

Nuptial feeding is reflected in tissue nitrogen isotope ratios of female katydids

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Summary

1. During copulation male katydids transfer a protein-rich nuptial gift to females, which is ingested by the female. We hypothesized that female katydids are built at least partly from proteins assimilated from nuptial gifts.

2. We measured the ratio of nitrogen isotopes ($\delta^{15}\text{N}$) in the diet and tissue of sexually reproducing *Isophya kraussii* and obligate parthenogenetically reproducing *Poecilimon intermedius*. We used the $\delta^{15}\text{N}$ of muscles as an indicator of the most recent diet and the $\delta^{15}\text{N}$ of the cuticula as an indicator of the larval diet.

3. In free-ranging *I. kraussii*, muscular and cuticular $\delta^{15}\text{N}$ of adult males was low, suggesting a plant diet. Cuticular $\delta^{15}\text{N}$ did not differ between sexes, indicating that immatures of both sexes fed on similar diets. Female muscles were significantly more enriched in nitrogen-15 than female cuticula and also than male muscles, suggesting that adult females incorporated animal proteins and immature females plant material.

4. Female *I. kraussii* that were kept with males in captivity had higher $\delta^{15}\text{N}$ in muscular tissue than in cuticula. Muscular $\delta^{15}\text{N}$ of these females was also higher than that of muscle and cuticula of females held isolated from males, suggesting that incorporated nitrogen originated from nuptial gifts.

5. In parthenogenetically reproducing *P. intermedius* muscular and cuticular $\delta^{15}\text{N}$ was almost identical, suggesting no additional uptake of insect proteins in the absence of nuptial gifts.

6. We therefore conclude that female *I. kraussii* substantially assimilate spermatophylax compounds for both egg production and body homeostasis.

Key-words: Homeostasis, *Isophya*, nitrogen source, spermatophore, stable isotopes

Functional Ecology (2006) **20**, 656–661
doi: 10.1111/j.1365-2435.2006.01154.x

Introduction

Copulating male katydids transfer a nuptial gift, a spermatophore, to females and while the males' sperm enter the female reproductive tract, females feed on the spermatophore (review in Gwynne 2001). The spermatophore consists of two parts: the sperm-containing ampullae and the gelatinous spermatophylax, produced by glands in the male reproductive tract. Two functions of nuptial feeding have been discussed. Firstly, spermatophylaxes may protect the ejaculate because it prevents females from feeding on the ampullae containing the sperm (mating effort hypothesis). Secondly, the offspring may benefit from the nutrients provided and therefore

the spermatophylax represents paternal investment (paternal investment hypothesis). Both hypotheses have been under dispute (Gwynne 1984, 1986, 1988a,b; Wickler 1986; Quinn & Sakaluk 1986), and during the past decades evidence has accumulated for both hypotheses in different species and under different situations (Wedell 1994; Gwynne 2001). Nevertheless, female katydids may as well assimilate spermatophore substrate and, consequently, spermatophores may as well support female body homeostasis (homeostasis hypothesis, Boggs & Gilbert 1979).

The vast majority of phaneropterine katydids feeds on plants, classifying them as herbivorous animals (except for some scavenging North American species; see Gangwere 1961). On the other hand, spermatophores consist mainly of proteins (Heller *et al.* 1998). Therefore, nuptial feeding is exceptional from a dietary perspective

as female katydids ingest protein-rich animal tissue originating from their mating partner in addition to or instead of plant carbohydrates. Thus, the more often female katydids mate and the larger nuptial gifts are, the more a female's diet is dominated by animal protein. Recently, it was shown for *Isophya kraussii*, Brunner von Wattenwyl 1878, that the energy content of male spermatophores is more than twice the amount of energy females expend per day (Voigt, Kunz & Michener 2005). Thus at a remating interval of *c.* 2 days, female *I. kraussii* could live from an exclusive spermatophore diet and still meet all their daily energy requirements, including egg production and body homeostasis. We propose that – depending on the relative spermatophylax size and the mating rate – nuptial feeding may contribute substantially to the diet of female katydids and, consequently, female bodies should be made at least partly of material originating from spermatophylaxes. If nuptial gifts contribute to female body homeostasis, females should have a higher enrichment of nitrogen-15 relative to nitrogen-14 in soft tissue, i.e. muscle, compared with males or females not feeding on spermatophores. We tested this hypothesis by measuring the ratios of nitrogen isotopes given as $\delta^{15}\text{N}$ (nitrogen-15 to nitrogen-14 in relation to an international standard) in two katydids species of the genera *Isophya* and *Poecilimon*.

Since the fundamental work of DeNiro & Epstein (1981) on the distribution of nitrogen isotopes in animal tissues and diets, nitrogen isotope ratios have been frequently used as tracers for trophic positions (e.g. Cabana & Rasmussen 1994; insect: Voigt & Kelm 2006) or the origin of proteins in an animal's diet (e.g. Herrera *et al.* 2001, 2002). DeNiro & Epstein (1981) pointed out that the $\delta^{15}\text{N}$ in animal tissues is related to the $\delta^{15}\text{N}$ in the animal's diet and this observation was commonly translated into the phrase 'you are what you eat ... plus a few per mil'. It has been noted that there are several ways how nitrogen-15 can be enriched in organisms, i.e. organisms can preferentially excrete light nitrogen-14 or enzymes may process nitrogen-15 at slower rates than nitrogen-14 (e.g. DeNiro & Epstein 1981; Ehleringer, Rundel & Nagy 1986). At the base of the food chain, plants typically have low nitrogen isotopic values relative to animal tissue (Nadelhoffer & Fry 1994). Thus, animals consuming animal tissue have a higher enrichment of nitrogen-15 than, for example, plant-feeding animals. Following this line, individuals that feed at a higher trophic level (e.g. females feeding more on animal tissue than on plants) can be distinguished from individuals feeding at a lower trophic level (e.g. males feeding exclusively on plants) based on their nitrogen-isotope signature. According to Ehleringer *et al.* (1986) the enrichment of nitrogen-15 between adjacent trophic levels averages 3.4‰, but can range from 2 to 5‰.

As the enrichment of nitrogen-15 increases with the trophic level, we predicted the following for free-ranging plant-feeding *Isophya kraussii* (Ingrisch 1976; Ingrisch & Köhler 1998):

- (a) The nitrogen isotope signature of the cuticula is similar in both sexes, because the adult cuticula originates mostly from material assimilated by the plant-feeding immature and because the metabolically inert cuticula maintains its isotopic composition (Webb, Hedges & Simpson 1998).
- (b) In males, the nitrogen-isotope signature of the cuticula and muscle ought to be similar as immature and imago males feed on a similar diet. By contrast, female muscles are more enriched in nitrogen-15 than their cuticula because the isotopic composition of the latter reflects the plant diet of the nymph, whereas the isotopic composition of muscles is related to the diet of the adults, which includes nuptial gifts.
- (c) Owing to nuptial feeding, female muscles are more enriched in nitrogen-15 than male muscles.

Secondly, we made the following predictions for captive female *I. kraussii* that were held either with or without males. Similar to the above predictions, we expected (d) cuticula of both sexes to reflect the diet of the nymphs and thus be similar in their isotopic signature; and (e) we predicted that the $\delta^{15}\text{N}$ of cuticula and muscle ought to be similar in female adult katydids that do not feed on nuptial gifts. Since females that are kept with males consume nuptial gifts, we predicted (f) that the $\delta^{15}\text{N}$ in muscle tissue of these females is higher than that of muscles of females that were kept without males or (g) in muscles of males.

Thirdly, we made the following predictions for obligate parthenogenetically reproducing free-ranging *Poecilimon intermedius*, Fieber 1853. Since males are absent in this species (Heller & Lehmann 2004) and therefore nuptial feeding impossible, we predicted (h) that female *P. intermedius* ought to have a similar $\delta^{15}\text{N}$ in cuticula and muscles.

Methods

FIELD SITES AND MATERIAL

We collected 16 female and 12 male adult *Isophya kraussii* in July 2001 from a population close to the town of Hetzles in the district of Upper Franconia (Bavaria/Germany) and removed small pieces of thorax muscles and cuticula from the hind tibia. As first adults occur at the end of May and the presence of imago *I. kraussii* peak in early July in southern Germany (Detzel 1998), we assumed that all females have mated several times before collection. We sampled material from five plant species on which *I. kraussii* was feeding (five plant individuals from each species): *Daucus carota* (Asteraceae), *Trifolium* sp. 1 (Fabaceae), *Trifolium* sp. 2 (Fabaceae), *Campanula* sp. (Campanulaceae), *Galium verum* (Rubiaceae). As katydids mostly feed on the flowers of the plant (Ingrisch 1976), we collected the floral parts for later isotope analysis.

We kept a group of 14 male *I. kraussii* in captivity on a diet of the above-mentioned plants. After 1 week, we

put each male with a single female so that the courtship and spermatophore transfer was initiated. After mating we removed the spermatophore and separated the sperm containing ampullae from the nutritious spermatophylax. All spermatophylaxes were dried to constant mass at 70 °C in a drying oven and then prepared for nitrogen isotope analysis.

FEEDING EXPERIMENTS WITH FEMALES

We collected 22 immature female *Isophya kraussii* and 10 adult males in June 2003 from the Bile Karpaty in Slovakia (compare Cejchan 1983). After all females moulted into adults we assigned them to two different feeding regimes. The first group consisted of 10 individuals and was kept together with males. The second group consisted of 12 individuals and was kept without males. Both groups of females were offered the same plant diet consisting of various plants species, e.g. *Daucus carota* and *Taraxacum officinale*. During the course of the experiment two females of the group held together with males escaped from the cage. Since these individuals starved, tissues were not analysed isotopically. After 1 month, we collected muscular tissue from the thorax and cuticula from one hind tibia for isotopic analysis from all other individuals. All samples were kept frozen until analysis.

POECILIMON INTERMEDIUS

We collected 10 female *P. intermedius* in the Bile Karpaty, Czech Republic. From each individual, we collected muscular tissue from the thorax and one hind tibia of each individual for isotopic analysis. All samples were kept frozen until analysis.

NITROGEN ISOTOPE ANALYSIS

Cuticular samples were cleaned from external contaminants with a 1:1 methanol : chloroform solvent. All samples were dried until constant mass at 70 °C. Dried samples were combusted and the resultant gases (N₂) sequentially measured in a CE 1110 elemental analyser connected via a continuous flow system to a Thermo Finnigan Delta Plus isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany) in the Stable-Isotope Laboratory of the Department of Geology, University of Erlangen-Nürnberg, Germany. Samples collected from captive *I. kraussii* and free-ranging *P. intermedius* were analysed with EuroVector Euro EA elemental analyser coupled to a GV instruments Iso-Prime in continuous flow mode at the Stable Isotope Laboratory at Boston University (USA). Accuracy of the measurements was monitored by analysis of international standards (IAEA-N1 and N2). Precision was better than ±0.1‰ (1σ). Boston University used internal lab standards calibrated to air. Precision on replicate sample analyses was better than ±0.2‰ (1σ). Nitrogen isotope ratios are expressed in the δ notation in parts

per thousands (‰) using the following equation for conversion:

$$\delta^{15}\text{N}(\text{‰}) = \left[\left(\frac{^{15}\text{N}/^{14}\text{N}}{\text{sample}} / \left(\frac{^{15}\text{N}/^{14}\text{N}}{\text{standard}} - 1 \right) \right) \times 10^3 \right]$$

We refer to the dietary shift in nitrogen isotope concentration as the difference in δ¹⁵N between the diet and the consumer tissue. This term is synonymous with the term diet-consumer enrichment in δ¹⁵N used by other authors.

STATISTICAL ANALYSIS

For calculations we used the software Excel (Microsoft Inc., Version 97) and for statistical analyses SPSS (SPSS Inc. 1998). For pair-wise comparisons, such as differences δ¹⁵N of muscle and cuticula within the same individuals, we used paired *t*-test and for tests between independent groups one-way ANOVA. Since we expected an increase in relative nitrogen-15 abundance owing to the consumption of animal proteins, we performed one-tailed tests if not otherwise stated. Mean values are expressed ± 1 SD. Prior to statistical analyses, we tested whether the underlying assumptions for parametric tests are fulfilled. We calculated the statistical power for the following predictions: (a) δ¹⁵N of cuticula is not different between the two sexes of *I. kraussii*, (b) δ¹⁵N of cuticula and muscle is not different in male *I. kraussii*, (d) δ¹⁵N of cuticula and muscle are not different in female *I. kraussii* kept without males, (e) δ¹⁵N of cuticula and muscle are not different in female *I. kraussii* kept without males, and (h) δ¹⁵N of cuticula and muscle is not different in obligate parthenogenetically reproducing *P. intermedius*. Calculations were performed with the software nQuery® Adviser (Elashof 1995) on the basis of preliminary data on nitrogen-isotope ratios in *I. kraussii* showing a mean δ¹⁵N of 3.3 ± 0.9‰ (*n* = 5) for muscles and 3.8 ± 1.3‰ (*n* = 5) for cuticula, respectively, and that individuals that consume predominantly animal tissue such as nuptial gifts have a δ¹⁵N that is higher by 3.4‰ than that of individuals feeding at a lower trophic level (Ehleringer, Rundel & Nagy 1986). During the first field season, some of the collected samples turned out to be insufficient in volume for isotopic analysis. Therefore the number of compared samples may deviate from the total number of collected samples since only paired samples were included in the statistical analysis.

Results

ISOTOPIC SIGNATURE OF FREE-RANGING ISOPHYA KRAUSSII

Free-ranging immature and adult *I. kraussii* fed on various plant species at the site of collection (see list in Table 1). The average δ¹⁵N of these plants equalled -0.4‰ (± 0.1 SD). Free-ranging female *I. kraussii* had no insect remains in their crop (*n* = 16). We expected the cuticula of adult *I. kraussii* to reflect the herbivorous

Table 1. Mean nitrogen isotope ratio ($\delta^{15}\text{N}$, ‰) of plants found at the site where *Isophya kraussii* was studied ($n = 5$ individuals for each plant species)

Plant	Mean $\delta^{15}\text{N}$	SD
<i>Daucus carota</i>	0.34	0.82
<i>Cardamine pratensis</i>	-2.01	2.27
<i>Trifolium</i> sp. 1	0.06	0.55
<i>Trifolium</i> sp. 2	-0.56	0.55
<i>Campanula</i> sp.	-0.88	0.57
<i>Galium verum</i>	0.61	0.36
Mean \pm SD	-0.4 \pm 0.9	

feeding habit of the nymphs. The $\delta^{15}\text{N}$ of the cuticula averaged 2.2‰ (± 2.9) in male and 2.8‰ (± 1.2) in female *I. kraussii*, which was not significantly different (one-way ANOVA, $F_{1,26} = 0.81$, $P = 0.38$, power = 99%; Fig. 1). Enrichment of nitrogen-15 between diet and consumer cuticula averaged 2.9‰. In contrast to the cuticular $\delta^{15}\text{N}$, female muscles were significantly more enriched in nitrogen-15 by 3.1‰ than male muscles (one-way ANOVA, $F_{1,19} = 24.3$, $P < 0.001$; Fig. 1). Female muscles were also significantly more enriched in nitrogen-15 than their cuticula (paired t -test, $t_{11} = 6.3$, $P < 0.001$), whereas both tissues had similar isotope signatures in males (one-tailed paired t -test, $t_7 = 1.4$, $P = 0.20$, power = 99%). The $\delta^{15}\text{N}$ of spermatophylaxes averaged 2.1‰ (± 0.9 ; $n = 14$), which was approximately 2.9‰ lower than the $\delta^{15}\text{N}$ of female muscle and almost identical to the $\delta^{15}\text{N}$ in male muscles.

ISOTOPIC SIGNATURE OF CAPTIVE *ISOPHYA KRAUSSII* FEMALES UNDER DIFFERENT FEEDING REGIMES

Captive female *I. kraussii* that did not feed on nuptial gifts had a mean $\delta^{15}\text{N}$ of 2.9‰ (± 1.0) in cuticula and of 3.1‰ (± 0.4) in muscle tissue, which was not significantly different according to a paired t -test ($t_{11} = 0.4$,

$P = 0.69$, power = 99). By contrast *I. kraussii* females that fed on spermatophores had a mean $\delta^{15}\text{N}$ of 4.2‰ (± 0.7) in muscle tissue, which was significantly higher than the mean cuticular $\delta^{15}\text{N}$ (mean 2.8 \pm 0.5‰; paired t -test: $t_9 = 5.3$, $P = 0.001$). The mean $\delta^{15}\text{N}$ of cuticula did not differ among individuals of different feeding regimes (one-way ANOVA: $F_{1,21} = 0.10$, $P = 0.75$, power = 99%), whereas the $\delta^{15}\text{N}$ of muscle tissue differed between individuals of feeding trials (one-way ANOVA: $F_{1,21} = 6.2$, $P = 0.022$).

ISOTOPIC SIGNATURE OF FREE-RANGING *POECILIMON INTERMEDIUS*

In obligate parthenogenetically reproducing *P. intermedius* $\delta^{15}\text{N}$ averaged 1.0 \pm 0.5‰ in cuticula and 1.2 \pm 0.8‰ in muscle tissue, which was not significantly different (paired t -test: $t_9 = 1.25$, $P = 0.24$, power = 99%).

Discussion

We investigated whether nuptial feeding leads to sex-specific differences in the $\delta^{15}\text{N}$ of the herbivorous katydid *Isophya kraussii*, predicting that female katydids feeding substantially on nuptial gifts should be more enriched in nitrogen-15 than individuals not feeding on nuptial gifts. As expected, the cuticular $\delta^{15}\text{N}$ did not differ between the sexes, suggesting that both sexes feed on the same diet as nymphs. Muscular tissue of free-ranging females, however, were significantly more enriched in nitrogen-15 than their cuticula, whereas both tissues had similar isotope signatures in males. Since this difference could be attributable to sex-specific plant diets with varying nitrogen isotope signatures (Table 1), we performed an experiment in which all katydids were provided with the same plant diet, but one group of females was kept with and the other without males. As predicted, the $\delta^{15}\text{N}$ of muscular tissue of female individuals feeding on nuptial gifts was more enriched in nitrogen-15 than muscular tissue of females lacking the opportunity to feed on nuptial gifts. Cuticula of female individuals of either group was similar. We therefore conclude that by feeding on animal proteins, free-ranging female adults fed at a higher trophic level than female nymphs or than adult males, supporting the idea that female katydids use spermatophylax substrate not only for egg production but also for body homeostasis. Additional support for this hypothesis is drawn from the obligate parthenogenetically reproducing species *Poecilimon intermedius* (G. U. C. Lehmann and A. W. Lehmann, unpublished data). Since males are absent in this species (Heller & Lehmann 2004), female *P. intermedius* can not feed on nuptial gifts. As predicted by the absence of nuptial feeding, cuticula and muscular tissue of female *P. intermedius* did not differ significantly.

A comparison of $\delta^{15}\text{N}$ between the normal herbivorous diet and the tissue of *I. kraussii* indicates that the enrichment in nitrogen-15 across one trophic level equals approximately 3‰. This is similar to the

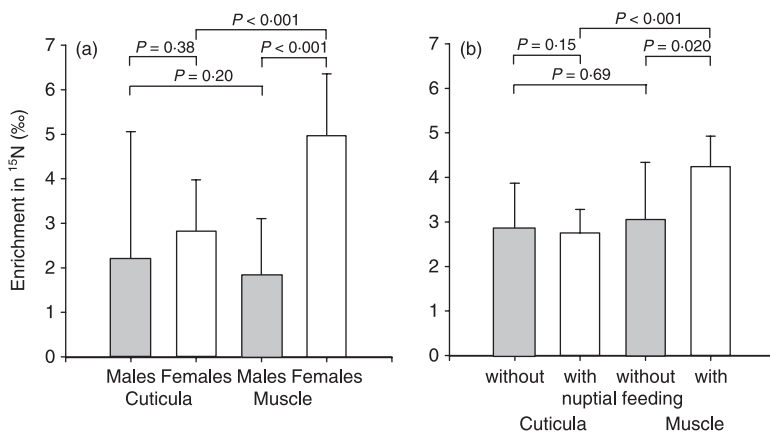


Fig. 1. Differences in mean nitrogen isotope ratios (parts per thousand difference from nitrogen-15/nitrogen-14 ratio in ambient air, $\delta^{15}\text{N}$) between (a) sexes of free-ranging *Isophya kraussii* and (b) captive females held either in presence or in absence of males. Bars indicate mean values (\pm SD).

diet-consumer enrichment that was previously found in six other insect species with a mean of $2.7\text{‰} \delta^{15}\text{N} (\pm 1.4)$ (data from DeNiro & Epstein 1981; Webb *et al.* 1998; Ponsard & Amlou 1999; Markow, Anwar & Pfeiler 2000; Oelberman & Scheu 2002; Voigt & Kelm 2006). We found almost the same difference in $\delta^{15}\text{N}$ in muscular tissue of free-ranging males and females (3.2‰). Both diet-consumer enrichments of nitrogen isotopes fall close to the expected isotopic difference of adjacent trophic levels (e.g. Ehleringer *et al.* 1986). Since female *I. kraussii* were never observed feeding on insects nor were any insect remains found in their intestinal tract, we infer from the nitrogen isotope signature that adult females probably derived most nitrogen from nuptial gifts. Based on $\delta^{15}\text{N}$ data, proteins of female bodies originated substantially from male nuptial gifts. Theoretically muscular isotope signatures could be affected by nitrogen imbalance (e.g. fish, Gaye-Siessegger *et al.* 2004; mammals, Voigt & Matt 2004). For example, male katydids may get into nitrogen stress when they produce proteinous spermatophores, and uncopulated females when they oviposit eggs that were built from proteins of their own body. However, nitrogen imbalance or stress usually leads to an enrichment of nitrogen-15 (Hobson, Alisaikas & Clark 1993; Vanderklift & Ponsard 2003), which would obscure differences with individuals feeding on animal proteins, such as nuptial feeding female katydids.

In addition to using stable carbon isotopes as a qualitative measure of the fate of spermatophore compounds, for example egg production (e.g. O'Brien, Fogel & Boggs 1999; O'Brien, Boggs & Fogel 2004), nitrogen isotopes may be used as a quantitative measure for nitrogen origin in nuptial feeding insects (see also Fischer, O'Brien & Boggs 2004).

In summary, adult female *I. kraussii* could be classified as omnivorous animals. Regarding their feeding habits adult females contrast with nymphs and adult male conspecifics, suggesting that the digestive physiology is different between sexes and possibly also between immature and adult females. Based on the observation that female *I. kraussii* never fed on insects and that female bodies were more enriched in nitrogen-15, we conclude that bodies of female *I. kraussii* are substantially made of substrate derived from male nuptial gifts. Thus, female katydids may use spermatophores for homeostasis, i.e. body maintenance as well as for egg production, suggesting an advantage of nuptial feeding for females in addition to that previously envisaged by the paternal investment hypothesis (see also Boggs & Gilbert 1979). Sex-specific differences in $\delta^{15}\text{N}$ of somatic tissue are possibly a widespread phenomenon in insects with nuptial feeding.

Acknowledgements

For help in the field, feeding captive animals and for collecting animals, we thank Holger Braun, Frieder Neuhäuser-Wespy, Rohini Balakrishnan, Angela Bruns,

Volker Runkel, Detlev Kelm, Katja Rex and Kirsten Jung. We are also most grateful to Doris Fichte and especially Christiane Stemmer who prepared many samples for isotope analysis. We thank Klaus Reinhold, Heribert Hofer, Sylvia Ortmann, Ruth Thomsen and Arne Lehmann for comments on the manuscript.

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Received 21 December 2005; revised 5 May 2006; accepted 22 May 2006

Editor: Carol Boggs