Testing the efficiency of three $^{15}$N-labeled nitrogen compounds for indirect labeling of grasshoppers via plants in the field

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Accepted: 17 May 2005

Key words: Acrididae, ammonium, Chorthippus biguttulus, Chorthippus parallelus, Dactylis glomerata, glycine, Holcus lanatus, multi-trophic interactions, Omocestus viridulus, stable isotope $^{15}$N, Trifolium repens

Abstract

In field studies of plant–insect herbivore interactions it is often difficult to establish which herbivore has fed on a particular plant. We investigated the suitability of three different $^{15}$N-labeled nitrogen compounds (ammonium, nitrate, and glycine) for indirect marking of three grasshopper species [Omocestus viridulus (L.), Chorthippus parallelus (Zett.), and Chorthippus biguttulus (L.) (Orthoptera: Acrididae)] through labeling their food plants in the field. In two short-term experiments grassland plots of 1 m$^2$ were separately labeled with either one of the different nitrogen compounds. Grasshoppers were caged on three food-plant species [Dactylis glomerata L., Holcus lanatus L. (Poaceae), and Trifolium repens L. (Fabaceae)] present in these plots for 72 h. Significantly enriched $\delta^{15}$N values in grasshoppers were found in all plant/grasshopper combinations. Enrichment in grasshoppers was positively correlated with the enrichment of plants and labeling with nitrate resulted in highest $^{15}$N enrichment. In a long-term experiment, individuals of C. biguttulus were placed in a cage covering an area of 1 m$^2$ for 37 days, with sampling of grasshoppers at regular intervals. $\delta^{15}$N values of the grasshopper and a common food plant, D. glomerata, increased steadily over time, up to 40-fold by the end of the experiment. Our results demonstrate that $^{15}$N-labeling of plants is an appropriate tool for the investigation of insect–plant interactions under natural conditions.

Introduction

Insects play an important role in natural grasslands as primary consumers, pollinators, and predators (Price, 1997; Weisser & Siemann, 2004). Herbivorous insects, which constitute about half of all insect species, influence nutrient cycling not only directly through the consumption of biomass, but also indirectly, for example by affecting plant–plant competition through their influence on plant resource allocation (Weisser & Siemann, 2004). One major problem in the study of plant–insect interactions is the impossibility to infer food web relations from the typical insect sampling methods. When insects are caught using sweep nets, suction samplers, or Barber traps, they can be identified taxonomically, but host-plant use cannot be assessed directly. In Central Europe, inferring host-plant use from the literature is often possible for specialist herbivores of well-studied taxa such as butterflies, which only feed on one or a few closely related food plants. However, for specialists of lesser-known taxa and for generalists, the literature record is incomplete. The situation is much worse for the tropics, where only a few studies have been conducted to determine the host specificity of insect herbivores (Novotny et al., 2002). An additional problem with generalist herbivores is that food plant preferences may differ between habitats and populations, making it impossible to infer from the literature alone whether a certain plant species is really fed upon by a particular herbivore in a particular habitat.

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Possible methods to establish food plant relationships in the field are direct observations (Illich & Winding, 1989), crop or feces analyses (Bernays & Chapman, 1970; Cherrill, 1989), or rearing the herbivores on various food plants (Novotny et al., 2002). The identification of food plants in the gut or the feces of certain insect herbivores requires numerous plant tissue preparations. Similarly, detecting food plants by field observations is a time-consuming enterprise because it is not sufficient to observe that an insect rests on a particular plant. To establish a trophic link it has to be found feeding at the time of the observation. Finally, rearing herbivores on many different plant species is very laborious. Even if the herbivore community of only one single plant species is sought, the work load can be enormous. Alternative methods that establish food-web relationships with less time investment are therefore desirable.

For mark and recapture studies with insects, a variety of methods such as dust and pollen marking have already been effectively used in the field, but these techniques are not applicable for determining insect-food-plant relationships. Another modern technique in the field of marking insects is the use of vertebrate proteins to study, for example, the dispersal of biological control agents or parasitoids of a certain host species, but this technique has not yet been verified for labeling host plants (see references in Hagler & Jackson, 2001).

The use of tracer elements to mark organisms at lower trophic levels is a relatively new technique to investigate multitrophic interactions (Hagler & Jackson, 2001). One of the tracer elements that has been proposed and already tested in a number of food web studies is the stable isotope $^{15}$N (Ostrom et al., 1997; Nienstedt & Poehling, 2000, 2004a, 2004b; Steffan et al., 2001). It is nonradioactive and persists for a long time in a system after application. So far, testing the suitability of $^{15}$N in insect research has been confined to laboratory or mesocosm studies; observations from natural ecosystems are missing.

In the present study, we examine the suitability of three nitrogen compounds enriched with the stable isotope $^{15}$N for indirectly marking grasshoppers via their host plants in the field. As target herbivores we used three generalist grasshopper species common in natural montane Central European grasslands. Grasshoppers constitute an important component of the phytophagous insect community in grasslands and are responsible for a large part of total herbivory (Köhler et al., 1987). Grasshoppers feed mainly on grasses, but there is observational and experimental evidence that they also feed on forbs and legumes (Illich & Winding, 1989; Köhler, 2001; Pfisterer et al., 2003). To what extent nongrass plant species constitute part of the regular diet of grasshoppers in the field is, however, barely known.

In this study, we asked the following questions: (1) Does the labeling of host plants under natural conditions with the stable isotope $^{15}$N result in significant enrichment of the grasshoppers when they only feed for a short time period on the plant? (2) Does enrichment of the grasshoppers increase with the time of exposure on the host plant? (3) Are there differences between the different N compounds or host plants in the efficiency of the method?

Materials and methods

Study area and general design

The study was conducted in three hay meadows in the Thüringer Schiefergebirge/Franconian Forest (50°27’N and 11°28’E), a plateau-like mountain range in central Germany. Main elevations of the sites varied between 575 and 705 m above sea level. The meadows were cut twice a year, not grazed, and not fertilized for at least the last 10 years. The vegetation in the three field sites was dominated by the perennial grasses Dactylis glomerata L., Anthoxanthum odoratum L., and Holcus lanatus L., and the forb Taraxacum officinale Wiggers. In 2003 we conducted two short-term experiments and one long-term experiment with grasshoppers. Table 1 gives an overview of the plant and grasshopper species combinations used in the experiments.

We selected the two grass species D. glomerata and H. lanatus, and the legume Trifolium repens L. as target food plants, as these plant species are potential food plants of grasshoppers in grasslands (Ingrisch & Köhler, 1998). We chose three grasshopper species (Acrididae, Orthoptera) that are common and dominant in the study area (Köhler & Renker, 2004) and generalist feeders (Ingrisch & Köhler, 1998; Köhler, 2001) on grasses and forbs. The selection of both grasshoppers and plant species was made in order to represent the most abundant and dominant species for the time at which each of the three experiments was conducted.

For the first short-term experiment in May, we chose Omocestus viridulus (L.) (orthoptera: Acrididae), a grasshopper that emerges earliest in the season and is the dominant Orthopteran species at the time (Köhler & Renker, 2004). Target plant species for O. viridulus were D. glomerata and T. repens (Table 1). For the second short-term experiment in August, we selected Chorthippus parallelus (Zett.) (orthoptera: Acrididae), and target plant species were the grasses D. glomerata and H. lanatus. Finally, for the long-term experiment carried out from August to September, Chorthippus biguttulus (L.) was the target grasshopper species and the target plant species was D. glomerata (Table 1).

We caught all grasshoppers with sweep nets on the same day the experiments started and kept them in “Fauna
Labeling grasshoppers with $^{15}$N in the field

boxes (Savic, Belgium) until the beginning of the experiments. *Omocestus viridulus* was in the third instar, and *C. parallelus* and *C. biguttulus* were in the adult stage at the time they were caught.

**Short-term experiments**

In the two short-term experiments (May and August 2003, Table 1), we labeled the entire plant community of three adjacent 1 m$^2$ plots using three different nitrogen compounds: ammonium, nitrate, and glycine (Sigma Aldrich Chemie GmbH, Germany). We produced three solutions consisting of all three nitrogen compounds in equal parts (9.66 mM $^{15}$NH$_4$Cl, 9.66 mM $^{15}$NO$_3$, or 9.66 mM Glycine). $^{15}$N tracer supplements were estimated to obtain plant δ$^{15}$N target values of about 100‰. In each of the three solutions, one of the nitrogen compounds was labeled with stable isotope $^{15}$N (each >98.9 atom% $^{15}$N) whereas the other two compounds were not labeled. The three solutions only differed in the $^{15}$N-labeled nitrogen compound. Each of the three solutions was injected into one of the three 1 m$^2$ sampling plots to a soil depth of 3 cm using spine syringes, resulting in 210 injections per plot. Assuming a 2 cm diffusion radius (McKane et al., 2002), we injected 2-ml of $^{15}$N labeled solution at each injection point, leading to a total of 55.35 mg added $^{15}$N m$^{-2}$. The first labeling experiment took place from May 22 to 24, 2003 at site 1 and the second labeling experiment took place from August 4 to 6, 2003 at site 2 (Table 1).

On May, 10 individuals each of *D. glomerata* and *T. repens* were randomly selected in each of the three plots in site 1 (Table 1). A perforated plastic bag (23.0 × 42.0 cm; Carl Pollmann, Germany) was placed over the plant and fixed with a plastic ribbon. The bag was then cut open at the top, and one nymph of *O. viridulus* was placed inside before the bag was tightly closed again with another plastic ribbon.

In August, 10 individuals of *D. glomerata* and *H. lanatus* were selected in each of the three plots in site 2 (Table 1). *Chorthippus parallelus* was already adult at the time of the experiment and therefore five individuals of each sex were used for each plot. In both short-term experiments, grasshoppers were left for 72 h on the plants after they were removed and processed for further analysis (see below).

**Long-term experiment**

The long-term experiment was conducted in order to investigate the $^{15}$N enrichment of grasshoppers in comparison to enrichment in plants over an extended time period of 37 days (August 12 to September 18). A 1-m$^2$ plot on site 3 was labeled with nitrogen solution that consisted of the three labeled N compounds, ammonium, glycine, and nitrate in equal parts [mix: 9.66 mM $^{15}$NH$_4$Cl, 9.66 mM $^{15}$NO$_3$, and 9.66 mM glycine (each >98.9 atom% $^{15}$N)] (Table 1). Thus, in this experiment, all three N compounds were labeled. The labeling procedure was otherwise the same as described above.

A cage (1 × 1 × 0.6 m) was set up on the labeled plot and 70 individuals of the grasshopper species *C. biguttulus* were placed inside. To monitor enrichment in plants, we chose

### Table 1 General design, labeled nitrogen compounds, and plant–herbivore combinations used in the various experiments

<table>
<thead>
<tr>
<th>Site</th>
<th>Target plant species</th>
<th>Grasshopper(species instar)</th>
<th>Labeled nitrogen compound</th>
<th>No. valid replicates$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>First short-term experiment, May 2003</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site 1</td>
<td><em>D. glomerata</em></td>
<td><em>O. viridulus</em> (3rd instar)</td>
<td>Ammonium</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nitrate</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glycine</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td><em>T. repens</em></td>
<td><em>O. viridulus</em> (3rd instar)</td>
<td>Ammonium</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nitrate</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glycine</td>
<td>7</td>
</tr>
<tr>
<td>Second short-term experiment, August 2003</td>
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<td></td>
</tr>
<tr>
<td>Site 2</td>
<td><em>D. glomerata</em></td>
<td><em>C. parallelus</em> (adult)</td>
<td>Ammonium</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nitrate</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glycine</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td><em>H. lanatus</em></td>
<td><em>C. parallelus</em> (adult)</td>
<td>Ammonium</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nitrate</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glycine</td>
<td>7</td>
</tr>
<tr>
<td>Long-term experiment, August/September 2003</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site 3</td>
<td><em>D. glomerata</em></td>
<td><em>C. biguttulus</em> (adult)</td>
<td>Mix of ammonium, nitrate, and glycine</td>
<td>2–8 per sampling day</td>
</tr>
</tbody>
</table>

$^a$10 individuals were used originally but some of the animals died or escaped from the bags.
the plant species *D. glomerata*, which covered >70% of the surface area in the plot. Samples of *D. glomerata* (mixed sample taken from different places within the cage) and *C. biguttulus* (2–8 individuals) were taken at days 1, 5, 8, 13, 21, and 37 after the release of the grasshoppers into the cage.

**15N analysis of plants, insects, and feces**

After each experiment, grasshoppers were immediately frozen at −20 °C. All corresponding plant individuals under the plastic bags were clipped 2 cm above ground and dried separately in a drying oven for 48 h at 70 °C. In the short-term experiment in August the feces of *C. parallelus* was also collected from the plastic bags and treated the same way as the plant material.

Background natural abundance 15N of the investigated plant species was determined by collecting the appropriate plant species in July 2003. Background natural 15N abundance of grasshoppers was determined using field-caught grasshoppers, collected at the same sites as the experimental animals (n>14 for each species). The grasshopper samples for each species were pooled for analysis.

For 15N analysis, a mix of leaves from each dried plant individual was ball milled and an aliquot of 3 mg was put into tin capsules and analyzed with an isotope ratio mass spectrometer (IRMS, Delta C Finnigan MAT, Bremen, Germany). The feces was treated likewise. The insects were lyophilized (Novalyphe-NL 150, Savant) and prior to ball milling, they were treated with liquid nitrogen. This was necessary because there is a high risk of burning the fat contents in insects during ball milling, which would disturb further analyses (J Rothe, pers. comm.). One milligram of each homogenized individual grasshopper was analyzed. Plants, animals, and feces measurements of 15N enrichment (=δ15N) obtained by IRMS are expressed in per mill (in ‰) compared to the international 15N standard.

**Data analysis**

Prior to statistical analyses, all data were checked for normality and heteroscedasticity. ANOVA tests were used to analyze 15N enrichment in plants as a function of the nitrogen compounds used. To analyze 15N enrichment in grasshoppers, we used analysis of covariance (ANCOVA), using the 15N enrichment of the host plant as covariate. For all models, we used Type III Sums of Squares (SPSS, 2003). All means are given as mean ± SE.

### Results

**Short-term experiments**

In the May experiment, the remaining number of replicates varied between three and nine in the *D. glomerata* treatments and between six and eight in the *T. repens* treatments because of some mortality of grasshoppers (Table 1). In the August experiment, the remaining number of replicates in each *D. glomerata* treatment was eight and in the *H. lanatus* treatments it varied between six and seven (Table 1).

In the short-term experiment in May, both *D. glomerata* and *T. repens* were strongly enriched in 15N after the 3 days of the experiment compared to natural abundance levels (Table 2, Figure 1). Enrichment was on average 10–12

### Table 2

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Nitrogen compound</th>
<th>δ15N values [%]</th>
<th>Natural abundance δ15N values [%]</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n¹ Range</td>
<td>Mean ± SE</td>
<td>n² Mean ± SE</td>
<td>t</td>
</tr>
<tr>
<td>First short-term experiment, May 2003</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>D. glomerata</em></td>
<td>Ammonium</td>
<td>10.33–495.97</td>
<td>373.24 ± 74.97</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>83.47–533.45</td>
<td>354.75 ± 140.44</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>87.88–370.32</td>
<td>209.59 ± 27.66</td>
<td>6</td>
</tr>
<tr>
<td><em>T. repens</em></td>
<td>Ammonium</td>
<td>5.54–44.08</td>
<td>19.54 ± 5.57</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>11.69–47.80</td>
<td>27.49 ± 4.60</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>13.26–37.01</td>
<td>22.60 ± 3.47</td>
<td>7</td>
</tr>
<tr>
<td>Second short-term experiment, August 2003</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>D. glomerata</em></td>
<td>Ammonium</td>
<td>83.92–373.62</td>
<td>195.06 ± 30.19</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>98.50–748.97</td>
<td>493.66 ± 79.54</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>120.73–712.40</td>
<td>343.02 ± 77.38</td>
<td>7</td>
</tr>
<tr>
<td><em>H. lanatus</em></td>
<td>Ammonium</td>
<td>163.03–371.47</td>
<td>273.22 ± 29.18</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>245.27–687.38</td>
<td>473.82 ± 61.35</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>74.63–325.99</td>
<td>192.41 ± 41.34</td>
<td>7</td>
</tr>
</tbody>
</table>

¹Number of valid replicates; ²Number of replicates for the control plants.
times higher in the grass than in the legume (cf. A Kahmen, C Renker, SB Unsicker & N Buchmann, unpubl.). Maximum values of δ^{15}N were 553‰ for D. glomerata and 47‰ for T. repens (Table 2). For both D. glomerata and T. repens there were no significant differences between the three nitrogen treatments (ANOVA: D. glomerata: F_{2,15} = 2.452, P = 0.120; T. repens: F_{2,18} = 0.760, P = 0.482). In the grasshopper O. viridulus, significant increases in δ^{15}N compared to control individuals were found both for D. glomerata and for T. repens (t-tests for grasshoppers: t_{Dactylis} = 2.98, P = 0.008; t_{Trifolium} = 2.54, P = 0.019; test value background natural {^{15}N} abundance of O. viridulus = 1.19), even though {^{15}N} enrichment in the legume was much smaller (Figure 1). For D. glomerata, enrichment in grasshoppers was correlated with enrichment of the plant they were feeding on (ANCOVA, effect of plant enrichment: F_{1,14} = 5.628, P = 0.034; Figure 3), and grasshopper enrichment differed significantly between the three nitrogen compounds (ANOVA, effect of nitrogen compound: F_{2,14} = 5.482, P = 0.019). For T. repens, grasshopper enrichment was not influenced by plant enrichment (ANOVA, effect of plant enrichment: F_{1,15} = 0.243, P = 0.629; Figure 3), and there were no significant differences in grasshopper enrichment between the nitrogen treatments (ANOVA, effect of nitrogen compound: F_{2,15} = 0.741, P = 0.493).

In the August experiment, both D. glomerata and H. lanatus showed strong enrichment (Table 2, Figure 2), which was largest with nitrate (ANOVAs, D. glomerata: F_{2,21} = 5.056, P = 0.016; H. lanatus F_{2,17} = 10.304, P = 0.001). Enrichment in the grasshopper C. parallelus was significant (t-tests for grasshoppers: t_{Dactylis} = 6.551, P<0.001; t_{Holcus} = 5.557, P<0.001; test value background natural {^{15}N} abundance of C. parallelus = 2.78). Grasshopper enrichment was positively related to enrichment in D. glomerata (ANOVA, effect of plant enrichment: F_{1,20} = 22.530, P<0.001; Figure 3), but no differences in grasshopper enrichment between the three nitrogen compounds were found (ANOVA, effect of nitrogen compound: F_{2,20} = 1.458, P = 0.256). For H. lanatus, there was a significant effect of host plant enrichment on grasshopper enrichment (ANOVA, effect of plant enrichment: F_{1,16} = 7.920, P = 0.012; Figure 3), but there were no differences in δ^{15}N values between the nitrogen compounds (ANOVA, effect of nitrogen compound: F_{2,16} = 1.914, P = 0.180). In the August experiment, {^{15}N} enrichment in the grasshopper C. parallelus was positively correlated with the amount of {^{15}N} enrichment in feces collected after 72 h of grasshopper feeding (r^2 = 0.176, P = 0.012, n = 35; Figure 4).

**Long-term experiment**

Enrichment of the target plant species D. glomerata as well as the grasshopper species C. biguttulus steadily increased over the 37 days of the experiment (Figure 5). Already 5 days after the start of the experiment, there was a significant enrichment of {^{15}N} in C. biguttulus individuals (one sample t-test: t = 8.0, d.f. = 6, P<0.001). On the last day of the experiment both the plants and the grasshoppers had increased their δ^{15}N value 40-fold in comparison to the start of the experiment.

**Discussion**

Labeling plants with the stable isotope {^{15}N} has already been used in a number of studies in order to investigate food web relations (Ostrom et al., 1997; Nienstedt & Poehling,
...In these studies, plants were labeled under standardized laboratory conditions. In our experiment, we labeled a natural plant community in the field to test how efficient $^{15}$N labeling is as a method to detect plant–insect interactions in the field. Our results show that marking a plant community with the stable isotope $^{15}$N in the field under natural conditions results in detectable labeling of common herbivores that feed on these plants. In the short-term experiment, we found a detectable enrichment in $^{15}$N for the group mean of both tested grasshopper species $O$. viridulus and $C$. parallelus already after 72 h, on all the plant species tested. The long-term experiment showed that enrichment increases with time in both the plant community and the herbivores. Thus, our results suggest that labeling target plant species with $^{15}$N is a suitable method to determine whether a certain insect herbivore has fed on a particular plant species.

On average, the $\delta^{15}$N values of grasses were 10–12 times higher than the corresponding values for the legume $T$. repens. The relatively small N enrichment rates in $T$. repens can be explained by the plants’ potential to host symbiotic N-fixing bacteria which makes legumes largely independent from soil N (Vitousek et al., 2002). Even though $^{15}$N enrichment was lower in the legume, we found significant enrichment in $\delta^{15}$N in all treatments with $T$. repens. The highest values for $^{15}$N enrichment in both plants and grasshoppers were on average reached in the nitrate treatment although the differences with the other treatments were not always significant. Nitrogen compounds can be transformed in the soil prior to plant uptake. Also, using $\delta^{15}$N labels to determine quantitative differences in the uptake of different nitrogen compounds from the soil requires further calculations, including the concentration of the respective nitrogen compounds in the soil (A Kahmen, C Renker, SB Unsicker & N Buchmann, unpubl.). Consequently, our data give little indication of the actual contributions of different nitrogen compounds to plant nutrition. For the purpose of this study, however, it was sufficient to simply determine the $^{15}$N label in the plant. Because of the consistently good results we suggest that nitrate be the preferred N-compound for future $^{15}$N-labeling studies in the field. Labeling plants under laboratory conditions will, of course, further improve the marking efficiency of the plant.

There was variation in $^{15}$N enrichment between the individual grasshoppers. There are several reasons for this variation: grasshoppers were caught in the field immediately before experiments started and therefore differed in age, sex, and physiological state, all of which might have influenced the consumption rates of the individuals. That consumption plays a crucial role for enrichment is seen in the analysis of the feces data, even though feces weight is only a crude measure of grasshopper consumption. The more plant material was ingested by the grasshopper, the higher the enrichment of the animal (Figure 4). As a consequence of the variability between individuals, the regressions between enrichment of the grasshoppers and enrichment of the plant individual on which they were placed were not always significant when looking at each plant/grasshopper combination separately (ANCOVA results, Figure 3). The differences between individuals can also be seen in the results of the long-term experiment. While enrichment increased on average (Figure 4), there...
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were also differences between the individuals in their actual $\delta^{15}$N.

One important advantage of tracer experiments with $^{15}$N is that food web relations are immediately established as soon as insects are found to be enriched with the stable isotope. Even a small amount of labeled plant material consumed by the herbivores is sufficient to name the particular plant species as an actual food plant of this herbivore. The data on feces weight show that with just 10 mg of plant material egested, enrichment is already significantly different from the background control level (Figure 4). This differs from observational studies, where insects need to be caught ‘in action’ to conclude that a particular plant is part of the diet of a particular herbivore. In the case of immobile insects, such as leaf miners that are limited to only one food plant individual for their whole developmental time period, observational studies are an adequate method to proof a feeding relation. For mobile insect species such as grasshoppers or adult herbivorous beetles, the tracer experiment provides much clearer evidence for food–plant associations. In the long-term experiment that we conducted, the values for $^{15}$N in both the plants and the grasshoppers increased 40-fold after 37 days. The steady

Figure 3 $\delta^{15}$N enrichment of the two host-plant species *Dactylis glomerata* and *Holcus lanatus* (black bars) and adults of *Chorthippus parallelus* (grey bars) as a function of the nitrogen compound used in the 2nd short-term experiment in August 2003. (A) *D. glomerata* and (B) *H. lanatus*. The error bars represent standard errors. Means that are not significantly different from one another (ANOVA, Tukey post hoc test) are indicated by the same letter. Differences in enrichment between plants and grasshoppers were tested using a paired sample t-tests ($P<0.05$).

Figure 4 Enrichment in the grasshopper *Chorthippus parallelus* as a function of the amount of feces collected after 72 h in the short term experiment in August 2003. The graph includes the data points of all three nitrogen compounds for the two grass species, *Dactylis glomerata* and *Holcus lanatus*.

Figure 5 Time-course of $^{15}$N enrichment in the long-term experiment with *Dactylis glomerata* and *Chorthippus biguttulus*. Sample sizes for the grasshoppers (circles): day 1, $n = 2$; day 5, $n = 7$; day 8, $n = 7$; day 13, $n = 7$; day 21, $n = 7$; day 37, $n = 8$. Each data point for the grass (triangles) represents the $^{15}$N value for one mixed sample of *D. glomerata* leaves. The error bars represent standard errors.
increase in enrichment over time is encouraging for future studies that aim to label the predators, parasitoids, and the hyperparasitoids of these grasshoppers. After some time, the grasshoppers are likely to be so strongly enriched that it is conceivable to indirectly label also the next (two) trophic levels with this method.

The downside of tracer experiments with $^{15}$N is that they are still constrained by the costs of analysis and the throughput facilities of the specialized laboratories in which the samples are analyzed. However, the costs per sample continue to fall and the number of laboratories in which stable isotope analyses can be performed is growing. Thus, tracer experiments are likely to be not only more effective in the future, but also more cost-effective.

Labeling an entire plant community, each plant species in turn, will of course still be a major task. The insects that are suspected to be herbivores of a plant need to be placed in (large) cages, otherwise an enormous number of individuals would have to be screened to find the individuals that actually fed on the target plant species. Nevertheless, clever experiments with large cages where target plants are labeled in the laboratory will mimic natural conditions quite closely and open up a new avenue for future studies of food web interactions.

Acknowledgements

The authors would like to thank Heike Geilmann for technical assistance, Ilka Egerer, Viviane Sternkopf, Anett Oswald, Markus Lange, and Anke Renker for their help in the field and in the lab, and Jan Rothe, Günter Köhler, and Markus Wagner for their advice on the methods. We also thank three anonymous reviewers for their advice on the manuscript. The project was funded by BMBF BIOLOG (project DIVA-Jena,01LC0013) and by LUBOM Thüringen.

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