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Research article

Measuring the trophic ecology of ants using stable isotopes

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Abstract. Ants are prominent components of most terrestrial arthropod food webs, yet due to their highly variable diet, the role ants play in arthropod communities can be difficult to resolve. Stable isotope analysis is a promising method for determining the dietary history of an organism, and has the potential to advance our understanding of the food web ecology of social insects. However, some unique characteristics of eusocial organisms can complicate the application of this technique to the study of their trophic ecology. Using stable isotopes of N and C, we investigated levels of intraspecific variation both within and among colonies. We also examined the effect of a common preservation technique on $\delta^{15}N$ and $\delta^{13}C$ values. We discuss the implications of our results on experimental design and sampling methods for studies using stable isotopes to investigate the trophic ecology of social insects.

Keywords: Ant ecology, stable isotopes, trophic level, *Linepithema, Solenopsis, Lasius, Aphaenogaster.*

Introduction

Ants are ubiquitous and important components of nearly every terrestrial ecosystem (Hölldobler and Wilson, 1990). Trophically, the family Formicidae is remarkably variable including "herbivores" (e.g. leaf cutter ants), highly specialized predators (e.g. army ants, Hirosawa et al., 2000), and a preponderance of omnivores (Blüthgen et al., 2003; Davidson et al., 2003). Recently, studies have utilized stable isotopes to elucidate the trophic ecology of ants including temporal variation in diet (Fisher et al., 1990; Mooney and Tillberg, in press), the nature of ant-plant symbioses (Treseder et al., 1995; Sagers et al., 2000; Solano and Dejean, 2004; Tillberg, 2004; Trimble and Sagers, 2004), and the community ecology of ants (Blüthgen et al., 2003; Davidson et al., 2003; Tillberg and Breed, 2004).

Understanding the ecological role of eusocial species using stable isotopes poses a unique challenge. Unlike solitary organisms, an ant colony's dietary input is the result of the foraging efforts of numerous individuals. Certain characteristics of eusociality, such as overlapping generations, behavioral specialization within the colony, food-sharing among nestmates (Børgesen, 2000), and differing dietary requirements between workers and larvae (Cassill and Tschinkel, 1999) complicate the matter of assessing the trophic ecology of the whole colony. Furthermore, recently ingested food held in the crop might bias workers' signatures if the unassimilated diet is processed along with the rest of the ant body (Blüthgen et al., 2003); this is tantamount to contaminating a consumer's sample with material from its prey.

Methodological constraints may further complicate the use of stable isotopes for trophic studies of social insects. For example, often only one worker or one colony is measured to represent the trophic position for the whole species in a community. Ant diets may vary both within and among seasons in relation to colony size, life cycle, and periods of brood production (Horstmann, 1972; Portha et al., 2002). Therefore, inadequate replication may bias an estimate of a species' trophic position. Sample collection, storage, and preparation are also of primary concern in stable isotope ecology. Ideally, specimens used for stable isotope analysis would be dried immediately after capture to prevent rotting, and subsequently kept in desiccant until processing. However, immediate desiccation is not always an option, and collection into ethanol (EtOH) is a common method in the field to preserve specimens for DNA work or point mounting (King and Porter, 2004). As such, it is important to know if and how this treatment alters isotopic values to determine whether specimens stored in ethanol should be used for isotopic analysis. In this study we address some outstanding questions regard-

ing the utility and methodology of stable isotopes for the investigation of the trophic ecology of eusocial organisms, specifically ants. To determine levels of within-colony variation in isotopic values, we investigated the isotopic ratios of nitrogen and carbon in workers versus brood from two species of ground-foraging ants. We also examined whether whole samples of workers should be processed, or if the abdomen should be removed from the rest of the body prior to analysis. To examine intraspecific variation among colonies, we sampled multiple colonies of each of five ant species. Our final methodological goal was to determine if storage in 95% ethanol has an effect on $\delta^{15}N$ and $\delta^{13}C$ values.

Methods

Background on stable isotopes for trophic ecology

The application of stable isotope techniques to ecological questions has opened a new frontier in our understanding of animal diets (Herrera et al., 2002; Callaham et al., 2000; Ben-David et al., 2004), animal movement and migratory patterns (Lott, 2003), food web structure (Ostrom et al., 1997; Oelberman and Scheu, 2002; Post, 2002), and ecophysiology (Yoneyama, 1997; Bort et al., 1998). Stable isotope analysis takes advantage of naturally-occurring differences in the ratios of heavy to light isotopes in biologically relevant elements, such as carbon and nitrogen. The delta value of the element is calculated by comparing the ratio of the heavy:light isotope to an element specific standard as follows:

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

The delta or 'δ' values are expressed in 'permil' or '‰' units. A change in the ratio of heavy:light isotopes due to some biological process is 'fractionation'; an increase in the heavy:light isotope ratio is 'enrichment' and a decrease in this ratio is 'depletion'. Two of the more commonly used isotopes in ecological studies are ^{13}C and ^{15}N.

Carbon isotopic values of consumers typically reflect the carbon isotopic values of their diet, with a small (0.5‰ to 1‰) enrichment per trophic exchange (DeNiro and Epstein, 1978; Macko et al., 1982; Fisher et al., 1990; Ostrom and Fry, 1993; Lajtha and Michener, 1994). The major source of variation in carbon is due to differing carbohydrate synthesis pathways among types of plants, resulting in markedly different $\delta^{13}C$ values. For example, C4 plants tend to have $\delta^{13}C$ values ranging from –9 to –17‰, while C3 plants typically have values ranging from –20 to –32‰. Carbon is most useful for inferring the basal resource of a consumer or food chain when the $\delta^{13}C$ values of possible resources are distinct.

Nitrogen isotopic values of consumers are typically enriched relative to their diet. Initial measurements from a broad diversity of organisms found a general pattern of 3 to 4‰ enrichment with each trophic exchange (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Owens, 1987; Cabana and Rasmussen, 1994). Recent work suggests that the amount of enrichment that occurs between diet and consumer may vary due to a number of factors including age, C:N ratio of the diet, taxonomic identity, physiological status, etc. (Vanderklift and Ponsard, 2003). Nitrogen fractionation is useful for understanding trophic position of organisms in food webs.

Sample preparation

Unless otherwise noted (see *Effect of storage in ethanol below*), samples were prepared and analyzed as follows. For each collection, 10-15 workers were collected with aspirators and frozen within two hours of collection, after which they dried in an oven at 50 °C. After drying, all samples were weighed into tin capsules using a Mettler & Toledo microbalance. To attain a massive enough sample for analysis, individual replicates of workers consisted of the head, thorax, and legs of 10–15 individuals per colony. All analyses were performed at the University of California – Davis Stable Isotope Facility using a Europa Hydra 20/20 continuous flow Isotope Ratio Mass Spectrometer.

Intracolonial variation

To examine within colony variation between brood and workers, and variation between workers' abdomens and heads/thoraces, we collected colonies of *Lasius alienus* (N = 15) and *Aphaenogaster rudis* (8) from Trelease Woods, Champaign C., Illinois, in June and July 2004. For the abdomen vs. head/thorax comparison, we separated the gaster and petiole (abdominal segments 2–7, these segments contain the abdominal organs including the crop) from the bodies of the ants and processed both the abdomens and the heads/thoraces from the same set of ants as separate samples. We used paired t-tests to compare $\delta^{13}C$, $\delta^{15}N$, and C:N of brood versus workers and abdomen versus head/thorax for each species. Then, we tested for differences in $\delta^{15}N$ and $\delta^{13}C$ between the *L. alienus* and *A. rudis* at the species level using MANOVA followed by Fischer's PLSD.

Intraspecific variation

To examine intraspecific variation in $\delta^{15}N$ among colonies within a population, we collected workers from five species including *Aphaenogaster rudis* (N = 8 colonies), *Lasius alienus* (N = 15), *Linepithema humile* (N = 10), *Pheidole obscurithorax* (N = 10), and *Solenopsis invicta* (N = 10). *Aphaenogaster rudis* and *L. alienus* were collected from Trelease woods, Champaign Co., Illinois. *Linepithema humile*, *P. obscurithorax*, and *S. invicta* were collected at Herradura, Formosa Prov., Argentina in November, 2003.

Effect of storage in ethanol

We measured the effects of storage in 95% EtOH on isotopic ratios by collecting ant workers either from foraging trails or from nest structures from nine species at the UC Elliot Chaparral Reserve in San Diego Co., California, and 13 species from two locations in Argentina (Reserva Ecologica Otamendi, Buenos Aires Prov.; Villa Ocampo, Santa Fe Prov.) in October and November 2003 (Table 1). In addition to preparing samples as described above (*Sample preparation*), we put an additional 10–20 workers directly into 95% EtOH. After storage in EtOH for six months, the ants were placed into clean vials, dried, and processed in the same manner as the previous samples. We used paired t-tests to compare $\delta^{13}C$, $\delta^{15}N$, and C:N of samples dried and processed immediately to the isotopic values of samples processed after 6 months in EtOH.

Results

Intracolonial variation

For both *L. alienus* and *A. rudis*, workers had higher $\delta^{13}C$ values than brood from the same colony (*Lasius*: DF = 14, T = –8.08, P < 0.0001; *Aphaenogaster*: DF = 7, T = –2.648, P = 0.03). The mean difference between brood and workers was 1.3‰ and 0.8‰ for *Lasius* and *Aphaenogaster*, respectively. *Lasius alienus* workers had significantly higher $\delta^{15}N$ than *L. alienus* brood (DF = 14, T = –4.037, P < 0.001), but there was no difference in $\delta^{15}N$ between *A. rudis* workers and brood (P > 0.1) (Fig. 1). The C:N ratios varied between brood and workers for both species (*L. alienus*: brood = 7.2, workers = 4.3, DF = 14, T = 11.16, P < 0.0001; *A. rudis*: brood = 5.6, workers = 3.9, DF = 7, T = 5.63, P < 0.001).

For both species, worker heads/thoraces had higher $\delta^{13}C$ values than worker abdomens (*L. alienus*: DF = 11, T =

Table 1. Species and collection locations of ants used to investigate the effect of storage in 95% EtOH on $\delta^{15}N$ and $\delta^{13}C$.

Location	Ant
San Diego, CA, USA	*Crematogaster californica*
San Diego, CA, USA	*Messor andrei*
San Diego, CA, USA	*Forelius mccookii*
San Diego, CA, USA	*Solenopsis xyloni*
San Diego, CA, USA	*Linepithema humile*
San Diego, CA, USA	*Pogonomyrmex subnitidus*
San Diego, CA, USA	*Dorymyrmex insanus*
San Diego, CA, USA	*Camponotus dumetorum*
San Diego, CA, USA	*Pheidole vistana*
Reserva Otamendi, Argentina	*Pseudomyrmex phyllophilus*
Reserva Otamendi, Argentina	*Solenopsis (Diplorhoptrum)*
Reserva Otamendi, Argentina	*Pheidole obscurithorax*
Reserva Otamendi, Argentina	*Cephalotes* sp. A
Reserva Otamendi, Argentina	*Acromyrmex* sp. A
Reserva Otamendi, Argentina	*Pogonomyrmex naegelli*
Reserva Otamendi, Argentina	*Crematogaster* sp. A
Reserva Otamendi, Argentina	*Solenopsis richteri*
Reserva Otamendi, Argentina	*Dorymyrmex* sp. A
Reserva Otamendi, Argentina	*Pogonomyrmex coarctatus*
Reserva Otamendi, Argentina	*Linepithema humile*
Reserva Otamendi, Argentina	*Pseudomyrmex "falvidus"* complex sp. A
Villa Ocampo, Argentina	*Linepithema humile*
Villa Ocampo, Argentina	*Solenopsis invicta*

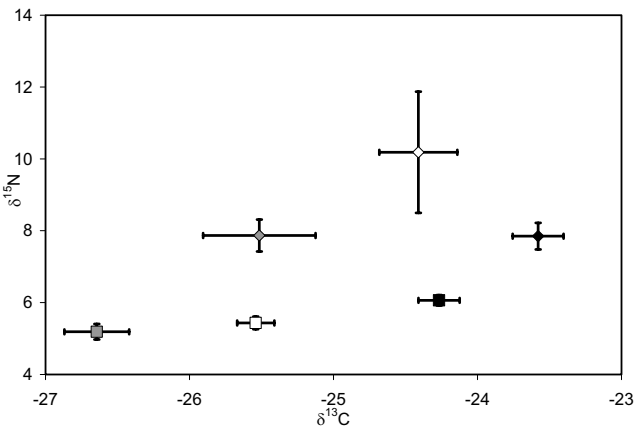

Figure 1. $\delta^{15}N \pm SE$ and $\delta^{13}C \pm SE$ for *Aphaenogaster rudis* brood (◇), workers (◆), and abdomens (◈), and *Lasius alienus* brood (□), workers (■), and abdomens (▣). Brood and workers for both species had different $\delta^{13}C$ values, but only *L. alienus* had a difference in $\delta^{15}N$ between workers and brood. Abdomens for both species had significantly depleted $\delta^{13}C$ values relative to the head/thorax. *Lasius alienus* abdomen and head/thorax differed in $\delta^{15}N$, but there was no difference between these body segments in *A. rudis*.

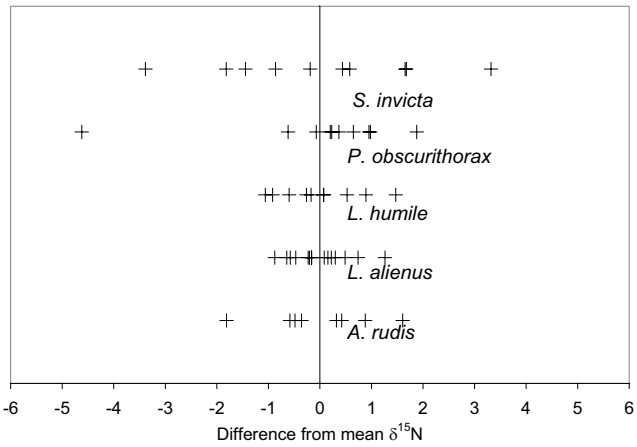

Figure 2. The range of colony $\delta^{15}N$ data plotted relative to each species' mean $\delta^{15}N$.

13.55, P < 0.0001; *A. rudis*: DF = 3, T = 8.05, P < 0.01). For *Lasius alienus*, worker heads/thoraces also had higher $\delta^{15}N$ values than abdomens (DF = 11, T = 9.01, P < 0.0001), but there was no difference in $\delta^{15}N$ between abdomens and heads/thoraces for *A. rudis* (Fig. 1). C:N ratios varied between abdomen and head/thorax for both species (*L. alienus*: abdomen = 8.7, head/thorax = 4.3, DF = 13, T = 5.99, P < 0.0001; *A. rudis*: abdomen = 7.1, head/thorax = 3.9, DF = 3, T = 8.99, P < 0.01).

Multivariate analysis of $\delta^{15}N$ and $\delta^{13}C$ detected a significant difference between the species (Wilk's Lambda = 0.29, F = 33.092, P < 0.0001). Subsequent univariate analysis found differences between the species in both $\delta^{15}N$ (Fisher's PLSD: DF = 28, F = 44.59, P < 0.0001) and $\delta^{13}C$ (Fisher's PLSD DF = 28, F = 12.81, P < 0.01) (Fig. 1).

Intraspecific variation

All species had a range of colony-level $\delta^{15}N$ values of at least 2.5‰, with a maximum of approximately 6.0‰ (*S. invicta* and *P. obscurithorax*). Standard deviation from the species' mean ranged from ± 0.7‰ (*L. alienus*) to ± 1.9‰ (*S. invicta*) (Fig. 2).

Table 2. Results of paired t-test comparing $\delta^{15}N$, $\delta^{13}C$, and C:N ratio of samples dried immediately after collection to samples stored in EtOH for six months.

Control vs. EtOH Storage	Mean difference	DF	t-value	P-value
$\delta^{15}N$	0.17 ‰	51	1.76	0.08
$\delta^{13}C$	0.61 ‰	51	3.77	**0.0004**
C:N ratio	0.7	51	–8.92	**<0.0001**

Effect of storage in ethanol

Storage in 95% EtOH for six months resulted in a 0.61‰ shift of $\delta^{13}C$. Delta-^{15}N of control ants and ants stored in EtOH for six months were not different. Ethanol storage resulted in a 0.7 decrease in C:N ratio (Table 2).

Discussion

These results demonstrate that the application of stable isotope techniques to social insect trophic ecology require careful consideration of design and methodology. Isotopic values varied considerably both within and among colonies of the same species. Furthermore, we show that a common preservation technique for insects may bias $\delta^{13}C$ values of specimens.

Delta-^{15}N and $\delta^{13}C$ values varied significantly within colonies. The brood of both ant species we investigated had lower $\delta^{13}C$ values, and higher C:N ratios relative to the workers. While these differences between brood and workers may be due to a difference in dietary carbon resources between workers and brood, or to ontogenetic differences in fractionation of carbon, it is also possible that these mean values are a result of the composition of the samples analyzed. For ant workers, we separated abdomens prior to analysis to remove the possibility that recently-ingested diet held in the crop would bias the whole sample. However, it was not possible to divide the abdomen from the rest of the body in the larvae samples. Thus, any diet items remaining in the larval digestive tract were included with the measurement of the whole larvae. If recent diet items fed to the larvae had high C:N ratio or lower $\delta^{13}C$ values, then this could account for some of the difference between workers and brood in $\delta^{13}C$ and C:N.

Alternatively, ant abdomens, which are the location of lipid storage in the fat body, may have depleted $\delta^{13}C$ values relative to the rest of the ant's body. Lipids, which are carbon-rich (high C:N), also tend to have lower $\delta^{13}C$ values than other body tissues in birds (Thompson et al., 2000) and fish (Sotiropoulos et al., 2004). We found ant larval values for $\delta^{13}C$ to be midway between adult abdomen and head/thorax values; analysis of whole workers may result in $\delta^{13}C$ values indistinguishable from $\delta^{13}C$ values of the larvae.

Patterns of within colony variation between brood and workers for $\delta^{15}N$ were different between *Aphaenogaster rudis* and *Lasius alienus*. While *A. rudis* brood and workers did not differ, *L. alienus* workers were enriched in $\delta^{15}N$ compared to their brood. This could be due to differences in $\delta^{15}N$ of resources assimilated by workers compared to brood. Alternatively, the possibility remains that this difference may be a result of processing whole larvae but removing the abdomen in the workers. However, if the latter explanation accounted for the entire difference, we would have expected brood and worker signatures to be different in both species, similar to the results for $\delta^{13}C$ and C:N. That $\delta^{15}N$ was not different between *A. rudis* brood and workers suggests that the difference we found in *L. alienus* has a biological basis and is not due solely to sample processing.

Abdomens had significantly different $\delta^{13}C$ and C:N ratios compared to ant bodies in both species. Abdomens had approximately twice the relative carbon content of the rest of the body. There was also a difference in $\delta^{15}N$ between abdomen and body for *L. alienus*, but not in *A. rudis*. These results suggest that the contents of the abdomen, which includes the digestive tract and any undigested food therein, could significantly alter whole specimen $\delta^{15}N$, $\delta^{13}C$, and C:N values in ants. Our results are similar to Blüthgen et al. (2003) who measured differences in $\delta^{13}C$ and $\delta^{15}N$ for *Oecophylla* that had recently ingested honeydew.

Lasius alienus and *A. rudis* appear to have differing diets; mean $\delta^{13}C$ and $\delta^{15}N$ values were significantly different for both species. Delta-^{15}N of *A. rudis* was 1.8‰ higher than *L. alienus*, and $\delta^{13}C$ of *A. rudis* was 0.7‰ higher than *L. alienus*. Both enrichments are consistent with a difference in trophic position between these ant species. Taken together, these data suggest that *A. rudis* takes more prey from higher trophic levels than *L. alienus*.

Our estimates of relative trophic position for *L. alienus* and *A. rudis* are based on replication at the colony level for both species at one site. This level of replication is important given the dietary variation present among colonies of the same species. In our study of five different species, we found substantial intraspecific variation in $\delta^{15}N$ (Fig. 2), allowing us to quantify potential differences in dietary history among colonies. We found a difference in $\delta^{15}N$ of 6.0‰ among some colonies suggesting that estimates of trophic position in a single species can span up to two trophic levels (DeNiro and Epstein, 1981; Cabana and Rasmussen, 1994). Tillberg and Breed (2004) analyzed ten individual ants from nine colonies of *Paraponera clavata* and found low variation in $\delta^{15}N$ within colonies among nestmates. However, colony means differed by as much as 2.4‰. Mooney and Tillberg (2005) also report considerable variation in $\delta^{15}N$ among colonies of the same species. Adequate intraspecific replication captures the range of dietary input among colonies and gives a more complete picture of the dynamic and variable trophic ecology of a species.

Ethanol storage has an effect on $\delta^{13}C$ values and also appears to change the C:N ratio of ant samples. By soaking in a solvent, the fat bodies of the ants may have been dissolved, resulting in a shift of the $\delta^{13}C$ values. After 6 months in EtOH, we did not detect a significant change in $\delta^{15}N$ values. Our $\delta^{13}C$ and $\delta^{15}N$ findings are similar to Ponsard and Amlou (1999) who found a significant effect of ethanol storage on $\delta^{13}C$, but not $\delta^{15}N$, in *Drosophila* after both ten days and six weeks. Thus, carbon values may be unreliable in specimens stored in ethanol, but useful information may still be gained about the trophic ecology of social insects stored in 95% ethanol by measuring $\delta^{15}N$.

Our results indicate that studies using stable isotopes to investigate the trophic ecology of social insects must be designed to account for intraspecific variation. Specifically, sampling only workers may ignore important dietary inputs of a colony, and using only one colony may ignore considerable within species variation among colonies (see also Tillberg and Breed, 2004). We also propose that researchers investigating

ant diet using stable isotopic techniques keep the specimens alive and without dietary input for a short period (12 h) to clear the digestive tract before processing. If this is not possible, then it may be necessary to remove the abdomen from specimens. Using just the head and thorax will give a better indication of the source of assimilated carbon and nitrogen, whereas including the abdomen might skew results heavily towards recent feeding. However, an important pitfall of removing the abdomen is that the mass balance of the whole organism may be compromised if there are significant differences between head/thorax and abdomen akin to differences in isotopic signatures among tissues in larger organisms. Finally, only individuals that have been processed immediately after collection should be used for analysis of $\delta^{13}C$. Our data suggest that specimens stored in EtOH can be used to investigate $\delta^{15}N$.

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