

ARTICLE

Nutritional challenges of feeding a mutualist: Testing for a nutrient–toxin tradeoff in fungus-farming leafcutter ants

Antonin J. J. Crumière¹  | Sophie Mallett¹ | Anders Michelsen²  |
Riikka Rinnan²  | Jonathan Z. Shik^{1,3} 

¹Section for Ecology and Evolution,
Department of Biology, University of
Copenhagen, Copenhagen, Denmark

²Terrestrial Ecology Section, Department
of Biology, University of Copenhagen,
Copenhagen, Denmark

³Smithsonian Tropical Research Institute,
Balboa, Panama

Correspondence

Antonin J. J. Crumière
Email: antonin.crumiere@gmail.com

Funding information

European Research Council, Grant/
Award Number: ERC-2017-STG-757810

Handling Editor: Matthew L. Forister

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

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2022 The Authors. *Ecology* published by Wiley Periodicals LLC on behalf of The Ecological Society of America.

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 staphyla 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 bactopectone (BD), bactotryptone (BD), and trypticase 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 (mm²) based on the final circumference line drawn around the outer border of the fungus using threshold contrast-adjusted grayscale images (with pixel² = 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, 216 Warminster, USA) for 24 h. We stored samples at -20°C in Ziploc bags with silica gel and transported them back to Copenhagen where we performed nutritional analyses and identified 44 plant species from the 87 samples using DNA barcoding of the Internal Transcribed Spacer 1 (Crumière et al., 2021; Shik et al., 2020).

Macronutrient and tannin measurements

Protein and carbohydrate concentrations of plant fragments were determined from plant fragments as part of the broader study described above (Crumière et al., 2021; Shik et al., 2020). Briefly, 30–100 mg of freeze-dried plant samples were placed into centrifuge tubes, plunged into liquid nitrogen, and ground using a plastic pestle. We acquired near-infrared (NIR) reflectance spectra for each of the 87 samples using an Antaris II FT-NIR Analyzer (Thermo Scientific) from 4000 to $10,000\text{ cm}^{-1}$ (2500–1000 nm) at a resolution of 16 cm^{-1} and $2\times$ 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 ($\pm\text{SD}$) 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 (\sim protein + carbohydrate + protein² + carbohydrate² + protein \times carbohydrate) for each tannin concentration (0, 400, and 800 μ g/ml) and for both response variables. We used the results to interpret FNN heatmaps. We next used a GLM analysis to test the model \sim 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 ($\log[\text{mg/g}] + 1$) 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 (\sim protein + carbohydrate + protein² + carbohydrate² + protein \times 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 μ g/ml reduced hyphal growth area across all diet treatments by ca. 55% ($\pm 31\%$ SD) relative to baseline (Figure 1a; Appendix S1: Figure S1A, Tables S5 and S6). Doubling the tannic acid concentration to 800 μ g/ml further reduced the overall growth area by ca. 69% ($\pm 25\%$ 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

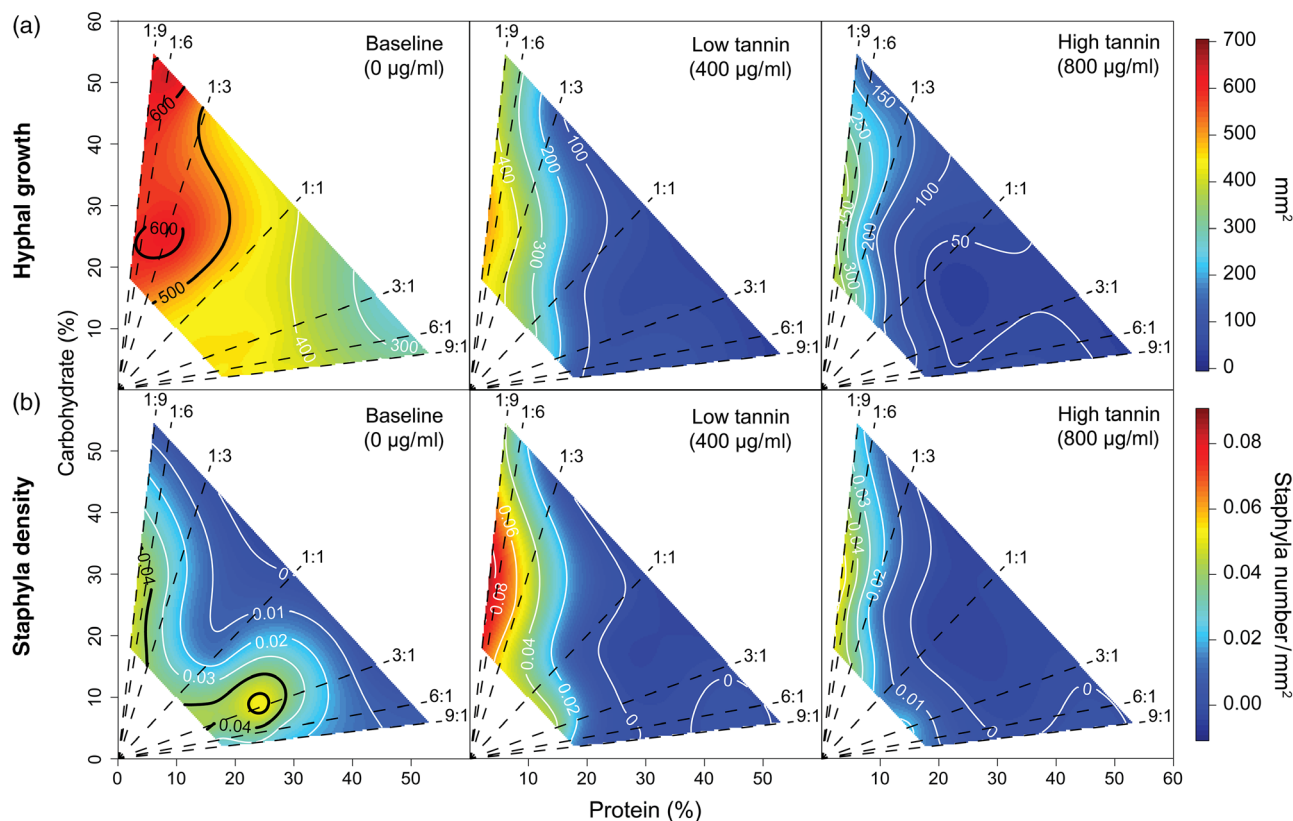


FIGURE 1 Testing for interactive effects of protein, carbohydrates, and tannins on *Leucoagaricus gongylophorus* performance. (a) In the absence of tannic acid, hyphal growth is maximized across a wide range of carbohydrate (ca. from <10% to >50%) as long as protein concentrations remain lower than 20%. 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 staphylo 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 staphylo density relative to baseline conditions. Fundamental nutritional niches for each trait are defined as the respective areas contained within the upper 75% performance isoclines 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

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 ($27.6 \text{ mg/g} \pm 27.5 \text{ SD}$) than leaves ($73.8 \text{ mg/g} \pm 77.9 \text{ SD}$), 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). Therefore, while tannin concentrations do not appear to be the decisive factor governing leafcutter foraging decisions, they may limit the palatability of flower fragments that were 1% of total fragment biomass (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

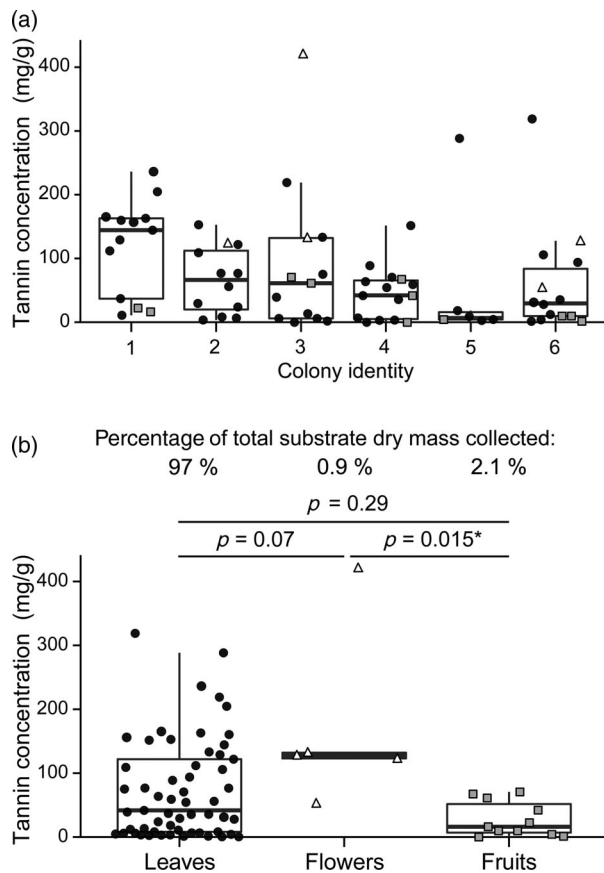


FIGURE 2 Tannin concentrations in foraged plant fragments sampled across six *Atta colombica* colonies and three substrate types (leaves, flowers, fruits). (a) Tannin concentrations (expressed in mg of tannins per g of plant fragment dry mass) did not vary significantly across leafcutter ant colonies. (b) Substrate types had significantly different tannin concentrations, with fruit fragments ($n = 11$) containing significantly lower tannin concentrations than flower fragments ($n = 5$), but not leaf fragments ($n = 57$)

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 ($F_{5,41} = 1.97$, $p = 0.103$; 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

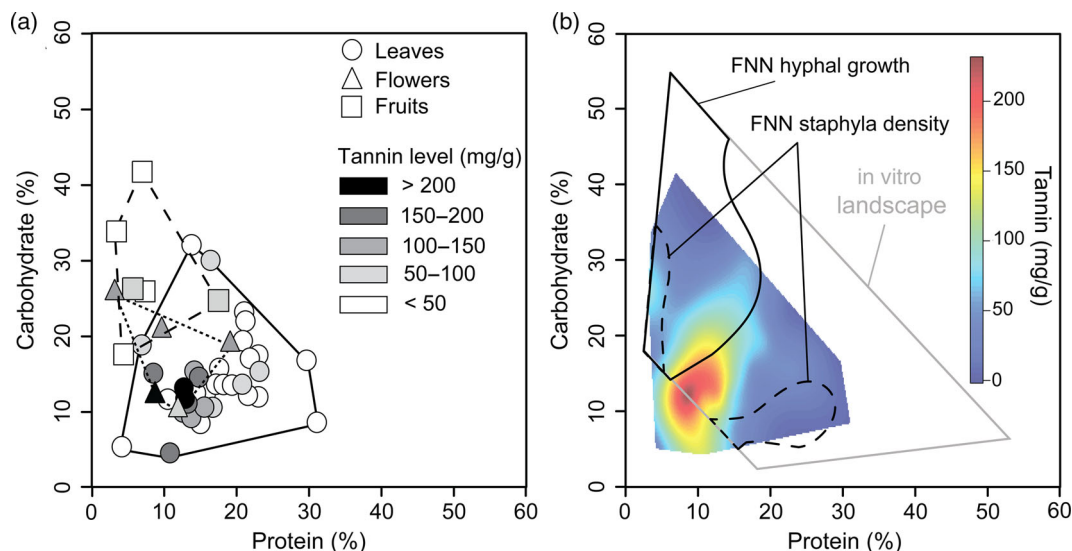


FIGURE 3 Testing for a nutrient–tannin foraging tradeoff. (a) Individual substrates are shaded in grayscale based on their tannin concentrations and arrayed across a nutritional landscape based on their protein and carbohydrate concentrations. This species-level data set includes 36 distinct types of leaf fragments, five flower fragments, and six fruit fragments. (b) The cultivar’s *in vitro* protein–carbohydrate fundamental nutritional niches (FNNs) for hyphal growth and staphyla density are overlaid over a heatmap representation of protein: carbohydrate:tannin concentrations of plant fragments foraged by free-ranging colonies. This visualization enables us to assess whether the highest tannin concentrations overlap with the nutritional blends maximizing cultivar performance and therefore constrain the ability of leafcutter ants to target their cultivar’s FNN needs

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 (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. gongylophorus* fungal cultivar (Crumière 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. Crumière, Sophie Mallett, and Anders Michelsen performed experiments and collected samples and data. Antonin J. J. Crumière, Sophie Mallett, and

Riikka Rinnan analyzed data. Antonin J. J. Crumière, Sophie Mallett, and Jonathan Z. Shik interpreted the data. Antonin J. J. Crumière and Jonathan Z. Shik designed the study. Antonin J. J. Crumière and Jonathan Z. Shik wrote the original draft.

DATA AVAILABILITY STATEMENT


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

ORCID

Antonin J. J. Crumière  <https://orcid.org/0000-0003-2214-2993>

Anders Michelsen  <https://orcid.org/0000-0002-9541-8658>

Riikka Rinnan  <https://orcid.org/0000-0001-7222-700X>

Jonathan Z. Shik  <https://orcid.org/0000-0003-3309-7737>

REFERENCES

- Appel, H. M. 1993. “Phenolics in Ecological Interactions: The Importance of Oxidation.” *Journal of Chemical Ecology* 19(7): 1521–52. <https://doi.org/10.1007/BF00984895>.
- Arenas, A., and F. Rocés. 2016. “Learning Through the Waste: Olfactory Cues from the Colony Refuse Influence Plant Preferences in Foraging Leaf-Cutting Ants.” *Journal of Experimental Biology* 219(16): 2490–6. <https://doi.org/10.1242/jeb.139568>.
- Arenas, A., and F. Rocés. 2017. “Avoidance of Plants Unsuitable for the Symbiotic Fungus in Leaf-Cutting Ants: Learning Can Take Place Entirely at the Colony Dump.” *PLoS One* 12(3): e0171388. <https://doi.org/10.1371/journal.pone.0171388>.
- Arnoldi, J.-F., S. Coq, S. Kéfi, and S. Ibanez. 2020. “Positive Plant–Soil Feedback Trigger Tannin Evolution by Niche Construction: A Spatial Stoichiometric Model.” *Journal of Ecology* 108(1): 378–91. <https://doi.org/10.1111/1365-2745.13234>.
- Barbehenn, R. V., and P. C. Constabel. 2011. “Tannins in Plant–Herbivore Interactions.” *Phytochemistry* 72(13): 1551–65. <https://doi.org/10.1016/j.phytochem.2011.01.040>.
- Barbehenn, R. V., A. Jaros, G. Lee, C. Mozola, Q. Weir, and J.-P. Salminen. 2009a. “Hydrolyzable Tannins as “Quantitative Defenses”: Limited Impact against *Lymantria Dispar* Caterpillars on Hybrid Poplar.” *Journal of Insect Physiology* 55(4): 297–304. <https://doi.org/10.1016/j.jinsphys.2008.12.001>.
- Barbehenn, R. V., A. Jaros, G. Lee, C. Mozola, Q. Weir, and J.-P. Salminen. 2009b. “Tree Resistance to *Lymantria Dispar* Caterpillars: Importance and Limitations of Foliar Tannin Composition.” *Oecologia* 159(4): 777–88. <https://doi.org/10.1007/s00442-008-1268-7>.
- Barry, T. N. 1989. “Condensed Tannins their Role in Ruminant Protein and Carbohydrate Digestion and Possible Effects upon the Rumen Ecosystem.” In *The Roles of Protozoa and Fungi in Ruminant Digestion*, edited by J. V. Nolan, R. A. Leng, and D. I. Demeyer, 153–69. Armidale: Penambul Books.
- Behmer, S. T. 2009. “Insect Herbivore Nutrient Regulation.” *Annual Review of Entomology* 54(1): 165–87. <https://doi.org/10.1146/annurev.ento.54.110807.090537>.

- Behmer, S. T., S. J. Simpson, and D. Raubenheimer. 2002. "Herbivore Foraging in Chemically Heterogeneous Environments: Nutrients and Secondary Metabolites." *Ecology* 83(9): 2489–501. [https://doi.org/10.1890/0012-9658\(2002\)083\[2489:HFICHE\]2.0.CO;2](https://doi.org/10.1890/0012-9658(2002)083[2489:HFICHE]2.0.CO;2).
- Bennett, R. N., and R. M. Wallsgrave. 1994. "Secondary Metabolites in Plant Defence Mechanisms." *New Phytologist* 127(4): 617–33. <https://doi.org/10.1111/j.1469-8137.1994.tb02968.x>.
- Berish, C. W. 1986. "Leaf-Cutting Ants (*Atta cephalotes*) Select Nitrogen-Rich Forage." *American Midland Naturalist* 115(2): 268–76. <https://doi.org/10.2307/2425863>.
- Bernays, E. A. 1998. "Evolution of Feeding Behavior in Insect Herbivores." *BioScience* 48(1): 35–44. <https://doi.org/10.2307/1313226>.
- Chaves, N., and J. C. Escuder. 1999. "Variation of Flavonoid Synthesis Induced by Ecological Factors." In *Principles and Practices in Plant Ecology: Allochemical Interactions*, edited by I. K. M. M. Dakshini and C. L. Foy, 267–85. Boca Raton, FL: CRC Press.
- Cherrett, J. M., R. J. Powell, and D. J. Stradling. 1989. "The Mutualism between Leaf-Cutting Ants and their Fungus." In *Insect-Fungus Interactions*, edited by N. Wilding, N. M. Collins, P. M. Hammond, and J. F. Webber, 93–120. London: Academic Press.
- Coley, P. D. 1983. "Herbivory and Defensive Characteristics of Tree Species in a Lowland Tropical Forest." *Ecological Monographs* 53(2): 209–34. <https://doi.org/10.2307/1942495>.
- Coley, P. D., J. P. Bryant, and F. S. Chapin. 1985. "Resource Availability and Plant Antiherbivore Defense." *Science* 230(4728): 895. <https://doi.org/10.1126/science.230.4728.895>.
- Constabel, P. C., K. Yoshida, and V. Walker. 2014. "Diverse Ecological Roles of Plant Tannins: Plant Defense and beyond." In *Recent Advances in Polyphenol Research*, edited by A. Romani, V. Lattanzio, and S. Quideau, 115–42. Oxford: Wiley-Blackwell.
- Crumière, A. J. J., A. James, P. Lannes, S. Mallett, A. Michelsen, R. Rinnan, and J. Z. Shik. 2021. "The Multidimensional Nutritional Niche of Fungus-Cultivar Provisioning in Free-Ranging Colonies of a Neotropical Leafcutter Ant." *Ecology Letters* 24(11): 2439–51. <https://doi.org/10.1111/ele.13865>.
- Crumière, A. J. J., S. Mallett, A. Michelsen, R. Rinnan & J. Z. Shik 2022a. "Data from: Nutritional Challenges of Feeding a Mutualist: Testing for a Nutrient-Toxin Tradeoff in Fungus-Farming Leafcutter Ants." Dryad, dataset. <https://doi.org/10.5061/dryad.sxksn0347>.
- Crumière, A. J. J., S. Mallett, A. Michelsen, R. Rinnan, and J. Z. Shik. 2022b. "Data from: Nutritional Challenges of Feeding a Mutualist: Testing for a Nutrient-Toxin Tradeoff in Fungus-Farming Leafcutter Ants." Zenodo, software. <https://doi.org/10.5281/zenodo.5643760>.
- Crumière, A. J. J., C. J. Stephenson, M. Nagel, and J. Z. Shik. 2020. "Using Nutritional Geometry to Explore How Social Insects Navigate Nutritional Landscapes." *Insects* 11(1): 53.
- De Fine Licht, H. H., M. Schiott, A. Rogowska-Wrzęsinska, S. Nygaard, P. Roepstorff, and J. J. Boomsma. 2013. "Laccase Detoxification Mediates the Nutritional Alliance between Leaf-Cutting Ants and Fungus-Garden Symbionts." *Proceedings of the National Academy of Sciences* 110(2): 583–7. <https://doi.org/10.1073/pnas.1212709110>.
- Dethier, V. G. 1954. "Evolution of Feeding Preferences in Phytophagous Insects." *Evolution* 8(1): 33–54. <https://doi.org/10.2307/2405664>.
- Dussutour, A., and S. J. Simpson. 2009. "Communal Nutrition in Ants." *Current Biology* 19(9): 740–4. <https://doi.org/10.1016/j.cub.2009.03.015>.
- Dyer, L. A., C. S. Philbin, K. M. Ochsenrider, L. A. Richards, T. J. Massad, A. M. Smilanich, M. L. Forister, et al. 2018. "Modern Approaches to Study Plant–Insect Interactions in Chemical Ecology." *Nature Reviews Chemistry* 2(6): 50–64. <https://doi.org/10.1038/s41570-018-0009-7>.
- Ekambaram, S. P., S. S. Perumal, and A. Balakrishnan. 2016. "Scope of Hydrolysable Tannins as Possible Antimicrobial Agent." *Phytotherapy Research* 30(7): 1035–45. <https://doi.org/10.1002/ptr.5616>.
- Endara, M.-J., and P. D. Coley. 2011. "The Resource Availability Hypothesis Revisited: A Meta-Analysis." *Functional Ecology* 25(2): 389–98. <https://doi.org/10.1111/j.1365-2435.2010.01803.x>.
- Feeny, P. 1976. "Plant Apparency and Chemical Defense." *Recent Advances in Phytochemistry* 10: 1–40. https://doi.org/10.1007/978-1-4684-2646-5_1.
- Garg, S. K., H. P. Makkar, K. B. Nagal, S. K. Sharma, D. R. Wadhwa, and B. Singh. 1992. "Oak (*Quercus incana*) Leaf Poisoning in Cattle." *Veterinary and Human Toxicology* 34(2): 161–4.
- Glendinning, J. I. 1992. "Effect of Salivary Proline-Rich Proteins on Ingestive Responses to Tannic Acid in Mice." *Chemical Senses* 17(1): 1–12. <https://doi.org/10.1093/chemse/17.1.1>.
- Hagerman, A. E., C. T. Robbins, Y. Weerasuriya, T. C. Wilson, and C. McArthur. 1992. "Tannin Chemistry in Relation to Digestion." *Journal of Range Management* 45(1): 57–62. <https://doi.org/10.2307/4002526>.
- Hansen, A. H., S. Jonasson, A. Michelsen, and R. Julkunen-Tiitto. 2006. "Long-Term Experimental Warming, Shading and Nutrient Addition Affect the Concentration of Phenolic Compounds in Arctic-Alpine Deciduous and Evergreen Dwarf Shrubs." *Oecologia* 147(1): 1–11. <https://doi.org/10.1007/s00442-005-0233-y>.
- Herz, H., B. Hölldobler, and F. Roces. 2008. "Delayed Rejection in a Leaf-Cutting Ant after Foraging on Plants Unsuitable for the Symbiotic Fungus." *Behavioral Ecology* 19(3): 575–82. <https://doi.org/10.1093/beheco/arn016>.
- Hölldobler, B., and E. O. Wilson. 2010. *The Leafcutter Ants: Civilization by Instinct*. New York, NY: W. W. Norton & Co. Ltd.
- Howard, J. J. 1987. "Leafcutting Ant Diet Selection: The Role of Nutrients, Water, and Secondary Chemistry." *Ecology* 68(3): 503–15. <https://doi.org/10.2307/1938455>.
- Howard, J. J. 1988. "Leafcutting and Diet Selection: Relative Influence of Leaf Chemistry and Physical Features." *Ecology* 69(1): 250–60. <https://doi.org/10.2307/1943180>.
- Howard, J. J. 1990. "Infidelity of Leafcutting Ants to Host Plants: Resource Heterogeneity or Defense Induction?" *Oecologia* 82(3): 394–401. <https://doi.org/10.1007/BF00317488>.
- Hudson, T. M., B. L. Turner, H. Herz, and J. S. Robinson. 2009. "Temporal Patterns of Nutrient Availability around Nests of Leaf-Cutting Ants (*Atta colombica*) in Secondary Moist Tropical Forest." *Soil Biology and Biochemistry* 41(6): 1088–93. <https://doi.org/10.1016/j.soilbio.2009.02.014>.
- Kao, Y. Y., S. A. Harding, and C. J. Tsai. 2002. "Differential Expression of Two Distinct Phenylalanine Ammonia-Lyase Genes in Condensed Tannin-Accumulating and Lignifying Cells of

- Quaking Aspen." *Plant Physiology* 130(2): 796–807. <https://doi.org/10.1104/pp.006262>.
- Lee, K. P., S. J. Simpson, F. J. Clissold, R. Brooks, J. William, O. Ballard, P. W. Taylor, N. Soran, and D. Raubenheimer. 2008. "Lifespan and Reproduction in *Drosophila*: New Insights from Nutritional Geometry." *Proceedings of the National Academy of Sciences* 105(7): 2498. <https://doi.org/10.1073/pnas.0710787105>.
- Lees, G. L., N. H. Suttill, and M. Y. Gruber. 1993. "Condensed Tannins in Sainfoin. 1. A Histological and Cytological Survey of Plant Tissues." *Canadian Journal of Botany* 71(9): 1147–52. <https://doi.org/10.1139/b93-135>.
- Machovsky-Capuska, G. E., D. Priddel, P. H. W. Leong, P. Jones, N. Carlile, L. Shannon, D. Portelli, A. McEwan, A. V. Chaves, and D. Raubenheimer. 2016. "Coupling Bio-Logging with Nutritional Geometry to Reveal Novel Insights into the Foraging Behaviour of a Plunge-Diving Marine Predator." *New Zealand Journal of Marine and Freshwater Research* 50(3): 418–32. <https://doi.org/10.1080/00288330.2016.1152981>.
- Machovsky-Capuska, G. E., A. M. Senior, S. J. Simpson, and D. Raubenheimer. 2016. "The Multidimensional Nutritional Niche." *Trends in Ecology & Evolution* 31(5): 355–65. <https://doi.org/10.1016/j.tree.2016.02.009>.
- Martin, M. M., M. J. Gieselmann, and J. S. Martin. 1973. "Rectal Enzymes of Attine Ants. α -Amylase and Chitinase." *Journal of Insect Physiology* 19(7): 1409–16. [https://doi.org/10.1016/0022-1910\(73\)90171-6](https://doi.org/10.1016/0022-1910(73)90171-6).
- McKey, D., P. G. Waterman, C. N. Mbi, J. S. Gartlan, and T. T. Struhsaker. 1978. "Phenolic Content of Vegetation in Two African Rain Forests: Ecological Implications." *Science* 202(4363): 61–4.
- Mehansho, H., L. G. Butler, and D. M. Carlson. 1987. "Dietary Tannins and Salivary Proline-Rich Proteins: Interactions, Induction, and Defense Mechanisms." *Annual Review of Nutrition* 7(1): 423–40. <https://doi.org/10.1146/annurev.nu.07.070187.002231>.
- Meyer, S. T., F. Roces, and R. Wirth. 2006. "Selecting the Drought Stressed: Effects of Plant Stress on Intraspecific and within-Plant Herbivory Patterns of the Leaf-Cutting Ant *Atta colombica*." *Functional Ecology* 20(6): 973–81. <http://www.jstor.org/stable/4139334>.
- Mole, S., and P. G. Waterman. 1988. "Light-Induced Variation in Phenolic Levels in Foliage of Rain-Forest Plants: II. Potential Significance to Herbivores." *Journal of Chemical Ecology* 14(1): 23–34. <https://doi.org/10.1007/bf01022528>.
- Nichols-Orians, C. 1991a. "Condensed Tannins, Attine Ants, and the Performance of a Symbiotic Fungus." *Journal of Chemical Ecology* 17(6): 1177–95. <https://doi.org/10.1007/BF01402942>.
- Nichols-Orians, C. 1991b. "Differential Effects of Condensed and Hydrolyzable Tannin on Polyphenol Oxidase Activity of Attine Symbiotic Fungus." *Journal of Chemical Ecology* 17(9): 1811–9. <https://doi.org/10.1007/BF00993730>.
- Nichols-Orians, C. 1992. "The Acceptability of Young and Mature Leaves to Leaf-Cutter Ants Varies with Light Environment." *Biotropica* 24(2): 211–4. <https://doi.org/10.2307/2388675>.
- Nichols-Orians, C. M. 1991c. "The Effects of Light on Foliar Chemistry, Growth and Susceptibility of Seedlings of a Canopy Tree to an Attine Ant." *Oecologia* 86(4): 552–60. <https://doi.org/10.1007/BF00318322>.
- Nichols-Orians, C. M. 1991d. "Environmentally Induced Differences in Plant Traits: Consequences for Susceptibility to a Leaf-Cutter Ant." *Ecology* 72(5): 1609–23. <https://doi.org/10.2307/1940961>.
- Nichols-Orians, C. M., and J. C. Schultz. 1990. "Interactions among Leaf Toughness, Chemistry, and Harvesting by Attine Ants." *Ecological Entomology* 15(3): 311–20. <https://doi.org/10.1111/j.1365-2311.1990.tb00813.x>.
- Nychka, D., R. Furrer, J. Paige, and S. Sain. 2017. "fields: Tools for Spatial Data." <https://github.com/NCAR/Fields>.
- Powell, R. J. 1984. "The Influence of Substrate Quality on Fungus Cultivation by Some Attine Ants." Doctor of Philosophy. Exeter: University of Exeter.
- Raubenheimer, D. 1992. "Tannic Acid, Protein, and Digestible Carbohydrate: Dietary Imbalance and Nutritional Compensation in Locusts." *Ecology* 73(3): 1012–27. <https://doi.org/10.2307/1940176>.
- Raubenheimer, D. 2011. "Toward a Quantitative Nutritional Ecology: The Right-Angled Mixture Triangle." *Ecological Monographs* 81(3): 407–27. <https://doi.org/10.1890/10-1707.1>.
- Raubenheimer, D., and S. J. Simpson. 1990. "The Effects of Simultaneous Variation in Protein, Digestible Carbohydrate and Tannic Acid on the Feeding Behaviour of Larval *Locusta migratoria* (L.) and *Schistocerca gregaria* (Forsk.) I. Short-Term Studies." *Physiological Entomology* 15(2): 219–33. <https://doi.org/10.1111/j.1365-3032.1990.tb00510.x>.
- Raubenheimer, D., S. J. Simpson, and A. H. Tait. 2012. "Match and Mismatch: Conservation Physiology, Nutritional Ecology and the Timescales of Biological Adaptation." *Philosophical Transactions of the Royal Society B: Biological Sciences* 367(1596): 1628–46. <https://doi.org/10.1098/rstb.2012.0007>.
- RStudioTeam. 2020. "RStudio: Integrated Development for R." RStudio, PBC. <http://www.rstudio.com/>.
- Schiött, M., A. Rogowska-Wrzesinska, P. Roepstorff, and J. J. Boomsma. 2010. "Leaf-Cutting Ant Fungi Produce Cell Wall Degrading Pectinase Complexes Reminiscent of Phytopathogenic Fungi." *BMC Biology* 8(1): 156. <https://doi.org/10.1186/1741-7007-8-156>.
- Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. "NIH Image to ImageJ: 25 Years of Image Analysis." *Nature Methods* 9(7): 671–5. <https://doi.org/10.1038/nmeth.2089>.
- Shik, J. Z., and A. Dussutour. 2020. "Nutritional Dimensions of Invasive Success." *Trends in Ecology & Evolution* 35(8): 691–703. <https://doi.org/10.1016/j.tree.2020.03.009>.
- Shik, J. Z., E. B. Gomez, P. W. Kooij, J. C. Santos, W. T. Wcislo, and J. J. Boomsma. 2016. "Nutrition Mediates the Expression of Cultivar–Farmer Conflict in a Fungus-Growing Ant." *Proceedings of the National Academy of Sciences* 113(36): 10121–6. <https://doi.org/10.1073/pnas.1606128113>.
- Shik, J. Z., P. W. Kooij, D. A. Donoso, J. C. Santos, E. B. Gomez, M. Franco, A. J. J. Crumière, et al. 2020. "Nutritional Niches Reveal Fundamental Domestication Trade-Offs in Fungus-Farming Ants." *Nature Ecology & Evolution* 5: 122–34. <https://doi.org/10.1038/s41559-020-01314-x>.
- Simpson, S. J., and D. Raubenheimer. 2001. "The Geometric Analysis of Nutrient–Allelochemical Interactions: A Case Study Using Locusts." *Ecology* 82(2): 422–39. [https://doi.org/10.1890/0012-9658\(2001\)082\[0422:TGAONA\]2.0.CO;2](https://doi.org/10.1890/0012-9658(2001)082[0422:TGAONA]2.0.CO;2).
- Simpson, S. J., and D. Raubenheimer. 2012. *The Nature of Nutrition: A Unifying Framework from Animal Adaptation to Human Obesity*. Princeton, NJ: Princeton University Press.

- Waghorn, G. 2008. "Beneficial and Detrimental Effects of Dietary Condensed Tannins for Sustainable Sheep and Goat Production—Progress and Challenges." *Animal Feed Science and Technology* 147(1): 116–39. <https://doi.org/10.1016/j.anifeedsci.2007.09.013>.
- Wint, G. R. W. 1983. "The Effect of Foliar Nutrients upon the Growth and Feeding of a Lepidopteran Larva." In *Nitrogen as an Ecological Factor*, edited by J. A. Lee, S. McNeill, and I. H. Rorison, 301–20. Oxford: Blackwell Scientific.
- Wirth, R., H. Herz, R. J. Ryel, W. Beyschlag, and B. Hölldobler. 2003. "Herbivory of Leaf-Cutting Ants." In I. T. Baldwin, M. M. Caldwell, G. Heldmaier, O. L. Lange, H. A. Mooney, E.-D. Schulze and U. Sommer, *Ecological Studies*, Vol 164. Berlin: Springer-Verlag Berlin Heidelberg.
- Zhu, C., M. Lei, M. Andargie, J. Zeng, and J. Li. 2019. "Antifungal Activity and Mechanism of Action of Tannic Acid against *Penicillium digitatum*." *Physiological and Molecular Plant Pathology* 107: 46–50. <https://doi.org/10.1016/j.pmpp.2019.04.009>.
- Zhu, J., L. J. Filippich, and J. Ng. 1995. "Rumen Involvement in Sheep Tannic Acid Metabolism." *Veterinary and Human Toxicology* 37(5): 436–40.

SUPPORTING INFORMATION

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

How to cite this article: Crumière, Antonin J. J., Sophie Mallett, Anders Michelsen, Riikka Rinnan, and Jonathan Z. Shik. 2022. "Nutritional Challenges of Feeding a Mutualist: Testing for a Nutrient–Toxin Tradeoff in Fungus-Farming Leafcutter Ants." *Ecology* 103(6): e3684. <https://doi.org/10.1002/ecy.3684>