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LETTER

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The multidimensional nutritional niche of fungus-cultivar provisioning in free-ranging colonies of a neotropical leafcutter ant

Antonin J. J. Crumière[1] | **Aidan James**[1] | **Pol Lannes**[1] | **Sophie Mallett**[1] | **Anders Michelsen**[2] | **Riikka Rinnan**[2] | **Jonathan Z. Shik**[1,3]

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

[2]Department of Biology, University of Copenhagen, Copenhagen, Denmark

[3]Smithsonian Tropical Research Institute, Balboa, Ancon, Panama

Correspondence
Antonin J. J. Crumière, Section for Ecology and Evolution, Department of Biology, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark.
Email: antonin.crumiere@gmail.com

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Abstract

Foraging trails of leafcutter colonies are iconic scenes in the Neotropics, with ants collecting freshly cut plant fragments to provision a fungal food crop. We hypothesised that the fungus-cultivar's requirements for macronutrients and minerals govern the foraging niche breadth of *Atta colombica* leafcutter ants. Analyses of plant fragments carried by foragers showed how nutrients from fruits, flowers and leaves combine to maximise cultivar performance. While the most commonly foraged leaves delivered excess protein relative to the cultivar's needs, in vitro experiments showed that the minerals P, Al and Fe may expand the leafcutter foraging niche by enhancing the cultivar's tolerance to protein-biased substrates. A suite of other minerals reduces cultivar performance in ways that may render plant fragments with optimal macronutrient blends unsuitable for provisioning. Our approach highlights how the nutritional challenges of provisioning a mutualist can govern the multidimensional realised niche available to a generalist insect herbivore.

KEYWORDS
ecophysiology, fundamental and realised niches, fungus, herbivory, leafcutter ants, nutritional geometry

INTRODUCTION

Natural selection is predicted to favour traits enabling consumers to acquire nutritionally balanced diets (Stephens & Krebs, 1986). For insect herbivores, such nutrient regulation poses major challenges. First, plant foods tend to contain carbon in far higher concentrations than other limiting resources such as nitrogen and phosphorus (Sterner & Elser, 2002). Second, each mouthful of ingested plant tissue is likely to contain valuable macronutrients (e.g. carbohydrates, proteins, lipids) and other essential components (e.g. vitamins, minerals) but also a mix of recalcitrant compounds (e.g. cellulose) and toxins (e.g. tannins) (Behmer, 2009). Third, insects are seldom limited by a single nutrient at a time, and the value of a given plant resource thus depends on ratios and concentrations of multiple interacting nutrients (Simpson & Raubenheimer, 2012). Nutritional geometry (NG) has provided new approaches for studying these multidimensional dietary challenges (Machovsky-Capuska et al., 2016; Shik & Dussutour, 2020) and has shown that organisms have diverse strategies for prioritising specific nutrients when foraging for and consuming imbalanced foods (Dussutour et al., 2010; Lee et al., 2008). We applied NG approaches to study nutritional regulation strategies in free-ranging colonies of the leafcutter ant *Atta colombica*. These ants are ecologically important neotropical herbivores and belong to a lineage that is unique among

Aidan James and Pol Lannes contributed equally to this work.

the ants in collecting plant fragments to provision a domesticated fungal food crop (*Leucoagaricus gongylophorus*) rather than ant nestmates (Hölldobler & Wilson, 2010; Weber, 1972).

Nutritional geometry studies have tended to focus on the blends of macronutrients contained in foods (Dussutour & Simpson, 2009; Krabbe et al., 2019) and less on macronutrient–mineral interactions (Nie et al., 2015) even as over 25 mineral elements are essential for life (Frausto da Silva & Williams, 2001; Kaspari & Powers, 2016). For instance, leafcutter ants concentrate Mg and Ca in their cuticle as a protective armour (Li et al., 2020) and Zn as a hardening agent in their mandibles (Edwards et al., 1993) while also preferentially foraging for Na-rich substrates (Chavarria Pizarro et al., 2012) and avoiding vegetation with elevated Mn and Al (Berish, 1986). Plants are typically assumed to contain minerals in sufficient abundance to meet the requirements of insect herbivores (Behmer, 2009), but mineral concentrations vary widely across plant species and tissues within individual plants (Han et al., 2011; Joern et al., 2012). Minerals also tend to exhibit thresholds beyond which limitation becomes toxicity (Höss et al., 2010; Ji et al., 2011) and they can even be sequestered by plants to deter herbivores as quantitative chemical defences (Boyd, 2007; Jansen et al., 2002; Kaspari, 2020). We thus hypothesised that minerals in vegetation can inhibit farming performance when leafcutter ants provision them in excess of their fungal cultivar's tolerances and requirements.

Leafcutter ants have multiple opportunities for such regulation. First, each plant substrate has a specific nutritional profile (Figure 1a) and colonies can likely target different nutritional blends by foraging among leaves, fruits, flowers and across plant species (Figure 1b; De Fine Licht & Boomsma, 2010; Shik et al., 2021). Indeed, a single *A. colombica* colony can forage across 126 plant species (53 families) and gather up to 370 kg of plant dry mass per year (Wirth et al., 2003). These ants are thus extreme generalists compared to the majority of insect herbivores that consume a few plant families (Bernays & Graham, 1988). The next phase occurs when gardener ants within underground fungus cultivation chambers macerate vegetation fragments and add a mixture of enzyme-rich faecal droplets (Figure 1c) to promote fungal hyphal growth and the production of nutrient-rich hyphal tips called gongylidia (packaged in bundles called staphyla; Figure 1d; Quinlan & Cherrett, 1979; Schiøtt et al., 2010). We further conjecture that (1) colonies forage across plant substrates to acquire a realised nutritional niche (RNN) that targets their cultivar's fundamental nutritional niche (FNN) for maximal crop performance (i.e. hyphal growth and staphyla production; Figure 1e; Shik et al., 2016; Shik et al., 2021) and (2) optimised nutritional provisioning is necessary to farm the cultivar at scales needed to sustain massive colonies comprising thousand to millions of individuals (Quinlan & Cherrett, 1979; Shik et al., 2018).

Recent laboratory-based experiments with nutritionally defined diets have shown that (1) *A. colombica* colonies tightly regulate protein foraging at low levels while allowing carbohydrate intake to fluctuate and (2) the cultivar is more sensitive to fluctuations in protein than carbohydrates, with reduced growth and survival when protein concentrations exceed *c.* 20% total substrate dry mass (Shik et al., 2021). However, studies of free-ranging leafcutters have shown that some colonies preferentially forage N-rich leaves and thus likely target proteins built from N-rich amino acids (Berish, 1986; Mundim et al., 2009). This mixed evidence of protein regulation is likely due to the chemical complexity of field-collected vegetation relative to the controlled protein:carbohydrate diets used to assess cultivar's nutritional needs in the laboratory. Specifically, we predicted that the minerals that likely vary across vegetation fragments, but which remain at low levels in laboratory diets, can influence the cultivar's metabolic performance and thus its ability to access nutrients (Shah et al., 2010; Zhang & Elser, 2017).

Here, we sought to explain how *A. colombica* leafcutter ants navigate a lowland Panamanian rainforest landscape of taxonomically and chemically diverse plant substrates, and whether the multidimensional foraging strategies of ant workers are mediated by the FNNs of their fungal cultivar *L. gongylophorus*. We first determined the cultivar's FNN dimensions across interacting gradients of two macronutrients (protein and carbohydrates) and 10 minerals (Al, Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn). We next quantified RNNs by identifying and nutritionally analysing the vegetation fragments sampled from the mandibles of laden *A. colombica* foragers in the field. By overlaying RNNs atop of cultivar's FNNs, we sought to determine the decisive nutrients and minerals regulated by leafcutter ants when provisioning their cultivars.

MATERIALS AND METHODS

Fungal isolation and in vitro experiments

We isolated staphylae from *L. gongylophorus* fungus gardens of two laboratory-grown *A. colombica* colonies (IDs: AC-2012-1, AC-2014-2) collected in Soberanía Park (Panama) and maintained at University of Copenhagen (Denmark) in the dark at 23–25°C and 72%–75% humidity. Staphylae were transferred to 60-mm petri dishes containing autoclaved potato dextrose agar media (PDA; VWR). See supporting protocols in Supporting Information for complete procedures for fungal isolation and culturing.

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 and 1:9 Pr:C) arrayed across three protein+carbohydrate concentrations (4, 8 or 25 g/L Pr+C; Table S1). We added protein using bactopeptone (BD), bactotryptone

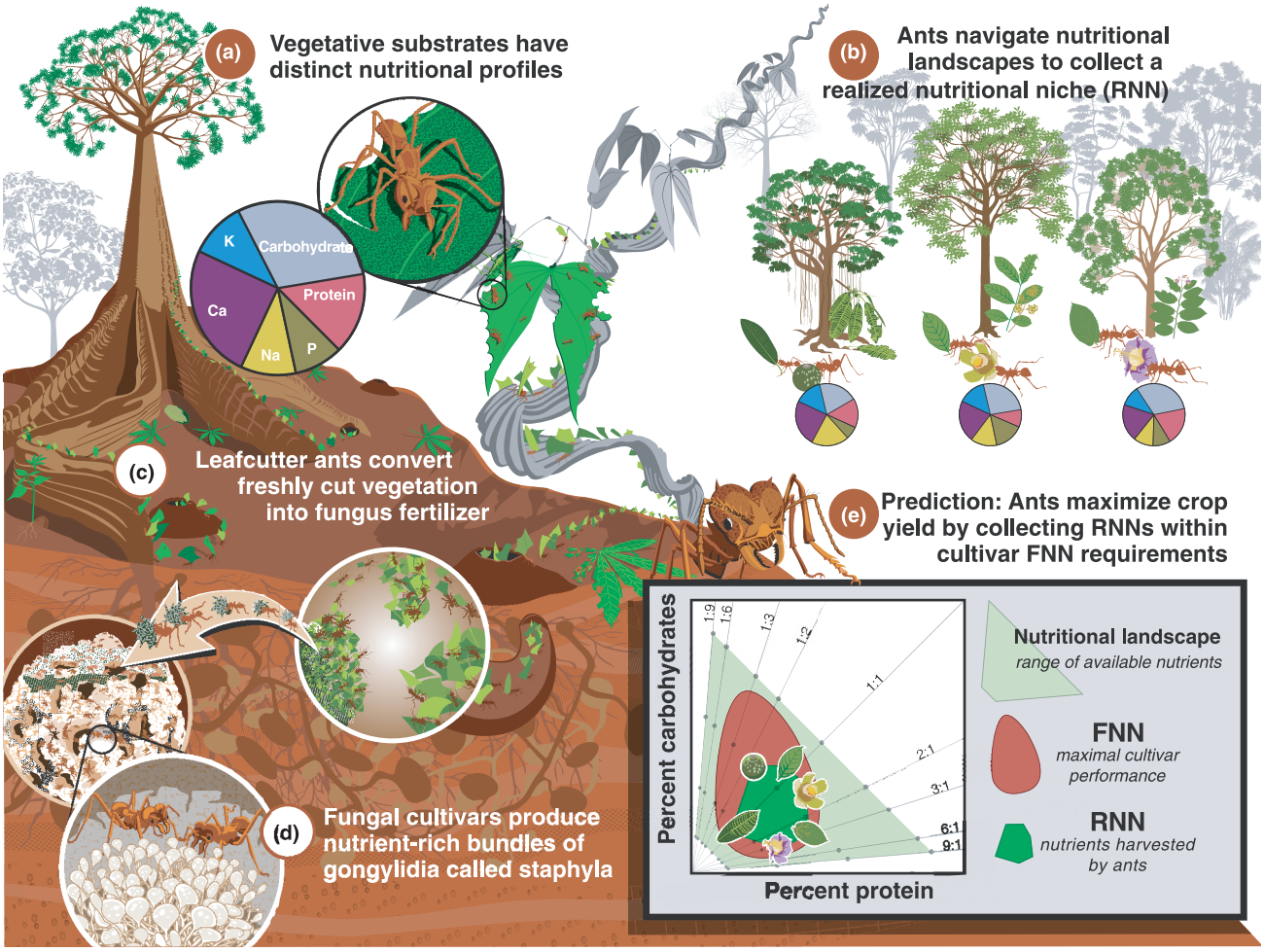

FIGURE 1 A niche-based framework for testing the hypothesis that leafcutter ants navigate tropical forests to collect plant substrates that target their fungal cultivar's nutritional needs. (a) Foragers can select among plant substrates (e.g. leaf, fruit, flower) that have distinct blends of protein, carbohydrates and minerals. (b) Colonies can regulate nutritional intake by foraging across hundreds of plant species to acquire a realised nutritional niche (RNN). (c) Gardener ants convert foraged plant fragments into a nutritional mulch used to provision their fungal cultivar. (d) These nutrients promote hyphal growth and the production of edible nutrient-rich hyphal tips called gongylidia (packaged in bundles called staphylae). (e) We can study the ants' nutrient-provisioning strategy in two steps. We first define the cultivar's fundamental nutritional niche (FNN) by measuring its performance when isolated onto petri dishes and grown across nutritional gradients, shown here as the light-green trapezoid ranging across protein:carbohydrate ratios (1:9 to 9:1 Pr:C) and protein + carbohydrate concentrations (4 diagonal rows of individual diet treatments (grey dots) with negative slopes ranging from 4 to 25 g/L Pr+C). The red region indicates a hypothetical FNN of maximal cultivar performance. We then quantify the RNN (dark green polygon) from nutrients contained in plant fragments foraged by free-ranging colonies. We array each plant fragment type based on their percent protein and carbohydrates and test the prediction that ants maximise cultivar performance by providing an RNN whose dimensions overlap with the cultivar's FNN. The illustrations are by Damond Kyllo

(BD), and trypticase peptone (BD), carbohydrates using sucrose (Mamone) and starch (Sigma-Aldrich), and combined these ingredients with bacteriological agar (VWR) and double-distilled water. Media were autoclaved at 121°C and then plated under laminar flow in 10 ml amounts per sterile 60-mm petri dish, before being UV-exposed for 30 min. Fungus from PDA cultures was aseptically inoculated onto each plate (n = 5 plates/diet) using a flame-sterilised 4-mm diameter steel cylinder. Plates were then sealed and stored at 23.5°C in the dark for 56 days during which we regularly checked plates and excised contaminated areas. If plates were heavily contaminated, we removed them from the experiment and inoculated new replicates.

We next assessed how 10 minerals impact fungal performance over 70 days by adding the following compounds to the previously described media in solution: Al (aluminium sulphate hydrate ($Al_2(SO_4)_3{\cdot}H_2O$), Alfa Aesar), Ca (calcium chloride ($CaCl_2$), Sigma-Aldrich), Cu (copper sulphate pentahydrate ($CuSO_4{\cdot}5H_2O$), Sigma), Fe (iron sulphate heptahydrate ($FeSO_4{\cdot}7H_2O$), Sigma), K (potassium chloride (KCl), Sigma), Mg (magnesium sulphate ($MgSO_4$), VWR), Mn (manganese chloride tetrahydrate ($MnCl_2{\cdot}4H_2O$), Sigma-Aldrich), Na (sodium chloride (NaCl), Merck), P (85% phosphoric acid (85% H_3PO_4), Alfa Aesar) and Zn (zinc sulphate heptahydrate ($ZnSO_4{\cdot}7H_2O$), Sigma-Aldrich; Table S1). For some media (Al, Cu, Fe, P and Zn), the addition of

high-mineral concentrations before sterilisation prevented media from solidifying after autoclaving. This was likely due to acidic pH at high temperature which hydrolysed the agar (Kanazawa & Kunito, 1996). Adding minerals to these diets after they were autoclaved allowed the media to solidify like all other diet treatments. Since it was not possible to add specific elements in isolation, minerals containing focal elements were selected from standard published protocols with the aim of avoiding minerals that supplemented other limiting nutrients. Nevertheless, some mineral-specific effects were likely unavoidable. For instance, the effects of calcium on cultivar performance may vary across mineral salts (e.g. $CaCl_2$ or $CaSO_4$) due to the presence of different secondary anions. Such interactions represent an exciting future extension of this approach.

We initially performed a pilot study to identify experimentally relevant concentration ranges for each mineral, inoculating and incubating plates as described above over 70 days. We used diet treatments including all seven Pr:C ratios at the 8 g/L Pr+C concentration, with eight concentrations for each mineral (n = 3 replicates/condition+3 baseline replicates with no mineral/condition; n = 1,890 plates). We chose three representative concentrations for each mineral: baseline (no mineral added), highest growth and highest concentration enabling growth (Figure S1). We then expanded the experiment to macronutrient concentrations of 4 and 25 g/L for the seven Pr:C ratios for the three concentrations for each mineral (n = 3 replicates/condition+3 baseline replicates/condition; n = 1,260 plates).

Measuring fungal performance

After the defined period of growth, we outlined the outer edge of fungal expansion and photographed each plate using a Canon EOS 7D Mark II camera mounted on a fix stand. We used ImageJ (v1.52a; Schneider et al., 2012) to estimate fungal expansion (area, mm^2) based on the final circumference line drawn around outer border of the fungus using threshold contrast-adjusted greyscale images (with $pixel^2$ = 0.02). We counted staphylae directly from plates viewed under a dissecting microscope. We used the pheatmap package (v1.02.12; Kolde, 2015) in RStudio v3.6.2 (RStudioTeam, 2020) to plot hyphal growth across the seven Pr:C ratios and 16 mineral concentrations for the 8 g/L Pr+C dilution (Figure S1). We used the fields package (v10.3; Nychka et al., 2017) in RStudio to plot cultivar hyphal growth and staphyla density across all diet treatments and dilutions and visualise the interactive effects of nutrients and mineral elements on fungal FNN dimensions. We set the topological resolution of the response surfaces with $\lambda = 0.001$ as the smoothing parameter (Figures 2 and 3; Figures S2 andS3). While cultivar performance was measured across growth media with nutrients added in g/L, nutrients in field-collected plant fragments were expressed as % dry biomass (as is explained in the next sections). To achieve a common currency for comparing these datasets, cultivar FNNs were plotted on nutritional landscapes where % protein and carbohydrates mass were expressed relative to the total dry biomass of the growth media including non-nutritive components like agar.

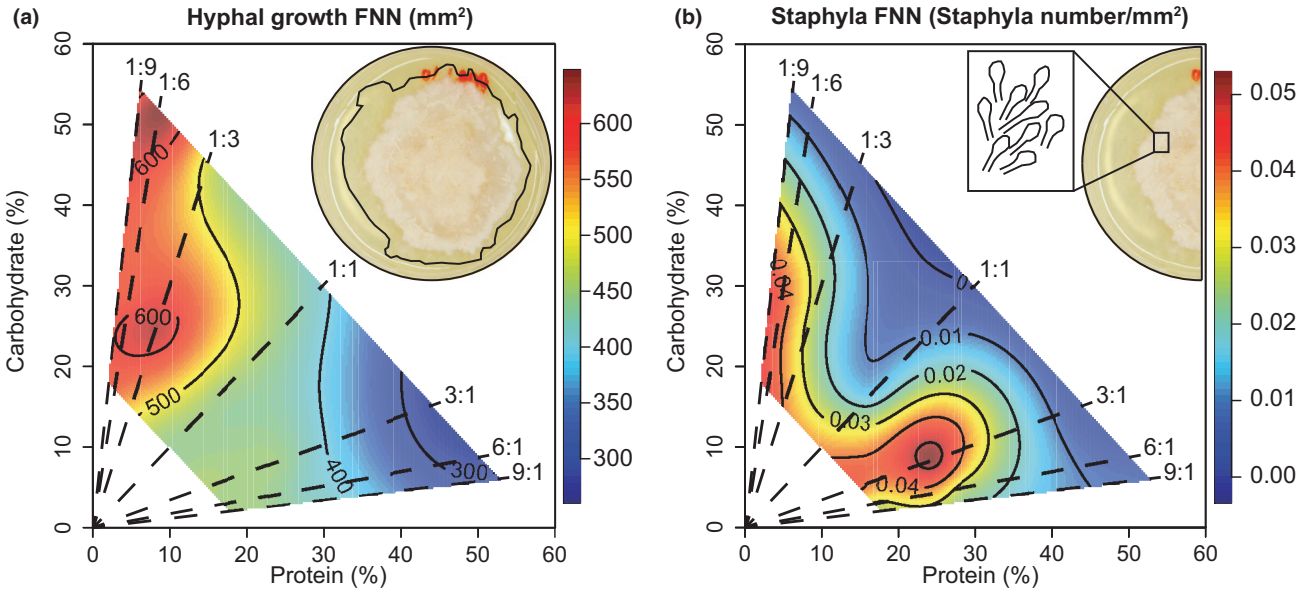

FIGURE 2 Quantifying the macronutrient fundamental nutritional niche (FNN) of the *Leucoagaricus gongylophorus* fungus cultivated by *Atta colombica* leafcutter ants. (a) Hyphal growth and (b) staphyla density could both be maximised when provided carbohydrate-biased media, and both traits declined when protein-biased provisioning exceeded 30%. Staphyla density exhibited a second FNN peak at elevated protein concentrations (up to 30%) and relatively lower carbohydrate concentrations (up to 20%). Nutritional landscapes were generated by isolating *L. gongylophorus* from an *A. colombica* colony and performing in vitro experiments with nutritionally defined media varying in protein:carbohydrate ratios (from 1:9 to 9:1 Pr:C) and protein + carbohydrate concentrations (4, 8 and 25 g/L Pr+C)

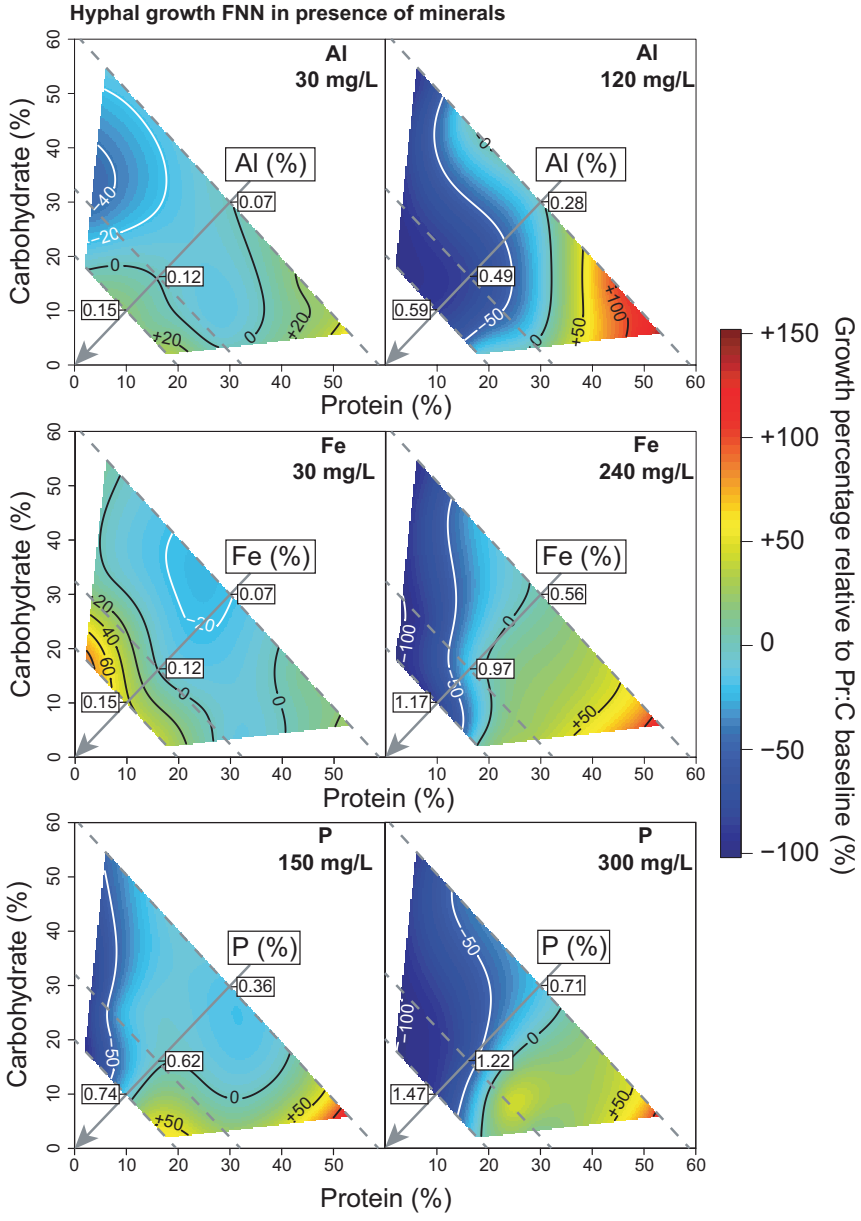

FIGURE 3 Quantifying the interacting effects of minerals and macronutrients on fungus-cultivar growth. Three minerals (Al, Fe and P) expanded the fundamental nutritional niche towards elevated growth in protein-rich conditions relative to baseline conditions without these minerals. Here, we calculated relative growth percentage using the difference between cultivar's final growth area in the presence of each mineral relative to the same macronutrient condition without the mineral. The diagonal grey arrow indicates the gradient of mineral percent relative to protein and carbohydrate percent in diets. Two mineral concentration addition treatments are shown. White isoclines indicate reduced growth relative to the macronutrient baseline, and black isoclines indicate increased growth. Seven other tested minerals (Ca, Cu, K, Mg, Mn, Na and Zn) induced varying degrees of toxicity for the cultivar across the gradient of protein and carbohydrate availability (see Figure S3)

Substrate collections from free-ranging *A. colombica* colonies

To determine *A. colombica* RNNs, we located six colonies of *A. colombica* in the lowland tropical rainforest at Soberanía National Park, Panama during wet season (a period of high ant activity) from 2 May to 29 June 2019 (Table S2). Vouchers of *A. colombica* ants were deposited in the Museo de Invertebrados Fairchild, Universidad de Panama. We laid on trash bags next to the most active foraging trail close to each colony's main nest entrance and collected plant substrates from laden returning foragers. Each collection event was performed by two observers over 1.5 h (between 9:00 and 12:00 AM) and was repeated for each colony over three non-consecutive days ($N = 9$ collection hours per colony). Each collection event included three 30-min sampling periods, after which all collected substrates were placed into Ziploc bags and stored in a cooler. Between each 30-min plant-fragment sampling period, we counted the number of laden returning foragers on trails during three 10-min observation periods (using a manual counter). In this way, we

estimated colony foraging activity in units of mean number of laden workers per minute. This sampling showed that colonies foraged 2166 (±283 SD) total fragments per 30 min, indicating that RNN samples comprised *c*. 40% (±9% SD) of each colony's total foraging effort (Figure S4). Back at the laboratory, we used a dissecting stereoscope to separate and weigh plant fragments into plant morphospecies groups based on venation, thickness and pilosity of leaf fragments, and the morphology of flower and fruit fragments. Substrates were further separated per colony and collection day, and then freeze-dried with a BenchTop Pro freeze-dryer (SP Scientific) for 24 h. We weighed these dry samples and stored them at −20°C in Ziploc bags with silica gel.

DNA barcoding identification of plant samples

We homogenised freeze-dried plant samples in 10% Chelex (Sigma) and extracted DNA following 30 min of incubation at 100°C. We amplified by PCR the *Internal Transcribed Spacer 1* (*ITS1*; ~276 bp) genetic marker using primers containing both generic M13 sequences (used for subsequent sequencing performed by Eurofins Genomics) and *ITS1*-specific Trac01 sequences (M13F-Trac01F 5' TGTAAAACGACGGCCAGTGATATCCRTTGCC GAGAGTC 3'; M13R-Trac01R 5' CAGGAAACAGC TATGACGAAGGAGAAGTCGTAACAAGG 3'). We performed a blast-n with the DNA sequences in the NCBI database and attributed species identification to the best hit (based on *E*-value and percent identity). When a given sequence obtained more than one equally possible result, we restricted the identification to the genus level. We identified 44 plant species from the 87 samples that we initially categorised into morphospecies (Table S3).

Protein and carbohydrate composition of plant samples

To quantify macronutrient RNNs, we used near-infrared reflectance spectroscopy (NIRS) to estimate concentrations of protein (from total nitrogen) and carbohydrates (water-soluble carbohydrates+starch) for the 87 initially identified sample types. We placed freeze-dried plant fragments in centrifuge tubes, plunged them into liquid nitrogen and homogenised them using a plastic pestle. We then used these homogenised samples to acquire NIRS spectra using an Antaris II FT-NIR Analyzer (Thermo Scientific) from 4.000 to 10.000 cm^{-1} (2.500 to 1.000 nm) at a resolution of 16 cm^{-1} and 2× gain. We used the standard default instrument calibration with no sample as the reference measurement. Each spectrum acquisition was the mean of 32 monochromatic scans. We calculated a mean from three replicate spectrum acquisitions (each following sample repacking) for each sample. We selected a representative subset of samples for wet chemical analyses using principal component analysis (PCA) on centred NIRS spectra for the 87 samples after pre-processing using first derivative model on SIMCA software (Umetrics). We selected samples for further chemical analyses according to their position on PCA axes (farthest away from the centre of the data and within the large cluster of scores; Næs et al., 2002), and depending on whether we had sufficient biomass to meet the requirements for chemical analyses.

We used a CN analyser (Eurovector) coupled to an isotope ratio mass spectrometer (Isoprime) to quantify total nitrogen from 3 to 4 mg of ground samples. We then estimated the quantity of crude protein by multiplying total nitrogen by 6.25. While this is a standard conversion for estimating crude protein in literature (Felton et al., 2009), this approach does not account for variation in the metabolic accessibility of proteins in plant material (e.g. some are bound in recalcitrant fibres or by secondary metabolites; Wallis et al., 2010). We used the crude protein approach given that much remains unknown about how the derived suite of *L. gongylophorus* enzymes interacts with the macerating action of leafcutter ant workers to digest recalcitrant plant materials and help the cultivar accessing proteins that may not be available to other herbivores (De Fine Licht et al., 2010).

We estimated total non-structural carbohydrates (*hereafter* 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 homogenised plant material, respectively. We used peach powder as a positive control and water as a negative control in these analyses. We used these empirically determined data to build partial least squares regression prediction models of the percentage of total protein and carbohydrates using the first derivative of the NIRS spectra in SIMCA software (Umetrics; Wold et al., 2001). See Table S4 for details about subsequent model validation approaches.

We used barcoding results to combine conspecific samples by calculating mean protein and carbohydrate values and used these data to generate RNNs for each of the six colonies (Figures S5 and S6) and to generate a composite RNN for the population of *A. colombica* in Soberanía Park (Figures 4 and 5). We defined RNNs as the region bounded by each general plant substrate type (leaf, fruit and flower). The macronutrient RNN from one of the colonies (colony 4) was previously published (Shik et al., 2021) as part of a comparative analysis of fungus-farming ants and is included here in a different conceptual context and as part of a much-expanded dataset about mineral–macronutrient foraging ecology of *A. colombica*.

We next estimated the intake target selected by *A. colombica* colonies, defined as the nutritional blend selected by a colony that in principle maximises cultivar's performance, and against which excess or deficient

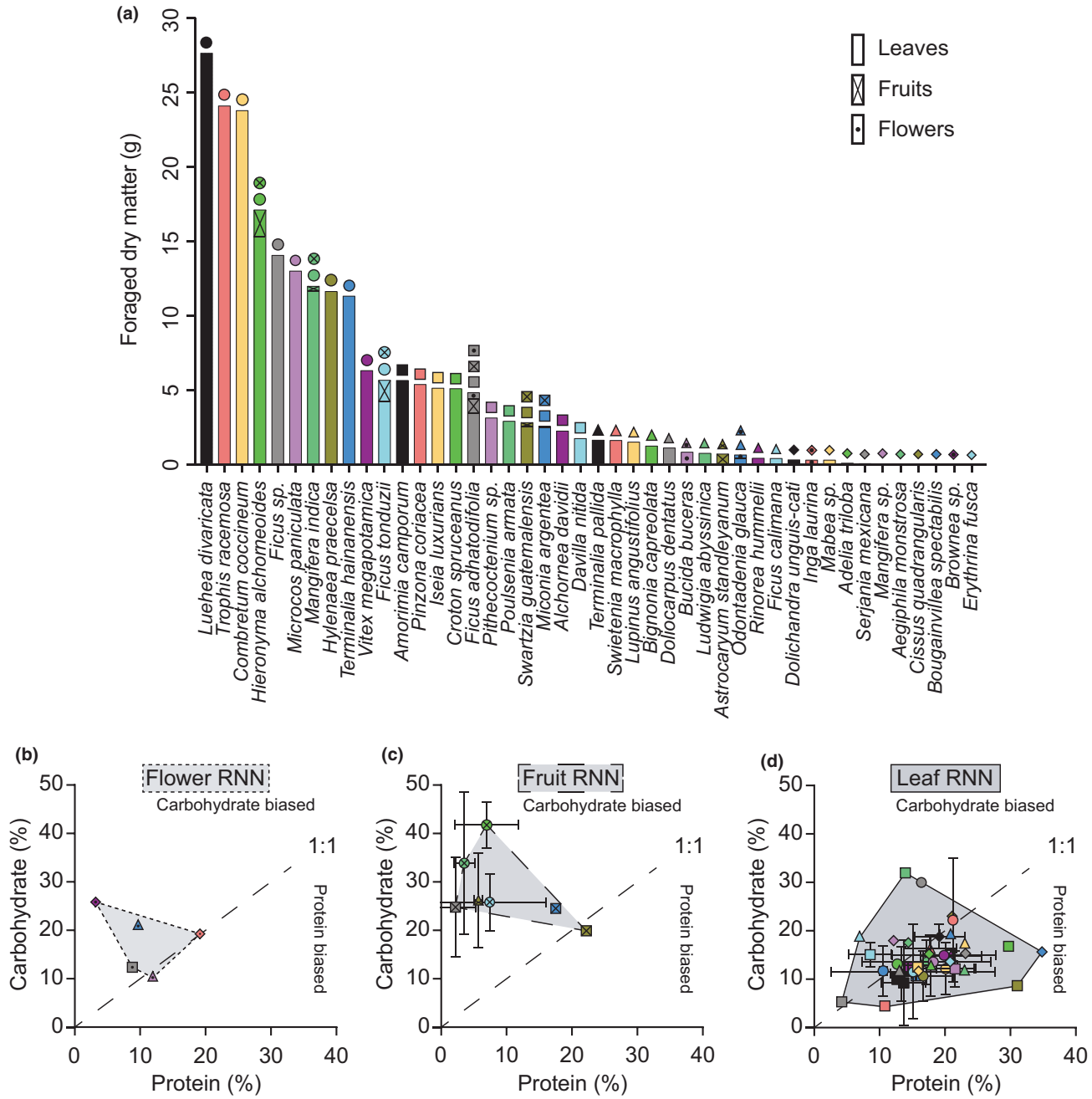

FIGURE 4 The plant fragments foraged by free-ranging leafcutter ants yielded a broad protein–carbohydrate realised nutritional niche (RNN), but each substrate type (flower, fruit and leaf) had distinct RNN dimensions. (a) Colonies of *Atta colombica* foraged mostly on leaves but also collected fruits and flowers from a total of 44 plant species. (b–d) Each species of flower, fruit and leaf fragment could be arrayed across a nutritional landscape based on their percent protein and carbohydrate content. The flower and fruit fragments foraged by leafcutter ants yielded carbohydrate-biased RNNs and leaf fragments yielded a protein-biased RNN

intake can be inferred (Behmer, 2009; Shik & Dussutour, 2020). We calculated this macronutrient intake target by translating the substrate collection data into realised levels of foraged protein and carbohydrates using arithmetic means weighted relative to total biomass (Chambers et al., 1995). For each substrate, we multiplied the percent protein and carbohydrates by the associated dry biomass (M). We then summed these values for each colony-observation period and divided this by the summed dry biomass of all substrates corresponding to colony-observation period. We used the formula illustrated here for protein:

$$\text{Protein \% IT day 1} = \frac{\left(\left(\text{Protein \%}_1 * M_1\right) + \left(\text{Protein \%}_2 * M_2\right) + \ldots\right)}{\left(M_1 + M_2 + \ldots\right)}.$$

We then calculated colony-level intake targets by averaging intake targets across the three observation periods for

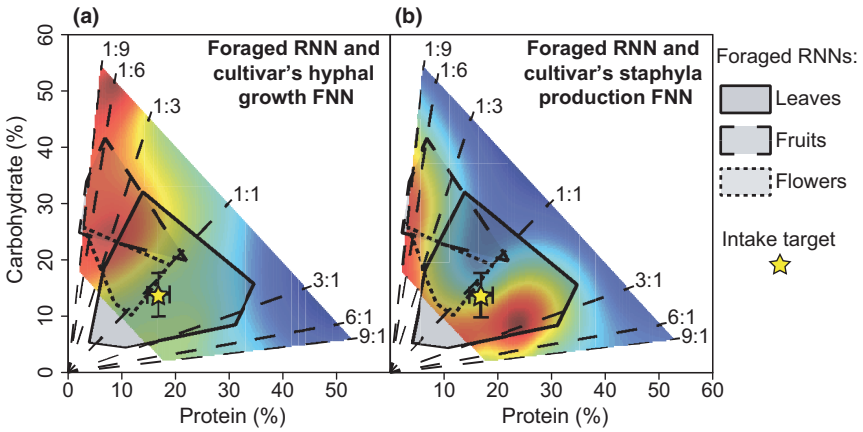

FIGURE 5 Leafcutter foragers collect realised nutritional niches (RNNs) whose dimensions match the fundamental nutritional niches (FNNs) of their cultivar. (a) The RNN polygons of flowers (n = 5 species) and fruit fragments (n = 7 species) overlapped with the carbohydrate-rich FNN for maximal hyphal growth, even as the RNN polygon of leaves (n = 40 species) extended beyond optimal protein concentrations. (b) The leaf RNN overlapped with the protein-rich FNN for maximal staphyla density, and the fruit and flower RNNs overlapped with the carbohydrate-rich FNN for maximal staphyla density. The overall macronutrient intake target was slightly protein-biased (>1:1 Pr:C) and was governed by leaf nutrients that contributed the majority of the foraged biomass. These results reflect the composite foraging of six *Atta colombica* colonies observed during 54 collection hours. Results for individual colonies are provided in Figures S5 and S6.

each colony and calculated a composite *A. colombica* intake target by averaging across the six colony-level intake targets (Table S5).

Elemental composition of plant fragments

We used inductively coupled plasma optical emission spectrometry (ICP-OES; Agilent 5100, Agilent Technologies) to compare the elemental composition of Al, Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn in plant substrates (Chen et al., 2020; Głazowska et al., 2018). Apple powder (known standard) and MilliQ water (negative control) were analysed as reference samples. See supporting protocols in Supporting Information for details about sample preparation. For each sample, we used three technical replicates to calculate a mean value for each element. For each substrate type collected for each plant species, we calculated a mean (±SD) for each element. Colony element intake targets were estimated by calculating the mean of the three weighted means (one for each day of collection) as described in previous paragraph (Figure S7; Table S5). We then mapped substrate mineral concentrations across the gradients of protein and carbohydrate concentrations in plant fragments using the fields package v10.3 (Nychka et al., 2017) in RStudio with the topological resolution set to $\lambda = 0.001$ (Figure S8). To test for possible macronutrient–mineral foraging regulation, we extracted the outlines of the dark read areas (i.e. isoclines representing the highest 5% mineral concentrations) for each mineral from Figure S8B and plotted them relative to the composite macronutrient intake target selected by ants. We then measured the distance (in units of pixels) between the centre of the macronutrient intake target and the closest outer edge of each isocline of maximal mineral concentration using ImageJ.

Statistical analyses

Statistical analyses were performed in RStudio v1.2.5042 (RStudioTeam, 2020). We log-transformed variables when necessary to improve normality. We used least-square regressions and ANOVA to assess the underlying significance and interactions of both linear and quadratic terms for carbohydrate and protein. Results of these tests were used to support the interpretation of FNN heatmaps showing variation in fungus hyphal growth area and staphyla density across the 21 protein and carbohydrate baseline diet combinations (Tables S6 and S7). To support the interpretations of FNN heatmaps for each mineral element, we used least-square regressions and ANOVA to assess the underlying significance and interactions of both linear and quadratic terms for carbohydrate, protein and mineral effects on hyphal growth area across 63 protein:carbohydrate:mineral diet combinations (Tables S8–S10). To determine possible macronutrient–mineral foraging regulation, we used Pearson tests to assess whether maximal mineral tolerance of the cultivar increased with (1) maximal mineral content of plant substrates or (2) the distance between maximal mineral concentrations and macronutrient intake target (Figure 6b,d). Corresponding datasets and R scripts are available in Dryad (https://doi.org/10.5061/dryad.6t1g1jx01) and Zenodo (https://doi.org/10.5281/zenodo.5160258), respectively (Crumière et al., 2021a, 2021b).

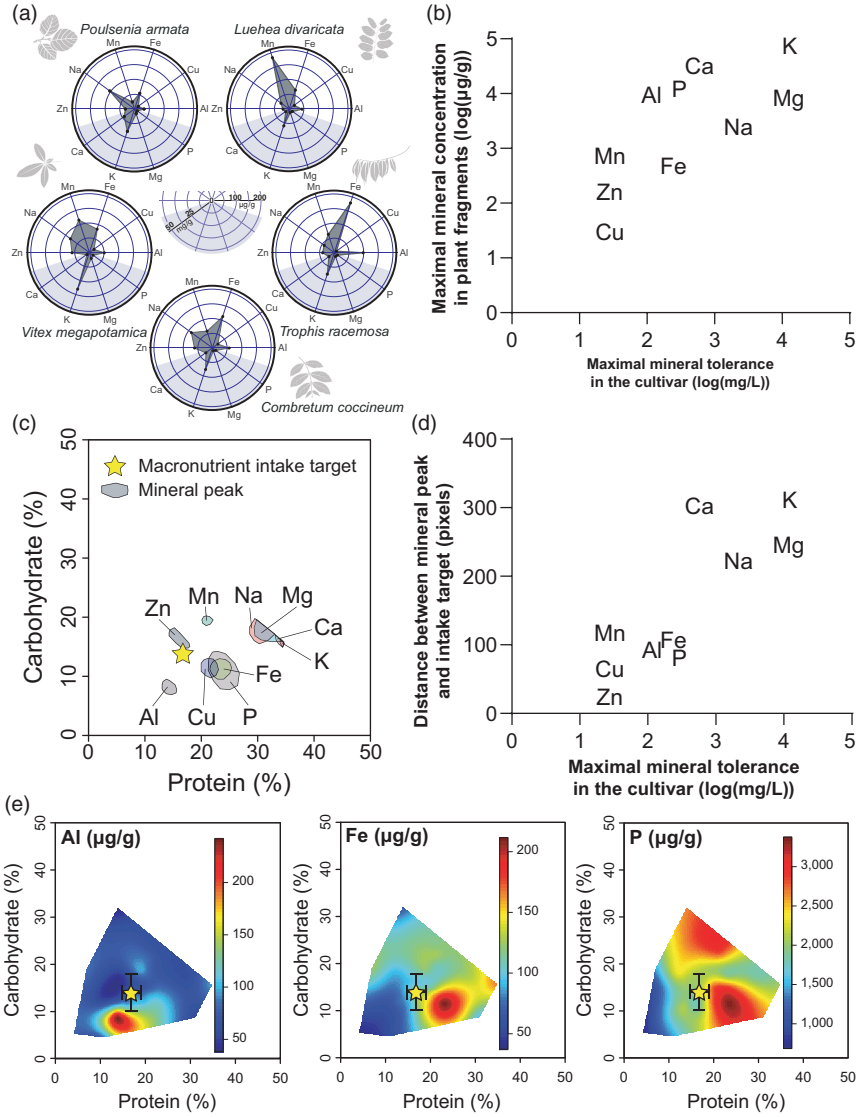

FIGURE 6 Testing for interactions between the mineral profiles and the macronutrient realised nutritional niches (RNNs) of foraged plant fragments. (a) Leaf mineral profiles of five foraged plant species illustrate the variation in concentrations of 10 minerals observed across the 44 plant species. The minerals Ca, K, Mg and P (blue shaded region of radial plots) are expressed in concentrations of mg/g, and Zn, Na, Mn, Fe, Cu and Al are expressed in µg/g (white region). (b) The cultivar's maximal in vitro tolerance for each mineral increases with the mineral's maximal concentration in foraged plant fragments (Pearson correlation test: $r = 0.72$; $p = 0.02$). (c) Maximal mineral concentrations in foraged leaf fragments (dark red areas extracted from Figure S8B) are overlaid across a gradient of protein and carbohydrates and interpreted relative to the Pr:C intake target. (d) The most toxic mineral elements (Cu, Mn and Zn) are located nearer to the macronutrient intake target compared to the least toxic minerals (Ca, K, Mg and Na). This was assessed by measuring the distance between the maximal concentration of each mineral and the macronutrient intake target. This distance was found to be positively correlated with the respective maximal mineral concentration the cultivar could tolerate in vitro (Pearson correlation test: $r = 0.83$; $p = 0.003$). (e) The three elements enhancing the cultivar's in vitro protein tolerance also tend to reach their highest concentrations in the most protein-biased leaf fragments foraged by ants. Leaf illustrations in panel A are by Damond Kyllo

RESULTS

Minerals shape the cultivar's macronutrient requirements

We first established a performance baseline that quantified the cultivar's FNNs for hyphal growth and staphyla density across an in vitro macronutrient gradient of protein and carbohydrate (Pr:C) availability. The results echoed recent findings (Shik et al., 2021) and included lower nutritional concentrations to visualise cultivar's FNN across a broader range of plant substrates. Maximal hyphal growth occurred across a broad carbohydrate gradient representing up to 60% of total diet dry mass and with carbohydrate-biased Pr:C ratios ranging from 1:9 to 1:1 Pr:C (i.e. red area in Figure 2a; Figure S2A; Tables S6 and S7). Staphyla density was maximised across a narrower range of

carbohydrates (up to 40%) but a wider range of protein than hyphal growth (up to 30%), and had two distinct peaks, the first in a carbohydrate-biased region below 1:3 Pr:C and the second in a protein-biased region below 6:1 Pr:C (red areas in Figure 2b; Figure S2B–C; Tables S6 and S7). These results indicate that both fungal traits are more sensitive to fluctuations in protein than carbohydrates, and that colonies have opportunities to use targeted doses of protein to selectively promote staphyla production.

We next examined mineral effects on cultivar growth relative to the macronutrient baseline. We focused on hyphal growth as staphylae were absent from most mineral addition plates. Three minerals (Al, Fe and P) increased cultivar growth in the previously toxic protein-rich media (Figure 3; Tables S8–S10). Other minerals either caused general toxicity effects by narrowing cultivar's FNN dimensions (Mn, Cu and K) or reducing cultivar growth across all protein and carbohydrate combinations (Ca, Mg, Na and Zn; Figure S3; Tables S8–S10). Fluctuations in mineral concentrations can thus reduce cultivar's growth performance and may render plant fragments with seemingly optimal macronutrient blends unsuitable for cultivar provisioning.

Macronutrient RNN targeted by free-ranging leafcutter ants

We next explored whether and how the cultivar's FNN governs nutrient foraging strategies of free-ranging leafcutters. We first quantified RNN dimensions in terms of protein and carbohydrates based on collections of 44,533 plant fragments (dry mass 220.38 g) from 44 plant species (Figure 4a; Table S3). Colonies exploited similar numbers of plant species, although no species was common to all six colonies and most species were foraged at low levels (Figure 4a; Figures S9 and S10; Table S11). Colonies could target distinct RNN dimensions by collecting different substrate types as flower and fruit fragments (Figure 4b,c) provided RNNs that were carbohydrate-biased relative to the protein-biased RNN provided by leaf fragments (Figure 4d). These foraged plant fragments generally provided a broad RNN that overlapped with the cultivar's FNNs for maximal hyphal growth and staphyla density (Figure 5).

And yet, the leaf fragments that comprised 96.2% of the overall foraging effort (Figure 4a; Figure S10) provided an RNN with protein levels that can potentially reduce cultivar growth performance (Figure 5a). Additionally, since leaves were the most foraged substrate type, they also governed each colony's intake target, such that leafcutter foragers selected protein levels beyond the cultivar's FNN for maximal hyphal growth, but near the protein-biased RNN for maximal staphyla density (Figure 5; Figures S5 and S6).

Optimal foraging requires multidimensional nutritional regulation

We next examined whether and how colonies regulate their mineral foraging. We predicted that since trace elements (Cu, Mn and Zn) are usually required at low levels to mediate an array of metabolic processes, their excess would more strongly inhibit cultivar performance relative to the more abundant flux minerals (Ca, K, Mg and Na) that function as ions moving across cell membranes (Kabata-Pendias, 2010; Kaspari, 2020). Foraged mineral profiles varied widely across plant fragments (Figure 6a; Table S11) and across leafcutter colonies (Figure S7; Table S5). Despite this variation, trace minerals tended to occur at lower concentrations in foraged fragments (micrograms per gram of plant tissue) than flux minerals (up to milligrams per gram of plant tissue). As predicted, increasing concentrations of trace minerals also more rapidly inhibited cultivar performance than the flux minerals, with Cu, Mn and Zn inducing toxicity effects at concentrations of 60 mg/L, but with Ca, K, Mg and Na being tolerated at concentrations exceeding 600 mg/L (Figure 6b; Figure S1).

Building upon these results, we tested a hypothesis assessing whether and how the cultivar's tolerance to these mineral elements is mediated by macronutrients (Figure 3). Individual foraging insect herbivores are known to tolerate higher toxin concentrations when consuming foods that also contain optimal macronutrient blends close to their intake target (Simpson & Raubenheimer, 2001). We extended this hypothesis to leafcutter ants, focusing on the leaf fragments that represent the majority of foraged substrates (Figure 4a). We found that concentrations of the more toxic trace minerals (Cu, Mn and Zn) in foraged leaves tended to peak closer to the macronutrient intake target compared to the less toxic flux minerals (Ca, K, Mg and Na) that peaked in more protein-biased substrates (Figure 6c,d; Figure S8). This suggests that negative effects of mineral surplus may be mitigated when cultivar receives optimal macronutrient blends.

We finally explored variation in the three minerals (Al, Fe and P) that enhanced the cultivar's protein tolerance (Figure 3) and found that they were more abundant in protein-biased leaf fragments (Figure 6e). This suggests that these minerals are associated with expanded RNNs enabling colonies to access carbohydrates from plant fragments that would otherwise contain protein at levels toxic to the cultivar. More generally, this result highlights that successful farming systems require multidimensional nutrient regulation and suggests a wealth of unexplored reciprocal adaptations in ants and fungal cultivars to navigate this nutritional landscape of foraging and provisioning.

DISCUSSION

This study extends NG beyond the foraging challenges of individuals targeting their own FNN needs (Raubenheimer & Simpson, 2003), and beyond individuals provisioning kin or conspecifics (Dussutour & Simpson, 2009), to a case where insect foragers collect plant fragments to provision a fungal mutualist. We provide a hypothesis-testing framework for disentangling these nutritional challenges and for explaining how the physiological needs of a microbial symbiont shape the niche breadth of its host. Our results support that (1) ants regulate nutritional intake by foraging across plant species and substrate types to collect a RNN whose dimensions overlap with their cultivar's FNN and (2) this provisioning must be optimised in multiple nutritional dimensions.

We also find evidence of a paradox underpinning the generalist herbivory of leafcutter farming systems—that colonies forage across hundreds of nutritionally variable plant species despite provisioning a cultivar with narrow tolerance for variation in key nutritional dimensions. For instance, a plant fragment can contain carbohydrate concentrations that maximise cultivar growth and still be unsuitable for cultivar provisioning if its protein concentration exceeds *c*. 20%. Quantifying higher-order nutritional interactions may help resolve this paradox since this same protein-rich plant fragment may actually be suitable if it contains a range of Al, Fe or P that enhances the cultivar's protein tolerance. The capacity of leafcutter farming systems to capitalise on natural variation in plant fragment mineral-macronutrient concentrations would add to an impressive list of other derived farming strategies. These include adaptations in the *L. gongylophorus* fungus enabling it to express enzymes that detoxify fresh vegetation (De Fine Licht et al., 2013) and degrade complex carbohydrates (De Fine Licht et al., 2010; Kooij et al., 2011).

The behavioural and physiological integration of specialised ants with their fungal mutualists is also likely critical for nutrient regulation (Shik et al., 2018). For instance, a specialised caste of gardener ants ingests fungal gongylidia and deposits faecal droplets vectoring nitrogen-rich compounds (Martin & Martin, 1970) and enzymes across the fungal garden to help the growing cultivar digest and assimilate freshly deposited plant fragments (Schiøtt et al., 2010; Shik et al., 2018). As they produce this nutritional mulch, it is reasonable to predict that gardeners dynamically detect the needs of their fungal mutualist and provision it with a narrowed version of the broad RNNs collected by foragers (Arenas & Roces, 2016; Herz et al., 2008). The critical regulatory decision points may thus depend on the cues (Green & Kooij, 2018; Khadempour et al., 2021) that enable ants to detect their cultivar's immediate nutritional needs and then adjust provisioning.

Leafcutter ants can potentially regulate nutritional intake more effectively than unitary generalist herbivores as each colony can recruit thousands of ants to simultaneously sample many chemically diverse plants (Csata & Dussutour, 2019). And yet, this distributed foraging also poses unique nutritional challenges. For instance, gardener ants in the nest must detect feedback from their cultivar about its nutritional needs (Arenas & Roces, 2016; Green & Kooij, 2018) and then communicate this information to foragers (Herz et al., 2008; Saverschek et al., 2010) and to other workers that often transport large numbers of potentially suboptimal fragments directly into trash heaps (Hart & Ratnieks, 2002; Hudson et al., 2009). These behaviours provide analogies to the 'pre-ingestive' and 'post-ingestive' stages of nutrient regulation seen in unitary herbivores and may provide opportunities to mediate RNN dimensions (Behmer, 2009).

The nutritional contributions of bacteria within the farming symbiosis are also increasingly coming into focus, as they fix nitrogen (Sapountzis et al., 2015), metabolise citrate (Sapountzis et al., 2018), detoxify plant secondary metabolites (Francoeur et al., 2020) and potentially assist mineral provisioning (Khadempour et al., 2020). Leafcutter ants are also the crown group of a monophyletic clade of over 250 fungus-farming ant species that scavenge mostly insect frass, tiny decaying wood pieces, flower bits and occasionally mineral-rich insect cuticles for cultivar provisioning (De Fine Licht & Boomsma, 2010; Shik et al., 2021). The approach developed in this study provides a means of linking physiological traits of these diverse cultivars with the specific multidimensional nutritional challenges faced by foraging workers in diverse environments. Moreover, next steps include linking provisioned RNN dimensions with the nutritional quality of the fungal cultivar and assessing whether colonies can capitalise on this to produce a nutritionally flexible crop that targets the colony's specific nutritional needs.

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AUTHORS' CONTRIBUTIONS

A.J.J.C. and J.Z.S. designed the study and experiments. A.J.J.C., S.M., A.J., P.L., A.M. and R.R. performed the experiments and collected the samples and data. A.J.J.C.

analysed the data. A.J.J.C. and J.Z.S. interpreted the data and wrote the original draft.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Dataset and R script used for this study are available in Dryad (https://doi.org/10.5061/dryad.6t1gljx01) and Zenodo (https://doi.org/10.5281/zenodo.5160258) respectively.

ORCID


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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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