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Published in:
Ecology

DOI:
10.1002/ecy.2431

Publication date:
2018

Document version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Disentangling nutritional pathways linking leafcutter ants and their co-evolved fungal symbionts using stable isotopes

JONATHAN Z. SHIK,1,2,5 WINNIE RYTTER,1 XAVIER ARNAN,3 AND ANDERS MICHELSEN4

1Centre for Social Evolution, Department of Biology, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark
2Smithsonian Tropical Research Institute, Apartado 0843-03092 Balboa, Ancon, Republic of Panama
3CREAF, Cerdanyola del Vallès, ES-08193 Catalunya, Spain
4Terrestrial Ecology Section, Department of Biology, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark

Abstract. Leafcutter ants are the ultimate insect superorganisms, with up to millions of physiologically specialized workers cooperating to cut and transport vegetation and then convert it into compost used to cultivate co-evolved fungi, domesticated over millions of years. We tested hypotheses about the nutrient-processing dynamics governing this functional integration, tracing 15N- and 13C-enriched substrates through colonies of the leafcutter ant Atta colombica. Our results highlight striking processing efficiencies, including rapid conversion (within 2 d) of harvested nutrients into edible fungal tissue (swollen hyphal tips called gongylidia) in the center of fungus gardens, while also highlighting that much of each colony’s foraging effort resulted in substrate placed directly in the trash. We also find nutrient-specific processing dynamics both within and across layers of the fungus garden, and in ant consumers. Larvae exhibited higher overall levels of 15N and 13C enrichment than adult workers, supporting that the majority of fungal productivity is allocated to colony growth. Foragers assimilated 13C-labeled glucose during its ingestion, but required several days to metabolically process ingested 15N-labeled ammonium nitrate. This processing timeline helps resolve a 40-yr old hypothesis, that foragers (but apparently not gardeners or larvae) bypass their fungal crops to directly assimilate some of the nutrients they ingest outside the nest. Tracing these nutritional pathways with stable isotopes helps visualize how physiological integration within symbiotic networks gives rise to the ecologically dominant herbivory of leafcutter ants in habitats ranging from Argentina to the southern United States.

Key words: 13C; 15N; attine ants; carbon and nitrogen isotopes; nutritional ecology; tropical rainforest.

INTRODUCTION

Social insects channel vast amounts of resources through their colonies at a global scale (Brian 1978, Del Toro et al. 2012, Griffiths et al. 2018). However, while ant foraging is a conspicuous sight in most terrestrial habitats (Lanán 2014), the fates of resources inside ant nests are rarely observed (Tschoinkel 1991, 2011). Moreover, while the basic details of colony growth are well known, from queen-laid eggs, across several larval instars, pupation, and the adult worker life cycle (Oster and Wilson 1978), the underlying nutrient processing dynamics are described for few of the >14,000 extant ant species. Dietary tracer experiments using foods labeled with heavy isotopes of carbon, phosphorus and nitrogen have enabled researchers to trace the flow of labeled resources as they flow among colony members inside nests where allocation dynamics are difficult to directly observe (e.g., Howard and Tschoinkel 1981, Feldhaar et al. 2010, Hölldobler and Kwapich 2017).

Radioactive tracers were the primary tool in isotopic research about resource allocation within colonies for over 60 yr (Wilson and Eisner 1957, Golley and Gentry 1964, Markin 1970, Sorensen and Vinson 1981), but stable isotope natural abundance studies of nitrogen (15N) and carbon (14C) are now commonly used to infer dietary habits when foraging dynamics occur out of sight (Davidson et al. 2003), when species are either rare (Jacquemin et al. 2014) or are members of diverse communities (e.g., Blüthgen et al. 2003, Smith and Suarez 2010, Penick et al. 2015), and when colonies are distributed across large spatial (Tillberg et al. 2007, Wilder et al. 2011) and temporal scales (Mooney and Tillberg 2005, Yang 2006, Roeder and Kaspari 2017). Stable isotope enrichment experiments also provide powerful tools for visualizing nutrient exchange among symbiotic partners (Kiers et al. 2011), making such experiments useful in ant ecology since ants often rely on nutrients derived from hemipterans (Shik et al. 2014e), plants (Sagers et al. 2000, Fischer et al. 2005, Pinkalski et al. 2018), and microbes (Feldhaar et al. 2007, Pinto-Tomás et al. 2009, Sapountzis et al. 2015).

Ecology of farming productivity

Leafcutter ants of the genus Atta are ideally suited for isotopic experiments because they farm a co-evolved fungal symbiont for food, harvesting fresh vegetation and using it to produce fungal crops in massive underground nests that can feed millions of workers (Hölldobler and Wilson 2010). Fungal symbionts are fully integrated parts of the leafcutter ant digestive system that begin to process harvested resources when gardener ants deposit mixtures of chewed vegetation and digestive enzymes on top of the fungus garden (Möller et al. 2011). Fungal symbionts (De Fine Licht et al. 2013), gardener ants (Quinlan and Cherrett 1979), and developing...
ant larvae (Erthal et al. 2007) then collectively convert this 
composted substrate into structural fungal hypha and edible 
gongylidia, swollen hyphal tips that concentrate nutrients 
and grow in bundles called staphyla (Martin et al. 1969, 
Quinlan and Cherrett 1979, Mueller et al. 2001, Schiøtt et al. 2010). We measured these production dynamics with novel sampling resolution, allowing foragers in laboratory colonies of A. colombica to harvest isotopically-enriched substrate, and then traced two isotopically labeled compounds (13C-enriched glucose and 15N-enriched ammonium nitrate) through symbiotic networks across over 800 samples spanning 20 d. Below, we outline how this methodology enabled us to test hypotheses about nutrient integration through the 
fungus garden (across layers of hyphae, within edible tissues, 
and disposal in the trash), allocation among ant consumers 
(adult and immature castes), and processing within individ-
ual ants (transported or assimilated).

Based on the timing of isotopic enrichment within hyphae 
at vertical layers of fungus, we first tested a fungus layers 
hypothesis, previously inferred from patterns of enzyme 
activity in leafcutter fungus gardens of serial downward 
nutrient integration within the garden (Møller et al. 2011, 
De Fine Licht et al. 2013). A vertical processing dynamic 
implies an organizing principle whereby workers systemati-

cally deposit fresh vegetation at the top of the fungus 
garden to initiate its use in the cultivation process. We next 
compared enrichment across fungal tissues to test a fungus food 
hypothesis: nutrient integration is targeted towards food 
production (edible gongylidia) rather than biomass of the 
non-differentiated hypha surrounding the gongylidia. We 

further explored waste disposal dynamics, sampling trash 
piles to quantify overall processing rates of nutrients follow-
ing their integration into the fungus garden. Since fungal 
cultivars grow best on specific nutritional blends (Shik et al. 2016), we tested a waste disposal hypothesis, that a potential 
mechanism of meeting their cultivar’s nutritional needs is 
that ant farmers select specific nutrients from the composted 
substrate initially provided to their cultivars through nutrient-specific disposal of harvested substrates.

**Nutrient allocation**

Transicioning from the fungal cultivar to the ant con-

sumers, we next traced labeled compounds as they were 

ingested and allocated among physiologically specialized ant 
castes. Foraging ants are generally assumed to be main-
tained primarily by carbohydrates (Markin 1970, Sorensen 

and Vinson 1981), but they must forage to also satisfy nutri-
tional requirements of non-foraging nestmates, including 

larvae whose growth depends on protein acquisition (Dussu-
tour and Simpson 2008a). Still, most nutrient allocation 
decisions may actually occur inside the nest, as ants regurgi-
tate ingested liquids from specialized abdominal storage 

organs (hereafter ‘gasters’) and share them with nestmates 

(Cook and Davidson 2006). Thus, ingestion does not guar-
antee assimilation in ants, and we hypothesized that carbo-
hydrates would be preferentially retained by adult workers 

and proteins would be shunted through the fungus garden 

and towards developing larvae.

We tested this allocation hypothesis by comparing isotope 
enrichment of two types of nutrients among ant castes: a 


carbohydrate (13C-enriched glucose), and a source of the 
nitrogen used to build proteins (15N-enriched ammonium 
nitrate). We tested the prediction among ant consumers that 

adult workers (foragers and gardeners) have higher mean 

13C values and developing brood (larvae and pupae) have 

higher mean 15N values. We then compared enrichment 
timelines across castes to test whether garden-inhabiting 
castes (gardeners and larvae) assimilate nutrients received 
directly from returning foragers or only later, ostensibly after 
they had been processed through the fungus garden.

**Nutrient processing**

Like microbial symbionts of other insects (e.g., bees, 
Engel et al. 2012, termites, Poulsen et al. 2014), fungal culti-
vars and their associated bacteria convert difficult to digest 
compounds (e.g., plant cellulose, Moreira-Soto et al. 2017) 
and then traced two isotopically labeled compounds 
(Dennett et al. 2010, De Fine Licht et al. 2013). We tested two 
resource processing hypotheses about whether and when for-
aging leafcutter ants assimilate ‘wild caught’ resources, sepa-
rating ant gasters prior to isotope analyses to distinguish 
between two types of processing dynamics: fungus-first 
(ingested nutrients transported in the gaster) and forager-
first (ingested nutrients directly assimilated in head-thorax 
tissue) (as per Tillberg et al. 2006, Feldhaar et al. 2010). We 
compared the timing of enrichment across ant tissues, 
assuming that simultaneous ingestion (gaster enrichment) 
and assimilation (head-thorax enrichment) indicates for-
ager-first nutrient processing without intermediate process-
ing by fungal cultivars. We then tested whether these 
processing dynamics depend on compound digestibility, 
with forager-first processing of glucose (e.g., it can be 
directly used to fuel metabolic respiration or converted to 
glycogen and stored in fat body cells, Arrese and Soulages 
2010), and fungus-first processing of the less readily metabo-
lized compound ammonium nitrate. Finally, we compared 
nutrient processing between foragers and gardeners, a non-
foraging caste we predicted would have greater reliance on 
fungus-first resource acquisition.

**Methods**

**Colonies of Atta colombica**

We established queenless subcolonies (hereafter colonies) 
from five large queenright colonies of the leafcutting ant 
Atta colombica collected in Panama from 2009 to 2012 and 
maintained at the University of Copenhagen in a climate-
controlled room (25°C, 70% RH, minimal daylight). For
four months prior to the experiment, colonies were fed
leaves, apples, and rice three times per week (provided in
small removable trays) and were housed under inverted bea-
kers in open plastic nest boxes (38 x 28 cm) with fluon-
coated walls that remained connected via tygon tubing to
the queenright nest chamber in the central nest box
(Appendix S1). This was done in order to avoid isotopic
contamination in the central nest box, and mimicked natural
colonies where colonies typically have many nest chambers
connected directly or indirectly to a central chamber con-
taining the queen and can regulate the flow of resources and
nestmates among chambers. Experimental colonies were
separated from the central nestbox just prior to the start of
the experiment. Examination of trash piles and fungus gar-
dens during the experiment, and demographic analyses
performed after the experiment indicated colonies experienced
low worker mortality (i.e., few dead workers were found)
and high fungus garden stability (i.e., colonies continuously
produced new fungus) during the 20-d isotopic sampling
period (albeit with diminished larvae numbers by the end
because colonies lacked queens), with (mean ± SE) 20.7 ± 5.3 g fungus (dry mass), 11,520 ± 2,401 workers,
131 ± 55 larvae, and 1,599 ± 423 pupae per colony
(Appendix S2: Table S1).

Isotopically enriched diets

We provided five colonies with isotopically enriched diet
and traced the single pulse of 13C and 15N enrichment from
this foraging event through colonies over 20 d. We modified 1:3 and 3:1 protein:carbohydrate (P:C) agar-based diets
from Dussutour and Simpson (2008b) (with a 60 g/L protein
plus carbohydrate dilution), to be enriched with 13C (D-glucose:
13C6H12O6, Sigma-Aldrich) and 15N (ammonium
nitrate: 15NH415NO3, Sigma-Aldrich). For detailed recipe
information, see Appendices S1 and S2: Table S2. We used
these diets as the means of isotopic enrichment because ants
harvest a variety of plant-based resources in nature, includ-
ing plant nectar (Littledyke and Cherrett 1976) and fallen
fruit (Evison and Ratnieks 2007), and because the diets gave
us precise and replicable control over the amount of isotopic
enrichment. Moreover, these diets enabled ants to success-
fully integrate the nutrients into their farming systems, with
ants licking the diets and also cutting pieces and planting
them on their gardens (Shik et al. 2016). Isotopic analyses
of 13C and 15N (Atom Percent Excess, APE values, see
below) indicated enrichment for the 1:3 P:C diet of 1.9% 13C
and 6.9% 15N, and enrichment values for the 3:1 P:C diet
of 2.4% 13C and 4.2% 15N (see Appendices S1 and S2: Table S2
for details). These enrichment values were found, in pilot tri-
als, to optimize isotope detection in colony components.

On Day 1 of the experiment, colonies were allowed to for-
age between diets with 1:3 and 3:1 P:C ratios for 24 h and
select their own P:C intake target (Behmer 2009). Workers
harvested substantial amounts of enriched diets (± SE): 46.5 (± 17.9)% of the initial weight and 0.64 (± 0.26) g dry
mass of 1:3 P:C diet and 19.2 (± 11.0)% of the initial weight
and 0.29 (± 0.16) g dry mass of 3:1 P:C diet (Appendix S2:
Table S1). This initial diet harvest was measured for each
colony and used as a covariate in subsequent statistical analyses
of isotope enrichment. Following the Day 1 pulse, colonies
were fed unenriched 1:3 and 3:1 P:C diets (Days 2–6) and
bramble leaves (Days 7–20) whose 13C and 15N levels were
at the natural abundance level (Appendix S1).

Sample collection

We sampled 5 colonies on the day before the isotopic pulse
(Day 0, natural abundance), and again on days 1, 2, 4, 8, and
20 following the pulse (Appendix S1). Nests were fit with
removable ‘collection windows’ enabling non-disruptive sam-
ping within the fungus garden (Fig. 1A). On each sampling
day, we collected fungal hyphae (with gongylidia removed)
from top, middle, and bottom layers of the garden (as per
Moller et al. 2011), and collected gongylidia (packed in tiny
0.5 mm diameter) bundles called staphylos from the middle
garden layer where they were most abundant (De Fine Licht
et al. 2014). We removed trash pile at each feeding event and
analyzed homogenized trash pile samples, when available, on
each sampling day. We collected adult ants in two groups: for-
agers (large and medium-sized workers collected outside the
nest) and gardeners (small ants collected inside fungus cham-
bers) (Wilson 1980, Forti et al. 2012). Prior to isotopic anal-
yses, these ants were anesthetized at 4°C and divided into
gaster and head-thorax samples. We also collected larvae and
pupae in the middle layer of fungus gardens where they were
most abundant, analyzing whole bodies in single samples as
they could not be readily separated into gaster and head-
thorax samples as in the adults. Overall, from each colony at
each sampling event, we collected the following samples for
isotopic analyses: foragers (n = 4) and gardeners (n = 2), lar-
vae (n = 3), pupae (n = 3), fungal hypha at three layers
(n = 3 per layer), fungal gongylidia (n = 1 in the middle
layer), and trash pile (n = 2), to yield 900 planned isotopic
samples (Appendices S1 and S2: Table S3), and 840 actual
isotopic samples (Appendix S2: Table S4A, B).

Stable isotope analyses

Samples were dried at 60°C for ≥24 h, homogenized,
weighed into tin capsules, and analyzed for 15N/14N and
13C/12C using a Eurovector CN analyzer (Pavia, Italy) cou-
pled to an Isoprime (Cheadle Hulme, UK) mass spectrom-
ter. Natural abundances of 15N and 13C provided a baseline
for interpreting subsequent enrichment, and were deter-
mined from Day 0 samples using the equations provided
below (Fischer et al. 2005, Fry 2006), where peach leaves
(NIST RM 1547) were used as the internal spectrometry cal-
ibration standard for N and C (as per Brand et al. 2014),
and where reference gas was calibrated against international
standards IAEA C5, CH6, CH7, N1, N2 and USGS 25, 26,
32:

\[
\delta^{15}N = \left( \frac{[{^{15}N/^{14}N}_{\text{sample}}]}{[^{15}N/^{14}N}_{\text{standard}}} - 1 \right) \times 1,000
\]

\[
\delta^{13}C = \left( \frac{[^{13}C/^{12}C}_{\text{sample}}]}{[^{13}C/^{12}C}_{\text{standard}}} - 1 \right) \times 1,000
\]

We next calculated Atom Percent (at%) of 15N and 13C as
the percentage of heavy isotope moles of N or C in a sample,
and Atom Percent Excess (APE) as the at% $^{15}$N or $^{13}$C of enriched samples above the Day 0 natural abundance:

$$\text{APE}^{15}\text{N} = \text{at%}^{15}\text{N}_{\text{sample}} - \text{at%}^{15}\text{N}_{\text{Natural abundance}}$$

$$\text{APE}^{13}\text{C} = \text{at%}^{13}\text{C}_{\text{sample}} - \text{at%}^{13}\text{C}_{\text{Natural abundance}}$$

For statistical analyses, we calculated excess $\mu$g $^{15}$N and $\mu$g $^{13}$C per gram dry mass of each sample (hereafter $^{15}$N and $^{13}$C) from the APE and the sample dry mass:

$$\mu\text{g}^{15}\text{N per g dry mass}^{-1} = \text{APE} \times \text{sample mass}^{-1} \times 1,000$$

$$\mu\text{g}^{13}\text{C per g dry mass}^{-1} = \text{APE} \times \text{sample mass}^{-1} \times 1,000$$

**Statistical analyses**

**Ecology of farming productivity.**—Fungus layers: We performed a mixed model analysis using the lme function in the nlme package (Pinheiro et al. 2018) in R 3.2.4 (R Development Core Team 2016) to compare enrichment timelines of $^{15}$N and $^{13}$C across layers of fungal hyphae where time (Day 0, 1, 2, 4, 8, 20; a categorical variable), nutrient ($^{15}$N and $^{13}$C), layer (top, middle, bottom) and their interactions were fixed factors. Mean hyphal enrichment values were analyzed to generate a balanced model. Waste disposal: We used a mixed model to analyze trash-pile samples for $^{15}$N and $^{13}$C enrichment, with time, nutrient, and their interaction as fixed factors, initial diet harvest as a covariate, and sample ID nested in colony ID as a random factor.

To facilitate direct statistical comparisons of $^{15}$N and $^{13}$C enrichment, data were standardized by calculating Z scores separately for $^{13}$C and $^{15}$N data. Significant differences existed among $^{15}$N and $^{13}$C enrichment (i.e., significant ‘nutrient’ main or interaction effects), we plotted observed nutrient means (± SE), as they generated similar temporal patterns as Z-scores, and enabled comparison with other similar published results. Otherwise, we plotted the Z-scores combining the means of $^{15}$N and $^{13}$C data. In all cases, we interpreted significant differences using posthoc Tukey tests.

**Nutrient allocation.**—We compared $^{15}$N and $^{13}$C enrichment across adult ants and brood, performing mixed model analyses in SAS (V9.4, Proc GLIMMIX) with time (Day 1, 2, 4,
8, 20), nutrient, caste (forager, gardener, larva, pupae), and their interactions as fixed factors, initial diet harvest as a covariate, and caste nested in colony ID as a random factor. We analyzed adult ant head-thorax tissue as the allocation hypothesis focused on assimilated nutrients, and analyzed Z-scores, using posthock Tukey tests to interpret significant differences among castes within sampling days.

**Nutrient processing**—We used a mixed model (proc GLIMMIX) testing for differences among tissues within ants over time. We performed separate analyses for 15N and 13C enrichment, with time, caste (forager, gardener), and tissue (gaster, head-thorax) as fixed factors, and the random factors colony ID, time × colony ID, and individual ID nested in (time × colony ID). This analysis also modeled within-subject tissue effects (gaster vs. head-thorax) as a repeated measure for organs within individuals. Separate analyses for 15N and 13C enrichment were preferred for nutrient processing analyses, given the overall complexity of the model, and our focus on interpreting nutrient processing timelines within ants. To test for latency between ingestion (enrichment of gaster) and assimilation (enrichment of head-thorax), we used post-hoc Tukey tests to interpret significant differences within tissues across days and across tissues within sampling days.

**Results**

**Ecology of farming productivity**

Within minutes of placing diets inside nest boxes, foragers could be observed licking and cutting agar-based substrates, and then carrying them back to their nests (Fig. 1A). This initial diet harvesting effort significantly influenced subsequent fungus enrichment levels (Table 1). **Fungus layers:** Nutrients exhibited distinct downward enrichment timelines within and across vertical layers of the fungus garden (time × nutrient × layer interaction effects in Table 1, Fig. 1B). First, 15N trended upwards in both the top and middle layers on the day of harvest, becoming significantly enriched in the middle layer by day 2 and in the top layer by day 4. In contrast, 13C was directly integrated in the middle layer, where it became significantly enriched by the first day (Fig. 1B). Second, 15N levels remained steady in the middle layer over 20 d (Fig. 1B), while 13C became significantly depleted in the middle layer by Day 20 (Fig. 1B). Despite these middle layer depletion differences, neither isotope was detected at significant levels in the bottom layer over 20 d (Fig. 1B).

**Fungus food:** We detected rapid integration of nutrients into edible gongylidia in the middle layer within two days of its harvest (time × tissue interaction effects in Table 1, Fig. 2). Gongylidia were also significantly enriched relative to surrounding structural hypha by the second day (Table 1), and at equal levels for 15N and 13C (Table 1), indicating targeted conversion of both nutrients towards edible fungal food (Fig. 2). **Waste disposal:** Waste disposal also occurred rapidly on day 1 (Time effects in Table 1), with trash piles becoming maximally enriched at similar levels for 15N and 13C (Fig. 3). This indicates that foragers did not distinguish between nutrients when delivering substantial amounts of harvested resources directly to the trash. By day 20, we detected a slight uptick in trash enrichment (Fig. 3), potentially indicating the disposal of old fungus from the fungus garden.

**Nutrient allocation**

The allocation hypothesis was generally supported by caste-specific enrichment dynamics (Nutrient × caste interaction effects in Table 1), with foragers assimilating significant levels of 13C, but not 15N when harvesting substrate (Fig. 4), and with larvae showing total enrichment levels that were higher for 15N than for 13C (Fig. 4). However, larvae on day eight (consuming enriched diet), and then pupae on day 20 (the aging cohort of enriched larvae) became significantly enriched for both 15N and 13C relative to adult castes (Fig. 4), indicating farming systems generally shunted nutrients towards ant colony growth (time × caste interaction effects in Table 1). Caste-specific enrichment timelines also help refine nutrient transfer dynamics among nestmates. Specifically, an eight-day lag from when foragers harvested enriched diet to when larvae and gardeners became enriched (Fig. 4) indicates these within-garden castes did not directly assimilate resources regurgitated by returning foragers. Rather, they instead appeared to rely on cultivar-derived resources.

**Nutrient processing**

We found mixed support for the hypothesis that foragers nutritionally bypass their gardens (i.e., forager-first processing). As evidence of assimilation during resource harvest, foragers had a significant head-thorax pulse from day 0 to 1 for 13C, and a positive (although non-significant) trend for 15N (Fig. 5). However, a more complex picture emerges considering this assimilation in the context of all nutrients ingested while foraging, as gaster enrichment timelines differed from those of head-thorax tissue for both 15N and 13C (time × tissue interaction effects for both nutrients in Table 1). Distinct ingestion-assimilation timelines for 13C and 15N further indicate that the likelihood of forager-first processing varies across nutrients. For instance, foragers appeared to bypass their cultivars to assimilate glucose as their head-thorax tissues had consistently elevated 13C enrichment following consumption. The consistently higher 13C gaster enrichment (Fig. 5) is also consistent with known glucose processing and fat body storage dynamics. Processing dynamics for ammonium nitrate are more difficult to interpret, as foragers initially assimilated small fractions of ingested 15N and then gradually assimilated larger amounts over 20 d as it was simultaneously depleted from their gasters (Fig. 5).

Gardeners exhibited similar nutrient ingestion-assimilation trends as foragers, for instance with consistently higher 13C enrichment in gaster tissue relative to head-thorax tissue following ingestion (Appendix S3: Fig. S1), even as significant enrichment differences existed among these castes for both 15N and 13C (Caste effects for both nutrients in Table 1). These differences were likely driven by significantly delayed ingestion timeline of gardeners, as gardeners only exhibited significant assimilation for 13C eight days and 15N twenty days after the initial day 0 pulse (Appendix S3: Fig. S1).
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<td>Nutrient × caste</td>
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<td>4.38</td>
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<td>Time × nutrient × caste</td>
<td>12</td>
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<td>1.31</td>
<td>0.207</td>
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<td>Initial diet harvest</td>
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<td>15</td>
<td>36.77</td>
<td>0.0001</td>
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<tr>
<td></td>
<td>Time</td>
<td>5</td>
<td>35.86</td>
<td>2.40</td>
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<tr>
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<td>Tissue</td>
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<td>30.83</td>
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<td>0.193</td>
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<td>Time × tissue</td>
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<td>6.65</td>
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<tr>
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<tr>
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<td>35.90</td>
<td>0.0001</td>
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<tr>
<td></td>
<td>Time × caste × tissue</td>
<td>5</td>
<td>176.1</td>
<td>0.58</td>
<td>0.719</td>
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Notes: Fungus layers: We compared enrichment timelines of $^{15}$N and $^{13}$C across vertical layers of fungal hypha, using a mixed model where time (categorical variable: Day 0, 1, 2, 4, 8, 20), nutrient ($^{15}$N and $^{13}$C), and layer (top, middle, bottom) were fixed factors, initial diet harvest was a covariate, and sample ID nested within layer and then nested within colony ID were random factors. Fungus food: We compared enrichment in gongylidia relative to surrounding middle layer hypha, using a mixed model where time, nutrient, and tissue (hyphae, gongylidia), were fixed factors, initial diet harvest was a covariate and colony ID was a random factor. Waste disposal: We used a mixed model analysis comparing $^{15}$N and $^{13}$C enrichment in trash piles, with time, nutrition and their interaction as fixed factors, initial diet harvest as a covariate, and sample nested in colony ID was a random factor. Nutrient allocation: We used a mixed model analysis comparing isotope enrichment across castes, with time (excluding Day 0), nutrient, caste (forager, gardener, larva, pupa) and their interactions as fixed factors, initial diet harvest as a covariate, and caste nested in colony ID as a random factor. Nutrient processing: We examined $^{15}$N and $^{13}$C enrichment within ants, using separate models for $^{15}$N and $^{13}$C with time, caste (forager, gardener, larva, pupa) and their interactions as fixed factors, initial diet harvest as a covariate, and sample nested in colony ID was a random factor. This analysis also included within-subject tissue effects (gaster vs. head-thorax), using a repeated statement for organs within individuals.
This study clarifies the nutrient-processing dynamics enabling leafcutter ants to convert harvested substrates into fungal food, and thus helps visualize how these farming systems unlock plant primary production as dominant herbivores across tropical ecosystems. Our results helped confirm unresolved nutritional hypotheses (e.g., nutrients are rapidly integrated into edible gongylidia), rule out others (e.g., foragers do not directly provision gardeners and larvae), and provide a template for disentangling others (e.g., the order of nutrient exchange between gongylidia, larvae, and gardeners). We further highlight how specific nutrients are transferred among symbiotic partners depending on their physiological requirements (e.g., allocating $^{13}$C in adult ants and $^{15}$N in developing brood) and metabolic processing capabilities (e.g., forager-first assimilation of glucose, but not ammonium nitrate). We envision using this isotopic approach in field studies moving beyond identifying the substrates harvested by farming ants (Leal and Oliveira 2000, Seal and Tschinkel 2008) to mapping the underlying nutritional landscapes navigated by foragers.

**Ecology of farming productivity**

Fungal cultivar genomes exhibit a variety of metabolic processing adaptations resulting from millions of years of co-evolutionary selection as cultivated symbionts (De Fine Licht et al. 2014, Nygaard et al. 2016). We explore the in vivo performance consequences of this crop selection, quantifying conversion rates of harvested nutrients into fungal food. We found evidence that cultivars deliver rapid and targeted gongylidia production, with both nutrients shunted towards food production within 2 d, even as their overall downward processing rates differed within and across layers of the fungus garden. This rapid metabolic processing is consistent with our current understanding of the enzyme specialization of cultivars (De Fine Licht et al. 2010, Kooij et al. 2011, Seal et al. 2014), and with the enzyme vectoring by ants to detoxify (De Fine Licht et al. 2013) and digest (Moller et al. 2011) substrate even before it is deposited on gardens. Fast gongylidia production rates may also govern the high metabolic rates of gongylidia-bearing fungi relative to less specialized cultivars of other attines that only produce hyphae (Shik et al. 2014b). Additionally, the capacity for fast substrate decomposition may have made ancestors of extant attine cultivars good symbiotic partners, despite their unremarkable nutritional qualities relative to other free-living fungi (Mueller et al. 2001).

Despite the fundamental advantages of collective foraging, the task of provisioning ant nestmates with different nutritional requirements also provides complex challenges about which nutrients to harvest and in what blends (Dussutour and Simpson 2008a). Leafcutter foragers likely face even greater nutritional challenges as they provision completely unrelated fungal cultivars, saprophytes with very different nutritional requirements (Shik et al. 2016). And, while we hypothesized that ants would selectively dispose of less desirable crop producing nutrients prior to depositing substrate on the fungus garden, workers actually placed similar amounts of both nutrients directly in their trash piles (Fig. 3). Further study will be needed to determine whether this seemingly ‘wasted’ foraging effort stemmed from physical properties of agar diets, the high amount of available nutrients contained per gram of diet relative to a typical leaf fragment, or whether it was simply analogous to the large
FIG. 4. Comparing $^{15}$N and $^{13}$C enrichment across castes to evaluate the allocation hypothesis. Letters indicate significantly different (Tukey test, $P < 0.05$) enrichment values or groupings across castes within sampling days. The text 'n.s.' indicates no significant enrichment differences among castes within the sampling day. Head-gaster tissue was analyzed for adult ants and whole bodies were analyzed for larvae and pupae. Enrichment means ($\pm$ SE) are provided in units of µg $^{15}$N, $^{13}$C/g.

FIG. 5. Testing forager-first and fungus-first models of nutrient processing in forager ants for (A) $^{15}$N-enriched ammonium nitrate (blue lines) and (B) $^{13}$C-enriched glucose (gold lines). Ants were subdivided prior to isotope analyses, to compare timelines of enrichment (means $\pm$ SE) reflecting nutrient transport in gaster tissue (dashed lines) and nutrient assimilation in head-thorax tissue (solid lines). We used post-hoc Tukey tests to interpret significant differences ($P < 0.05$) within tissues relative to day 0 (letters indicate significance groupings) and across tissues within sampling days (asterisks, where *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) indicate significant differences). Gaster tissue $^{15}$N-enrichment on day 20 did not differ significantly from enrichment on day 0 (tukey result excluded for clarity).
piles of unused leaf fragments often generated outside nest entrances by leafcutter colonies in nature (Wirth et al. 2003).

**Nutrient allocation**

Despite the many ecological advantages of farming fungus (e.g., access to a stable resource supply), the nutritional challenges of a fungal diet can be inferred from the rarity of fungivory across the ant phylogeny (von Beeren et al. 2014). Our results provide evidence that leafcutter ants may overcome these challenges by targeted allocation of fungal-derived nutrients to specific castes. Specifically, while brood were highly enriched for $^{15}$N, supporting a prediction of the allocation hypothesis, brood also had high $^{13}$C-enrichment levels, supporting a general trend of allocating nutrients to colony growth. Further study will be needed to link specific nutrients fueling colony growth with the labeled compounds provided in diets, since larvae appeared to consume the metabolic byproducts of fungal cultivars rather than liquids supplied by returning foragers.

**Nutrient processing**

Nutrient-specific enrichment timelines of gasters (ingestion) and head-thorax tissue (assimilation) shed light on the underlying metabolic processing dynamics. First, $^{13}$C-enriched timelines support forager-first assimilation of harvested glucose as their head-thorax tissue remained consistently more enriched than pre-harvest baseline over 20 d. Moreover, since their gasters remained even more $^{13}$C-enriched than their head-thorax tissues (Fig. 5), the ants likely converted much of the ingested glucose to glycogen and stored it in abdominal fat body cells (Roma et al. 2006). In contrast, the delayed assimilation of $^{15}$N until day 20 was potentially consistent with both fungus-first and forager-first hypotheses, although both explanations imply pre-processing of ammonium nitrate by microbial symbionts. Specifically, ants may have relied on cultivars to convert ammonium nitrate into edible gongylidia, which they then consumed by day 20 (fungus first), or the ants’ own digestive systems, aided perhaps by their recently characterized resident communities of symbiotic gut microbes (i.e., Sapountzis et al. 2015), may have gradually metabolized the ammonium nitrate, making it available to their ant hosts over time (forager first). The forager-first hypothesis seems likely, since the gradual transfer of $^{15}$N from the ants’ alimentary canals to head-thorax tissue (Fig. 5), implies a reliance on metabolic work performed by microbial gut symbionts. The fungus-first hypothesis might thus be more aptly called the ‘symbiont-first hypothesis’.

These ingestion-assimilation results also help resolve a 40-yr old debate in the attine literature about the primacy of fungus in leafcutter ant diets (Littledyke and Cherrett 1976, Stradling 1978, Wetterer 1994, Mueller et al. 2001, Silva et al. 2003, Ryttner and Slik 2016). First, while Littledyke and Cherrett (1976) initially confirmed ingestion of plant sap by foraging leafcutter ants, they analyzed entire ant bodies and could thus not distinguish between assimilated nutrients, and liquids shared with nestmates or vectored directly to fungus gardens. Our results highlight the dynamic nature of resource-exchange dynamics within leafcutter symbioses, as workers appear to nutritionally bypass their fungal cultivars depending on their ability to metabolize the ingested compound, and whether they are a caste that forages outside the nest. Thus, while our results highlight remarkable functional integration among symbiotic partners, they also highlight that fungal cultivars may only partially meet their farmers’ nutritional needs. Moving forward, it will be important to explore how these production dynamics vary when these broad-ranging generalist foragers encounter taxonomically (Wirth et al. 2003), nutritionally (Kooij et al. 2011), and biochemically (Howard 1988) diverse plant substrates, and when cultivars are farmed across ecological gradients (Mueller et al. 2011).

**ACKNOWLEDGMENTS**

We thank Jack Howe for colony husbandry advice, Luigi Pontieri, Kevin Grimm, Consuelo Arellano and David Nash for statistical guidance, and John Bruun Andersen for assistance with colony ‘sampling windows’. Christian Peeters, Jacobus Boomsma and Panos Sapountzis provided valuable comments. I.Z.S was supported by a Postdoctoral Fellowship from a Marie Curie International Incoming Fellowship (327940 INSEAME), and by the Centre for Social Evolution at the University of Copenhagen. X.A. was supported by a Ramón y Cajal research contract by the Spanish Ministry of Economy and Competitiveness (RYC-2015-18448).

**LITERATURE CITED**


services and disservices (Hymenoptera: Formicidae). Myrmecological News 17:133–146.

tion by instinct. Norton & Company, New York, New York, USA.
Kooij, P. W., M. Schi
Quinlan, R. J., and J. M. Cherrett. 1979. The role of fungus in the diet of the leaf-cutting ant Atta cephalotes (L.). Ecological Ento
mology 4:151–160.


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