 0.1 a	2.4 ± 0.4 a	2.3 ± 0.3 ab	6.4 ± 1.2 a
Fat body	–3.0 ± 0.8 a	1.7 ± 0.5 a	15.7 ± 5.6 b	–	–	–

See Table 1.

MATERIALS AND METHODS

The isotopic composition of certain organs at different development stages were studied in the larvae and adults of the European rhinoceros beetle *Oryctes nasicornis* (L., 1758) (Scarabaeidae) and the darkling beetle *Uloma rufa* Piller et Mitterpacher, 1783 (= *Uloma perroudi* Mulsant et Guillebeau, 1855) (Tenebrionidae). The insects were collected in June 2012, in the sawdust waste piles of a woodworking plant in the environs of Shuya, Ivanovo Province (56°51′N, 41°22′E). The main criterion for choosing the study objects was the uniform isotopic composition of their food substrate (decomposing sawdust). The insects were dissected into individual tissues and organs: head, legs, sclerotized integuments, wings, thoracic muscles, fat body, foregut wall, and Malpighian tubules (Tables 1, 2). Fragments of integuments were obtained from the ventral surface of the first abdominal segments of *U. rufa* and the larvae of *O. nasicornis*, and from the dorsal surface of the abdomen of adult *O. nasicornis*.

In addition, we studied the changes in the isotopic composition during the life cycle of the confused flour beetle *Tribolium confusum* (Jacquelin du Val, 1861) (Tenebrionidae) from the laboratory culture. Two food substrates were used in the experiment: wheat grits (C3 type of photosynthesis, $\delta^{13}C$ about –27‰) and corn grits (C4 type of photosynthesis, $\delta^{13}C$ about –12‰). The larvae were kept at 28°C and a relative air humidity of 40%. Samples were taken at all the stages of the life cycle except the egg. The larvae were divided into four age groups by their size and duration of development (L1–L4). A special group was that of pupae (P). Among the adult beetles, freshly eclosed young adults with their integuments still soft (YA) were processed separately from mature adults (MA).

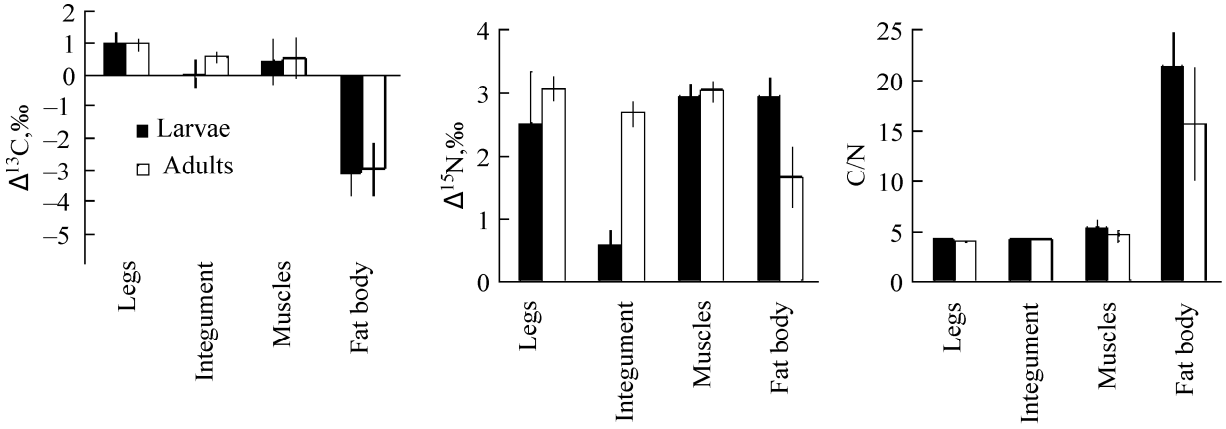

Fig. 1. The carbon and nitrogen isotopic composition ($\Delta^{13}C$, $\Delta^{15}N$) and the C/N mass ratio of some body parts of the larvae and adults of *Oryctes nasicornis*: means and standard errors, $n = 5$.

Before measurements the insects were kept for 24 h without food to allow them to empty their intestines. Since in this case we wanted to observe the ontogenetic changes in the isotopic composition of the entire organism, whole insects were homogenized for analysis.

The tissue samples of *O. nasicornis* and *U. rufa* and whole specimens of *T. confusum* were dried at 50°C for 48 h. The dried specimens of *T. confusum* were ground with mortar and pestle before analysis. Isotope analysis of insects and food substrate samples ($n = 4$–5) was performed using a Flash 1112 Elemental Analyzer and a Thermo Delta V Plus isotope ratio mass spectrometer at the Joint Usage Center of Severtsov Institute of Ecology and Evolution. The carbon and nitrogen isotopic composition was expressed as deviation δ from the international reference standard (vPDB and atmospheric N), in parts per thousand (‰):

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

where X is the element in question (carbon or nitrogen), and R is the molar ratio of its heavy and light isotopes. The analytical error of determining the nitrogen and carbon isotopic composition did not exceed ± 0.2‰. Besides the isotopic composition, the carbon and nitrogen content (%C, %N) and the mass ratio of these elements (C/N) were determined for each sample.

The trophic substrates were considerably different in the nitrogen and carbon isotopic composition. The $\delta^{13}C$ and $\delta^{15}N$ values were −25.6 ± 0.1 and −1.3 ± 0.2‰ for sawdust; −26.9 ± 0.1 and −2.8 ± 0.1‰ for wheat grits; −11.5 ± 0.1 and 4.0 ± 0.1‰ for corn grits, respectively. For convenience in comparing the results of different experiments, we used the difference between the isotopic composition of insects and that of the corresponding food: $\Delta = \delta_{consumer} - \delta_{food}$. This difference is commonly known as the "trophic fractionation factor" (Martinez del Rio et al., 2009).

The data were statistically processed by ANOVA and correlation analysis using the Statistica 8 software (StatSoft, Tulsa, USA). The confidence level corresponded to $p < 0.05$. The values are reported as the mean ± the standard error.

RESULTS

The isotopic and elemental composition of certain tissues and body parts of the larvae (Table 1) and adults (Table 2) of *O. nasicornis* were significantly different. The fat body had the lowest $\Delta^{13}C$ values both in the larvae and in the adults. The C/N ratio was significantly higher in the fat body than in other tissues (Fig. 1). There was a strong negative correlation between the $\Delta^{13}C$ value and the C/N ratio (Fig. 2). Thus, the low $\Delta^{13}C$ value in the fat body was probably related to the higher lipid content. The remaining tissues and body parts of rhinoceros beetles were similar in their carbon isotopic composition and in most cases only weakly differed from the $\delta^{13}C$ value of the substrate ($\Delta^{13}C$ close to 0), with the exception of the wings (Table 2) which were somewhat enriched in ^{13}C ($\Delta^{13}C = 1.8 \pm 0.4$‰). The fat body was not developed in the larvae and adults of *U. rufa*. Neither larvae nor adults revealed any significant differences in the carbon isotopic composition and the C/N ratio among different tissues and organs, even though there was a distinct negative correlation between $\Delta^{13}C$ and the C/N

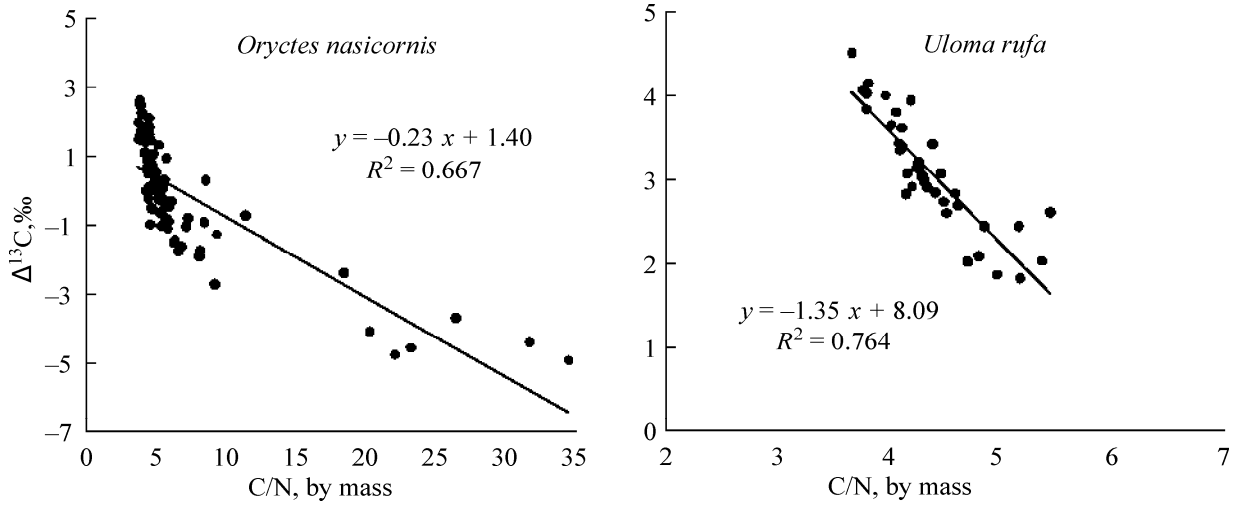

Fig. 2. Correlation between the $\Delta^{13}C$ values and the C/N mass ratio in the tissues of the larvae and adults of *Oryctes nasicornis* and *Uloma rufa*: equations of linear regression and the fraction of explained variance.

ratio in the tissues (Fig. 2). The $\Delta^{13}C$ values in all the examined tissues of *U. rufa* were considerably greater (by 1–3‰) than in *O. nasicornis* (Tables 1, 2).

Oryctes nasicornis and *U. rufa* showed practically no difference in the nitrogen isotopic composition. In the rhinoceros beetle larvae, the highest content of ^{15}N ($\Delta^{15}N$ about 3.0‰) was recorded in the muscles and the fat body (Table 1). The only sample significantly different from these tissues was that of the integuments (0.6 ± 0.3‰). In the adults, the highest $\Delta^{15}N$ values were recorded in the wings, legs, muscles, and gonads. The fat body, Malpighian tubules, and elytra were depleted in ^{15}N (Table 2). Similarly, in *U. rufa* the highest $\Delta^{15}N$ values were recorded in the muscles and gonads, and the lowest, in the integuments of the larvae and the elytra of the adults (Tables 1, 2). In all the cases, there were no significant differences in the isotopic composition between the muscles and the legs.

Considerable similarity in the isotopic composition of the muscle tissues of the larvae and adults was observed in both species, although other organs revealed certain differences in the nitrogen isotopic composition (Fig. 1). On the whole, the results of our study of two beetle species from natural populations indicated that the adults largely preserved the isotopic signatures characteristic of the larvae.

The study of the laboratory population of *T. confusum* allowed us to follow the changes in the carbon and nitrogen isotopic composition of individuals at different stages of development. Two-way ANOVA confirmed the significant influence of the life cycle stage and the type of trophic substrate on the $\Delta^{13}C$ and $\Delta^{15}N$ values (Table 3). With both types of diet, the value of $\Delta^{13}C$ gradually decreased as the larvae developed, and reached the minimum (1–2‰ lower than that of L1) before pupation, in the pupa or in the mature adult. The C/N ratio gradually increased during larval development, reaching the maximum in the pupa or L4 (6.5–6.8). Regardless of the diet, there was a strong negative correlation between the $\Delta^{13}C$ value and the C/N ratio in the tissues of *T. confusum* (Fig. 3). The $\Delta^{15}N$ value varied less significantly (within 0.8–1.2%) and generally increased during development; it reached the maximum at the mature adult stage, but the difference between the stages was

Table 3. The influence of the life cycle stage and the trophic substrate on the carbon and nitrogen isotopic composition ($\Delta^{13}C$ and $\Delta^{15}N$ values) and the C/N mass ratio in the tissues of *Tribolium confusum*: two-way ANOVA

Factor	d.f.	$\Delta^{13}C$		$\Delta^{15}N$		C/N	
		F	*p*	*F*	*p*	*F*	*p*
Substrate	1	40.0	< 0.001	290.4	< 0.001	0.1	0.715
Stage	6	23.1	< 0.001	15.1	< 0.001	11.8	< 0.001
Stage × Substrate	6	4.0	< 0.001	2.9	0.012	2.3	0.039

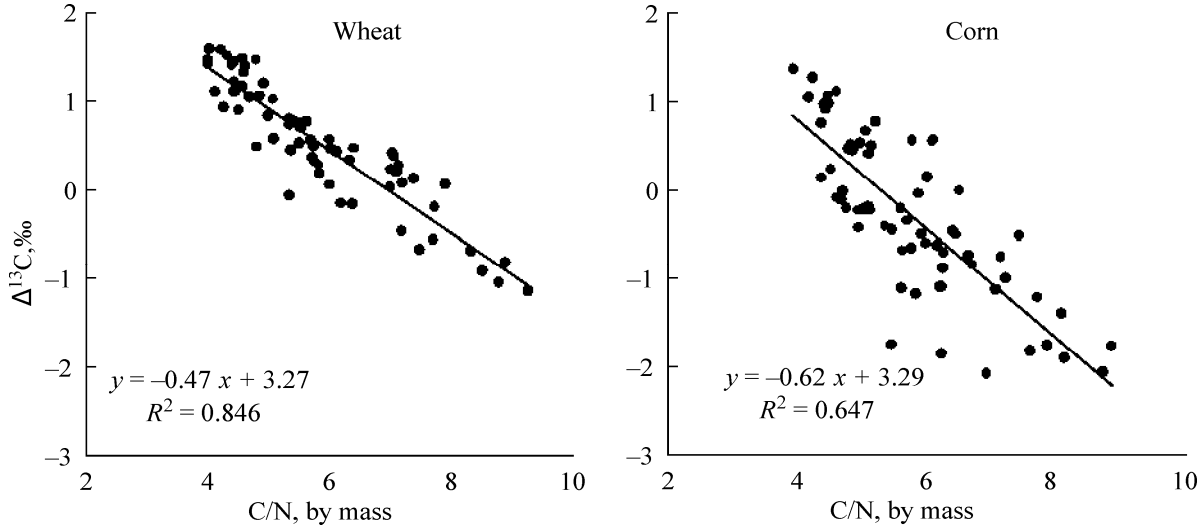

Fig. 3. Correlation between the $\Delta^{13}C$ value and the C/N mass ratio in the tissues of *Tribolium confusum* reared on wheat and corn grits (data for all the stages examined): equations of linear regression and the fraction of explained variance.

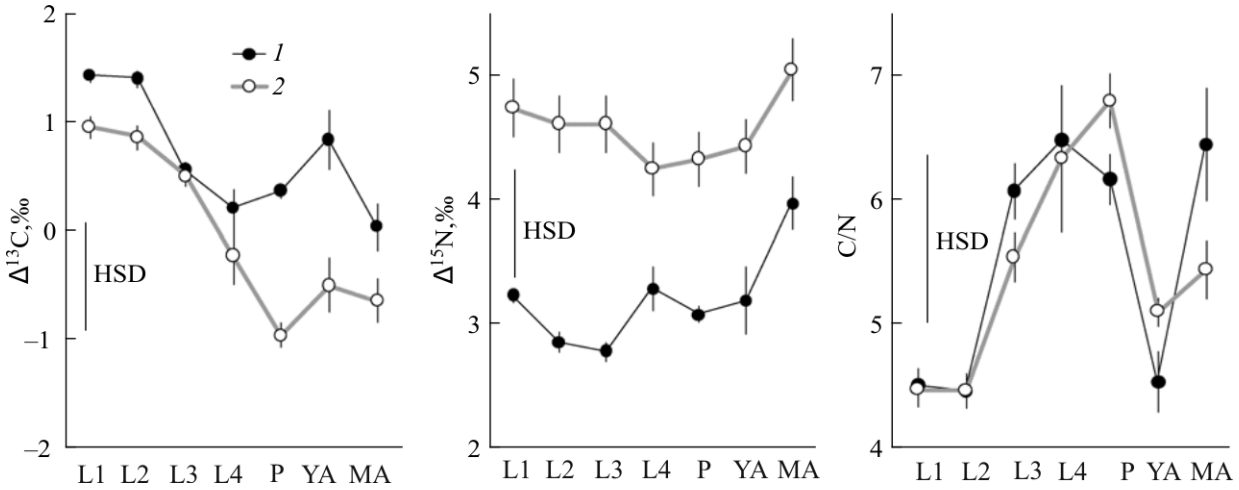

Fig. 4. Changes in the isotopic composition ($\Delta^{13}C$ and $\Delta^{15}N$ values) and the C/N mass ratio at different stages of *Tribolium confusum* reared on wheat (*1*) and corn grits (*2*): means and standard errors, $n = 6–12$. HSD shows the honestly significant difference (Tukey's test for unequal samples); for designations of stages, see text.

usually non-significant (Fig. 4). The $\Delta^{15}N$ values averaged for the entire experiment were significantly higher in the insects feeding on corn grits (4.6 ± 0.1‰) than in those feeding on wheat grits (3.2 ± 0.1‰).

DISCUSSION

It is well known that the carbon and nitrogen isotopic composition varies between the different body parts of animals. However, the problem of selecting the best reference tissue for isotope analysis, which is important for ecological studies, is still unsolved. In most studies of small soil-dwelling insects, analysis was performed with whole specimens, sometimes with the gut content removed. However, in case of larger insects, the use of wholly homogenized specimens does not seem feasible, since the relative mass of the arthropod exoskeleton increases with the body size (Schwanwitsch, 1949). The exoskeleton consists of inert compounds including chitin, biocarbonates, and waxes, whose isotopic composition is often different from that of the metabolically active tissues (Tiunov, 2007; Maraun et al., 2011; Semenyuk and Tiunov, 2011). Therefore, the use of wholly homogenized specimens may result in an unpredictably biased estimate of the isotopic composition. The muscle tissues are metabolically active and do not accumulate inert compounds; therefore, their isotopic signature may be considered to be typical of the whole organism. Unfor-

tunately, it is technically difficult or impossible to obtain muscle tissue samples from very small insects and other invertebrates.

In the studies of the trophic structure of communities using stable isotope analysis, the greatest attention is usually focused on $\delta^{15}N$ value, since this value reflects the trophic level of an organism (Scheu and Falca, 2000; McCutchan et al., 2003). Our results indicate that the $\delta^{15}N$ values are similar in the muscles and the legs, whereas the total range of $\delta^{15}N$ values of the main body parts and tissues of one specimen seldom exceeds 1.0‰. These findings agree with the data obtained for lady beetles (Gratton and Forbes, 2006), aphids (Perkins et al., 2013), and orthopterans (Webb et al., 1998; Wehi and Hicks, 2010). Exceptions to this rule were the integuments and elytra which had lower $\delta^{15}N$ values, possibly due to the high content of chitin depleted in ^{15}N (Webb et al., 1998). On the contrary, the wings of the adults of *O. nasicornis* (but not of *U. rufa*) were strongly enriched in ^{15}N. This fact may be related to the presence of elastomeric proteins in the wing cuticle, in particular resilin that has a relatively large fraction of aspartic acid and alanine enriched in ^{15}N (Popp et al., 2007; van Eldijk et al., 2012).

The strong negative correlation between the $\Delta^{13}C$ and C/N values in all the species studied (Figs. 2 and 4) suggests that the lipid content may be one of the principal factors determining the intraspecific variation of the carbon isotopic composition in insect tissues. This assumption is confirmed by data of other researchers (Tarroux et al., 2010; Perkins et al., 2013). A greater lipid content in the tissues leads to a higher C/N ratio and a lower value of $\Delta^{13}C$, since lipids are depleted in ^{13}C (DeNiro and Epstein, 1978). Thus, lipid extraction which is widely used in the studies of aquatic invertebrates (Post et al., 2007) may be also a useful method of preparing insect tissue samples for isotope analysis. This approach is sometimes used in soil zoology (Abd El-Wakeil, 2009). However, since the lipids contained in the prey constitute an important energy resource for the consumers, lipid extraction may hamper the reconstruction of trophic links of predatory invertebrates (Tarroux et al., 2010). In addition, it should be remembered that most of the isotope-based studies of the terrestrial (and soil) food webs carried out so far did not use the lipid extraction.

The isotopic composition of *O. nasicornis* and *U. rufa* showed very little difference between the larval and adult tissues (Fig. 1). This fact indicates that the isotopic composition of tissues does not change drastically during metamorphosis, although this cannot be ascertained by studying beetles from natural populations. In the laboratory experiment with *T. confusum* we observed quite insignificant changes in the nitrogen isotopic composition during individual development: the range of mean $\Delta^{15}N$ values was 1.2‰ on the wheat diet and 0.8‰ on the corn diet. The metamorphosis itself was not accompanied by pronounced changes in the $\Delta^{15}N$ value (Fig. 4). The value of $\Delta^{13}C$ changed somewhat more significantly during development, probably due to the changes in the relative amount of fat tissue. A decrease in the C/N ratio and an increase in the mean $\Delta^{13}C$ value in the "young" adults of *T. confusum* may be explained by expenditure of the fat reserves during the pupal stage. When the adult beetle starts feeding and replenishes its fat reserves, the C/N ratio increases again while the $\Delta^{13}C$ value decreases (Fig. 4).

Our study provided new data on the trophic fractionation of carbon and nitrogen isotopes in beetles. The $\Delta^{15}N$ value in the muscle tissues of larval and adult *U. rufa* and *O. nasicornis* was almost the same and averaged about 3‰ (Tables 1 and 2), which agreed perfectly with the expected ^{15}N accumulation per one trophic level (Mc-Cutchan et al., 2003; Tiunov, 2007). On the contrary, the carbon isotopic composition ($\Delta^{13}C$) of the tissues of *U. rufa* and *O. nasicornis* was considerably different, even though the two species co-occurred in the same habitat. It may be assumed that the main diet of the darkling beetles *U. rufa* consisted not of wood debris but of the microflora inhabiting them. In this case, the darkling beetle tissues enrichment with ^{13}C (by 2–4‰ as compared to the sawdust) would correspond exactly to the increase in the $\delta^{13}C$ value in the tissues of soil-dwelling saprophages as compared to the plant litter (Potapov et al., 2013). Therefore, darkling beetles should be regarded as "false xylophages" (Chauvin, 1953). The larvae of Scarabaeidae, including *O. nasicornis*, form advanced symbiotic associations with the cellulose-decomposing intestinal microflora, which are facilitated by specific morphological adaptations, namely the midgut caeca and the hindgut fermentation sac. This symbiosis allows the larvae to utilize the structural components of plant tissues as the source of carbon (Striganova, 1980). Correspondingly, the $\Delta^{13}C$ value in rhinoceros beetles is close to zero, which is typical of phytophagous insects (Spence and Rosenheim, 2005). However, we could not reliably determine the trophic links

of the beetles in our material, since they were collected in the field.

In the laboratory experiment with *T. confusum* the isotopic composition of the diet was precisely known. The flour beetles feeding on wheat grits reached the adult stage 12 days faster, the total duration of development being about two months. This was probably related to higher protein content in wheat: the mass fraction of nitrogen in wheat grits was two times larger than in corn grits (2.3 ± 0.1% and 1.2 ± 0.1%, respectively). The highest trophic fractionation of nitrogen isotopes were recorded on the diet with low protein content; the mean $\Delta^{15}N$ value on the corn diet was 1.5‰ greater than that on the wheat diet (Fig. 4). The dependence of $\Delta^{15}N$ on the food quality (in particular, protein content) was repeatedly demonstrated in laboratory experiments, but the reasons and mechanisms of this dependence are still insufficiently known (Webb et al., 1998; Martinez del Rio et al., 2009; Robbins et al., 2010; Semenina and Tiunov, 2011). The $\delta^{15}N$ value was considerably higher in corn grits than in wheat grits (4.0 and −2.8‰, respectively). Thus, our data contradict the assumption that the $\Delta^{15}N$ value should decrease as the $\delta^{15}N$ value of the food increases (Caut et al., 2009; Hussey et al., 2014). The $\Delta^{13}C$ value of *T. confusum* varied during development from −1 to +1.5‰ regardless of the diet, which agrees with the published data on low trophic fractionation of carbon isotopes (Webb et al., 1998; McCutchan et al., 2003).

CONCLUSION

Variation in the $\delta^{13}C$ and $\delta^{15}N$ values of different organs and tissues should be taken into account during analysis of the isotopic composition of large insects. In the adults and larvae of two beetle species in our material, the ranges of $\delta^{13}C$ and $\delta^{15}N$ values within one specimen reached 4.8 and 2.4‰, respectively. In our opinion, the results of isotope analysis of muscle tissue samples without additional lipid extraction would be the easiest to interpret. The isotopic compositions of the legs and muscle tissues were quite similar in the beetle species studied; therefore, legs of arthropods can also be used for isotope analysis. Metamorphosis was not accompanied by considerable changes in the nitrogen isotopic composition of beetle tissues. The relatively greater changes (in the range of 2‰) in the $\delta^{13}C$ values during development were mostly related to changes in the mass fraction of the lipids.

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