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Published in:
Ecology

DOI:
10.1002/ecy.3684

Publication date:
2022

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

Document license:
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Citation for published version (APA):
Nutritional challenges of feeding a mutualist: Testing for a nutrient–toxin tradeoff in fungus-farming leafcutter ants

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Abstract
The biochemical heterogeneity of food items often yields tradeoffs as each bite of food tends to contain some nutrients in surplus and others in deficit, as well as other less palatable or even toxic compounds. These multidimensional nutritional challenges are likely to be compounded when foraged foods are used to provision others (e.g., offspring or symbionts) with different physiological needs and tolerances. We explored these challenges in free-ranging colonies of leafcutter ants that navigate a diverse tropical forest to collect plant fragments they use to provision a co-evolved fungal cultivar. We tested the prediction that leafcutter farmers face provisioning tradeoffs between the nutritional quality and concentration of toxic tannins in foraged plant fragments. Chemical analyses of plant fragments sampled from the mandibles of Panamanian Atta colombica leafcutter ants provided little support for a nutrient–tannin foraging tradeoff. First, colonies foraged for plant fragments that ranged widely in tannin concentration. Second, high tannin levels did not appear to restrict colonies from selecting plant fragments with blends of protein and carbohydrates that maximized cultivar performance when measured with in vitro experiments. We also tested whether tannins expand the realized nutritional niche selected by leafcutter ants into high-protein dimensions as: (1) tannins can bind proteins and reduce their accessibility during digestion, and (2) in vitro experiments have shown that excess protein provisioning reduces cultivar performance. Contrary to this hypothesis, the most protein-rich plant fragments did not have highest tannin levels. More generally, the approach developed here can be used to test how multidimensional interactions between nutrients and toxins shape the costs and benefits of providing care to offspring or symbionts.

KEYWORDS
fungus, herbivory, leafcutter ants, nutritional geometry, plant secondary metabolites, tannins
INTRODUCTION

Nutritional ecologists have classically faced challenges of defining the biochemical quality of foods relative to the multidimensional physiological needs of consumers (Bernays, 1998; Dethier, 1954; Raubenheimer et al., 2012). These challenges must be overcome to understand the nutritional tradeoffs that emerge when consumers ingest foods containing many nutrients in varying degrees of deficit or surplus, or the nutrient–toxin tradeoffs that emerge when these foods are also chemically defended (Behmer, 2009). In recent years, the field of nutritional geometry (NG) has provided a research agenda and an empirical toolbox for rigorously testing these nutritional ecology hypotheses that naturally encompass multiple niche dimensions (Simpson & Raubenheimer, 2012). One recent NG innovation allows researchers to seamlessly integrate results of controlled laboratory-based experiments and field-based approaches in fluctuating environments (Machovsky-Capuska, Senior, et al., 2016; Raubenheimer, 2011; Shik & Dussutour, 2020). First, we can define a consumer’s fundamental nutritional niche (FNN) by measuring performance when the organism is confined to a suite of nutritionally defined diets in the laboratory (Lee et al., 2008; Shik et al., 2016). Second, we can measure the organism’s realized nutritional niche (RNN) by collecting and nutritionally analyzing the foods it forages in the field (Raubenheimer, 2011). When combined, these results yield a “nutritional landscape” on which the RNN is compared with the FNN to assess whether and how the organism meets its multidimensional nutritional needs (Crumière et al., 2021; Shik et al., 2020).

These nutritional approaches can also be extended to a broad range of species interactions. For instance, organisms must often forage for RNNs that satisfy the FNN needs of other individuals with different nutritional needs. This is the case when parental birds provision their offspring or social insect workers provision their siblings (Crumière et al., 2020; Dussutour & Simpson, 2009; Machovsky-Capuska, Priddel, et al., 2016). Leafcutter ants face additional provisioning challenges as foragers belong to colonies with up to millions of workers that cooperatively select, cut, and transport fresh plant fragments back to the nest where nestmates convert them into a nutritional mulch used to provision a co-evolved fungal cultivar, Leucoagaricus gongylophorus that they rely upon for food (Hölldobler & Wilson, 2010). The ecological success of these farming systems depends in part on the ability of leafcutter ant farmers to forage nutritionally diverse plant fragments from up to hundreds of plant species and attain RNN dimensions that target FNNs for maximal productivity of their fungal cultivar (Crumière et al., 2021; Shik et al., 2020).

The ability of herbivores to achieve optimized RNNs is often mediated by foraging tradeoffs between nutrients and toxins in the vegetation they consume (Behmer, 2009; Mole & Waterman, 1988; Wint, 1983). For leafcutter ants, these tradeoffs must be examined relative to the fungal cultivar’s physiological tolerances and requirements (De Fine Licht et al., 2013; Howard, 1987; C.M. Nichols-Orians & Schultz, 1990; Powell, 1984). We used a cultivar-centered approach to test whether and how multiple nutrients interact with plant toxins to shape the foraging behaviors of free-ranging colonies of the leafcutter ant Atta colombica in a Panamanian rainforest. We focused our analysis on tannins, which are among the most abundant plant secondary metabolites (Barbehenn & Constabel, 2011), and which are part of a larger group of toxic phenolic compounds produced by plants that also include flavonoids, lignans, and quinones (Bennett & Wallsgrove, 1994; Constabel et al., 2014). Tannins are known to generally inhibit fungal growth by disrupting the plasma membranes of fungal cells (Ekambaram et al., 2016; Zhu et al., 2019) and binding to proteins and therefore rendering them metabolically inaccessible (Arnoldi et al., 2020; Powell, 1984). The deleterious effects of tannins are therefore closely linked to the amounts and ratios of limiting nutrients in plant material (Behmer, 2009; Simpson & Raubenheimer, 2001).

If tannins can reduce the performance of L. gongylophorus (Howard, 1988; C. Nichols-Orians, 1991a; Powell, 1984), it is reasonable to conjecture that leafcutter foragers avoid tannin-rich plants, and that this aversive foraging behavior constrains the ability of colonies to regulate nutritional intake relative to the cultivar’s needs. Currently, the effects of tannins on leafcutter foraging remain ambiguous. Some evidence suggests that leaf selection is influenced by nutrient quality and not tannin content (Howard, 1987, 1990), but other studies conclude that leafcutter foragers avoid nutritionally beneficial leaves if they contain high tannin concentrations (Cherrett et al., 1989; Howard, 1988; C. Nichols-Orians, 1991c, 1991d, 1992). This ambiguity has resulted, in part, because previous studies have tended to explore tannin effects independently of plant nutrients or relative to a single macronutrient (e.g., protein) (Howard, 1988; Nichols-Orians, 1991a; Powell, 1984), or have not related ant foraging behaviors to the cultivar’s physiological requirements for nutrients or tolerance to tannins (Howard, 1990; Nichols-Orians, 1991c).

Recent NG studies of L. gongylophorus isolated on Petri dishes indicate that optimized nutritional provisioning hinges on interactions among at least two nutrients: protein and carbohydrates (Crumière et al., 2021; Shik et al., 2020). This provisioning challenge is defined by the cultivar’s broad tolerance to variable carbohydrate concentrations, but sharp declines in growth performance as protein levels rise beyond low concentrations of ca. 15%
(Crumière et al., 2021). Despite these negative effects of excess protein, free-ranging colonies of A. colombica also tend to forage mostly leaf fragments with protein levels higher than 15% (Crumière et al., 2021). We therefore predicted that the ability of tannins to bind proteins enhances the suitability of plant fragments that would otherwise contain excess protein. In this way, tannins may enable leafcutter colonies to expand their RNN and exploit carbohydrates from a broader pool of foraged plant materials.

When interpreting an RNN it is also important to consider that organisms typically have several performance traits whose expression can be optimized by distinct FNNs (Lee et al., 2008; Shik et al., 2016). Leafcutter ants can nutritionally provision their cultivar to maximize at least two performance traits: (1) hyphal growth rates and (2) the density of swollen hyphal tips called gongylidia that concentrate nutrients and enzymes and grow in clusters called staphylae (Martin et al., 1973; Schiøtt et al., 2010). These two performance traits have partially overlapping FNNs, with both traits maximized by carbohydrate-biased protein:carbohydrate (P:C) ratios, and with staphylae density also being maximized at protein-biased P:C ratios and higher protein concentrations (Crumière et al., 2021; Shik et al., 2020). Given that hyphal growth and staphyla density exhibit different tolerances and requirements for nutritional provisioning, we also examined whether these traits exhibit different responses to tannins.

We tested for a nutrient–tannin tradeoff in three steps. First, we used in vitro experiments with L. gongylophorus to quantify how protein, carbohydrates, and tannins interact to shape cultivar growth and staphyla density. Second, we chemically analyzed plant fragments sampled from the mandibles of laden leafcutter foragers to quantify variation in tannin foraging rates across colonies and substrate types. Third, we integrated results from the laboratory and the field to test whether plant tannin concentrations constrain a colony’s ability to forage protein and carbohydrate blends that maximize cultivar performance.

**METHODS**

**Fungus isolation and culturing**

We isolated L. gongylophorus fungus from the middle layer of the fungus garden of an A. colombica colony (AC-2012-1) collected from Soberanía Park in Panama and maintained at the University of Copenhagen in Denmark. We used a sterile dissecting needle to transfer staphyla to 60-mm Petri dishes containing autoclaved potato dextrose agar medium (PDA; VWR). Petri dishes were sealed with parafilm and incubated at 23.5°C for 1 week in the dark. Clean fungal cultures were then transferred to new Petri dishes with PDA, sealed, and incubated again for 2 weeks. We isolated from these plates, repeating the procedure, and letting these cultures grow for an additional 3 weeks. We used these isolates to estimate the growth rate of L. gongylophorus in a no-choice experiment with seven protein:carbohydrate diets (9:1, 6:1, 3:1, 1:1, 1:3, 1:6, 1:9 P:C) arrayed across three protein + carbohydrates concentrations (4, 8 or 25 g/L P + C) (Appendix S1: Table S1; Crumière et al., 2021) and repeated at three tannin concentrations (0, 400, and 800 μg/ml).

We added protein using equal amounts of bactopeptone (BD), bactotryptone (BD), and trypsinase peptone (BD), and carbohydrates using equal amounts of sucrose (Mamone) and starch (Sigma-Aldrich), and combined these macronutrients with bacteriological agar (VWR) and double-distilled water. We used tannic acid (Sigma) as the tannin source, adding it homogeneously into wet growth medium. This tannin application mimicked the work of gardener ants inside the nest that prepare plant fragments by: (1) maceration that physically bursts plant cells and are likely to release tannins from vacuoles and cell wall (Barbehenn & Constabel, 2011; Kao et al., 2002; Lees et al., 1993), and (2) creating an enzymatic mulch by mixing plant fragments with their own fecal droplets (De Fine Licht et al., 2013). Medium was autoclaved at 121°C and then aliquoted in 10 ml increments into sterile 60-mm Petri dishes under laminar flow. These plates were then exposed to UV light for 30 min. Fungus from PDA cultures was aseptically inoculated onto each plate (n = 5 plates/diet; total = 315 plates) using a flame-sterilized 4-mm diameter steel cylinder. We then sealed and stored these plates in the dark at 23.5°C for 56 days while regularly checking plates and excising contaminated areas.

**Measuring cultivar performance**

After 56 days, we outlined the outer edge of cultivar growth using a marker and photographed each plate using a Canon EOS 7D 336 Mark II camera mounted on a stand. We used ImageJ software (v1.52a; Schneider et al., 2012) to estimate hyphal growth area (mm$^2$) based on the final circumference line drawn around the outer border of the fungus using threshold contrast-adjusted grayscale images (with pixel$^2 = 0.02$). We counted staphylae directly from plates viewed under a dissecting microscope. We used the “fields” package v10.3 (Nychka et al., 2017) in RStudio to plot cultivar hyphal growth and staphyla production (density and mean number per plate) across the in vitro protein:carbohydrate:tannin landscapes with topological resolution of response surface $\lambda = 0.001$. To facilitate a comparison with nutritional data attained from
field-collected substrates, nutritional concentrations in growth medium were converted from g/L to % of total protein and carbohydrate mass relative to the total dry biomass of the growth media including non-nutritive components such as agar (as per Shik et al., 2020).

Collecting and identifying foraged plant fragments

We hand-collected 44,533 plant fragments from the mandibles of laden returning foragers from six A. colombica colonies between 9:00 AM and 12:00 AM in the wet season (May–June 2019) in Soberanía Park in Panama, as part of a larger study on nutritional foraging strategies in A. colombica (Crumière et al., 2021). We lay down on trash bags next to each colony’s most active trail close to the nest entrance. Two observers sampled each colony for, in total, 9 h of collection during three 1.5-h observation periods (N = 54 collection hours across six colonies). Each sampling session included three 30-min sampling periods, with plant fragments placed into Ziploc bags and quickly placed in a cooler. Back at the laboratory, we sorted substrates under a dissecting microscope into categories of fruits, flower petals or leaf fragments and then into morphospecies groups resulting in 87 samples. Substrates were organized per colony and collection day and then weighed before and after being dried in a BenchTop Pro freeze-dryer (SP Scientific) from 4000 to 10,000 cm⁻¹ (2500–1000 nm) at a resolution of 16 cm⁻¹ and 2× gain and the standard built-in reference of the instrument as reference measurement. For each sample, three spectrum acquisitions each composed of 32 monochromatic scans were performed to calculate a mean spectral average value. Samples were physically homogenized between each replicate measurement. We selected 30 samples for further chemical analyses to build prediction models for protein, carbohydrate, and tannin concentrations using principal component analysis (PCA) of the 87 NIR spectra after preprocessing using the first derivative model on SIMCA software (Umetrics) (Appendix S1: Table S2). We selected samples to maximize the diversity of spectra shown in the PCA and to ensure that sufficient biomass was available for chemical analysis.

We used a CN analyzer (Eurovector, Pavia, Italy) coupled to an isotope ratio mass spectrometer (Isoprime, Cheadle, UK) to quantify total nitrogen from ca. 3.5 mg of ground samples and estimated the quantity of total protein by multiplying total nitrogen by 6.25 (Crumière et al., 2021). We estimated carbohydrates by quantifying water-soluble carbohydrates with a Total Carbohydrate Assay Kit (Sigma-Aldrich) and starch with a Total Starch Assay Kit (Megazyme) using 25 and 50 mg of homogenized plant material, respectively (Crumière et al., 2021). We used peach powder as a positive control and water as a negative control in these analyses. Condensed tannin concentrations were quantified using the vanillin:HCl method (Hansen et al., 2006; Appendix S1: Table S3). We added 50 mg of dried, milled, and homogenized plant powder to 2.5 ml methanol, mixed this in a rotary shaker for 20 min at 230 rpm, and then centrifuged for 10 min at 4000 rpm. The extracts were then immediately transferred to test tubes in a temperature-controlled water bath of 30°C, incubated for 20 min in the dark, and then analyzed using a spectrophotometer.

We retained 73 samples that had no missing concentration values for protein, carbohydrate, or tannins from the full 87 sample data set described above (Appendix S1: Table S3). This “morphospecies-level” data set was used to account for variation in the nutrient–tannin signature measured for a given substrate type when sampled across colonies. We used DNA-barcoding IDs to generate “species-level” nutrient–tannin values when determining the composite A. colombica RNN, calculating means (±SD) for pooled samples of each of the 47 substrate types (leaves of 36 species, flowers of five species, fruits of six species; Appendix S1: Table S4). We used the “fields” package v10.3 (Nychka et al., 2017) in RStudio to overlay tannin concentrations over the protein and carbohydrate landscape generated by these 47 substrates with topological resolution of response surface $\lambda = 0.001$.

We used these FNN and RNN response surfaces to test the hypothesis that tannins limit the ability of leafcutter ants to forage plant fragments with optimal blends of protein and carbohydrates in three steps. First, we measured cultivar FNNs for hyphal growth and staphyla density, using ImageJ software (v1.52a; Schneider et al., 2012), and defined by the area (in units of pixels²) within their respective maximum
75% performance isoclines. Second, we used the same 75% isocline threshold to denote the plant fragments providing the highest foraged tannin levels. Third, we calculated the percent overlap between the cultivar’s performance FNNs and the highest realized tannin foraging levels by dividing the overlapping area by the FNN area for both performance traits and multiplying the value by 100.

Statistical analyses

Statistical analyses were performed in RStudio v1.2.5042 (RStudioTeam, 2020). We first used a GLM analysis to test for linear and quadratic variation in response variables (hyphal growth, staphyla density) across the independent variable “diet treatment” that included seven P:C ratios (9:1, 6:1, 3:1, 1:1, 1:3, 1:6, 1:9 P:C) and three P + C concentrations (4, 8 or 25 g/L P + C). We tested the model (~protein + carbohydrate + protein^2 + carbohydrate^2 + protein × carbohydrate) for each tannin concentration (0, 400, and 800 μg/ml) and for both response variables. We used the results to interpret FNN heatmaps. We next used a GLM analysis to test the model ~ colonies + substrate type and examine whether the response variable “tannin concentration” varied between the independent variables “field-collected substrate type” (leaf, flower, fruit) and “sampled colony” (N = 6 colonies). Prior to this analysis, we log-transformed the tannin concentrations (log[mg/g] + 1) of the 73 plant fragment samples to improve normality and verified the assumption of homogeneity of variances using Bartlett tests. We followed up significant main effects with Tukey tests to perform pairwise comparisons. We also performed a Spearman test with these 73 samples to test for a correlation between tannin concentration (mg/g) and total dry mass (mg) of each substrate foraged by ants. We finally performed a GLM analysis to test for linear and quadratic variation in the response variable “tannin concentration” (mg/g) across gradients of protein and carbohydrate concentrations generated by the foraged plant fragments. We tested the model (~protein + carbohydrate + protein^2 + carbohydrate^2 + protein × carbohydrate) using the species-level data set. All raw data and corresponding R scripts are available (Crumière et al., 2022a,b).

RESULTS

Do plant toxins mediate the cultivar’s FNN dimensions?

In baseline conditions without tannic acid, the cultivar’s hyphal growth performance increased linearly toward higher carbohydrate concentrations and decreased linearly toward higher protein concentrations (Figure 1a; Appendix S1: Figure S1A, Tables S5 and S6). The addition of tannic acid at 400 μg/ml reduced hyphal growth area across all diet treatments by ca. 55% (±31% SD) relative to baseline (Figure 1a; Appendix S1: Figure S1A, Tables S5 and S6). Doubling the tannic acid concentration to 800 μg/ml further reduced the overall growth area by ca. 69% (±25% SD) relative to baseline (Figure 1a; Appendix S1: Figure S1A, Tables S5 and S6). These deleterious effects of tannic acid caused especially pronounced reductions in the FNN’s protein dimension (Figure 1a). In both tannin treatments, hyphal growth declined as protein concentrations increased with negative linear slopes that were six-fold steeper than baseline (Appendix S1: Table S6). Additionally, the carbohydrate by protein interaction was never significant (Appendix S1: Table S6), suggesting that the growth-promoting effects of carbohydrates did not mitigate these tannin-mediated reductions in protein tolerance.

Staphyla density similarly increased linearly across a wide range of carbohydrates under baseline conditions and had comparatively narrowed FNNs in the protein dimension when tannins were added (Figure 1b; Appendix S1: Figure S1B,C, Tables S5 and S6). In absolute terms, staphyla mostly disappeared from cultures at protein levels exceeding ca. 15% in the low tannic acid treatment and at protein levels exceeding 10% in the high tannic acid treatment (Figure 1b). Non-significant protein by carbohydrate interactions indicated that higher carbohydrate levels did not offset this tannin-mediated protein sensitivity (Appendix S1: Table S6). Therefore, contrary to the prediction that tannins enhance protein tolerance: (1) the addition of tannic acid restricted the already narrow range of protein on which the cultivar could grow, and (2) these deleterious effects increased with increasing tannin concentrations.

Do tannin concentrations vary across foraged plant fragments?

To test the hypothesis that colonies avoid foraging substrates with high tannin concentrations, we first examined the prediction that colonies forage high tannin substrates at lower levels. In contrast with this prediction, tannin concentrations of individual substrates were not correlated with their total foraged biomass (Spearman correlation test: rho = -0.08; p = 0.48, Appendix S1: Figure S2), and tannin foraging levels did not differ statistically across observed leafcutter colonies (F_{5,65} = 2.05; p = 0.08; Figure 2a; Appendix S1: Figure S3A). More generally, a study performed in the same locality indicated
that the foraged leaves we sampled did not appear limited to a subset of the local tree community with low tannin concentrations (Coley, 1983).

Tropical forest leafcutter ants are hypothesized to avoid plant allelochemicals when possible by foraging for flowers and fruits (Feeny, 1976) rather than leaf fragments that typically dominate their harvest (Wirth et al., 2003). We therefore next compared tannin concentrations across substrate types. Tannin concentrations differed across foraged substrate types ($F_{2,65} = 4.41; p = 0.016$; Figure 2b; Appendix S1: Figure S3B). However, whereas fruit fragments tended to contain lower tannin concentrations (27.6 mg/g ± 27.5 SD) than leaves (73.8 mg/g ± 77.9 SD), this pairwise difference was not significant ($p = 0.29$; Figure 2b; Appendix S1: Figure S3B). This was likely to be because colonies foraged mostly leaf fragments relative to fruit fragments (97% and 2.1%, respectively, of foraged biomass) with a greater diversity of tannin concentrations (Figure 2b; Appendix S1: Figure S3B). Flower fragments exhibited higher tannin concentrations than fruit fragments (Figure 2b, Appendix S1: Figure S3B), although this difference was mediated by an outlier that had a tannin level of 421.4 mg/g (Figure 2b; Appendix S1: Table S3). Despite this outlier, flower fragments tended to have tannin concentrations at the upper range of those measured in leaf fragments (Figure 2b; Appendix S1: Table S3).

Do tannins prevent leafcutter ants from optimally foraging macronutrients?

We next tested whether tannins prevent colonies from targeting a macronutrient RNN that matches their

FIGURE 1 Testing for interactive effects of protein, carbohydrates, and tannins on Leucoagaricus gongylophorus performance. (a) In the absence of tannic acid, hyphal growth is maximized across a wide range of carbohydrate (ca. from <10% to >50%) as long as protein concentrations remain lower than 20%. Tannic acid reduces hyphal growth across all protein:carbohydrate (P:C) treatments relative to baseline conditions with steeper growth reductions at 800 μg/ml than at 400 μg/ml. (b) In the absence of tannic acid, the cultivar has maximal staphyla density on carbohydrate-biased diets ranging below 40% carbohydrate and on protein-biased diets ranging below 20% carbohydrate and 30% protein. Tannic acid generally decreases the staphyla density relative to baseline conditions. Fundamental nutritional niches for each trait are defined as the respective areas contained within the upper 75% performance isolines shown here as black topography lines in baseline treatment heatmaps. Statistical support for interpretation of these heat maps is provided in Appendix S1: Tables S5 and S6.
cultivar’s FNN dimensions. We first arrayed foraged substrates on a nutrient–toxin landscape, which showed that colonies attained a broad macronutrient RNN by foraging across leaves, flowers, and fruits. This RNN covered a triangular region with vertices at 5% protein and 5% carbohydrates, 5% protein and 40% carbohydrates, and 30% protein and 10% carbohydrates (Figure 3a). Second, we overlaid tannin concentrations atop this RNN to test for gradients across the landscape of foraged plant macronutrients. Tannin concentrations did not vary significantly relative to the protein and/or carbohydrate content contained in plant fragments (mg of tannins per g of plant fragment dry mass) did not vary significantly across leafcutter ant colonies (Figure 3b). Third, we integrated results from the laboratory and field to qualitatively assess a key prediction of a nutrient–toxin tradeoff: that the highest foraged tannin levels prevent ants collecting nutritionally optimal plant fragments. Contrary to this prediction, the highest foraged tannin levels overlapped with only 2.8% of the cultivar’s FNN for maximal hyphal growth and do not overlap with the FNN for staphyla density (Figure 3b). These combined results indicate that tannins do not appear to systematically limit colonies from foraging nutrients that maximize fungal crop performance.

**DISCUSSION**

We used a nutritional landscape approach to test whether and how plant secondary metabolites constrain the ability of leafcutter ants to forage RNNs that maximize the performance of their co-evolved fungal cultivar. We first performed in vitro experiments showing that tannins uniformly reduce cultivar performance regardless of whether the cultivar is optimally provisioned with blends of protein and carbohydrates. Despite these strong deleterious effects, tannins do not appear to be a decisive factor governing foraging decisions of leafcutter ants in a tropical rainforest. First, colonies do not tightly regulate tannin intake as they select plant fragments varying widely in tannin content. Second, tannins do not appear to prevent colonies from selecting substrates with nutritional blends that maximize cultivar growth and staphyla density. These results also highlight that nutritional foraging challenges cannot be adequately understood by studying any single nutrient in isolation and provide a template for exploring how these multidimensional nutritional challenges apply to systems in which foraged resources are provisioned to other individuals with distinct physiological niches.

Whereas free-ranging leafcutter ants tend to forage mostly leaf fragments with protein-biased P:C ratios (Berish, 1986; Crumière et al., 2021), it appears that optimized fungal cultivation by leafcutter ants hinges upon targeted protein provisioning. This is because: (1) the cultivar’s in vitro performance declines steeply as protein levels increase, and (2) colonies tightly regulate protein (but not carbohydrates) when allowed to forage for nutritionally defined substrates in laboratory experiments (Shik et al., 2020). It was therefore surprising that tannins do not appear to play an important role in expanding the protein dimensions of foraged RNNs because we hypothesized that their protein-binding capacity would make protein-rich plant fragments more palatable (Arnoldi et al., 2020; Powell, 1984). Perhaps the multidimensional logic we applied to nutrients in the previous paragraph also extends to plant allelochemicals. This is because A. colombica colonies forage >100 plant species (Wirth et al., 2003) that contain diverse and highly variable concentrations of toxic compounds (e.g., flavonoids, lignans, and quinones; Bennett & Wallsgrove, 1994; Coley et al., 1985; Constabel et al., 2014), which can act
synergistically in nuanced ways to negatively impact herbivores (Dyer et al., 2018). Moreover, because tannin levels in plant can be dependent on soil nutrients (Endara & Coley, 2011; McKey et al., 1978) and abiotic factors such as light, temperature, CO₂, water availability, and ozone (Chaves & Escuder, 1999), it will be important to explore these dynamics across environmental gradients. Case in point, earlier studies found that the cultivar’s expression of an enzyme involved in tannin detoxification (polyphenol oxidase) depended on the type of tannin used. It was unaffected by tannic acid (a hydrolysable tannin) but was inhibited by quebracho (a condensed tannin) at high concentrations (Nichols-Orians, 1991a, 1991b). Both types of tannins can be toxic for their consumers at high concentrations (Nichols-Orians, 1991a, 1991b). Both types of tannins can be toxic for their consumers at high concentrations (Barbehenn & Constabel, 2011; Garg et al., 1992; Zhu et al., 1995), but they differ in their capacity to bind nutrients. Condensed tannins are polymers that efficiently precipitate dietary proteins (Barbehenn & Constabel, 2011; Barry, 1989), whereas hydrolysable tannins less effectively bind protein as they are mostly monomers or dimers readily degraded by microflora in vertebrates (Garg et al., 1992; Hagerman et al., 1992; Waghorn, 2008; Zhu et al., 1995). In this study, we used tannic acid in in vitro studies of the cultivar, because it is a hydrolysable tannin commonly used in feeding experiment in insects (Barbehenn et al., 2009a, 2009b; Behmer et al., 2002; Simpson & Raubenheimer, 2001). However, we measured condensed tannins in the plant fragments sampled from foragers in the field. Further studies of varied phenolic compound mixtures in varied ratios and concentrations will be important for linking the cultivar’s sensitivity to tannins and the ability of free-ranging colonies to collect tannin-rich plant fragments while navigating complex phytochemical landscapes in tropical rainforests.

The broad tannin tolerance in free-ranging leafcutter colonies must also be reconciled with laboratory results showing that individual leafcutter ants avoid sucrose solutions infused with tannins (Powell, 1984), and that colonies preferentially forage plant material enriched with the amino acid proline (Meyer et al., 2006) that is known to bind with tannins and reduce tannin toxicity in mammals (Glendinning, 1992; Mehansho et al., 1987). Key next steps will involve parsing details of secondary nutrient–toxin regulation that are not directly mediated by leafcutter foragers. For instance, gardener ants inside the nest are known to discard a subset of foraged tannin-rich plant fragments directly into trash piles (Hudson et al., 2009; Powell, 1984) where other workers can associate fungal toxins with specific waste-pile odors and then transmit a “delayed rejection” strategy throughout the colony (Arenas & Roces, 2016, 2017; Herz et al., 2008). Moreover, the L. gongylophorus cultivar exhibits an innate response to tannins with constitutive production of polyphenol oxidases that can detoxify tannins at low concentrations (Cherrett et al., 1989; De Fine Licht et al., 2013; Nichols-Orians, 1991a, 1991b). Ants are
involved in the detoxification mechanism by acquiring these polyphenol oxidases when ingesting gongylidia and then depositing them onto freshly mulched vegetation as fecal droplets (Appel, 1993; De Fine Licht et al., 2013; Powell, 1984).

Previous experiments with herbivorous locusts have shown that a multidimensional nutritional approach is needed to fully parse the deterrent effects of plant secondary metabolites and the physiological costs of their ingestion (Behmer et al., 2002; Raubenheimer, 1992; Raubenheimer & Simpson, 1990; Simpson & Raubenheimer, 2001). For instance, the negative effects of tannins can be mitigated if locusts were also allowed to forage near their performance-maximizing nutritional intake target (Raubenheimer & Simpson, 1990; Simpson & Raubenheimer, 2001). Moreover, tannins had important negative post-ingestive effects on nitrogen assimilation even though they did not affect nutritional foraging rates (Simpson & Raubenheimer, 2001). This parallels the results of the present study. However, the nutrient–tannin regulation by leafcutter ants also requires additional layers of nutritional feedback, from foragers that cut and transport plant fragments, to gardeners that convert this material into a nutritional mulch, to the fungal cultivar that probably signals the optimality of these provisioning decisions. In this sense, it is striking that leafcutter ants are able to maintain a broad foraging niche across hundreds of chemically heterogeneous plant species (Wirth et al., 2003) despite the apparently narrow physiological tolerance of their L. gongylphorus fungal cultivar (Crümére et al., 2021; Shik et al., 2020). More generally, the NG approach provides a unifying framework to study how diverse organisms navigate complex biochemical landscapes, whether they are foraging for themselves or to provision others with potentially non-overlapping nutritional needs.

ACKNOWLEDGMENTS

We thank Pol Lannes and Aidan James for assistance with fieldwork. We thank the Smithsonian Tropical Research Institute for logistical support during fieldwork. Fieldwork and sample transportation were carried under the research permit SE/A-24-19 and the export permit SEX/A-41-19 delivered by the Ministerio de Ambiente, Republica de Panama. This research was funded by a European Research Council Starting Grant (ELEVATE: ERC-2017-STG-757810) to Jonathan Z. Shik.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Antonin J. J. Crümére, Sophie Mallett, and Anders Michelsen performed experiments and collected samples and data. Antonin J. J. Crümére, Sophie Mallett, and Riikka Rinnan analyzed data. Antonin J. J. Crümére, Sophie Mallett, and Jonathan Z. Shik interpreted the data. Antonin J. J. Crümére and Jonathan Z. Shik designed the study. Antonin J. J. Crümére and Jonathan Z. Shik wrote the original draft.

DATA AVAILABILITY STATEMENT

Data (Crümére et al., 2022a) are available in Dryad at: 10.5061/dryad.sxksn0347. Code (Crümére et al., 2022b) is available on Zenodo at: 10.5281/zenodo.5643760.

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SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.