Application of CO_2 carbon stable isotope analysis to ant trophic ecology: preliminary results

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Abstract

Stable isotope analysis of animal tissues is commonly used to infer diet and trophic position. However, it requires destructive sampling. The analysis of carbon isotopes from exhaled CO_2 is non-invasive and can provide useful ecological information because isotopic CO_2 signatures can reflect the diet and metabolism of an animal. However, this methodology has rarely been used on invertebrates and never on social insects. Here, we first tested whether this method reflects differences in $\delta^{13}C\text{-}CO_2$ between workers of the Mediterranean ant *Crematogaster scutellaris* (Olivier, 1792) (Formicidae: Crematogastrini) fed with beet (C3) and cane (C4) sugar (*Beta vulgaris* L., Amaranthaceae and *Saccharum officinarum* L., Poaceae, respectively). We found that a significant difference can be obtained after 24 hours. Consequently, we used this technique on wild co-occurring ant species with different feeding preferences to assess their reliance on C3 or C4 sources. For this purpose, workers of *C. scutellaris*, the invasive garden ant *Lasius neglectus* (Van Loon, Boomsma & Andrasfalvy, 1990) (Formicidae: Lasiini) and the harvester ant *Messor capitatus* (Latreille, 1798) (Formicidae: Stenammini) were sampled. No significant differences in their carbon isotopic signatures were recorded, suggesting that in our study site no niche partitioning based on the carbon pathway occurs, with all species sharing similar resources. However, further analysis revealed that *M. capitatus*, a seed-eating ant, can be regarded as a C3 specialist, whereas *L. neglectus* and *C. scutellaris* are generalists that rely on both C3 and C4 pathways, though with a preference for the former. Our results show that this methodology can be successfully applied even to small animals like ants and can provide useful information on the diets of generalist omnivores.

Introduction

Stable isotopes analysis (SIA) of animal tissues is widely used in animal ecology to infer diet, determine trophic positions and define trophic niches (Fry, 2006; Boecklen et al., 2011). The most widely used isotopes are those of C and N ($\delta^{13}C$ and $\delta^{15}N$), which provide information on the main carbon source used by a consumer and its trophic level based on predictable isotope fractionation occurring from one trophic level to another (Post, 2002). Moreover, SIA is employed for the study of invasive species impacts (Balzani et al., 2016; Stellati et al., 2019) and interactions (Haubrock et al., 2019a, 2020) and to predict the effects of species reintroductions (Haubrock et al., 2019b). Applications in studies of insects include the determination of resource origin (Ouyang et al., 2015), movements (Madeira et al., 2013, 2014; Zhang et al., 2020) and feeding periods (Ouyang et al., 2014), as well as the study of nutrient uptake (Pollier et al., 2016) and allocation (Levin et al., 2017a,b). In ant ecology, SIA is used to study the trophic role of each species (e.g. Ottonetti et al., 2008), to infer how ant assemblages vary in different habitat conditions (Gibb & Cunningham, 2011), to estimate the importance of trophobiosis (Brewitt et al., 2015) and to study the effects of natural local baseline variations (Cronin et al., 2015) and colony parameters (Barriga et al., 2013) on isotopic signatures. This approach requires destructive sampling and is therefore difficult to use when dealing with rare species or when repeated measurements on the same subjects are desired (Hatch et al., 2002b). However, social insects are somewhat unique in that colonies can be considered the ecological unit of study and these can be repeatedly sampled overtime with relative ease, to assess the effect of changes in resource availability on their diet (Roeder & Kaspari, 2017; Shik et al., 2018).

Stable isotope analysis can also be performed on gas — a common technique in geochemistry in which C stable isotopes of CO_2 and CH_4 are employed to identify emission sources in natural or anthropised environments (e.g. Venturi et al., 2017, 2019, 2020 and references therein). In the biological sciences, isotopic gas measurements on respiration-derived CO_2 are relatively common. This approach is primarily used in medical studies in which changes in the isotopic composition of CO_2 (breath tests) are used to investigate human pathologies, physiology and nutrition, and to test the metabolic rate during stress exercises (McCue & Welch, 2016). Similarly, this approach can be applied to the animal sciences. The carbon isotope signature ($\delta^{13}C$) of exhaled CO_2 correlates with diet and metabolism as it reflects the signature of the catabolised substrate (Perkins & Speakman, 2001). Moreover, the $\delta^{13}C$ values in the breath indicate both the present and past diet and are used to calculate the proportion of lipids and carbohydrates metabolised by an animal (Hatch et al., 2002a,b). Comparing the $\delta^{13}C$ values of breath and body tissues allows for the detection of diet changes (Podlesak et al., 2005; Voigt et al., 2008a). Shifts in substrate oxidation during starvation or torpor/hibernation have been studied using breath stable isotopes in vertebrates (McCue & Pollock, 2013; Lee et al., 2017; Rosner & Voigt, 2018) and invertebrates (McCue et al., 2015). The reasoning behind these studies is that breath is highly metabolically active compared to tissues, which show slower turnover rates, thus reflecting the more recent diet (Perkins & Speakman, 2001). If breath and tissue signatures differ, a dietary shift is likely to have occurred over a certain temporal window (Podlesak et al., 2005; Voigt et al., 2008a). On the other hand, lipid $\delta^{13}C$ values are depleted with respect to carbohydrate $\delta^{13}C$ (Post et al., 2007), and this difference is recorded in breath isotope signatures (Voigt et al., 2008b,c). Therefore, animals metabolising their fat stores (due to starvation or torpor) will have lower breath $\delta^{13}C$ than non-fasted animals (McCue & Welch, 2016). The advantages of this methodology are that it (i) uses a non-invasive and non-destructive sampling technique, (ii) can be performed repeatedly on the same individual, and (iii) can be applied to studies of endangered or protected animals (Hatch et al., 2002b). However, most studies have focused their attention on birds and mammals, while investigations on invertebrates are less common (but see e.g. DeNiro & Epstein, 1978; Miller et al., 1985; Engel et al., 2009). To the best of our knowledge, no breath study has addressed the trophic ecology of ants.

Most ant species are believed to have an omnivorous and opportunist diet (Hölldobler & Wilson, 1990; Blüthgen & Feldhaar, 2010). Still, different food preferences can be found in different taxa. For example, Formicinae generally tend to feed on lower trophic levels (mainly homopteran honeydew or plant extrafloral nectaries) compared to Myrmicinae, which are more carnivorous, although generalisations are problematic (Fiedler et al., 2007; Blüthgen & Feldhaar, 2010; Brewitt et al., 2015). Food selection is determined by several other factors such as resource distribution and availability, food quality and quantity, handling time and colony nutritional requirements (Csata & Dussutour, 2019). Additionally, resource value can vary in different periods of the year, changing their profitability and, consequently, their uptake by ants (Kay, 2002). The application of SIA techniques can, therefore, provide cost-effective information on ant metabolism and can help to elucidate details of their trophic ecology.

The acrobat ant *Crematogaster scutellaris* (Olivier, 1792) is a Mediterranean Myrmicinae ant that nests in tree trunks (Santini et al., 2011). While it is commonly observed tending homopterans to obtain their carbohydrate honeydew, it is also known to be an avid predator of arthropods (Schatz et al., 2003; Frizzi et al., 2016). Indeed, SIA has revealed its high trophic level, suggesting a strong reliance on animal prey (Ottonetti et al., 2008). The invasive garden ant *Lasius neglectus* (Van Loon, Boomsma & Andrasfalvy, 1990) is a Formicinae ant considered invasive in Europe (e.g. Nagy et al., 2009). This species tends to monopolise aphid aggregations and to rely primarily on honeydew (Paris & Espadaler, 2009; Frizzi et al., 2018). While seed collection (with elaiosome consumption) is common, few genera, such as the Myrmicinae *Messor* and *Pheidole*, actually eat them (Blüthgen & Feldhaar, 2010). However, the harvester ant *Messor capitatus* (Latreille, 1798) often feeds on plant seeds (Cerdá & Retana, 1994), while *Pheidole pallidula* retrieves mostly insects and thus is more predaceous (Detrain, 1990).

In this study, carbon isotopes of CO_2 emitted from different ant species collected in the field were analysed to assess their diet and their degree of trophic specialisation on C3 or C4 sources.

First, the application of this method on ants was tested by comparing the $\delta^{13}C$ values in the CO_2 produced by laboratory colonies of *C. scutellaris* fed with different food sources. In particular, the colonies were fed with either raw beet sugar (C3 plant) or raw cane sugar (C4 plant), which are known to have distinct isotopic signatures (O'Leary, 1981; Boecklen et al., 2011). Then, the isotopic signatures of the gas produced by wild colonies of three ant species (*C. scutellaris, L. neglectus* and *M. capitatus*), which have different feeding preferences, were analysed. We hypothesised that if these species feed on distinct carbon pathways, a difference in CO_2 isotope signature should be detected.

Materials and Methods

In July 2019, we collected workers of the ant *C. scutellaris* from 10 natural colonies on the University Science Campus in Sesto Fiorentino or in the surrounding area (43°49’00’’N, 11°11’59’’E). The area is a managed suburban park made up by University buildings surrounded by overgrown fields, public gardens with ornamental trees (*Acer campestre, Celtis australis, Quercus robur, Quercus cerris, Quercus ilex, Fraxinus angustifolia*) and some sparse trees of *Populus sp., Morus sp.* and *Ailanthus altissima*. Samples from each colony were brought to the laboratory and subdivided into two groups of approximately 200 workers each. The ants were housed in plastic aquaria (10 x 20 x 30 cm) with Fluon®-coated walls to prevent escape. One group was fed with raw beet sugar (C3) and the other group was supplied with raw cane sugar (C4). Sugar and water were provided ad libitum for two weeks to let the ants equilibrate to their new diet.

After two weeks, 100 workers from each group were placed into closed 3-litre respiratory jars, which have a 3-way valve sealed on the cap. The jar walls were coated with Fluon® in the upper portion to prevent ants from reaching the valve. Four jars with no ants were used as controls. The jars were kept in a thermostatic chamber with controlled conditions to reproduce the daily (24 hours) light-dark cycle (16 h / 8 h). During the daytime, the temperature was set to a constant 27°C, and during the night it was 19°C. The air in the jars was sampled at the beginning of the experiment (t_0) and after 24 hours (t_{24}). At each time, aliquots of air (50 ml) were collected (after mixing the air in each jar) using a 60 ml plastic syringe connected to the valve and were stored in 1-litre Supelco Tedlar® gas sampling bags equipped with a push/pull lock valve.

In the second set of experiments in July–August 2019, we collected specimens of three co-occurring species, *C. scutellaris, M. capitatus* and *L. neglectus*, in the same area to assess the differences in their diets. For each species, we selected four natural colonies and collected 70–200 workers (depending on the species' individual size to obtain enough biomass) from each. Workers were immediately placed into sealed respiratory jars and air was collected according to the procedure described above. No food was provided during or before the experiment. At the end of the experiment, the ants were euthanised by freezing at -20°C and dried in an oven at 60°C for 48 h before being weighed using an electronic balance (accuracy: 0. 01 mg).

Carbon isotopic composition of CO_2 (expressed as $\delta^{13}C$-CO_2, ‰ vs. V-PDB standards) was analysed by Cavity Ring-Down Spectroscopy (CRDS) using a Picarro G2201-i Analyzer (CO_2 operational range: 100–4000 ppm). The Supelco Tedlar® gas sampling bags were directly connected to the analyser inlet via silicon connections. Statistical analyses were performed using R software version 3.6. CO_2 production rates were estimated by multiplying the mean difference in gas concentration within the 24 h by the jar volume (3 l) and dividing by 24 to obtain a rate per hour. The mean individual CO_2 production rate was calculated by dividing by the number of workers in the jar. Both estimations were corrected for the mean dry weight of the workers.

Linear mixed effects models and simple linear models were built for the first and second experiments, respectively. Separated models were constructed for t_0 and t_{24} using log-transformed CO_2 concentration and isotopic signature as the response variables and sugar type or species as predictors. The CO_2 in the respiration jars was a mixture of the gas produced by ants during the experiment and that of the air already present in the jar. To estimate the signature of the CO_2 produced by ants, Keeling plots (Keeling, 1958, 1961; Carleton et al., 2004) were used separately for each colony. Keeling plots are biplots of time-repeated measurements (at t_0 and t_{24}) with the $\delta^{13}C$ values on the y-axis and $1/[CO_2]$ on the x-axis. In the biplot, the y-intercept of the regression line between t_0 and t_{24} represents the case in which the CO_2 concentration is infinitely high and the ambient CO_2 is negligible. With the log-transformed data, an additional linear model with sugar or species (in lab or field, respectively) as the predictor was built.

According to the two-endpoint mixing model by Engel et al. (2009), the δ^{13}C values in the breath of an ant is:

$$\delta^{13}C_{breath} = p(\delta^{13}C_{A}) + (1-p)\,(\delta^{13}C_{B}) \qquad \text{(eq.1)}$$

where p is the proportion of C3 or C4 sources in the diet and the endpoints (A and B) are the isotopic signatures of ants fed exclusively with either C3 or C4 sugars, respectively. The latter were obtained by averaging the pure isotopic signatures estimated by the intercepts in the Keeling plots. This formula was used to produce the values expected for each species using different proportions of C3 or C4 sources. This formula was also used to calculate the δ^{13}C pure signatures for the three species of ants used in the second experiment. The average ant pure signatures were then compared to the threshold values obtained from eq. 1 using t-tests to identify species specialists for either C3 or C4 plants. We defined specialists as those consuming more than 90% of either C3 or C4 sources in their diet ($p > 0.9$ in eq. 1).

Results

For the lab experiments, the mixed effects models showed no difference in CO_2 concentrations at t_0 ($F_{2,9.76} = 3.04$, $P = 0.09$) as well as no difference in the $\delta^{13}C\text{-}CO_2$ values ($F_{2,12.71} = 0.36$, $P = 0.71$). At t_{24}, a significant difference was found for CO_2 concentrations ($F_{2,20} = 35.32$, $P < 0.001$), with significant differences between controls and each treatment ($P < 0.001$ for both) but no differences between cane and beet treatments ($P = 0.97$). Overall, the $\delta^{13}C\text{-}CO_2$ values differed among groups ($F_{2, 13.27} = 181.21$, $P < 0.001$; Figure 1). No differences between controls and cane treatment were found ($P = 0.84$), while the $\delta^{13}C\text{-}CO_2$ values measured in beet treatments significantly differed with respect to controls and cane treatment ($P < 0.001$). The average pure $\delta^{13}C\text{-}CO_2$ signatures of C3 and C4 sugar were estimated to be -24.56‰ and -11.41‰, respectively. The linear model of the Keeling plot intercepts revealed significant differences between the two treatments ($F_{1,17} = 485.74$, $P < 0.001$).

The linear model for the wild ants showed no difference in CO_2 concentrations at t_0 ($F_{3,11} = 0.95$, $P = 0.45$) as well as no difference in $\delta^{13}C\text{-}CO_2$ ($F_{3,11} = 1.84$, $P = 0.20$). At t_{24}, a significant difference was found for CO_2 concentrations ($F_{3,11} = 24.90$, $P < 0.001$), with all ant species differing from the controls ($P < 0.001$). The mean CO_2 production rate per g of dry weight was 1282.50 $\mu l\ h^{-1}\ g^{-1}$ for *C. scutellaris* (mean individual rate: 12.83 $\mu l\ h^{-1}\ g^{-1}$), 2888.75 $\mu l\ h^{-1}\ g^{-1}$ for *L. neglectus* (mean individual rate: 28.89 $\mu l\ h^{-1}\ g^{-1}$) and 598.29 $\mu l\ h^{-1}\ g^{-1}$ for *M. capitatus* (mean individual rate: 8.55 $\mu l\ h^{-1}\ g^{-1}$). Significant differences in $\delta^{13}C\text{-}CO_2$ were found at t_{24} ($F_{3,11} = 38.10$, $P < 0.001$; Figure 2). All species differed from controls ($P < 0.001$ for all), although no difference among species was found. Similar results were obtained from the linear model of the Keeling plot intercepts among species ($F_{2,9} = 3.41$, $P = 0.08$).

Using the isotope mixing model, we identified the threshold values for C3 or C4 specialists, which were δ^{13}C-CO_2 > -12.73‰ (C4) and δ^{13}C-CO_2 < -23.25‰ (C3). Intermediate values indicated generalist feeders. We found no C4 specialists (Figure 3), and all species had much more negative δ^{13}C-CO_2 values. Both *C. scutellaris* and *L. neglectus* had δ^{13}C-CO_2 values not different from the expected threshold value for C3 specialists (t-test $P > 0.7$ for both), with some colonies beyond that threshold. On the other hand, *M. capitatus* significantly differed from this threshold ($t_3 = -5.5331$, $P = 0.012$) and all colonies were beyond that value (Figure 3), suggesting an exclusive reliance on C3 sources.

Discussion

To the best of our knowledge, this is the first application of a breath test on social insects and one of few on insects overall (Miller et al., 1985; Engel et al., 2009). One of the main achievements of this study was that the methodology used for analysing the carbon isotopic composition of breathed CO_2 can easily be applied to small organisms like ants. Indeed, the isotopic signatures of *Crematogaster* ants fed with either C3 or C4 sugars were recorded to be significantly different after 24 h in the respiration chambers, demonstrating that the experimental setup was efficiently able to allow discrimination of distinct dietary carbon sources. The technique also provided insights into the feeding choices of the three species of ants collected in the field.

For the lab experiments, 24 hours proved to be a sufficient time for the ants to accumulate significant amounts of CO_2 in the respiration chambers relative to the control jars. After 24 hours, a significant difference in the isotopic signatures of ants fed with the two sugars was found and their $\delta^{13}C\text{-}CO_2$ values were in line with those expected based on the known isotopic content of the two sources. C3 plants produce sugars with a mean isotopic signature of -28‰, while C4 plants synthesise sugars with a mean $\delta^{13}C\text{-}CO_2$ of -13‰ (Ehleringer & Cerling, 2002; Fry, 2006). Significant differences between the two groups at t_{24} were also found when comparing the pure signatures estimated by the Keeling plots, with these estimates close to the reference values commonly reported in the literature for C3 and C4 (e.g. Fry, 2006). Unfortunately, the isotopic signature of the air in the control chambers, which reflected the composition of the air in the lab, was close to that of C4 sugar, and this is likely the reason why the two groups did not show significant differences. To prevent this issue, we recommend that future experiments are carried out by using pure chromatographic air to amplify the differences among treatments and controls as much as possible.

The results from ants collected in the field showed that the CO_2 production rate was consistent with the data reported in the literature and negatively correlated with worker dimensions, as previously found by Mason et al. (2015). Moreover, in our study site, there was no evidence of niche partitioning based on the carbon pathway, with all sampled species sharing similar resources. This result is confirmed by the pure breath signature estimates. As pointed out by Perkins & Speakman (2001), finding a strong difference in carbon signatures in the field can be difficult, especially for generalist animals like ants. However, though field-collected ants did not show a clear reliance on different carbon sources (i.e. C3 vs. C4 pathways) as found in other wild animals (Voigt, 2009; Voigt et al., 2013), the two-endpoint mixing model revealed further information on ant feeding preferences. Indeed, we could not classify any of the studied species as a selective C4 consumer (Engel et al., 2009), but the degree of reliance on C3 plants seems to vary across species. Both *C. scutellaris* and *L. neglectus* did not exceed the C3 threshold, suggesting that they use both C3 and C4 sources. Interestingly, however, some variability among colonies was present, as some of them seemed to be C3 specialists, while others evidently consumed some sources from the C4 pathway. Indeed, both species were collected on C3 trees (*Quercus* sp.) on which they were feeding on aphid honeydew, but predation on other insects is common, especially for *C. scutellaris* (Frizzi et al., 2016). Conversely, *Messor capitatus*, a seed-eating ant, showed a complete reliance on C3 plants for its diet. It is important to stress that in our study we did not characterised in detail the resources available to each colony, and therefore we cannot say how much the observed differences reflected an active choice or the local availability.

Studying omnivorous bats, Voigt et al. (2008a) pointed out that $\delta^{13}C$ of breath and tissues could present a discrepancy as a result of isotopic routing, i.e. the different destinations that macronutrients have in the consumer body. While breath signature is indicative of metabolism, tissue signature indicates the sources used for tissue synthesis. This implies that species from lower trophic levels (only one C source, from plants) will have similar $\delta^{13}C$ values in the tissues and breath, while omnivorous species (two C sources, from plants and from animal prey) will have higher (more positive) $\delta^{13}C$ values in the tissues than in the breath, the latter underestimating the protein contribution to the diet. Therefore, breath signatures are more related to the carbohydrate portion of the diet. This could explain the similarity found between *L. neglectus* and *C. scutellaris* breath. Although *C. scutellaris* preys on other insects, its main carbohydrate source is the plant-derived aphid honeydew, as well as for *L. neglectus* (Frizzi et al., 2016, 2018). One important point to carefully consider when dealing with the analysis of breathed CO_2 is that the $\delta^{13}C$ values reflects the catabolised substrate. If an animal is fasted and uses fat stores, its breath will reflect the carbon signature of the diet from which reserves were formed, which do not necessarily coincide with its more recent diet (Hatch et al., 2002a; Welch et al., 2006). Unfortunately, the nutritional status of the ants collected in the field was not known and, although there were no reasons for them to be fasting, it is possible that differences in their reliance on C3 resources are affected by different uses of fat stores (Hatch et al., 2002a; Welch et al., 2006). At the same time, it is important to stress that the small differences observed could also be due to different availabilities of C3/C4 sources around the nests of the sampled colonies.

The applicability of the method to this taxonomic group was facilitated by its social behaviour, which allows the handling of groups of individuals instead of single ants, providing information on the colony as an ecological unit (Lach et al., 2010). Indeed, given that a sufficient amount of breathed CO_2 must accumulate in the respirometric chamber and that a non-negligible volume of air must be sampled for the analysis, this approach can more easily process pools of individuals from the same colony rather than the breath of single small animals. In conclusion, we demonstrated that despite the important limitation of providing only one variable, breathing tests could provide useful information on the trophic behaviour of omnivorous generalists like ants. This study represents a starting point for further investigations of insect breath—particularly in herbivores, but also in other ants in tropical ecosystems, where C4 plants are more abundant, to better understand their trophic ecology.

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Figure legends

Figure 1. CO_2 concentrations (A) and carbon isotopic signatures (B) for controls and treatments (beet and sugar cane) after 24 hours. In each plot, the groups having different letters were significantly different after Tukey post hoc test.

Figure 2. CO_2 concentrations (A) and carbon isotopic signatures (B) for controls and ant species (*Crematogaster scutellaris*, *Lasius neglectus* and *Messor capitatus*) after 24 hours. In each plot, the groups having different letters were significantly different after Tukey post hoc test.

Figure 3. Estimated pure breath signatures of ant species (*Crematogaster scutellaris*, *Lasius neglectus* and *Messor capitatus*). The horizontal red lines show the threshold values for C3 (continuous) and C4 (dashed) specialists.