Stable isotopes: past and future in exposing secrets of ant nutrition (Hymenoptera: Formicidae)

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Abstract

Ants can utilize a large variety of food sources, and this ability contributes to their ecological and evolutionary success. Their broad dietary niche, however, in conjunction with their sociality, makes this group of animals notoriously difficult to study with respect to nutritional ecology. Natural-abundance-stable-isotope studies are a useful tool for assessing the trophic position of ants in food webs. In theory, they may also help to assess the relative contribution of food sources to their biomass, although such quantitative estimates have to be taken with caution. Consumers are typically enriched in $^{15}$N and sometimes $^{13}$C relative to their diet. The magnitude of enrichment may be influenced by feeding mode, diet quality and possibly nitrogen-recycling endosymbiotic bacteria. We provide an estimate of the enrichment of stable isotopes relative to a chemically defined diet for the ant *Camponotus floridanus* (Buckley, 1866) and show that their endosymbiotic bacteria do not significantly alter the enrichment process. The average enrichment of *C. floridanus* pupae to their diet was 3.0‰ for $^{15}$N and 1.1‰ for $^{13}$C.

Stable isotopes have also been used successfully for studying nutrient fluxes utilizing compounds enriched in either $^{15}$N or $^{13}$C as tracers. Such studies can help to elucidate nutrient fluxes, for example within an ant colony or between ants and other organisms such as plants. They may also clarify whether certain compounds can be metabolized by ants at all. Here we present an overview of topics and questions that can be addressed using stable-isotope methods. We discuss experimental design, sampling methods and potential pitfalls when applying stable-isotope techniques. We point out fields of research in ant biology that can be explored more extensively with stable-isotope analyses.

Key words: Stable isotopes, $\delta^{13}$C, $\delta^{15}$N, fractionation, isotope mixing model, nutrient flux, review.

Introduction – What can stable-isotope analyses tell us?

Stable isotopes can be utilized in two different approaches. First, naturally occurring differences in isotopic signatures of stable isotopes are used predominantly in ecological studies to assess trophic positions of organisms in food-webs or feeding preferences. Second, compounds marked with stable isotopes can be used as tracers for a direct measurement of nutrient fluxes between organisms or metabolic capacities, for example whether a precursor can be metabolized into a focal compound. In tracer experiments stable isotopes are replacing radioisotopes that are nowadays often more expensive and much harder to handle due to necessary precautions to avoid damage to health and environment.

Natural-abundance studies: Analyses of stable isotopes, in particular those of nitrogen (N) and carbon (C), provide useful tools for disentangling complex food-webs. The assessment of the trophic position of an organism in a food-web is based on the observation that the heavier isotope of N increases predictably with increasing trophic level (De Niro & Epstein 1981, Mingawa & Wada 1984, Fry 2006). In such studies, $\delta^{15}$N describes the relative proportion of the heavy $^{15}$N to light $^{14}$N isotopes, and $\delta^{13}$C the ratio of $^{13}$C to $^{12}$C. Stable-isotope analysis has produced important insights into marine and soil food webs (Post 2002, Chahartaghi & al. 2005, Illig & al. 2005), and the extent of intraguild predation among carnivorous arthropods (Rickers & al. 2006). Results of these studies sometimes contradicted former observational data, suggesting that omnivory, feeding on multiple trophic levels, was more prevalent in many ecosystems, and challenging the view that most organisms fit tightly into a particular trophic level.

Natural-abundance studies of stable isotopes are particularly suited for analyses of nutritional ecology of organisms whose feeding habits are cryptic or change over time,
as is often the case with omnivores such as ants. In contrast to solitary animals whose food intake may be easily observed, in ant colonies it is particularly difficult to quantify the relative contribution of the various food sources to the colony’s overall dietary input, i.e., the sum of items brought into the colony from multiple foragers that may have collected a variety of food sources. Food intake, i.e., the actual uptake and digestion of food by individual workers and larvae, cannot be deduced directly from the food input into the colony. Larvae are usually less constrained in food digestion due to a different suite of digestive enzymes present in larvae (RICKS & VINSON 1972, ERTHAL & al. 2007) and absence of the proventriculus that allows only liquids and minute particles to enter the digestive tract of adult ants (COOK & DAVIDSON 2006, overview in BLÜTHGEN & FELDHAAR in press). Thus, larger chunks of food may be broken down by larvae and nutrients may be transferred back to the workers via liquid larval secretions. Observational studies have shown that nutritional needs of a colony may change seasonally (MOONEY & TILLBERG 2005, YANG 2006) and with colony size and amount of brood present (MARKIN 1970, DUSSETOUR & SIMPSON 2009). Recently TILLBERG & al. (2007) utilized stable-isotope analysis to show that the trophic position of the Argentine ant, Linepithema humile (MAYR, 1868), subtly changes in invaded habitats temporally. Over a period of eight years, the δ15N signature of workers decreased considerably, showing that this ant feeds more strongly on plant-based resources. Within invaded habitats, the stable-isotope signatures resembled those of herbivores, rather than those of predators, as in native habitats (TILLBERG & al. 2007). Intraspecific variation in δ15N can also be pronounced in other ants, for example in the Australian Oecophylla smaragdina (FABRICIUS, 1775) (see BLÜTHGEN & al. 2003) or the neotropical Paraponera clavata (FABRICIUS, 1775) (see TILLBERG & BREED 2004). In the latter, strong variation in δ15N of prey items (e.g., herbivores and predators) may partly explain differences between colonies. Furthermore, as in other holometabolous insects, dietary requirements of ants change during ontogeny, with larvae depending on nitrogen-rich sources for growth, and adult workers relying more on a carbohydrate-rich diet (MARKIN 1970, BLÜTHGEN & FELDHAAR in press).

Apart from obtaining nectar and honeydew as metabolic carbohydrate source, ants have been traditionally regarded as being predominantly predacious animals, meeting their larval protein requirements by preying on other arthropods. However, observational evidence suggested that numerous ant species feed extensively on a wide variety of food sources. Plant-derived food sources of ants include self-cultivated fungal products and plant polysaccharides that are broken down by fungal enzymes of leaf-cutter ants as well as sugary excretions of plant-sap sucking insects (honeydew) or extrafloral nectar (HORSTMANN 1974, RICOGRAY 1989, RICO-GRAY 1993, SILVA & al. 2003, RICO-GRAY & OLIVEIRA 2007). Seeds, especially those carrying ant-attractive elaiosomes of myrmecochorous plants, and food bodies provided by ant-plants (Beltian bodies, Muellerian bodies), may differ from other plant-tissues in that they are enriched in nitrogen and especially lipid-content in comparison to leaf-tissue (HUGHES & al. 1993, HEIL & al. 1998, FISCHER & al. 2008). Recent studies utilizing stable-isotope analysis in ant communities in tropical rain forests (BLÜTHGEN & al. 2003, DAVIDSON & al. 2003), subtropical areas (TILLBERG & al. 2007) and temperate regions (FIEDLER & al. 2007, OTTONETTI & al. 2008) have indeed revealed that ant species known to collect large quantities of extrafloral nectar and honeydew (temperate Camponotus and tropical Tetraponera, Dolichoderus, Oecophylla, Camponotus, Polyrachis) showed consistently lower levels of δ15N compared to ant species known to be more predaceous within the same habitat. δ15N signatures of many ant species were as low as those of true non-ant herbivores. Given that honeydew excreted from phloem-feeding hemipterans may be enriched in both 13C and 15N compared to the phloem from which it was derived (shown for an aphid species by SAGERS & GOGGIN 2007), the importance of such indirect plant products may even be underestimated based on 15N. Because body-mass analyses of adults reflect what these ants have fed on as larvae, such nectari vorous ants were deduced to derive most nitrogen assimilated and directed toward growth during ontogeny directly from plants rather than from prey (BLÜTHGEN & al. 2003, DAVIDSON & al. 2003, COOK & DAVIDSON 2006). The conclusion that plant-derived nitrogen and not only carbon is fed to larvae, contrasts with earlier views that ant brood is usually raised with protein-rich prey. These findings contributed to solve what TOBIN (1994) had described as the “ant-biomass paradox”: The biomass of ants in samples of arthropod communities in tree crowns in tropical rain forests and some temperate habitats was so high that predation alone may not account for the ant biomass observed. It has been discussed that endosymbionts or bacteria in the gut of ants that provide their host with amino acids or play a role in nitrogen recycling contributing to the host’s biomass lead to lower δ15N signatures in their host ants (DAVIDSON & al. 2003, COOK & DAVIDSON 2006, FIEDLER & al. 2007). Strikingly, such bacteria have been described mostly from ants with arboreal lifestyle such as Camponotus, Polyrachis, Tetraponera, Dolichoderus, Pseudomyrmex or Cephalotes (JAFFÉ & al. 2001, FELDHAAR & al. 2007, STOLL & al. 2007, EILMUS & HEIL 2009). Certain metabolic processes such as deamination or transamination preferentially remove the lighter 14N-containing amino groups (MACKO & al. 1986). However, whether bacteria preferentially enrich or lose 15N depends not only on those reactions but also on the substrate and whether the substrate is limited or abundant (MACKO & al. 1986).

Stable-isotope analyses have also been applied successfully to uncover nutrient fluxes within more closed systems like ant-plant associations. Plants in symbiotic relationship with ants (myrmecophytes) offer nesting space (domatia) and usually also food. Thus, food flow is expected to be from plant towards the ant, as was shown in the Piper-Pheidole bicornis FOREL, 1899 association, where the specific partner ant nourishes nearly exclusively on the food bodies provided by the plant (FISCHER & al. 2002). Costs and benefits in ant-plant symbioses may vary over space and time as well as with the partners involved though. Most ant-plants can be colonized by more than one species of ant, possibly resulting in different outcomes of the ant-plant interactions varying from mutualism to parasitism. It is assumed that colonizing ants of higher trophic levels, i.e., those with higher 15N, are more beneficial to their plant hosts since they will obtain more of their nitrogen from preying on herbivores that attack their respective hosts in-
stead of relying on hosts for nitrogenous compounds (Tillberg 2004, Trimble & Sagers 2004). In a comparison of four different ant partners of the myrmecophyte Cordia alliodora, stable-isotope analysis confirmed observations that only two specific ant partners were indeed mutualists that defended the plant against herbivores. Similarly, Aztec species colonizing Cecropia obtusifolia differed strongly in the amount of nitrogen acquired from the host (Trimble & Sagers 2004).

The benefit that ants confer to plants may exceed the more obvious defence against herbivores and encroaching vines (Heil & McKey 2003) and extend to “feeding” the plant. The epiphytic ant-plant Dischidia major has been shown to be able to absorb nitrogen from debris and facaces accumulating in the leaf cavities inhabited by Philidris ants (Treseder & al. 1995). In their study, Treseder and co-workers (1995) compared natural abundance of isotopes of ant debris collected from the leaf pouches and leaves of D. major to leaves of D. nummularia, which lives on the same hosts but whose leaves are, on a regular basis, used only as supporting structures for carton nests of the ants. Leaves of D. major were enriched in 15N and depleted in 13C in comparison to leaves of D. nummularia and thus resembled more the stable-isotope signatures of ant debris. Similarly, most nitrogen taken up by the neotropical mymecophyte Maieta guanensis (see Solano & Dejean 2004) and possibly also Cecropia peltata (see Sagers & al. 2000) may be obtained from debris deposited by their ant partner. Inhabited plants had stable-isotope signatures closely resembling those of the ant-deposited debris, whereas unoccupied plants were significantly different. In Maieta plants 15N values were significantly lower, suggesting that the plants took up the majority of nitrogen from the soil (Solano & Dejean 2004). Unexpectedly, absence of the ants led to higher values of unoccupied Cecropia (Sagers & al. 2000), but reasons for this finding were not explained.

Stable isotopes as tracers in experimental studies: Tracer experiments represent an elegant way to confirm and measure nutrient or compound fluxes within individual ants, colony members, or an ant colony and another party (e.g., myrmecophytes or myrmecophilous insects). When tracers are accessed by only one partner (usually the ant) and subsequently detected in the other partner, such studies can yield direct evidence for nutrient exchange between partners in a mutualism. In contrast to measurements of natural abundance of stable isotopes, tracers allow direct quantification of the amount of nutrients transferred over time. Thus, the amount of nutrient taken up, the distribution of compounds either among individual ants or brood in the colony or percentage of compound transferred to another organism like a host plant can be measured.

Pioneering studies using radio-labelled tracers have been utilized to evaluate the spread of ant colonies (Kloft & al. 1965) and the number of workers within a colony using mark-release-recapture experiments (Stradling 1970). Tracer experiments have also yielded important insights into food flow within ant colonies. In his still widely cited paper, Markin (1970) showed experimentally that carbohydrate-rich food is retained mostly by adult workers whereas proteinous resources are distributed towards the brood and queen. Regulation of food intake into the colony was shown to depend on degree of hunger of colonies as well as on food type (Howard & Tschenkel 1980, 1981, Sorensen & al. 1985). Within colonies food flow from workers to brood was quantified in dependence of time after foraging and food type (Sorensen & al. 1981, Sorensen & Vinson 1981). These studies were the first to show that there must be a flow of information between foragers and brood on the nutritional needs in the respective colony.

Using radio-labelled precursors, Soroker and coworkers successfully demonstrated the synthesis of hydrocarbons and their active distribution among nestmates via trophallaxis or allogrooming in Cataglyphis niger (André, 1881) and Pachycondyla apicalis (Latrelle, 1802) (see Soroker & al. 1995, 1998). Likewise, suspected nutrient transfer from ants to the tubers of myrmecophytic Hydnophytinae (Rubiacae: Myrmecodia and Hydnophyllum) was shown unambiguously using tracer experiments (Huxley 1978, Rickson 1979, Benzing 1991). Researchers have now switched to using tracers labelled with stable isotopes, due to ready availability of a large variety of compounds, analytical facilities, lower cost and easier handling due to lower health risks during handling. Nitrogen transfers from ants to host plants were shown for the Piper-Pheidole bicornis mutualism (Fischer & al. 2003) as well as for Maieta guanensis inhabited by Pheidole minuta Mayr, 1878 (see Solano & Dejean 2004). Fischer and coworkers (2003) used 15N-labelled glycine in sucrose solution to ensure a ready collection of the substance as a food source. Likewise, tracer experiments with 15N-labelled urea have revealed that Blochmanna, the bacterial endosymbi- ont in the midgut epithelium of Camponotus and related genera, can upgrade host nutrition by providing essential amino acids derived from non-essential amino acids or recycled nitrogenic waste products (Feldhaa & al. 2007).

Basics of natural-abundance-stable-isotope analyses

In tracer studies, untreated individuals from the same colony or untreated plant parts can be used as controls to assess the baseline of a stable isotope before enrichment of a tracer. Absence of such a straightforward baseline makes natural-abundance studies much harder to interpret because, to infer differences in trophic niches within an ant community, stable-isotope signatures of an ant can only be interpreted relative to those of possible food sources and other ants sampled within the same ecosystem. Thus, individuals with the same trophic niche, for example predators of centipedes, from two entirely different ecosystems, may have similar relative enrichment of 15N but may differ strongly in their absolute 15N values due to ecosystem differences at lower trophic levels. Ecosystem values differed between rainforest sites where ants were studied in Borneo and Amazonia (Davidson & al. 2003). An extreme example for this phenomenon is the invertebrate community found in the Movile cave system in Southern Romania. Chemoautotrophic microbial mats form the food base for all higher trophic levels. As a result, all carnivorous invertebrates from inside the cave had by far lower 15N levels than terrestrial herbivores or detri- vores sampled nearby (Sarbi & al. 1996).

In addition to the need to delineate the respective ecosystem and to collect ample samples in order to obtain a baseline, the results of stable-isotope analyses can be strongly influenced by several other factors such as fractionation (see below), the choice of body part or developmental stage,
feeding mode or C:N ratio of the diet (Spence & Rosenheim 2005, Hood-Nowotny & Knols 2007 and references therein). It is always assumed that fractionation in ants is comparable to that of other arthropods, and thus a baseline for ants has never been measured so far. However, Spence & Rosenheim (2005) suggest that predictability of fractionation for arthropods based on their ecological niche and feeding strategy may be low as they found significant differences in the level of enrichment even in a narrowly defined group of herbivorous insects. Thus, even among different ant species, the degree of enrichment may vary. More critical to the outcome of a study is the sampling design for the individual ants. Stable-isotope signatures also depend on the decision whether whole individuals are used, potentially including uncontrolled amounts of undigested contents in the crop and gut (Davidson & al. 2003, Tillberg & Bree 2004), or whether only head and alitrunk were analysed (Blüthgen & al. 2003, Tillberg & al. 2006, Fiedler & al. 2007, Tillberg & al. 2007). The gaster may include large amounts of nitrogen-poor nectar or honeydew that may explain why nitrogen concentrations and 15N values can be much smaller compared to the rest of the body (Fig. 1). Selective sampling of outgoing workers (e.g., Davidson & al. 2003) may circumvent such problem, but this has yet to be shown definitively. However, in some camponotines, where foods can be held and digested in the head (Hansen & al. 1999), sampling of ant heads can also be misleading. Aside from crop contents that can be removed prior to analysis (Mooney & Tillberg 2005), gland contents may be present in considerable amounts in some ants but to a much lesser extent in others (Dos Nascimento & Morgan 1996). To the extent that such gland contents include N and C, they may influence levels of 15N and 13C signatures.

Fractionation: In many catabolic processes, the lighter isotopes are metabolized and then excreted preferentially over the heavier isotopes, leading to an enrichment of the latter in the structural body mass compared to the diet – a process called fractionation. Isotopic fractionation is based on discrimination of isotopes through kinetic effects in animal metabolism. Enzymatic processes are commonly faster for molecules bearing lighter isotopes (13C, 14N). Light isotopes are therefore enriched in animal excretions (15N) or respired CO2 (13C) (Peterson & Fry 1987). This process leads to concentration of heavier isotopes (13C, 15N) in the animal tissue relative to the diet (De Niro & Epstein 1978, 1981) (Box 1, Fig. 2). With higher position in the food chain, organisms contain on average a higher proportion of heavy isotopes. Typically a consumer is enriched by 3 - 4% of 15N relative to its diet, although considerable variation among different groups of animals has been found (De Niro & Epstein 1981, Mingawa & Wada 1984, Fry 2006, Tiuov 2007). Cross-taxon variation in the 15N increment across trophic levels (see above) can depend on mode of excretion (e.g., uricotelic in insects vs. ammonotelic in nematodes) (Ruess & al. 2004), food quality (Oelbermann & Scheu 2002, Ruess & al. 2004, Haubert & al. 2005), life-stage (young nymphs vs. adults in Hemimetabola) (Haubert & al. 2005). In contrast, the ratio of carbon isotopes changes usually very little (Gearing & al. 1984). 13C is often used to identify the carbon source of a primary consumer. Plants with C3-, CAM- and C4-pathways differ strongly in their 13C signal (Farquhar & al. 1989). Correspondingly, plant diets such as leaves or phloem sap have distinct signatures of 13C (Post 2002, McCutchan & al. 2003). Fractionation seems to be absent in sulfur isotopes (Peterson & al. 1986).

Using mass-flow equations: Stable isotopes are often used to compare the relative trophic position of different species qualitatively. However, they can also be used for a quantitative estimation of the elementary contribution of particular diets to the body mass of the target organism (Box 1, Fig. 3). Questions addressed would be, for example, an assessment of the relative contribution of nitrogen obtained from plant nectar versus prey, or the proportion of C3 versus C4 plant-derived carbohydrates to an ant’s diet. For a simple model based on two dietary sources, the following equation may be used:

Equation (1): $\delta^{15}N_0 = p_1 \cdot \delta^{15}N_1 + (1 - p_1) \cdot \delta^{15}N_2 + \Delta N_0$

where $\delta^{15}N_0$: nitrogen-isotope ratio of the study organism, $p_1$: proportion of nitrogen obtained from diet 1, $1 - p_1$: proportion of nitrogen obtained from diet 2, $\delta^{15}N_1$: nitrogen-isotope ratio of diet 1, $\delta^{15}N_2$: nitrogen isotopic shift through fractionation, $\Delta N_0$: isotopic shift through fractionation.

To obtain the nitrogen contribution of diet 1 ($p_1$), equation (1) can be rewritten as follows:

Equation (2): $p_1 = (\delta^{15}N_0 - \delta^{15}N_2 - \Delta N_0) / (\delta^{15}N_1 - \delta^{15}N_2)$

The same equation can be applied for any element. For carbon, replace $\delta^{15}N$ by $\delta^{13}C$ and $\Delta N$ by $\Delta C$.

The above equations, or some derived functions, have been employed by different authors, but some studies of ant nutrition neglected the term for fractionation. Ignoring $\Delta N$ or $\Delta C$ may lead to a substantial error of the dietary contribution ($p_1$), if fractionation is considerably large in relation to differences between diets. The most satisfactory solution would be to determine $\Delta N$ and $\Delta C$ for the target organism using experimentally controlled feeding of a known...
diet. However, a fractionation value typical for ants may help to provide a reasonable approximation. We applied the fractionation levels determined for *Camponotus floridanus* (Buckley, 1866) pupae (see Box 1) to the existing studies that did not incorporate fractionation, in order to estimate the potential error. Fisher and coworkers (1990) estimated the contribution of extrafloral nectar secreted by an orchid to six ant species using $\delta^{13}C$, and reported yearly average values ranging from 10.8 to 27.3%. Because of the large isotopic difference between the signature of the control ant species and the orchid, a plant with CAM pathway, the inclusion of the fractionation term ($\Delta_C = 1.1\%$) only slightly decreases the estimate to 9.9% - 24.9%. Changes in the estimated dietary contributions are more severe in a study of ant taxa using the contribution of extrafloral nectar secreted by an orchid is more similar to cockroaches, the signatures may even reflect that the honey functioned more as carbon source (composed of 37.8% C and only 0.08% N) and cockroaches more as nitrogen source (50.7% C, 11.9% N). Incorporating these values in the concentration-dependent mixing model provided by Phillips & Koch (2002) reveals that virtually all nitrogen could have been derived from cockroaches (> 90%) and most carbon (> 90%) from honey when only these two diets are considered in the model. However, when Bhatkar-agar is included in the analysis the results yield 94% overall carbon-body-mass contribution by honey, 24% by Bhatkar-agar and -17% by cockroaches. This analysis shows the methodological limitations of the dietary mixing model, particularly since the ants' value does not exactly plot between the diets. Negative, and thus meaningless, values in dual isotope mixing models occur when the consumer is not plotted in the triangle given by the three diets, and in this case the diets line up and do not form a broader triangle (Box 1, Fig. 3). Moreover, the uncertainty of the model also increases with variable fractionation and digestibility factors (Koch & Phillips 2002, Robbins & al. 2002). Evaluation of the differential contribution of diets based on stable isotopes is thus problematic even for more controlled diets in laboratory feeding trials, particularly when these diets differ in composition and digestibility. Further complications, for example cannibalism, can occur in the field as well as in laboratory experiments.

**Recommendations on how to perform natural-abundance studies of stable isotopes**

Currently ant researchers follow no standard protocol for natural-abundance studies. When planning a study based on stable-isotope analysis, a researcher should consider especially sampling method (i.e., storage of samples and choice of body part) and design (i.e., choice of control samples or reference samples). Recommendations on storage and which tissues to use were provided by Tillberg & al. (2006). Direct sampling into 95% ethanol in the field and subsequent drying of samples in the laboratory has proven to significantly alter $\delta^{13}C$ (by an average 0.61‰) and C:N ratios, while $\delta^{15}N$ did not change significantly (Tillberg & al. 2006). Short storage times and immediate drying, or freezing samples, may thus help to minimize this source of variation, particularly when carbon mass and $^{13}C$ are involved in the study. In earlier studies less attention was paid to whether whole ants were used or only parts of the ants' bodies. When ants are collected from the field using only alitrunk (and legs) of workers will minimize the risk that undigested food in the head, gut or crop content will strongly influence the results (Blothgen & al. 2003, Tillberg & al. 2006). Gland contents that differ in amount and substances among ant taxa may also influence results. However, killing ants by dropping them directly into alcohol and longer storage in alcohol should decrease the risk that samples are strongly contaminated with such secretions. When dropped into
Box 1: Measuring fractionation and effects of endosymbionts in *Camponotus floridanus*.

Fractionation can be estimated when the organism studied is raised on a single homogenous food source, in order to compare stable-isotope signatures of the diet with that of the focal organism.

We measured the isotopic fractionation for nitrogen ($\Delta N$) and carbon ($\Delta C$) in controlled feeding experiments for laboratory colonies of *Camponotus floridanus* ants (for exact feeding regimes and experimental conditions, see Feldhaar & al. 2007). Stable-isotope signatures were quantified for pupae of *C. floridanus* raised from eggs in worker groups fed with a chemically defined diet of known content (Straka & Feldhaar 2007). We used pupae to circumvent the problem that gut, gland, or head contents of individuals at other developmental stages may interfere with the analysis of body mass composition (see “Basics of natural-abundance-stable-isotope analyses” and Fig. 1). From each of eight large queenright colonies of *C. floridanus*, four groups of 150 randomly picked minor workers were separated into smaller containers ($20 \times 10 \times 10$ cm). Each of the 32 worker groups was provided with 45 eggs and with 45 first-instar larvae collected from the respective queenright colony. Pupae used in this study had been raised by the worker groups and were sampled from the colony 2 to 3 days after pupation.

Each of the four worker groups per colony was fed three times per week, but on different diets:

- **A** Chemically defined diet containing all trace metals, vitamins, growth factors and amino acids (AAs) essential for the ants. For exact composition and preparation of the diet see Straika & Feldhaar (2007).
- **AR** Same as A but with 100 µl of a solution containing 2% of the antibiotic Rifampicin mixed into the food every other week, in order to remove the endosymbiotic Blochmannia bacteria that are harboured in specialized midgut cells.
- **B** Chemically defined diet like A, except that essential AAs were omitted and compensated by higher amounts of non-essential AAs, so that the total amount of AAs was unchanged (Straka & Feldhaar 2007).
- **Control** Bhatkar-agar (Bhatkar & Whitcomb 1970), cockroaches and honeywater ad libitum.

In order to prevent the ants from supplementing their own diet by cannibalizing dead ants, corpses were removed daily. At the end of the experimental period larvae still present in the worker groups were counted to estimate the number of eggs or larvae cannibalized by the workers.

One pupa of each of the 32 worker groups was sampled and oven dried at 60°C for 48 hours. Likewise the different food items (artificial diets A, AR and B, cockroaches, honey-water and Bhatkar-agar of Control) were sampled and dried ($n = 3$ samples each). Food items were homogenized in a mortar and weighed on an electronic balance (Sartorius M25D, Göttingen, Germany). Pupae investigated in this study had masses ideally suited for isotope-ratio-mass spectrometry (0.4 to 2.2 mg dry weight per individual). For the subsequent sample combustion, food items and pupae were placed in tin capsules. Isotopic N and C composition of each sample was measured in comparison to standard gases (N$_2$ or CO$_2$, respectively) using an elemental-analyser-isotope-ratio-mass spectrometer (EA-IRMS). The analysis was performed in a dual element analysis mode with a Carlo Erba 1108 (Carlo Erba, Milano, Italy) elemental analyser coupled via a ConFlo III interface to a delta S mass spectrometer (Finnigan MAT, Bremen, Germany). Standard gases were calibrated with respect to international standards by using the reference substances N1 and N2 for the N isotopes and ANU sucrose and NBS 19 for the C isotopes (standards from the International Atomic Energy Agency, Vienna, Austria). Reproducibility and accuracy of the isotope abundance measurements were routinely controlled by measures of the test substance acetanilide (Gebauer & Schulze 1991). At least six test substances with different sample weights were routinely analysed within each batch of 50 samples. Maximum variation of $\delta^{13}$C and $\delta^{15}$N within and between batches was always below 0.2‰. N and C concentrations in the samples were calculated from sample weight and peak areas using a daily six-point-calibration curve based on the acetanilide measurements (Gebauer & Schulze 1991). Acetanilide has constant N and C concentrations of 10.36% and 71.09%, respectively.

On average, isotopic fractionation of *Camponotus floridanus* was $\Delta N = 3.0‰$ and $\Delta C = 1.1‰$ (Fig. 2), median across 33 pupae raised on artificial diets A, AR and B. These values are similar to the average levels across other animals, but could be used more appropriately for ants if fractionation levels are unknown but required for comparisons or quantitative modelling (see Fig. 3 and “Using mass-flow equations”).

The impact of endosymbionts on fractionation. We quantified the impact of *Blochmannia floridanus*, the endosymbiont of *Camponotus floridanus*, on $\delta^{15}$N signatures of its host. In spite of its strongly reduced genome, *Blochmannia* has retained the biosynthetic pathways for the synthesis of all essential amino acids (except arginine) in its genome (Gil & al. 2003), and in-vivo experiments have confirmed that the endosymbiont provides its host with essential amino acids (Feldhaar & al. 2007).
Fractionation of both $^{13}$C and $^{15}$N was relatively similar between diet A and diet B (Fig. 2, Mann-Whitney $U$-tests, $Z \leq 0.76$, $p \geq 0.45$, $n = 11 + 11$ pupae). Hence, the potential upgrading of non-essential amino acids by the endosymbionts showed no effect on isotope signatures. Moreover, isotopic signatures of pupae harbouring the endosymbiont (fed with diet A) in comparison to those cleared of the endosymbiont (diet AR) did not differ significantly ($Z \leq 0.89$, $p \geq 0.38$). Both results suggest that the activity of endosymbionts does not strongly affect the isotope composition of *C. floridanus*. When essential amino acids were omitted from the ants' diet (diet B), we assume that they were largely provided by the endosymbiont, although a certain amount may have been obtained by cannibalizing eggs or small larvae. Approximately one third of the provided brood was missing at the end of the experimental period. When worker groups were treated with antibiotics (diet AR), nearly half of the small brood items was missing, which may thus have contributed to a slightly higher $\delta^{15}$N. Cannibalism may thus have, at least partly, compensated for a possible effect of endosymbionts on fractionation (assumed to decrease $\delta^{15}$N). In conclusion, the impact of endosymbionts or beneficial gut microflora on $\delta^{15}$N in ants could be either negligible, or at least only minor in comparison to the impact of a food source itself, particularly when ants continuously retrieve food items under natural conditions.

Fig. 2 (to the right): Results of a controlled feeding experiment on *Camponotus floridanus* colonies in order to measure fractionation of nitrogen $\Delta_N$ and carbon $\Delta_C$ (median fractionation levels shown as numbers). Box-plots show medians, quartiles and range without outliers that appear as circles. Experimental treatments correspond to diets A, AR, B and Control (from left to right).

Fig. 3 (above): Stable-isotope composition of *Camponotus floridanus* pupae, cockroaches, Bhatkar-agar and honey water fed ad libitum. When average fractionation values from single-diet trials are applied (corrected values), the signature of the ants plots between honey and cockroach signatures. This suggests that these were used as main carbon source (esp. honey) and nitrogen source (esp. cockroaches) to raise the larvae. However, the role of Bhatkar-agar cannot be evaluated independently of that of the cockroaches as the three diets are lined up rather than forming a broader triangle that would better facilitate quantitative estimations using dietary mixing models.

alcohol, most ants will release gland secretions, a fact that can be observed readily as the ethanol turns colourful when submerging ants with huge quantities of such secretions, such as specimens of *Crematogaster (Physocrema)* (H. Feldhaar, unpubl). Other gland contents of insects may be washed out by the ethanol (STOW & al. 2007). The use of pupae would yield the most comparable results between different ant taxa, since neither gland products nor gut contents are present. However, pupae of many ant species will most likely be accessible to a myrmecologist only in the laboratory or by destroying nests. Moreover, isotopic values of pupae cannot be compared directly with those of adults, since there is often an increase from larvae and pupae to adult workers in $\delta^{13}$C and, although less pronounced, in $\delta^{15}$N (BLÜTHGEN & al. 2003). Additionally, some pupae are encased in silk and others are naked. If only one or few species are the target of a study, we thus recommend including several kinds of sampling in the analysis in order to control for such effects: measuring head and alitrunk, and separately measuring gasters with emptied crop. The optimal dry weight for isotopic analyses of ants may be about 1 mg (~ 0.2 - 5 mg), but this depends on the type of mass
spectrometer, since more and less sensitive spectrometers exist. If individual ants are too large for tin capsules, or if pooled samples of many individuals are intended, they should be ground and pooled to reduce a bias by specific body parts or certain individuals, respectively. Fluids such as nectar and honeydew may potentially be analyzed as well, although water needs to be filtered within the system. The main problem here is to have sufficient mass of C and N included in the sample of a limited maximum volume, so that prior evaporation by drying is often required. Honey that was analyzed in our study as a diet of *Camponotus floridanus* was still in a fluid form, although it had been oven-dried for many days.

When studies cover a broad range of habitats and regions, residual analyses have proven to be suitable to account for some regional variation (Fiedler & al. 2007). We recommend including samples from certain species other than ants from the same habitat as a baseline to control for variation across habitats. Plants and / or animals with relatively consistent diets such as specialized herbivores are most suited for this purpose. For instance, Blüthgen & al. (2003) often included leaves from host trees of arboreal ants to be used as covariate in analyses of intra- or intraspecific variation in stable-isotope composition of ants. Likewise a range of heriborous and predatory arthropods from each respective community can facilitate comparability of datasets obtained in different habitats and allows the placing of a focal ant taxon within the food web (Davidsson & al. 2003, Tillberg & al. 2007). Again, for other arthropods collected in the same habitat the question arises which tissues or body parts should be used for analysis. It should at least be considered to treat such arthropods in a similar way as the ants and remove the gut including its content.

Inclusion of the potential diets in the analysis will increase sampling and analytical efforts but can be very rewarding. This implies however, that the researcher actually observes what the ants feed on and, for example, fungal hyphae collected during leaf foraging and other food sources maybe cryptic (see discussion in Davidson & Cook 2008). Stable-isotope signatures of the diet allow a more fine-scaled placement of an ant within a food web, e.g., by gaining information on potential contribution of particular food sources that differ in N and C signature.

**Conclusion and future directions**

Stable-isotope analyses have recently contributed to a renaissance in the study of ant-nutritional ecology and have led to a more differentiated picture of basic ant-nutritional ecology. The most important finding was that the dietary niche breadth of Formicidae is broader than hitherto thought, including a large number of omnivores and ants feeding largely on various plant-derived food sources. Tracer studies have contributed to a better understanding of food flow within colonies or among ants and other organisms like plants or bacteria. However, scientists are only beginning to tap the manifold possible applications of stable-isotope analyses.

More comparative studies on trophic-niche partitioning within ant or arthropod communities are still needed in order to uncover differences in their nutritional ecology on a broad geographic scale. In the future studies on intraspecific variation of natural abundance of stable isotopes can contribute to a better understanding of shifts in nutritional ecology in correlation with colony size or range expansion (e.g., Tillberg & al. 2007).

Stable isotopes may be useful for the quantification of physiological costs of individual ants. In contrast to solitary insects, where longevity and fecundity is usually used as a proxy to measure fitness trade-offs, in social insects it is often only the ratio of surviving to dead workers that is measured, e.g., in studies on immunity. Metabolic costs such as a higher turnover rate of nutrients and faster recycling of internal nitrogen stores induced by investment into an immune reaction could probably be measured at a finer scale since these reactions should theoretically lead to a stronger enrichment in $^{15}$N.

To date, most studies on food flow within ant colonies were concerned with the flow of macronutrients only (such as the protein : carbohydrate ratio). The availability of a suite of compounds labelled with stable isotopes now allows monitoring of the flow of micronutrients within colonies with tracer studies. The possible presence of a "digestive caste" in ant colonies should be examined in greater detail. Larvae have been shown to differ from workers in their digestive capabilities (Sørensen & al. 1983). Such differences can have important implications for conflict resolution in colonies of social insects as was shown in social wasps where exchange of food between larvae and adults goes both ways and contributes to colony cohesion (Hunt & al. 1982). Likewise endosymbionts present in workers may still contribute to larval nutrition as nutrients could be metabolized and upgraded in the workers before the latter feed the larvae (Zientz & al. 2006).

Tracer studies can also be useful for uncovering the metabolic interdependence of ants and their symbionts, especially microsymbionts. Based on observations it was recently suggested that ants may feed on resources that cannot be broken down easily (or at all) without the help of gut microflora (Davidsson & Cook 2008, Blüthgen & Feldhaar in press) such as fungal material that may be consumed directly (Witte & Maschwitz 2008).

In conclusion, stable-isotope analyses have a promising potential in contributing important insights into nutritional ecology of ants in the widest sense – including information flow during foraging – at all levels of organization from an individual ant over ant colonies to communities.

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**Zusammenfassung**


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