

Measurement of predatory behaviour in cow dung-colonising insect larvae, using compound-specific ^{13}C -tracing of dietary fatty acids

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Abstract Studies of the trophic interactions between organisms in opaque environments where direct observation can be difficult, such as soil or leaf litter, often require the use of indirect inferential approaches. Here, the use of compound-specific ^{13}C -tracing of dietary biomarker fatty acids is evaluated as a method for studying predation by larvae of the dung-breeding fly, *Mesembrina meridiana* (Diptera: Muscidae); the technique was used to differentiate dung from high-enrichment ^{13}C -labelled prey in their gut contents. Potential prey, ^{13}C -labelled larvae of the dung-breeding fly, *Neomyia cornicina* (Diptera: Muscidae), were placed into unlabelled dung microcosms in the laboratory. A single 7-day-old *M. meridiana* larva was allowed to feed in each microcosm for 8 h. The magnitude of increases in the $\delta^{13}\text{C}$ values of fatty acids (*i*14:0, 14:0, *i*15:0, *a*15:0, 15:0, 16:0 and 18:0) in the gut contents, relative to those of *M. meridiana* deprived of prey, demonstrated the predation of *N. cornicina* larvae which were estimated to have constituted at least 35% of the average dietary wet mass of these *M. meridiana* larvae. The tracing of specific labelled compounds increased confidence in dietary assessment and helped to avoid systematic errors associated with compound-dependent efficiency of assimilation in the gut. The results demonstrate the potential value of this method in helping to elucidate trophic interactions in predator–prey

systems within opaque environments. The precision of the quantitative dietary estimation that arose from these isotopic data was superior to that generated using fatty acid distributional data, a widely used and evidentially independent line of evidence.

Keywords Cattle dung · Diptera · Gas chromatography–combustion–isotope ratio mass spectrometry · $\delta^{13}\text{C}$ · Fatty acids · *Mesembrina meridiana* · *Neomyia cornicina*

Introduction

The dung of large herbivores is often an abundant and nutritionally valuable resource in grazed ecosystems, which forms an ecologically distinct environment for a complex successional insect community, composed largely of Coleoptera and Diptera [12]. The presence of this diverse insect community within dung is of ecological and economic importance, since it promotes rapid dung decomposition, which helps to return nutrients to the soil, particularly nitrogen, a large proportion of which would otherwise be lost as ammonia [14, 23, 24]. Timely decomposition also may help to reduce dung-breeding pest-fly populations [16, 22, 25] and reduce the potential for the transmission of livestock endoparasites [23]. Dung-breeding insects may in turn be an essential resource for insectivorous birds and small mammals [15]. Hence, it is important to understand the factors that facilitate the establishment and activity of functional dung-colonising invertebrate communities.

Within the invertebrate community, a wide variety of interactions, such as competition, parasitism and predation, may have major effects on community structure and on the rate of dung decomposition. Predatory larvae, for example, were estimated to have reduced the populations of

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coprophagous larvae by 51% [13] and, in artificial cow pats, there was a 96–98% lower emergence of *Musca tempestiva* (Diptera: Muscidae) from fully colonised, compared with uncolonised, isolated pats [11]. In the latter case, staphylinid predation was the thought to be the primary cause of mortality. The problem faced by Kirk [11] in confirming predation is also a good example of how studying the interactions between components of the dung-colonising insect community can be difficult. Their impact is therefore usually estimated by inference. Hence, the development of reliable indirect methods for detecting and quantifying these interactions would be of value.

The aim of the present study was to evaluate the use of ^{13}C -labelling of prey to quantify trophic interactions within cow dung. It was also intended to compare the accuracy of such an isotope approach with a more established chemical method. The alternative approach chosen was based upon the principle that the distribution of lipids in the consumer reflects that of its diet. Candidate compounds for isotopic comparison in such work, must be abundant in both dung and prey and must be capable of substantial ^{13}C -labelling, whereas candidate compounds for the alternative, compound profiling, approach must have widely different concentrations in dung and prey. ^{13}C -labelled fatty acids have been shown to be appropriate candidate compounds for isotopic studies [2]. The abundance profiling approach need not have used the same compound class, but fatty acids are recognised for their characteristic profiles in insects [4, 7, 26] and dung [5]. On this basis, a suite of fatty acids (albeit a different one from that selected for isotopic dietary analysis) was also used for the comparator (compound profiling) approach. The predatory behaviour of the holarctic dung-visiting species *Mesembrina meridiana* (Diptera: Muscidae) was selected as the subject of quantitative dietary study. The large, conspicuously coloured adults of this species are commonly observed feeding at the dung surface, but have also been recorded on flowers and ripe fruit, from which it has been inferred that they also use plant sugars [8]. When fully developed, the larvae of this species are up to 20-mm long, making them the largest fly larva in cow dung in temperate habitats. Observations have shown that the larvae may be predatory [17]. Nevertheless, owing to their large, sluggish appearance and the classically coprophagous features of their buccopharyngeal armature, Séguy [20] and Portchinsky [19] suggested that the larvae are purely coprophagous, while Hammer [8] and Keilin & Tate [10] concluded that any predation may only be opportunistic. They are certainly capable of complete development in fresh dung without potential mesofaunal prey [8, 13]. This study, therefore, aimed to examine the feeding behaviour of *M. meridiana*, through the use of high ^{13}C -enrichment labelling of fatty acid fractions.

Materials and methods

All dung used in the experiments described originated from organically managed cattle grazed on ryegrass sward in the southwest of England. Adult *M. meridiana* flies were collected from the same cattle pastures by netting flies on dung pats. The adults were returned to the laboratory where they were maintained at 25 °C in 30×30×30-cm plastic cages, and supplied with sucrose and water ad libitum. The flies were allowed to lay eggs into fresh dung supplied to each cage. Larvae that hatched from these eggs were used at 7 days old as consumers in subsequent experiments.

Mesembrina meridiana gut transit time

As a first step, the gut transit time of *M. meridiana* was established so that an appropriate duration for the subsequent predation experiment could be identified. To evaluate this, larvae were fed fresh cow dung containing dysprosium (Dy), a rare earth element used as a marker in studies of the human gut because it is not absorbed from the lumen [21]. Assuming mass transport of dung along the gut, the amount of Dy in an insect given food with dissolved Dy would be expected to increase approximately linearly from the time it was moved from Dy-free food until one gut transit has been completed at which point the amount of Dy should plateau.

Dy as $\text{DyCl}_3 \cdot 6\text{H}_2\text{O}$ (2.087 g as a solution in 28 ml water) was added to fresh cow dung (6 kg) to produce a calculated Dy concentration of 149 ppm. The Dy solution was stirred into the dung for 15 min. Larvae of *M. meridiana*, obtained from adults collected in the field, were placed into individual pots of the Dy-labelled dung. Five replicate larvae were recovered after 25 min, 1, 2, 3.5, 5, 6.5 and 8 h. The experiment was repeated using larvae of 5, 6 and 7 days of age. At the end of the feeding period, the larvae were rinsed with water and frozen. Larvae were typically inanimate within 3 min. Frozen larvae were freeze-dried, weighed and digested in boiling 70% nitric acid (5 ml) until dry. The digested sample was redissolved volumetrically in 1% nitric acid (2 ml) and the concentration of Dy was determined in triplicate using inductively coupled plasma/atomic emission spectroscopy. The technique was similar to that used in the analysis of earthworm mineral content by Paoletti et al. [18].

^{13}C -labelling of prey

To label dung, 0.90 g of $\text{U6 } ^{13}\text{C}$ -glucose (99 atom%) was added to 1 kg of fresh dung as a solution in water (7 ml) with stirring. The mixture was incubated aerobically for 21 days at 20 °C and ambient humidity. Batches of ^{13}C -labelled dung were then stored at −18 °C until required,

when they were thawed at 25 °C, mixed to ensure homogeneity and placed in glass crystallising basins.

Larvae of the common coprophagous fly species, *Neomyia cornicina* were obtained from a laboratory colony maintained at the University of Bristol. To obtain ^{13}C -labelled *N. cornicina* larvae, adult flies were supplied initially with batches of fresh unlabelled dung for a period of <8 h. Eggs deposited during this time were collected carefully and placed onto 70 g of dung which had been ^{13}C -labelled as described above. ^{13}C -labelled *N. cornicina* were then recovered and used as prey 6 days after hatching (± 0.5 days).

Diet choice experiment

Eleven ^{13}C -labelled *N. cornicina* larvae were placed onto the surface of 50 g of unlabelled dung in a microcosm, composed of a 50-mm diameter glass crystallising basin. *M. meridiana* larvae (one per microcosm) were then added and allowed to feed in the microcosms for 8 h. Microcosms were placed in an illuminated incubator at 25 °C. At the end of this feeding period, all *M. meridiana* larvae were removed and rinsed in water. They were then killed by immersing their anterior ends in boiling water (whole body immersion was found to cause ejection of some of the gut contents). Entire guts were dissected immediately from the dead *M. meridiana* larvae. As far as was possible, fat body and other tissues adhering to the anterior and posterior ends of the gut were removed. The gut was placed onto a small patch of aluminium foil and freeze-dried.

Since the previous analysis described above had shown that the gut transit time of *M. meridiana* larvae was approximately 5 h at 25 °C by the end of the feeding trial, the gut contents of the *M. meridiana* larvae from the microcosms should not have contained material ingested before the experiments were started.

Five replicates of the experimental microcosm were undertaken. Control microcosms were also established under the same conditions as the experimental microcosms, but in which *N. cornicina* were absent. Samples of unlabelled dung and ^{13}C -labelled *N. cornicina* larvae of the same age as those offered to *M. meridiana* larvae were also freeze-dried and weighed.

Total fatty acid fractions

An appropriate mass of a *n*-nonadecanoic acid internal standard was added to all samples, and lipids were extracted using the method of Bligh & Dyer [1], modified into the form used by Dungait et al. [6]. The monophasic solvent was a mixture of potassium dihydrogen phosphate-buffered water at pH 7.2, MeOH and chloroform in a ratio of 4:10:5 (v/v/v). Residual water was removed when the

lipid extract, dissolved in chloroform, was passed through a mass (~0.4 g) of anhydrous sodium sulphate crystals in a Pasteur pipette plugged with dichloromethane (DCM)-extracted cotton wool. The drying crystals were washed in situ with chloroform (2 ml) before use. An aliquot of lipid residue in a closed culture tube was subjected to mild, base-catalysed hydrolysis using a solution of 0.5 M NaOH in 90% methanol (2 ml, 70 °C, 1 h). A total neutral lipid fraction was then extracted into DCM (3×2 ml) and, after acidification to between pH 2 and 3 using 1.0 M HCl_(aq), the acid fraction was extracted into chloroform (3×2 ml). Residual water was removed using anhydrous sodium sulphate, as above, and the solvent evaporated at 40 °C under a gentle stream of nitrogen.

Total fatty acid fractions were derivatised for instrumental analysis, by heating (50 °C) in 5% HCl_(dry methanol) (2 ml) in closed culture tubes. After 20 min, tubes were cooled and 5% NaCl_(water) (5 ml) was added, followed by hexane (1 ml). The hexane layer, containing the fatty acid methyl esters, was decanted and the remaining aqueous phase extracted with more hexane (2×1 ml). The combined hexane layers were washed with 2% KHCO_{3(aq)} (4 ml) and then dried over anhydrous sodium sulphate. The solvent was evaporated under a gentle flow of nitrogen and the residue dissolved in hexane for instrumental analysis.

Internal standard (*n*-nonadecanoic acid, 50 µg, dissolved in chloroform/methanol, 2:1 v/v, 250 µl) was added to approximately 250-mg freeze-dried dung, accurately weighed, followed by monophasic solvent (4 ml) for lipid extraction. From this point onwards, lipid extraction and preparation of the methyl esters of total fatty acid fractions preceded as for insect-derived samples described immediately above.

Identification of fatty acid methyl esters used gas chromatography/mass spectrometry (GC/MS; ThermoQuest Trace GC-MS). The GC was equipped with a Factor Four VF23-MS fused silica capillary column (high cyanopropyl-modified methyl polysiloxane; Varian Chrompack, 60 m×0.32 mm, 0.15-µm film thickness). The carrier gas was helium, supplied at a constant pressure (10 psi), with the GC oven temperature programmed to rest at 50 °C for 1 min then ramped from 50 to 100 °C at 15 °C min⁻¹, from 100 to 240 °C at 4 °C min⁻¹ then from 240 to 260 °C at 15 °C min⁻¹ and held at 260 °C for 10 min. The MS was set to scan the range *m/z* 50–650 in a total cycle time of 0.6 s. The ion source temperature was 200 °C; emission current, 300 µA; electron ionisation potential, 70 eV; the GC-MS interface was maintained at a temperature of 300 °C; helium was the carrier gas. Data were acquired and processed using the Xcalibur (ThermoFisher Scientific, Hemel Hempstead, UK) software suite. Fatty acid methyl esters in dung, prey and gut content samples were identified using their GC retention times and mass spectra. An external standard solution containing the saturated fatty acids myristic acid

(14:0), pentadecanoic acid (15:0), palmitic acid (16:0), margaric acid (17:0), steric acid (18:0) and nonadecanoic acid (19:0) was analysed at the beginning of each GC sequence. These chromatograms provided reference retention times for the peaks of saturated analytes. An adjustment for changes in GC conditions between runs could be made by comparison of the retention time of the internal standard, 19:0, in the external standard solution and as the internal standard in samples. Methyl-branched fatty acids (indicated by the prefix *i*, for *iso*-branched compounds, or *a*, for *anteiso*-branched compounds) were then identified on the basis that these compounds elute from polar phases before the unbranched isomer in the order *iso*-branched, *anteiso*-branched, straight chain [3]. Saturated fatty acid methyl esters have characteristic electron ionisation mass spectral fragmentation patterns [3] in which the molecular ion $[M]^+$ is prominent, as are the ions corresponding to loss of a portion of the methyl end of the alkyl chain $[M-(15+n(14))]^+$. The base peak ion is known as the McLafferty rearrangement ion; formed after cleavage of the parent molecule β to the carboxyl group, giving $[MeOC(OH)CH_2]^+$, m/z 74. Christie [3] gives details of how methyl-branched compounds (which have the same parent ion as the straight chain compound) can be identified from subtle differences in their mass spectra when compared to that of the straight-chain compound. He also shows how the monounsaturated analyte palmitoleic acid (16:1) can be identified even when its molecular ion ($2m/z$ units below that of 16:0) is in insufficient abundance for detection.

Fatty acid methyl esters were quantified against the internal standard using a GC (Hewlett Packard 5890) with a flame ionisation detector using the same GC conditions as for GC/MS identification except that hydrogen was used as a carrier gas.

Fatty acid methyl ester gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) analyses were performed using a GC of the same specifications as that for GC/MS. The GC was attached to a Finnigan MAT isotope ratio mass spectrometer (Delta S model: electron ionisation potential, 100 eV; emission current, 1 mA; three Faraday cup collectors m/z 44, 45 and 46; CuO/Pt Finnigan MAT Mark I Pt/CuO combustion interface at 850 °C. Delta XL or Delta XP models: same specifications except that the combustion interface was Cu/Ni/Pt maintained at 950 °C) Reference CO_2 was introduced directly into the source three times at the beginning and end of every run.

Statistical analysis

Multiple analysis of variance (ANOVA) was used initially to compare the abundances of the fatty acids in the two treatment groups and, to confirm the identity of the most dissimilar fatty acid groups, this was then followed-up by one-way ANOVA comparisons with an appropriate Bonferonni correction.

Results

Mesembrina meridiana gut transit time

The mass of Dy was quantified simply as mass per larva (assuming uniform gut volume). No improvement in the clarity of the result was obtained by adjusting for larval mass. The data show an approximately linear net accumulation of Dy until about 5 h and no major net change in Dy concentration thereafter (Fig. 1). No effect of age on gut transit time was evident over the range of ages examined.

Gut contents total fatty acid distributions

The relative abundances of the following fatty acids in fresh ryegrass-derived cow dung were significantly different from those of the same fatty acids in ^{13}C -labelled prey *N. cornicina* (Pillai's trace 1.0, $df=8, 1$, $P=0.045$, Fig. 2): *iso*-pentadecanoic acid (*i*15:0), palmitoleic acid (16:1), *iso*-margaric acid (*i*17:0) *ante*-margaric acid (*a*17:0), the coeluting margaric acid phytanic acid (17:0/Phy) and steric acid (18:0). The most important differences were in the 16:1 ($F_{2,12}=10.5$, $P<0.01$) and 18:0 species ($F_{2,12}=437.2$, $P<0.01$) components (Fig. 2). This confirmed that quantification of these six total fatty acid species in the dissected gut contents of *M. meridiana* had the potential to give an estimate of the diet they had consumed when given the choice between dung and *N. cornicina* prey. However, while suggestive, comparison of the relative concentrations of the total fatty acids in the *M. meridiana* gut contents could not unequivocally indicate whether more of the fatty acid in the guts were derived from dung or from prey and hence could not clearly demonstrate predation by *M. meridiana*. As a result, the compound-specific stable isotope labelling approach was

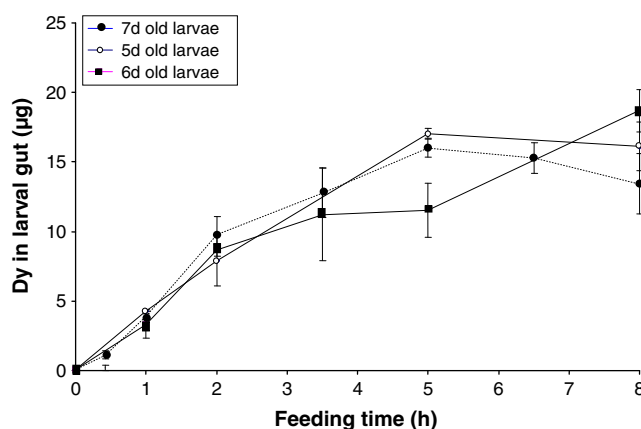


Fig. 1 Mean (\pm SE) gut Dy content (microgramme) of *M. meridiana* larvae over time, after being moved into dung with dissolved Dy

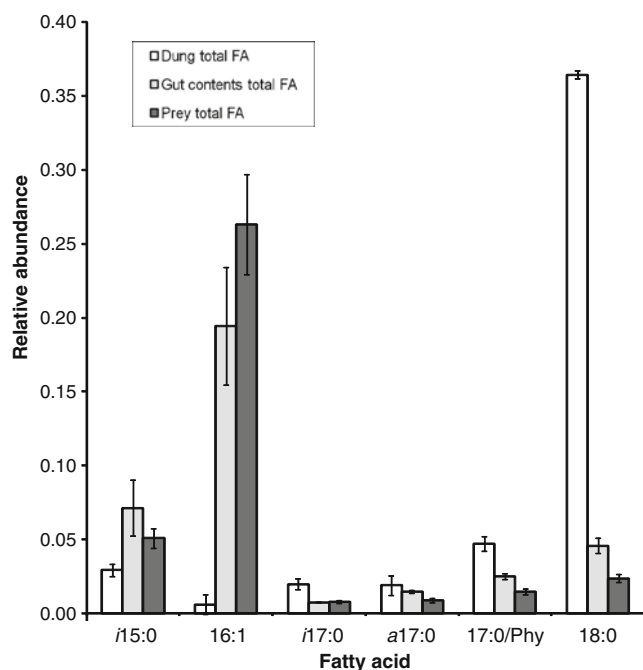
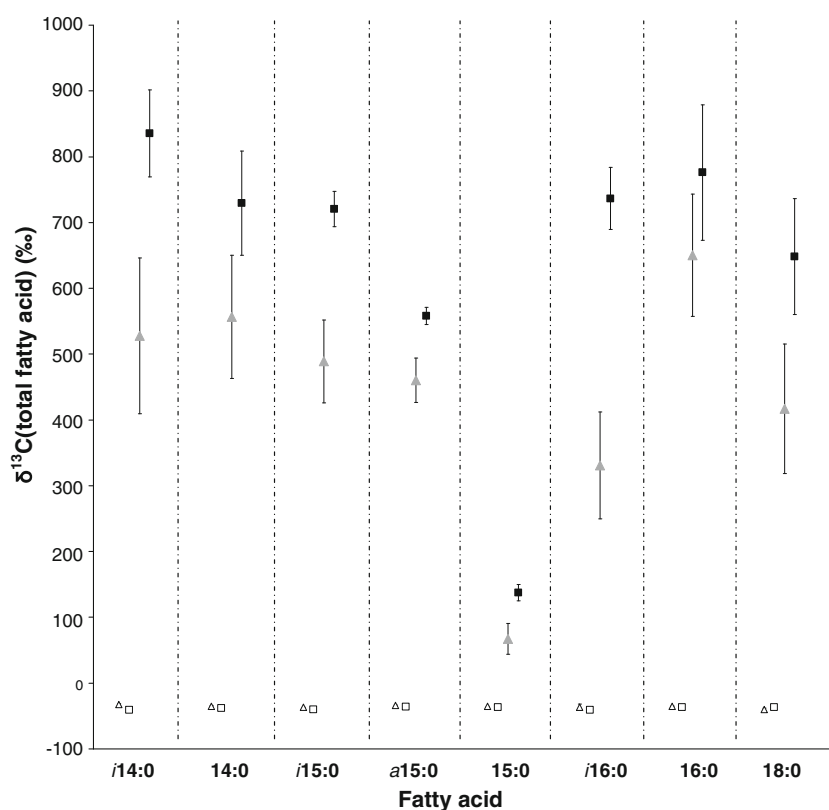


Fig. 2 Relative abundances (mean±SE) of total fatty acids in 7-day-old *Mesembrina meridiana* gut contents (grey bar) compared with those of dung (open bar) and *Neomyia cornicina* larvae (dark bar) total fatty acid fractions

required to improve on the fatty acid distribution approach and yield a quantitative diet estimation by *M. meridiana*.

Fig. 3 $\delta^{13}\text{C}$ (total fatty acid) values (mean±SE) of the gut contents of *Mesembrina meridiana* larvae offered a dung-only or dung-plus- ^{13}C -labelled *N. cornicina* diet, compared with the $\delta^{13}\text{C}$ (total fatty acid) values of each of the foods offered. Open squares dung, solid black squares prey, open triangles gut contents of *M. meridiana* offered only dung, closed grey triangles gut contents of *M. meridiana* offered both dung and *N. cornicina* larvae



Gut content total fatty acid $\delta^{13}\text{C}$ values

Dilution of the ^{13}C -label in prey larvae, due to feeding in unlabelled dung for 8 h, was not apparent. The average $^{13}\text{C}/^{12}\text{C}$ fractional abundance value for total fatty acids (a mass-balance average across all fatty acid components) from ^{13}C -labelled *N. cornicina* that had spent 8 h in unlabelled dung was 0.01884 ± 0.00017 (mean±SD), while for *N. cornicina* sampled from a culture of newly labelled larvae, it was 0.01824 ± 0.00246 (mean±SD). Comparison of the total fatty acid $\delta^{13}\text{C}$ values for *M. meridiana* gut contents and the gut contents of larvae fed only dung, with the values for the two food choices (Fig. 3), shows that the values recorded fell in the order that would be predicted if the diet had contained both unlabelled dung and ^{13}C -labelled prey, i.e.: $\delta^{13}\text{C}(\text{dung}) \approx \delta^{13}\text{C}(\text{gut[no prey]}) < \delta^{13}\text{C}(\text{gut[dung+prey]}) < \delta^{13}\text{C}(\text{prey})$. These $\delta^{13}\text{C}$ values were used to calculate the contributions of dung and prey biomass to the gut contents of the *M. meridiana*. Appropriate fatty acids for inclusion in diet composition estimates were selected on the basis of the significant spacing between the $\delta^{13}\text{C}(\text{dung})$ and $\delta^{13}\text{C}(\text{prey})$ values. Individual dietary composition estimates based on comparison of isotopic determinations for each of the eight eligible fatty acids considered are presented in Table 1. This shows that, in terms of dry mass, in four of the five replicates gut contents, there was more dung than prey biomass, but all

Table 1 Mean dung biomass ($f_{B,D}$) (\pm SD) as a proportion in the diet of five replicate 7-day-old *Mesembrina meridiana* offered the choice between ^{13}C -labelled *Neomyia cornicina* larvae and unlabelled dung for 8 h

<i>M. meridiana</i> larva replicate	Mean $f_{B,D}$	SD of estimates used
1	0.07	0.18
2	0.83	0.13
3	0.78	0.19
4	0.87	0.05
5	0.68	0.23
Overall mean \pm SD	0.64	0.33

M. meridiana larvae appear to have attacked and eaten at least part of one *N. cornicina* larva. As an overall average, predation was estimated to have provided at least 35% of the diet (Table 1).

Discussion

Here, the composition of the diet of *M. meridiana* was examined using two independent analytical chemistry methods. Firstly, the similarity of gut contents total fatty acid distributions to those of the two potential foods was considered. Secondly, the $\delta^{13}\text{C}$ values of an appropriate suite of total fatty acids in the gut contents were compared with those of the same set of fatty acids in the two potential foods. The first approach identified fatty acid biomarker components that gave overall differences between the fatty acid distributions. However, the fatty acid quantification approach, alone, did not have the power to conclude significantly upon the presence of any prey in the gut contents. In contrast, the compound-specific ^{13}C -labelling approach had quantitative power that was adequate to yield an estimation of the relative dietary importance of the two offered foods. This was also the experience of Chamberlain et al. [2], who established the technique of coupling compound-specific stable isotopic analysis of captive invertebrates with the ^{13}C -labelling of dietary options. Here, we have added to the method both the use of high enrichment ^{13}C -labelling and the analysis of the gut contents alone, rather than the entire consumer. The former improvement meant that major total fatty acids of *N. cornicina* larvae used in this study had $\delta^{13}\text{C}$ values 300–3,000‰ above those of unlabelled dung, enhancing the sensitivity of prey fatty acid detection relative to that which would have been possible using a ^{13}C -labelling technique similar to that of Chamberlain et al. [2].

Despite considerable care, it was likely that some gut tissue may have been included when sampling gut contents, and this would have resulted in the inclusion of unlabelled

fatty acids from the gut tissues [9] and, potentially, even from the surrounding fat body. Indeed, it is the fat body which gives *M. meridiana* larvae their distinctive yellow colour [17]. Being unlabelled, these fatty acids would have tended to increase the estimated amount of ingested dung, and would have therefore led to an overestimation of dung biomass ingested. The assessments of predation given are therefore a minimum estimate of its probable importance in the diet of *M. meridiana*.

In 8 h, all experimental *M. meridiana* larvae offered a mixture of dung and larvae appear to have attacked and eaten at least part of one *N. cornicina* larva and predation was estimated to have provided at least 35% of their average diet. Prey biomass accounted for 2.2% of the total wet mass of the dung/prey mixture offered. Hence, rather than consuming prey and dung in proportion with their respective abundances within the dung microcosms, *M. meridiana* appear to have effectively located and consumed *N. cornicina* larvae. For the purposes of reproducing the situation in natural cow pats, the prey loading in laboratory microcosms is considered to be comparable with the average living animal content of cow pats in the field, estimated to be 1.3% by Laurence [13]. The behaviour of *M. meridiana* as a facultative predator is therefore supported [8, 10, 13, 17] and in demonstrating this, this study highlights the effectiveness of compound-specific stable isotope analysis with high-enrichment ^{13}C -labelling for quantitative studies of trophic interactions in an otherwise opaque environment.

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