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Liquid foraging behaviour in leafcutting ants: the lunchbox hypothesis

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Optimal foraging theory makes clear predictions about the benefits of maximizing energetic returns per unit of foraging effort. However, predictions become less clear when animals belong to symbioses that would be destabilized by such foraging decisions. For instance, leafcutter ants are dominant herbivores in Neotropical ecosystems that harvest fresh vegetation and convert it into compost used to cultivate specialized fungus for food. Individual foragers have long been assumed to supplement their fungal diets by harvesting liquid nectar outside the symbiosis, although this has not been demonstrated in the field, and would probably destabilize the fine-tuned farming systems. By dissecting liquid storage organs in foragers of four sympatric Panamanian leafcutter ant species we found that liquid foraging is not a general strategy in the field. Moreover, while over 40% of these foragers returned to their nests without leaf fragments, these unladen ants were not more likely to carry liquids. Instead, we found support for a newly formulated 'lunchbox hypothesis' because most workers exited nests for foraging trips with midguts full of liquids that were depleted (assimilated and transferred to hindguts) if workers returned with a leaf fragment in the field or transported a load in laboratory experiments. Thus, in contrast to the destabilizing effects of external nectar foraging, these results provide a novel mechanism promoting symbiotic stability, as fungi provide fuel for foragers to harvest more substrate for fungal crop production.

Efficient food acquisition is a central challenge faced by organisms, and optimal foraging theory (OFT) explores the ways natural selection has fine-tuned foraging strategies to meet this challenge, maximizing food harvest while minimizing foraging time and exposure to predators (Stephens, Brown, & Ydenberg, 2007; Stephens & Krebs, 1986). OFT models typically weigh energetic dietary gains against energetic foraging costs and predict reduced fitness with excessively costly or unsuccessful foraging trips (Pyke, Pulliam, & Charnov, 1977). However, it is often not feasible to directly observe foraging dynamics including ecological processes related to ingestion (e.g. food capture and transport) and physiological processes related to digestion (e.g. food assimilation). This means that measuring foraging success, and thus testing OFT models, can be difficult even with inferential techniques (e.g. stable isotopes; Feldhaar, Gebauer, & Blüthgen, 2009).

Many animals are 'central place foragers' and a specific set of OFT models has helped researchers understand how they overcome issues such as local resource depletion when repeatedly returning harvested resources to a central nest or sleeping site (Orians & Pearson, 1979; Oster & Wilson, 1978; Stephens & Krebs, 1986). Ant colonies have provided model systems for testing OFT predictions because they are central place foragers that use diverse collective foraging behaviours to locate, defend and transport resources back to stationary nests (Lanan, 2014; Oster & Wilson, 1978; Roces & Núñez, 1993). Moreover, individual workers dynamically adjust foraging behaviours when harvesting resources with varied nutritional compositions (Dussutour & Simpson, 2008; Kay, 2002; Portha, Deneubourg, & Detrain, 2002) and physical properties (Robson & Traniello, 1998). For instance, many ants carry protein-rich insect prey in their mandibles and transport carbohydrate-rich liquids (e.g. nectar from plants or insect symbionts) within three connected specialized storage organs (crop, midgut, hindgut; Fig. 1; Engel, Fischer, Wäckers, & Völkl, 2001; Josens, Farina, & Roces, 1998).

In colonies where solid food is most commonly harvested, foragers returning full of liquids can appear 'unladen', or lacking harvested resources, and thus be erroneously considered energetic drains on their colonies. However, these liquid resources support an ant colony's 'social stomach', with a digestive adaptation called the proventriculus (Fig. 1c), enabling workers to regurgitate liquids and

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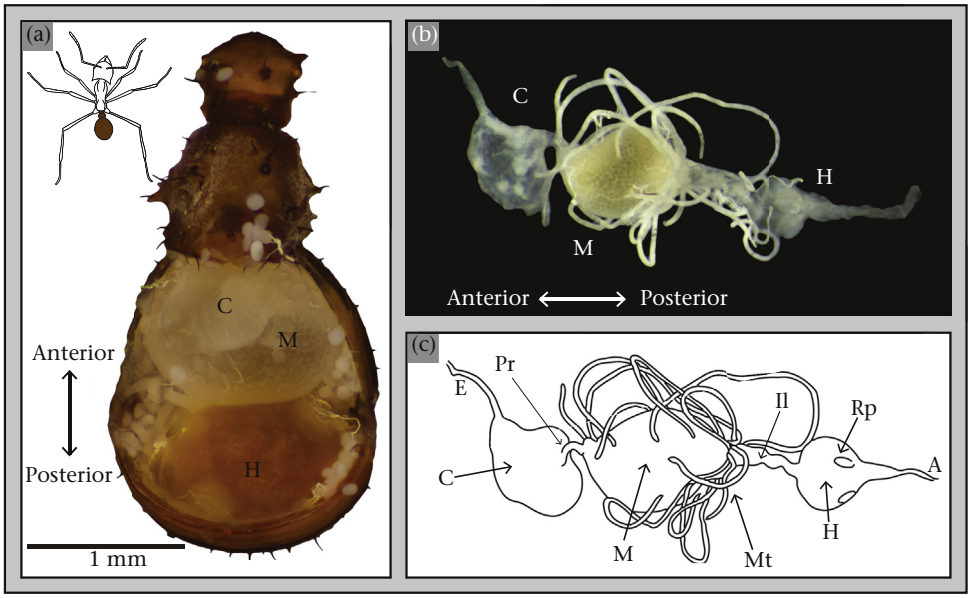

Figure 1. Leafcutter ant digestive system, highlighting the three connected storage organs (crop, midgut, hindgut) described in the text. (a) Dorsal view of storage organs in a partially dissected *Ac. echinatior* worker abdomen showing a full hindgut and midgut and empty crop (white spheres are fat body cells). (b) Lateral view of fully dissected *At. sexdens* digestive system showing a midgut full with the characteristic yellow fluid flanked by an empty crop and hindgut. (c) Schematic illustration of the digestive system highlighting key digestive organs: E, oesophagus; C, crop; Pr, proventriculus; M, midgut; Mt, Malpighian tubules; Il, ileum; H, hindgut; Rp, rectal pads; A, anus. Images viewed at 250X magnification.

share them with nestmates (Cook & Davidson, 2006; Eisner, 1957; Eisner & Brown, 1958). Leafcutter ants (Attini; genera *Atta* and *Acromyrmex*) are the most evolutionarily derived fungus-farming attine ants, cutting and transporting mostly fresh vegetation they prepare as compost to cultivate fungus crops for food in subterranean nests (Hölldobler & Wilson, 2010). Leafcutter foragers often transport loads optimized in size and shape to their individual foraging abilities (Lewis, Pollard, & Dibley, 1974; Wetterer, 1994, 1995). However, foraging trips also often appear unsuccessful as foragers return without carrying any vegetation (Araújo, Della Lucia, Lima, Souza, & Petternelli, 2002; Lewis et al., 1974, but see Kooij, Aanen, Schiott, Boomsa, 2014; Kooij, Rogowska-Wrzesinska et al., 2014). While these unladen foragers appear to present efficiency problems relative to OFT predictions, they have alternatively been hypothesized to lead nestmates to high-quality resources (Bollazzi & Roces, 2011; Jaffe & Howse, 1979; Roces & Núñez, 1993) or maintain foraging trails (Lewis et al., 1974).

Unladen leafcutter foragers have also frequently been assumed to transport, consume and assimilate liquid resources in the form of carbohydrate-rich plant nectar (Bass & Cherrett, 1995; Littledyke & Cherrett, 1976; Mueller, Schultz, Currie, Adams, & Malloch, 2001; Wirth, Herz, Ryel, Beyschlag, & Hölldobler, 2003), but no direct evidence has been provided that this happens routinely in the field. While many ant lineages are known to consume nectar or similar plant secretions (Hölldobler & Wilson, 1990), it has remained underappreciated that specialized fungivory may constrain opportunities to maintain a complementary generalist feeding strategy. Indeed, since the digestive system of *Acromyrmex* leafcutter ants appears to be specialized for vectoring fungal enzymes to new garden growth via faecal droplets (De Fine Licht et al., 2013; Kooij, Aanen et al., 2014; Kooij, Rogowska-Wrzesinska et al., 2014; Martin, 1970; Schiøtt, Rogowska-Wrzesinska, Roepstorff, & Boomsma, 2010), opportunistic foraging on other liquids would probably destabilize this fine-tuned system. Although it is possible that foragers do collect liquids, no study has involved dissections of ants to confirm their presence or absence in the storage organs of unladen workers returning to the nest (Fig. 1a).

We dissected individuals of four Panamanian rainforest leafcutter species to test the OFT prediction that unladen leafcutter ants actually represent successful foraging trips because they are more likely to harvest liquids. We initially established baseline levels of liquid storage in foragers collected as they left their nests, reasoning that if returning foragers had excess liquids above this baseline, they harvested them outside the nest. This led to the surprising observation that most foragers carried liquids in their midguts when exiting their nests, which, in turn led us to perform additional experiments testing a newly formalized 'lunchbox hypothesis'. Below, we develop this hypothesis within an OFT framework, outlining how it integrates digestive physiology, energetic foraging costs and symbiotic stability.

The lunchbox hypothesis provides an OFT prediction that foraging leafcutter ants leave nests with full midguts, which they deplete to fuel energetically costly foraging activities. These energetic costs include the cutting of leaves, which requires extreme mandibular forces (Roces & Lighton, 1995), foraging trips extending >200 m from the nest (Lewis et al., 1974) and the transport of heavy loads (Lighton, Bartholomew, & Feener, 1987) weighing more than double the body mass of a forager (Wetterer, 1994). Whereas nectar foraging would appear at odds with the specialized interplay between ant farmers and fungal crops, lunchbox dynamics would provide a powerful nutritional mechanism integrating the performance of symbiotic partners, as fungi would fuel ant foragers to perform foraging tasks needed to harvest resources that fuel fungal growth. We tested the lunchbox prediction that liquid depletion reflects task performance using a series of laboratory experiments manipulating foraging distance, load mass and leaf-cutting activity.

METHODS

Liquid Transport in Field-Collected Foragers

We observed foraging behaviour in a rainforest within Soberania National Park, Panama (9.15451°N, 79.73583°W) in May 2015, during the start of the rainy season, a period of high leafcutter

activity. We observed four sympatric leafcutter species: *Acromyrmex echinatior* (five colonies), *Acromyrmex octospinosus* (three colonies), *Atta colombica* (three colonies) and *Atta sexdens* (three colonies). This sampling effort is similar to that in other studies on leafcutter foraging dynamics (e.g. Burd & Howard, 2005; Lopes, Forti, & Camargo, 2004).

We counted laden and unladen foragers returning to nests in focal observations that were ≥30 min for *Acromyrmex* (*Ac. echinatior*, $N = 8$; *Ac. octospinosus*, $N = 4$) and 3 min for *Atta* (*At. colombica*, $N = 6$; *At. sexdens*, $N = 6$; see Appendix Table A1 for observation times and sample sizes of returning ants). Colonies of *Atta* (ca. 100 workers/min) had far more workers foraging per min than *Acromyrmex* (ca. 1 worker per min), which necessitated both shorter foraging observation windows and the use of video recordings to accurately capture the laden versus unladen worker foraging dynamics (Appendix Table A1). We thus counted *Acromyrmex* workers directly in the field and video recorded foraging *Atta* trails with an iPhone 4S, slowing the recordings to 15 frames/s in VLC media player (v2.2.1). We calculated the mean percentage of unladen returning foragers (not carrying any substrate in their mandibles) per observation period, per colony and per species (±SE).

We next tested whether unladen returning foragers were more likely to transport liquid resources, collecting foragers from three categories (laden returning, unladen returning and exiting the nest), and returning them to the laboratory in separate containers for dissections to determine empty/full status of liquid storage organs (crops, midguts, hindguts; Fig. 1). Soon after being returned to the laboratory, foragers were secured under water in wax-filled petri dishes under a dissecting microscope (160X magnification) and their abdomens opened with no. 5 watchmaker forceps. Full storage organs had a characteristic distended appearance and were usually filled with yellow fluid (Fig. 1). Since repeated dissections of workers were not possible, we used the following logic to infer that liquids were harvested outside the nest. First, we established baseline liquid storage dynamics by collecting exiting workers immediately upon leaving their nests for foraging trips. Second, if returning workers had more stored liquids than exiting workers, we inferred these represented liquids harvested outside the nest.

We dissected 8.33 ± 0.88 *Acromyrmex* foragers and 18.80 ± 1.29 *Atta* foragers per foraging category and per collection event (total ants dissected: *Ac. echinatior*: 161; *Ac. octospinosus*: 114; *At. colombica*: 200; *At. sexdens*: 195). We selected the 'typically' sized forager for each species (Hughes, Sumner, Van Borm, & Boomsma, 2003; Moreira et al., 2010), measuring the head width (HW in mm) from eye to eye for all dissected ants (*Ac. echinatior*: 2.2 ± 0.2; *Ac. octospinosus*: 2.3 ± 0.3; *At. colombica*: 2.3 ± 0.3; *At. sexdens*: 2.1 ± 0.3). Thus, while *Atta* species are distinct from other attines in having substantial intracolony variation in worker body size corresponding to task specialization (Hölldobler & Wilson, 2010; Wilson, 1980), we compared similarly sized workers across all colonies in the present study.

We used the GLIMMIX procedure in SAS (version 9.3, SAS Institute Inc., Cary, NC, U.S.A.) to perform mixed model analyses with an underlying Gaussian distribution testing whether the percentage of individuals with full storage organs varied with foraging status (laden, unladen, exiting), storage organ (crop, midgut, hindgut) and species identity (*Ac. echinatior, Ac. octospinosus, At. colombica, At. sexdens*). To account for multiple sampling events for some colonies, we included colony ID as a random factor nested within species. We evaluated significant results here and in all tests described below with post hoc multiple comparison tests, applying Holm–Tukey adjustment of observed P values to control for Type I error.

Liquid Transport and Foraging Activity: the Lunchbox Hypothesis

To test the lunchbox hypothesis, we performed a series of laboratory experiments on three intact *Ac. echinatior* colonies (Ae276, Ae342, Ae480) collected from Panama and maintained at the University of Copenhagen in a climate-controlled room (25 °C and 75% relative humidity) housed in plastic nestboxes (38 × 28 cm) with fluon-coated walls. We first dissected 102 foragers collected immediately after they exited the nests to establish baseline liquid transport frequencies (full versus empty) in crops (41% full), midguts (91% full) and hindguts (91% full). We then tested whether foragers depleted more liquids with increasing foraging distances, load weights and leaf-cutting activity.

In a full-factorial design, we first compared liquid transport status in foragers varying in load mass (no load versus 10 mg load) and foraging distance (2 m versus 10 m). Colonies were connected via a removable bridge to a 'middle box' that was connected to a foraging arena via clear 1 cm inner diameter Tygon tubing. We manipulated foraging distance by connecting middle boxes and foraging arenas with 2 m or 10 m lengths of tubing. Laden foragers had a 10 mg clay ball affixed to their dorsal thoracic spines (Lighton et al., 1987; Wetterer, 1995) while 'unladen' control foragers lacked a clay ball. The clay ball was gently affixed immediately after removing foragers from the empty foraging arena, and they were then placed back in the arena. We collected and dissected returning foragers as soon as they reached the middle box on their way back to the colony. We quantified the effects of experimental handling on liquid storage, comparing liquid storage in workers seconds after affixing the clay ball ($N = 30$) with control workers that had just left their nest to forage ($N = 30$). Handling treatment did not significantly affect the overall presence of stored liquids ($F_{2,174} = 0.01$, $P = 0.910$), nor did handling effects vary across organs ($F_{2,174} = 1.36$, $P = 0.261$).

Prior to experiments, we placed blackberry leaves (*Rubus* sp.) in the foraging arena for ≥5 h so colonies would establish foraging trails inside the tube. When collecting foragers for the experiment, we removed the leaves to avoid potential liquid harvest resulting in exiting foragers arriving at an empty foraging arena. We then collected foragers as they arrived in the arena to use for load mass manipulation. Although leafcutters are known to carry loads of >10 mg (Cherrett, 1972), the load mass we used approximated the average leaf fragment weight (9.3 ± 1.03 mg, $N = 24$) transported by laden *Ac. echinatior* foragers in the laboratory colonies. We used the GLIMMIX procedure as described above to test for differences in the percentage of individuals with full storage organs across load mass treatments (0, 10 mg), distance treatments (2, 10 m) and storage organs (crop, midgut, hindgut), with colony ID included as a random factor. As above, we modelled residual variance with storage organ as a repeated factor, with a covariance structure based on separate residual variances for each organ and residual covariance between two adjacent organs moving from the crop to midgut to hindgut.

We tested whether foragers depleted transported liquids when cutting leaves. We allowed foragers from five colonies (Ae332, Ae376, Ae430, Ae490, Ae507) to access the middle box containing a small branch with blackberry leaves. We collected foragers for dissection immediately after they finished cutting a leaf fragment ($N = 84$) and compared their liquid status to exiting control foragers collected just after leaving the nest ($N = 78$). We used the same statistical approach described above to test for differences in percentages of individuals with full storage organs (crop, midgut, hindgut) depending on leaf-cutting activity (cutting, exiting).

Ethical Note

Dissections were necessary to test the hypothesis of liquid foraging, since leafcutter ants have a thick, opaque cuticle (Fig. 1). However, dissected individuals represented an exceedingly small fraction of overall colony populations (e.g. 40 workers from colonies with millions of workers). Thus, the present study did not harm leafcutter ant colonies that were observed in the field (remain intact in Panama) or laboratory (maintained in Copenhagen), as all colonies continued to forage normally after the experiment, and in no case was the queen harmed. Prior to dissections, ants were secured unconscious underwater in a wax-filled dish, and workers were transferred to 95% ethyl alcohol as soon as observations were complete.

RESULTS

Liquid Foraging Behaviour in the Field

Unladen workers without any substrate in their mandibles were common across four leafcutter species, representing >41% of all 5252 returning workers observed during >500 min of field observations (Fig. 2). Colonies of *Ac. echinatior* had 1.17 ± 0.31 returning ants per min, 38.1% of which were unladen, while *Ac. octospinosus* had 0.96 ± 0.22 ants per min, 31.2% of which were unladen (Fig. 2). Colonies of *At. colombica* had 148.83 ± 30.57 returning ants per min, 48.1% of which were unladen foragers, while *At. sexdens* had an average foraging rate of 115.44 ± 35.19 returning ants per min, 44.7% of which were unladen (Fig. 2).

Liquid transport behaviours in the field did not vary significantly across leafcutter species (Table 1), but liquid transport varied across storage organs depending on foraging status (Table 1, Fig. 3). Specifically, foragers tended to exit nests with full midguts and return with depleted midguts when laden with vegetation (Fig. 3, Appendix Table A2). Moreover, foragers were more likely to transport liquids in their midguts than their crops (Fig. 3, Appendix Table A2), but the likelihood of transporting liquids in their midguts and hindguts was not significantly different (Fig. 3, Appendix Table A2), indicating low potential of transport for sharing with nestmates because digestion is unidirectional after material passes through the proventriculus. Laden returning foragers also tended to have fuller hindguts than midguts (Fig. 3, Table 1, Appendix Table A2), suggesting that during foraging the ants assimilated liquids from the midguts and transferred waste to the hindgut.

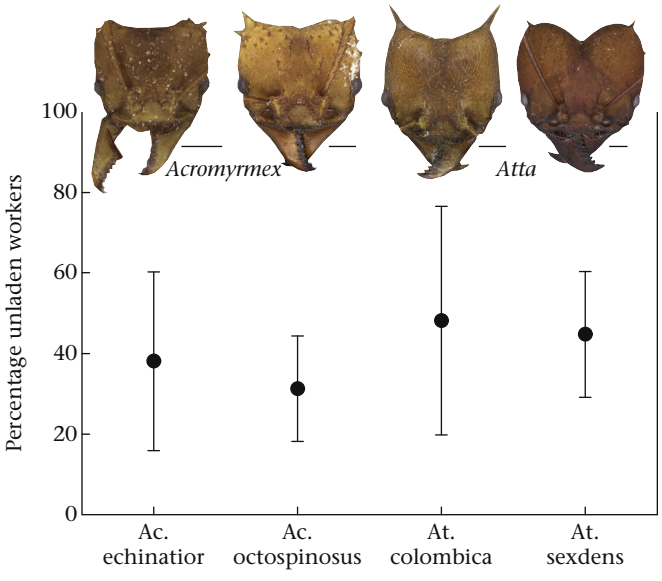

Figure 2. Field observations of foraging rates across four leafcutter ant species. Data points indicate percentage of workers returning to their nests unladen (without carrying vegetation in their mandibles). For observation times and sample sizes see Appendix Table A1. Means are given ± SD. Head size scale bars represent 0.5 mm. Ant images are from antweb.org.

Liquid Transport and Foraging Behaviour: the Lunchbox Hypothesis

Liquid transport behaviour in controlled laboratory experiments likewise varied across storage organs and foraging activities. First, as in the field, foragers more frequently carried liquids in their midguts and hindguts than in their crops (Table 1), with ca. 22% of foragers having crop liquids compared to ca. 83% of foragers having midgut liquids (Fig. 4). Second, experimentally laden workers transported liquids in all storage organs less frequently than unladen foragers, regardless of whether they foraged at 2 m or 10 m (Table 1, Fig. 4). Third, leaf-cutting activity did not significantly reduce overall liquid storage or midgut-specific liquid storage (Table 1).

DISCUSSION

This study tested whether the OFT prediction that individuals forage to maximize energetic returns holds when the foragers belong to a highly derived symbiosis that would probably be destabilized by such foraging decisions. Field observations and laboratory experiments confirmed that leafcutter ants do not forage for liquid nectar and thus question the decades-old assumption that they acquire nutrition from sources other than their tightly coevolved fungal cultivars. Moreover, while over 40% of the foragers in four sympatric leafcutter species returned to their nests without leaf fragments, these unladen ants were not more likely to carry liquids. Instead, we found support for the lunchbox hypothesis because most workers left nests with midguts full of liquids that were depleted (assimilated and transferred to hindguts) if workers returned with a leaf fragment in the field or transported a load in laboratory experiments. Thus, in contrast to the destabilizing effects of external nectar foraging, these results provide a novel mechanism promoting symbiotic stability, as fungi provide fuel for foragers to harvest more substrate for fungal crop production.

Such nutritional feedbacks probably govern the ecological success of the leafcutter symbiosis, dominant herbivores in habitats across the New World tropics and subtropics (Wirth et al., 2003). Specifically, leafcutter ants produce a highly 'domesticated' fungal cultivar *Leucoagaricus gongylophorus* that no longer grows freely in the wild (Mueller et al., 2001) and depends completely on vegetation foraged by ants for its food. Thus, if lunchbox resources fuel worker foraging, they are analogous to investment in hypha production by ancestral free-living basidiomycete fungi (Mueller et al., 2001) that extend hyphae to forage for decaying organic matter on the forest floor. More generally, organisms engaged in symbioses often lose critical functions (Gil, Sabater-Muñoz, Latorre, Silva, & Moya, 2002), and lunchbox dynamics provide a means of eliciting the replacement cooperative behaviour from symbiotic partners.

Littledyke and Cherrett (1976) performed a ground-breaking study of nutritional dynamics within leafcutter farming symbioses, using radiolabelled tracers (P^{32}, C^{14}) to indirectly track the flow of nutrients from harvested substrate to fungus cultivar to ant farmers. They found some evidence of liquid ingestion when a leaf fragment was placed directly on the fungus garden and when an exposed 'leafy shoot' was provided (Littledyke & Cherrett, 1976). However, liquid ingestion was not actually detected (or

Table 1
Results of mixed model analyses testing for variation in the percentage of leafcutter ant foragers with full storage organs (crop, midgut, hindgut) in both field observations in a Panamanian rainforest and in laboratory experiments

Effect	Numerator *df*	Denominator *df*	*F*	*P*
Field liquid transport				
Species	3	10.99	0.42	0.744
Foraging status	2	65.34	1.32	0.274
Species∗foraging status	6	65.21	0.27	0.949
Organ	2	45.45	121.17	0.0001
Species∗organ	6	53.83	1.44	0.215
Foraging status∗organ	4	50.94	5.10	0.002
Species∗foraging status∗organ	12	56.51	0.59	0.839
Distance×load mass experiment				
Load mass treatment	1	10.63	17.46	0.002
Foraging distance treatment	1	10.63	1.16	0.306
Load mass∗distance	1	10.63	2.55	0.139
Organ	2	7.91	202.91	0.0001
Load mass∗organ	2	7.91	1.25	0.337
Distance∗organ	2	7.91	0.02	0.980
Load mass∗distance∗organ	2	7.91	1.21	0.349
Leaf-cutting experiment				
Cutting treatment	1	12.84	0.99	0.338
Organ	2	7.204	52.95	0.0001
Cutting treatment∗organ	2	7.204	0.61	0.569

The analysis is based on field observations comparing liquid storage across four leafcutter species (*At. colombica*, *At. sexdens*, *Ac. echinatior*, *Ac. octospinosus*) and foraging statuses (laden: carrying a fragment of vegetation; unladen: empty mandibles; exiting: exiting nest). In the distance and load transport experiment, we compared liquid storage across load transport treatments (control, 10 mg) and distance treatments (2 m, 10 m). In the leaf-cutting experiment analysis, we compared liquid storage across leaf-cutting treatments (cutting: having just cut a leaf fragment; control: having just exited the nest).

barely detected) among the leafcutter workers cutting leaves in foraging arenas (Littledyke & Cherrett, 1976). It is also unclear whether any of the P^{32}, C^{14} tracers reflected liquid assimilated by adult workers or were simply processed as faecal droplets used to manure fungus gardens. Yet, the idea that foragers consume, transport and assimilate plant exudates while cutting and preparing leaves has been frequently used to assert that ants consume large amounts of nutrients from outside their farming

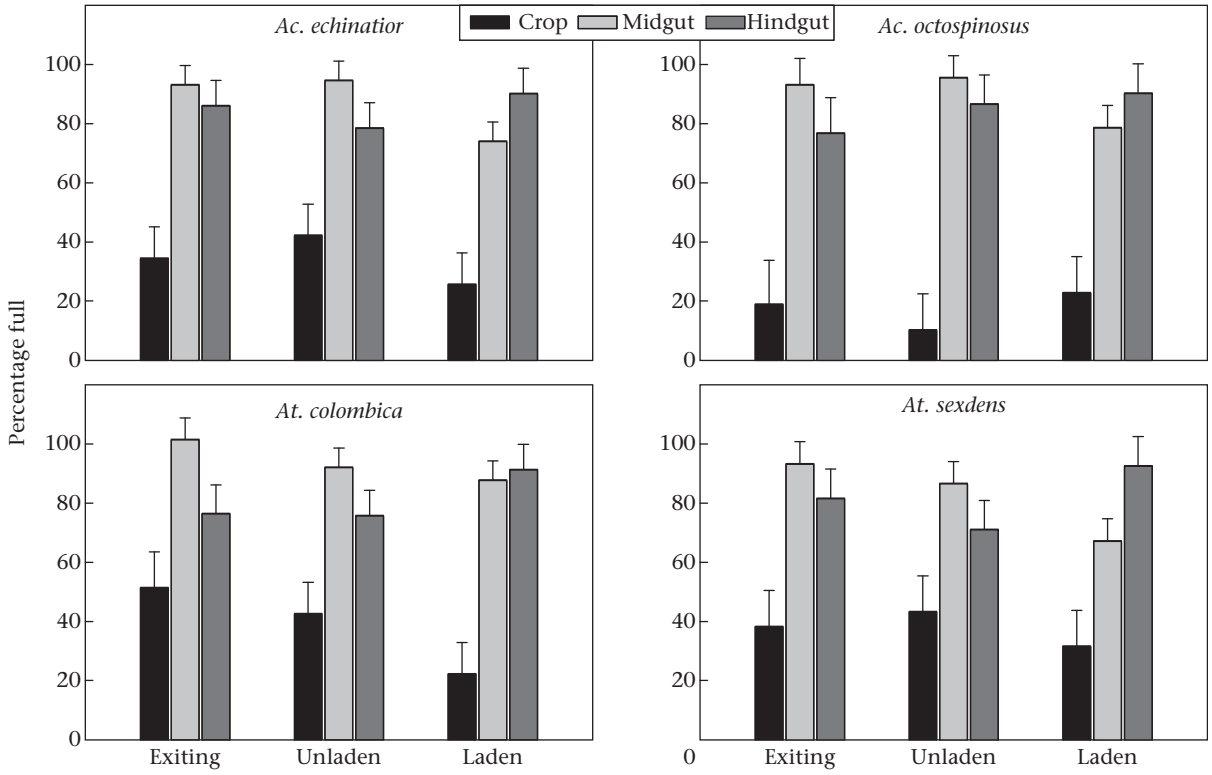

Figure 3. Similarities in liquid transport dynamics across four leafcutter ant species. Columns show percentage of full crops (black), midguts (light grey) and hindguts (dark grey) from dissections performed on a total of 670 field-collected foragers (*Ac. echinatior*: 161; *Ac. octospinosus*: 114; *At. colombica*: 200; *At. sexdens*: 195). Least square means are given +SE.

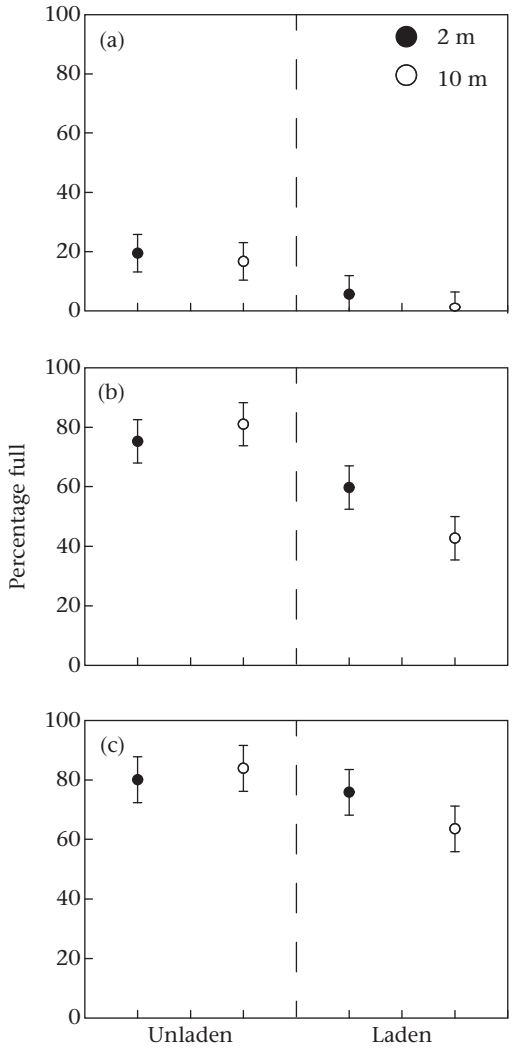

Figure 4. The effects of load carrying (0 mg versus 10 mg) and foraging distance (2 m versus 10 m) on liquid transport dynamics in (a) crops, (b) midguts and (c) hindguts of *Ac. echinatior* foragers in laboratory experiments. Least square means are given ± SE.

symbiosis (e.g. Bass & Cherrett, 1995; Mueller et al., 2001; Wirth et al., 2003).

Direct consumption by workers of nutritional subsidies from plants would imply that fungi do not provide sufficient nutrition for ant farmers (Bass & Cherrett, 1995; Mueller, 2002; Mueller et al., 2001), and runs counter to major recent advances towards understanding the specialized physiological interplay between ant farmers, their fungal cultivars and additional microbial partners. For instance, leafcutter ants receive enzymes from ingested fungus that are then passed unharmed through the digestive system and returned to the garden as faecal droplets to manure new garden substrate (De Fine Licht et al., 2013; Kooij, Aanen et al., 2014; Kooij, Rogowska-Wrzesinska et al., 2014; Martin, 1970; Schiøtt et al., 2010). Moreover, N-fixing rhizobiales bacteria have been discovered in the leafcutter fungus gardens (Pinto-Tomás et al., 2009) and the guts of workers (Sapountzis et al., 2015). Comparing these specialized digestive adaptations in leafcutter workers with their highly simplified crops and proventriculi (Caetano, 1990; Fig. 1) strongly supports the idea that leafcutter ants in natural populations lack plant nectar harvesting as a foraging strategy.

We next review prospects for the lunchbox hypothesis, which provides a novel strategy by which departing foragers can fuel their foraging by dynamically ingesting a short-term pulse of nutrition. First, while only load carriage was associated with liquid depletion in this study, ants may indeed use midgut liquids when cutting more recalcitrant leaves or foraging at greater distances, especially while climbing trees. Workers may also fuel energetically costly foraging activities with alternative metabolic fuels (e.g. glycogen from fat body cells; Roma, Mathias, & Bueno, 2006). Second, the contents of stored liquids were not identified, as leafcutter ants have a sieve-like organ preventing particles with diameter >10 μm from entering their alimentary canals (Mueller et al., 2001), and this prevented visual inspection of partially digested foods. Third, while we confirmed a within-nest origin for lunchbox liquids, a possible retention duration of 10 days (Erthal, Silva, & Samuels, 2004) makes the precise source difficult to ascertain. For instance, exiting workers may directly consume fungi or solicit regurgitated liquids from larvae. It will thus be important to identify both the chemical makeup of lunchbox liquids and the behaviours preceding their acquisition.

Moving forwards, lunchbox dynamics can be tested across diverse ant species facing diverse ecological challenges. For instance lunchbox liquids may be limited to ecological specialists with ample within-nest liquid supplies, such as leafcutter ants with fungi, species tending exudate-producing hemipterans inside their nests (Hölldobler & Wilson, 1990) or arid climate species with liquid storage castes (Rissing, 1984). However, such ecological specializations may also be unnecessary, as ants generally have larvae inside their nests that could provide foragers with regurgitated liquids (Hölldobler & Wilson, 1990). More generally, the lunchbox hypothesis provides a robust theoretical framework to link foraging energetics, digestive physiology and symbiotic performance.

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Appendix

Table A1
Foraging rates of four leafcutter ant species

Genus	Species	Observation time (min)	Total ants returning	Ants per min±SE	% Unladen returning
Acromyrmex	*echinatior*	374	365	1.17±0.31	46.5
	octospinosus	151	130	0.96±0.22	26.1
Atta	*colombica*	18	2 679	148.83±30.57	39.9
	sexdens	18	2 078	115.44±35.19	43.9

Table A2
Results of post hoc Tukey tests exploring significant main effects in analysis with combined species (Table 1)

Comparison		*df*	*t*	*P*
Overall organ effects				
Crop versus midgut		38.18	−15.46	**0.0001**
Crop versus hindgut		48.63	12.63	**0.0001**
Midgut versus hindgut		41.72	1.66	0.105
Foraging status by organ effects				
Exiting	Crop versus midgut	38.18	−8.88	**0.0001**
Exiting	Crop versus hindgut	48.63	−5.95	**0.0001**
Exiting	Midgut versus hindgut	41.72	2.68	**0.010**
Laden	Crop versus midgut	38.18	−8.45	**0.0001**
Laden	Crop versus hindgut	48.63	9.67	**0.0001**
Laden	Midgut versus hindgut	41.72	2.79	**0.008**
Unladen	Crop versus midgut	38.18	−9.48	**0.0001**
Unladen	Crop versus hindgut	48.63	−6.41	**0.0001**
Unladen	Midgut versus hindgut	41.72	2.80	**0.008**
Organ by foraging status effects				
Crop	Exiting versus laden	24.29	1.28	0.213
Crop	Exiting versus unladen	24.29	0.15	0.882
Crop	Laden versus unladen	24.08	−1.19	0.245
Midgut	Exiting versus laden	21.09	4.20	**0.0004**
Midgut	Exiting versus unladen	21.09	0.70	0.494
Midgut	Laden versus unladen	20.52	−3.72	**0.001**
Hindgut	Exiting versus laden	26.02	−1.74	0.093
Hindgut	Exiting versus unladen	26.02	0.36	0.723
Hindgut	Laden versus unladen	25.68	2.22	**0.035**

Pairwise Tukey post hoc tests followed mixed model GLM analyses testing for variation in the percentage of leafcutter ant foragers with full storage organs (crop, midgut, hindgut) in field observations. As species (*At. colombica*, *At. sexdens*, *Ac. echinatior*, *Ac. octospinosus*) did not differ in their liquid storage dynamics (Table 1), we performed a combined species analysis. Foraging statuses were laden (carrying a fragment of vegetation), unladen (empty mandibles) and exiting (exiting nest). Significant *P* values are in bold.