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Title: Fungal infection alters collective nutritional intake of ant colonies

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SUMMARY

In animals, parasitic infections impose significant fitness costs¹⁻⁶. Yet, infected animals can alter their feeding behavior to combat parasites⁷⁻¹². For instance, they can adjust nutrient intake to support immune function^{9,10}. However, parasites can manipulate animal foraging behavior to their own benefits¹³⁻¹⁶. How nutrition influences host-parasite interactions is not well understood. Until now, studies have mainly focused on the host, and less on the parasite^{9,12,17-23}. Here, we used the Nutritional Geometry Framework²⁴ to investigate the role of amino acids (AA) and carbohydrates (C), in a host-parasite system: the Argentine ant, *Linepithema humile*, and the entomopathogenic fungus, *Metarhizium brunneum*. First, using 18 diets varying in AA:C composition, we established that the fungus performed best on the high-amino acid diet 1:4. Second, we found that the fungus was able to reach this optimal diet when given various diet pairings, revealing its ability to cope with nutritional challenges. Third, we showed that the optimal fungal diet reduced the lifespan of healthy ants when compared to a high-carbohydrate diet, but had no effect on infected ants. Fourth, we revealed that infected ant colonies given a choice between the optimal fungal diet and a high-carbohydrate diet, chose the optimal fungal diet whereas healthy colonies avoided it. Lastly, by disentangling fungal infection from host immune response, we demonstrated that infected ants foraged on the optimal fungal diet following immune activation to maintain immune function and not as a result of parasite manipulation. Therefore, we revealed that infected ant colonies chose a diet that is costly for survival in the long term but beneficial in the short term, a form of collective self-medication.

Keywords: Host-parasite interactions, Nutritional Geometry, Social insects, Fungal pathogens, Immune system, Ants

RESULTS

In this study, our objective was to unravel the impact of diet composition on the outcome of a host-parasite interaction from the perspective of both the parasite and its host. First, we established the optimal diet composition of the fungal pathogen *Metarhizium brunneum* for two key nutrients, amino acids (AA), the building blocks of proteins, and carbohydrates (C). To this end, we confined the fungus to one of 18 artificial diets, varying in both their AA:C ratio and their total concentration AA+C (Data S1A and Data S1B). For each diet, we measured the extent of fungal expansion (growth rate: area mm²) after 14 and 30 days, when fungal conidiospores (hereafter abbreviated as spores) were harvested and counted. We found that the number of spores produced by the fungus per unit of surface depended on both AA:C and AA+C. The fungus produced the highest number of spores on the high-amino acid diet 1:4 with an intermediate concentration of 33g.L⁻¹ ($p < 0.001$, Figure 1A, Figures S1A and S1B, Data S2A). The fungus also grew to cover the largest area on this diet (Figures 1B, 1C and 1D) after both 14 days ($p < 0.001$, Figures S1C and S1D, Data S2B) and 30 days ($p < 0.001$, Figures S1E and S1F, Data S2C). Hence, the fungal reproduction, estimated from the total area covered by the fungus and the number of spores produced per unit of surface, was the highest on the high-amino acid diet 1:4 (Figure 1E, Data S2D). Since spore quality also affects fungal fitness, we investigated if viability differed between spores harvested on different diets (1:199, 1:4 and 2:1). Spores harvested on the optimal fungal diet 1:4 and transferred to a standard medium had the highest chance of germinating and producing new spores (Figure 1F, Data S2E).

We further tested if the fungus could combine AA and C from two highly imbalanced but complementary diets to maximize its fitness. Six different diet pairings were tested, each of them offering an amino acid-biased diet 2:1 and a carbohydrate-biased diet (1:199, 1:49, 1:24, 1:16, 1:12 or 1:8, Figure S2). After 30 days, the fungus produced the same number of spores per unit of surface in all diet pairings in the choice experiment as on the optimal fungal diet 1:4 in the previous no-choice experiment ($p = 0.157$, Figure 2A, Data S2F). Likewise, the overall area covered by the fungus in all diet pairings was the same as on the optimal fungal diet 1:4 (Figure 2B, Figure S3 and Data S2G for observations at day 14), yet the fungus was differently distributed over the two diets: the fungal area on the amino acid-biased diet (2:1) was always smaller than that on its paired carbohydrate-biased diet ($p < 0.001$, Data S2H). This result revealed that, by different growth patterns, the fungus

maximized spore production on any of the tested diet pairings, revealing its ability to use a wide range of diet compositions.

Nutrition is a key factor modulating the response of hosts to infection as diet composition affects pathogen resistance. For instance, AA shortage decreases survival after infection²⁵ while a diet supplemented with C promotes resistance to infection¹². Here, we investigated how the survival of healthy and *Metarhizium*-infected ants was affected by diet composition. We followed the survival of 200 ants kept solitarily for 64 days confined to either the high-carbohydrate diet 1:199 or the high-amino acid diet 1:4, after being exposed to the fungus or not. We showed that both infection ($p < 0.001$) and diet ($p = 0.01$) influenced the survival of the ants with a non-significant interaction between these two factors ($p = 0.10$, Figure 3A, Data S3A). Infected ants died faster compared to uninfected ants on both diets. The high-amino acid diet decreased drastically the survival of uninfected ants when compared to the high-carbohydrate diet ($p = 0.020$, Data S3B). In contrast, infected ants fed the high-amino acid diet lived as long as those fed the high-carbohydrate diet ($p = 0.840$, Data S3B). Thus, infection and the high-amino acid diet did not have a cumulative negative effect on survival, suggesting that feeding on the high-amino acid diet was harmless and potentially beneficial for infected ants.

We next tested whether infection would alter the collective foraging strategy of the colony. We offered both infected and uninfected colonies a choice between the high-carbohydrate diet 1:199 and the high-amino acid diet 1:4 once a week over a total of 5 weeks. Foraging activity (total number of ants visiting both diets) decreased throughout the experiment ($p < 0.001$, Figure 3B, Data S3C), but was not affected by the colony's infection status ($p = 0.615$, Figure 3B, Data S3C), even though infected colonies had a higher mortality than the uninfected ones ($p < 0.001$, Figure 3D, Data S3D). Uninfected colonies selected the high-carbohydrate diet while infected colonies preferentially targeted the high-amino acid diet ($p < 0.001$, Figure 3B, Data S3E). The slopes of the regression lines in Figure 3C represent the regulated AA:C ratio of infected and uninfected colonies. Infected colonies converged to a higher AA:C ratio (1:6) than uninfected colonies (1:12) ($p = 0.002$, Figure 3C, Data S3F). Interestingly, in this experiment, infected colonies modified their foraging strategies even if only a minority of workers were infected (on average 12% of the corpses showed infective fungal outgrowth).

The change in foraging strategy observed in infected colonies could either reflect a parasite manipulation towards the fungal optimal diet or a host strategy to compensate for the increased use of AA during the immune response^{23,26}. To disentangle these two hypotheses, we subjected the ants to either a full infection with live *Metarhizium* or only induced an antifungal immune response in the absence of live fungus by injection of fungal cell wall material (β -1,3 glucan) – a known elicitor of the ant immune system^{27,28}. Then, we compared their immune gene upregulation and foraging strategy in comparison to their respective controls (Triton X, saline-injected ants). Immune gene expression analysis confirmed that both, *Metarhizium* infection ($p = 0.01$) and β -1,3 glucan injection ($p < 0.001$) led to significant upregulation of antimicrobial peptides, in particular, *Hymenoptaecin*²⁹ (Figure 4A, Data S4A-S4D). Interestingly, when given a choice between the two diets 1:199 and 1:4, not only the *Metarhizium*-infected ants ($p = 0.04$, Figure 4B, Data S4E) but also immune-activated ants ($p = 0.009$, Figure 4B, Data S4F) converged to a higher AA:C ratio than their respective controls. No difference in foraging strategy was observed between the *Metarhizium*-infected and β -1,3 glucan injected ants ($p = 0.480$, Figure 4B). This experiment revealed that immune activation alone was enough to trigger a shift toward a higher AA:C ratio suggesting a compensatory mechanism for the increased need for AA for antimicrobial peptide production during the immune response.

DISCUSSION

Nutrition has a crucial impact on host-parasite interactions. To understand how diet composition affects infection we need to identify the nutritional requirements of both the pathogen and its host. Using Nutritional Geometry²⁴, we established that the optimal diet composition of the entomopathogenic fungus *M. brunneum* is more amino acids-biased than that of its ant host. The fungus performed best in terms of growth and reproduction on the high-amino acid diet 1:4. In nature, *M. brunneum* principally feeds on hemolymph and other host tissues^{30,31}. In insects, the proteins to carbohydrates ratio of the hemolymph varies between species³², with a reported 1:1 in silkworms³³, 1:3 in fire ants³⁴ and 1:6 in crickets³⁵. Hence, the optimal artificial diet composition for *M. brunneum*, as determined in our study, would appear to correspond to the hemolymph composition of ants, making these insects a perfect host for this generalist entomopathogenic fungus. Furthermore, we revealed that this fungus, comprising distributed hyphae without specialized centers, was able to acquire an optimal supply of multiple nutrients essential for its reproduction on diets of different

nutrient compositions. The ability to respond to complex nutritional challenges has been reported only once before in a non-neural system³⁶.

This optimal fungal diet stands in stark contrast to the optimal host diet, as Argentine ants are known to perform best on high-carbohydrate diets^{37,38}. Based on these diet optima, infected ants could be expected to suffer twice from a high-amino acid diet, first since an excess in amino acids is known to shorten lifespan in ants³⁷ and second since it should give the fungus optimal conditions to thrive to the detriment of its host. However, diet composition also plays a crucial role in maintaining the immune function of the host, such that deficiency or excess in one or multiple nutrients can have negative or positive consequences on immune status and resistance to pathogens²³. Thus, the effects of infection and diet composition on the ant host may not be additive. We found that the half-life of healthy ants decreased by almost 2-fold in response to the high-amino acid diet as compared to the high-carbohydrate diet. As expected under pathogen infection³⁹⁻⁴¹ exposure to our experimental dose of *Metarhizium* spores decreased the host' half-life by 5-fold, when the ants were on their optimal high-carbohydrate diet. Interestingly, however, ant mortality was only decreased by 2-fold on the high-amino acid diet, suggesting that the harm of infection was in fact less pronounced when the ants fed on their otherwise non-optimal diet. This suggests that the negative effects of the high-amino acid diet observed in healthy ants might have been compensated in infected ants by an increased need for amino acids to maintain an energetically costly immune response. Amino acids are indeed necessary for cellular immune defense mechanisms like the production of antimicrobial peptides, which are short immune effector proteins⁴² and the encapsulation and melanization responses⁴³⁻⁴⁵. Intake of high-protein diets have been shown to improve pathogen resistance and immune function in other insects⁹, and could therefore also provide a survival advantage for the ants under *Metarhizium* infection by improved immune efficacy²³. Notably, over the course of our whole experiment the longevity of ants was not different whether they were confined to the high-amino acid or the high-carbohydrate diet. We would like to speculate that this may result from opposing effects of the high-amino acid diet on (i) ants that need to raise an immune response, like in the early phase after exposure, when intake of amino acids can complement the need for proteins in the immune response, (ii) ants after the acute phase, when high-amino acid intake may have the same long-term negative effects as in healthy ants.

Given the positive effect that amino acid intake can have on immune function²³, we asked whether infected colonies would actively seek to increase their amino acid intake.

When infected and healthy colonies were given a choice between diets varying in amino acids and carbohydrates composition, we found that infected colonies converged to a higher amino acid to carbohydrates ratio than healthy colonies, that is, that they actively foraged for more amino acids than healthy colonies did. Notably, this change in collective foraging strategy was observed even if only a minority of colony members was infected. Yet, this result may not necessarily reveal that the foragers of infected colonies actively seek to increase amino acid intake to improve immunity, it could also represent parasite manipulation, where the fungus may try to manipulate its host to forage for its own optimal diet. Several pathogenic fungi are indeed known to generate sophisticated behavioral modification in their hosts. For instance, to improve spore dispersal, *Ophiocordyceps* and *Pandora* fungi change the ants' behavior by making them leave the nest and bite on vegetation overhanging the hosts' foraging trail^{13,14,46}. Despite intense studies^{30,39,47}, to our knowledge, no contribution has shown that any of the *Metarhizium* species modify their hosts' behavior. Moreover, even if the high-amino acid diet corresponds to the optimal diet of the fungus, we showed that confinement of infected ants on this diet did not worsen the infection. Hence, parasite manipulation seems an unlikely scenario to explain the results of our study. Instead, our findings suggest that the collective foraging strategy adopted in response to infection might be the result of a collective compensatory mechanism to maintain immune function, similar to adjustments of dietary intake toward amino acids following an infection observed at the individual level in solitary insects^{9,10,26}. For instance, in lepidopterans, infected larvae adjust their foraging behavior and actively feed on high-protein diets which were shown to improve pathogen resistance and immune function⁹. Our final experiment conducted at the individual level, indeed confirmed that foraging for a high-amino acid diet can be induced purely by immune stimulation, in the absence of live pathogen. *Metarhizium*-infection and immune stimulation with β -1,3 glucan⁴⁸ both caused an increase in the expression of the immune-effector *Hymenoptaecin*^{29,48} and altered the ants' foraging activity to a higher amino acid to carbohydrate ratio than their respective controls. As *Hymenoptaecin* requires amino acids for its production⁴⁸, these findings support our hypothesis that ants adjust their foraging strategy to compensate for the costs of immune function and offer a novel social immunity mechanism to promote colony-level health^{49,50}. Uptake of otherwise harmful diets might be a protective behavior used by the colony to combat an infection, a potential process of collective self-medication^{51,52}.

Taken together, our experiments highlight the intricacy that exists between social behavior, nutrition and infection resistance. In all organisms, including insects, foraging

behavior needs to be adjusted over time to maintain the nutrient balance in the face of environmental stressors including fungal infection²³. In solitary insects, concentrations of nutrients and signaling molecules in the hemolymph inform the nervous system about the physiological status of the individual which in turn directs its foraging behavior³². In social insects, nutrition is a collective process. Assessment and collection of food outside the nest is undertaken by a minority of workers, the foragers⁵³. The food collected by each forager is then distributed via repeated mouth-to-mouth exchange among members of the colony. The ecological and evolutionary benefits of this collective nutrition could, however, come at the cost of increasing the spread of pathogens through the colony *via* food exchanges. Collective foraging relies initially on the foragers' individual decision to eat or not eat specific food, which depends primarily on their nutritional status⁵⁴. For instance, a forager deprived of amino acids favors diets that offer these missing nutrients⁵⁴. Once the forager decides to eat, it often communicates its discovery by laying a chemical trail to encourage other workers to visit the same food⁵⁴. Here, we revealed that similarly to amino acid-deprived individuals⁵³, infected ants fed more often on a high-amino acid diet. Owing to amplification by indirect interactions through chemical communication, differences in the feeding behavior of foragers in response to infection drove the whole colony toward a high-amino acid diet. Increasing amino acid intake at the collective level even if only a minority of the workers are infected might fuel the immune system of all colony members and help to keep infection at bay during an emerging epidemic threat, as pathogen spread regularly occurs in colonies⁵⁵. This collective foraging strategy could be added to the list of behavioral mechanisms used by ants to prevent or slow down infection transmission which include: changes in the social network and spatial segregation⁵⁶, social withdrawal⁵⁷, hygiene behaviors including self and allogrooming^{2,12}, poison and resin use^{58,59} and corpse removal⁶⁰. In conclusion, our results reveal how ants collectively overcome immune challenges and pave the way for more studies investigating nutritional immunology at the social level.

STAR Methods

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Glucose	Sigma-Aldrich	CAS#50-99-7
Ethanol	Fisher scientific	CAS#64-17-5
Cholesterol	Sigma-Aldrich	CAS#57-88-5
Choline Chloride	Sigma-Aldrich	CAS#67-48-1
Acid Ascorbic	Sigma-Aldrich	CAS#50-81-7
Vanderzant Vitamin Mix	Fisher scientific	CAT#903244
Wesson Salt Mix	Fisher scientific	CAT#902851
Inositol	Sigma-Aldrich	CAS#87-89-8
Methionine	Sigma-Aldrich	CAS#63-68-3
Histidine	Sigma Aldrich	CAS#71-00-1
Valine	Sigma-Aldrich	CAS#72-18-4
Threonine	Sigma-Aldrich	CAS#72-19-5
Arginine	Sigma-Aldrich	CAS#74-79-3
Tryptophan	Sigma-Aldrich	CAS#73-22-3
Phenylalanine	Sigma-Aldrich	CAS#63-91-2
Leucine	Sigma-Aldrich	CAS#61-90-5
Isoleucine	Sigma-Aldrich	CAS#73-32-5
Lysine	Sigma-Aldrich	CAS#56-87-1
Fluon	Whitford	U-22101540
Malt Extract Agar	Sigma-Aldrich	70145
Triton™ X-100	Sigma-Aldrich	1056142500
Sodium hypochlorite	Sigma-Aldrich	CAS#9002-93-1
Zymosan-A	Sigma-Aldrich	CAS#58856-83-2
Serpin 27a	Sigma-Aldrich	N/A
Defensin	Sigma-Aldrich	N/A
Relish	Sigma-Aldrich	N/A
Hymenoptaecin	Sigma-Aldrich	N/A
Deposited data		
All Data	Supplementary Material	Data S1
Code	Supplementary Material	
Experimental models: Organisms/strains		
<i>Metarhizium brunneum</i> (strain Ma 275, KVL 03-143)	Jorgen Eilenberg Nicolai V. Meyling University of Copenhagen	N/A
Argentine ants <i>Linepithema humile</i>	Université Paul Sabatier - Toulouse	N/A
Software and algorithms		
R Core Team 2021	A language and environment for statistical computing. R Foundation for Statistical	URL https://www.R-project.org/ . ⁶¹

	Computing, Vienna, Austria.	
Antethoc	Author: Maud Combe maud.combe@univ-tlse3.fr	https://cran.r-project.org/web/packages/lme4/index.html
ImageJ	Schneider et al. ⁶²	https://imagej.nih.gov/ij/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact Enikő Csata (Eniko.Csata@ur.de).

Materials availability

The study did not generate new unique materials.

Data and code availability

- All data generated in this paper will be made publicly available upon acceptance.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Host ant

We used the Argentine ant, *Linepithema humile*, a widespread invasive species, that forms polygynous supercolonies with millions of individuals^{63,64}. Argentine ants are opportunistic and feed on a large variety of nutritional resources, such as protein-rich prey items, as well as carbohydrate-rich nectar and honeydew⁶⁵. Ants within the colony have their own nutritional needs, adults such as foragers and nurses mostly need carbohydrates as a source of energy, whereas larvae and queens rely heavily on proteins for growth and egg production respectively⁶⁶. Ant colonies were collected from a large supercolony that lacks nest boundaries in Toulouse (France) in 2018, following the guidelines of the Nagoya protocol for Access and Benefit Sharing. Ants were subdivided into experimental colonies

(see details below). When not used in experiments, we supplied each colony with water and a mixed diet of vitamin-enriched food⁶⁷. All experiments followed European and national law and institutional guidelines.

Fungal pathogen and conidiospore suspensions

We exposed experimental colonies of Argentine ants to the entomopathogenic fungus *Metarhizium brunneum* (strain Ma 275, KVL 03-143), previously known as *Metarhizium anisopliae*, but now separated as a sister species⁶⁸. The genus *Metarhizium* is used worldwide for invertebrate pest control^{69,70}. *M. brunneum* is a generalist entomopathogenic fungus that naturally infects several host species from different taxa⁴⁷ including ants⁶⁸. It has a broad geographic distribution and occurs naturally in the soil⁷¹. The conidiospores (hereafter spores) of entomopathogenic fungi, such as *M. brunneum*, penetrate the cuticle of the host with the help of several enzymes^{39,40}, and then the pathogen spreads into the hemolymph and kills the host within several days due to tissue penetration, toxins and nutrient depletion³⁹⁻⁴¹. Death occurs between 4 and 10 days, depending on the virulence of the fungi and the host species³.

METHOD DETAILS

Experiments and measures: In total 18 synthetic diets were prepared and filter-sterilized (filter: 0.22 µm polyethersulfone membrane). The diets varied in both the amino acids to carbohydrates ratio (AA:C) and total concentration of amino acids + carbohydrates (A+C). The amino acids content of the diets consisted of a mixture of the following essential amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine, whereas glucose was used as a digestible carbohydrate source. The fat content of the diet consisted of a mix of cholesterol and lecithin. Each diet contained the same quantity of fat and other nutrients (lecithin 0.034%, cholesterol 0.034%, Vanderzant Vitamin mix 0.1%, Wesson salt mix 0.1%, ascorbic acid 0.2%, inositol 0.05%, choline chloride 0.034%). All the nutrients were mixed in a 0.66% agar gel and this mixture was poured into Petri dishes (10 mL in a 55 mm x 15 mm Petri dish). The Petri dishes were then sealed with parafilm and stored at 4°C.

Experiment 1: No-choice diet experiment. In our first experiment, we established the optimal diet for the entomopathogenic fungus *M. brunneum*. The fungus was confined to 18 different diets, varying in their essential amino acids to carbohydrates ratios (AA:C, 2:1,

1:1, 1:1.5, 1:2, 1:3, 1:4, 1:6, 1:8, 1:12, 1:16, 1:24, 1:49, 1:99, 1:199, Table S1) or in their nutrient concentration (AA+C, control 1:4, 1:4/2, 1:4/4, 1:4/8, 1:4x2, Table S2). For each diet, 10 μl of a 2×10^7 spores ml^{-1} (hereafter abbreviated as spores) suspension was applied in the center of a Petri dish directly on the diet, and then the Petri dish was sealed with parafilm and incubated at 25°C. We run 6 to 20 replicates per diet leading to a total of 283 Petri dishes. The fungal growth was recorded using a camera (Lumix DMC-FZ1000) after 14 and 30 days. The fungal expansion (growth rate: area mm^2) was measured using the program ImageJ (NIH Image, v1.49g). The fungus was harvested with a sterile cell scraper after 30 days from each diet by taking three circular subsamples of 1.5 cm diameter at equal distances from the center of the dish. Then, the number of spores was counted from each subsample. The fungal growth never reached the edge of the Petri dishes in the experiment. Spores were counted with a hemocytometer (depth 0.1 mm, Sigma-Aldrich, catalog number: Z359629-1EA) and an automatic cell counter (Nexcelom Bioscience, Cellometer Auto M10). The correlation between the two counting methods (hemocytometer and cellometer) was highly significant ($p < 0.001$, $N = 55$).

Experiment 2: The effect of past diet on spore production. In the second experiment, we tested the viability of the spores harvested on different diets *i.e.* we measured their ability to germinate, grow and produce new spores on a standard rearing medium. We collected spores from the two most extreme diets (1:199 and 2:1) and from the optimal fungal diet 1:4. Then, 10 μl of 30 spores ml^{-1} suspension was applied in the center of a Petri dish (diameter 55 mm) directly on a standard rearing medium (10 mL Malt Extract Agar medium: agar 1.5%, malt extract 3%, mycological peptone 0.5%). Then, the Petri dishes were sealed with parafilm and incubated at 25°C. We replicated the experiment at least 5 times for each diet. Spores were harvested after 30 days from three subsamples of 1.5 cm diameter from each Petri dish and counted with a hemocytometer (depth 0.1 mm, Sigma-Aldrich, catalog number: Z359629-1EA).

Experiment 3: Choice diet experiment. In the third experiment, we allowed the fungus to select between two diets differing in their essential amino acids to carbohydrates ratio (AA:C). Following the results from the first experiment, we tested six diet pairings (1:199 vs. 2:1, 1:49 vs. 2:1, 1:24 vs. 2:1, 1:16 vs. 2:1, 1:12 vs. 2:1, 1:8 vs. 2:1). The Petri dishes (diameter 55 mm) were divided into two parts, one of the diets (5 mL) was poured on one side of the Petri dish. Once the first diet was solidified, the second diet (5 mL) was poured

on the other side of the Petri dish. 20 μl of a 2×10^7 spores ml^{-1} suspension was applied in a line, in the center of each Petri dish directly in contact with both diets (55 x 15 mm, Figure S2). The Petri dishes were sealed with parafilm and incubated at 25°C. We run 6 to 15 replicates per diet leading to a total of 63 Petri dishes. The fungal growth was recorded with a camera (Lumix DMC-FZ1000) after 14 and 30 days. The fungal expansion on each diet (growth rate: area mm^2) was measured using the program ImageJ (NIH Image, v1.49g). The fungus was harvested after 30 days from each diet by taking two subsamples from each diet, and the number of spores was counted for each subsample using a hemocytometer (depth 0.1 mm, Sigma-Aldrich, catalog number: Z359629-1EA).

Experiment 4: Survival of the host on different diets. In the fourth experiment, we studied how infected and uninfected hosts performed when confined to one of two different diets varying in their essential amino acids to carbohydrates ratios (AA:C, 1:199 and 1:4). Ants were infected with *Metarhizium* spores, suspended in 0.05% Triton-X. We applied 0.5 μl of 1×10^7 spores ml^{-1} suspension to each ant. Control ants received 0.5 μl of 0.05% Triton-X solution. In total 200 ants (100 infected and 100 control) were housed solitary, in Fluon-coated Petri dishes (55 x 15 mm) with moistened cotton (remoistened every day). Ants were supplied with a fresh diet three times a week. Ant mortality was checked daily, for 64 days. Dead ants were removed from the nest, surface sterilized, and monitored for 14 days for signs of *Metarhizium* infection. At the end of the experiment, all the remaining live ants were killed, surface sterilized, and monitored for *Metarhizium* outgrowth. We observed that 65.26% of the 100 corpses from infected groups and none of the 100 corpses from control groups produced *Metarhizium* spores. In *Metarhizium*-exposed ants, the proportion of corpses that produced spores did not differ significantly with diet ($p = 0.390$, high-carbohydrate diet 1:199 = 63.04%, high-amino acid diet 1:4 = 67.3%).

Experiment 5: Collective choice of infected and uninfected ants. In the fifth experiment, we determined whether infection affects the foraging strategies of ant colonies. Colonies of uninfected and infected ants were offered two diets 1:199 and 1:4 varying in their ratios of essential amino acids to carbohydrates (AA:C) for one hour. We investigated whether ants preferred one diet over the other, or whether they showed no preference depending on their infection state. We used 22 colonies in total, 12 were infected while 10 were uninfected. Each colony contained 1000 individuals, one queen and brood.

For each experimental colony, ants were installed in 3 test tube nests (15 cm in length, 1.3 cm in diameter) placed in a foraging arena (20 x 10 x 10 cm) with walls coated with Fluon to prevent ants from escaping. Between the choice assays, ant colonies were food-deprived and kept at room temperature ($25 \pm 1^\circ\text{C}$) under a 14:10 L:D photoperiod. To infect experimental colonies with *M. brunneum*, spores were diluted in 0.05% Triton solution (1×10^7 spores per mL). Then, 450 μl of this suspension was vortexed vigorously and deposited on a filter paper (7 cm diameter) which was placed in the foraging arena, *i.e.* outside the brood chambers (modified after⁷²). For the control groups, 450 μl of 0.05% Triton X solution was deposited on the filter paper, following the same procedure. We renewed these operations every three days for each treatment for 5 weeks (modified after⁷³). Hence, the workers were in contact with the fungal spores during the entire time course of the experiment.

Ants were starved for the whole duration of the experiment (5 weeks) except when we were running the diet choice assay once a week. The first assay started 3 days after exposure to the fungal spores. During an assay, two diets (1:199 and 1:4) were placed on two platforms (5 cm x 5 cm) tied to a Y-shaped bridge with two branches of equal length ($L = 5$ cm, 60 angle between the two branches) connected to the colony. For each assay, we added a colorant randomly to each solution and each examiner was blinded to the identity of the solution tested. The ants had access to both diets for one hour⁵⁴. The ant traffic on the two branches was recorded by a video camera (Canon LEGRIA HF G30) for one hour. To assess foraging effort, for all choice assays, we counted the number of ants traveling on each branch at a particular point (one centimeter from the choice point) every minute for one hour. Counting began as soon as the first ant climbed onto the bridge and lasted for 60 min⁵³. During the entire experiment, we also counted the number of dead ants once a week. During the experiment, a total of 625 infected and 485 uninfected ants died. The corpses were surface-sterilized and monitored for 14 days for *Metarhizium* outgrowth. We found that 11.68% of the corpses from infected colonies and none of corpses from control colonies produced *Metarhizium* spores. These last results prove that our infection method that mimics natural conditions worked successfully.

Experiment 6: Host compensation or parasite manipulation. In the last experiment, to test whether the changes observed in the collective foraging behavior of infected ants were caused by host compensation or pathogen manipulation, we stimulated the ants' immune system using β -1,3 glucan, a major component of the cell walls of pathogenic fungi and a

known elicitor of the ant immune system. Soluble β -1,3 glucan was obtained by suspending 5 mg of Zymosan-A (*Saccharomyces cerevisiae* wall fragments [Sigma-Aldrich]) in 1 ml of sterile physiological ant saline (as described in⁷⁴). The zymosan suspension was vortexed for 1 hr. at 3200 rpm before being centrifuged at 10000 rcf for 5 min. The supernatant, containing the soluble β -1,3 glucan⁷⁵ was then removed and stored at 4°C.

Before starting the experiment, a total of 68 individuals were collected in one colony (1000 individuals, one queen and brood) and were housed individually in Petri dishes (55 x 15 mm). Right after isolation, 21 individuals were placed gently one by one into a sponge harness. Using fine glass capillaries (with a spike to aid injection; inner diameter = 25 μ m [BioMedical Instruments, Germany]), a microinjector (parameters: p_i = 120 hPa, t_i = 0.3 s, p_c = 20 hPa [FemtoJet, Eppendorf, Germany]) and a micromanipulator (Luigs and Neumann, Germany) we injected 46 nl of the β -1,3 glucan solution (N = 21) through the intersegmental membrane of the ant's first tergite, into their haemocoel. Capillaries were cleaned between injections using 96% ethanol. 15 ants were microinjected in the same way with sterile physiological saline and served as controls⁷⁴. In addition, 16 ants were exposed to 0.5 μ l of a 1×10^7 spores ml^{-1} suspension of *Metarhizium* spores, and 16 control ants received 0.5 μ l of 0.05% Triton-X solution (for further details *see* Supplementary methods). Right after each treatment, ants were placed back in their Petri dishes without food for 24h. Based on several studies^{76,77}, the fungal conidiospores after contact with the insect cuticle, first adhere to the body surface within several hours and then germinate and penetrate the host cuticle within approximately 24-48 hours, and immune genes of ants upregulated 24h after *Metarhizium* exposure⁷⁸.

Individual ants were then offered two diets 1:199 and 1:4 varying in the ratio of essential amino acids to carbohydrates (AA:C) for one hour and were filmed with Ueye cameras (each filming 2 Petri dishes simultaneously). Every ant was observed for one hour from the moment we added the diets to the Petri dish. We counted the number of feeding events (*i.e.* contacts with the food that lasted more than 5 seconds) and their duration. The approximate regulated amino acids to carbohydrates ratio (AA:C) for each ant was derived from the foraging activity FA (number of feeding events) and was calculated using the following equation (inspired from [⁵⁴]):

$$\text{AA:C ratio} = ([\text{AA}_{1:4}] * \text{FA}_{1:4} + [\text{AA}_{1:199}] * \text{FA}_{1:199}) / ([\text{C}_{1:4}] * \text{FA}_{1:4} + [\text{C}_{1:199}] * \text{FA}_{1:199}).$$

As could be expected, the regulated amino acids to carbohydrates ratios computed from time spent feeding were highly correlated to the ratios estimated from the number of feeding events (Spearman correlation, $p < 0.0001$). We thus decided to present only the latter.

Following the feeding assay, individuals were frozen at -80°C . A candidate gene approach was used to test if the β -1,3 glucan injection and *Metarhizium* exposure elicited an immune response, using saline-injected ants and Triton-X-treated ants as their respective controls. We measured the expression levels of four immune genes commonly found in insects, including ants, in the 68 individuals tested (*Defensin 1*, *Hymenoptaecin*, *Relish*, *Serpin 27a*) by droplet digital PCR (ddPCR). *Defensin 1* and *Hymenoptaecin* are genes coding for antimicrobial peptides^{29,79}; *Relish* is required for a humoral immune response⁸⁰; and *Serpin 27a* (SPN-27a) is a gene involved in the melanization cascade⁸¹. Sample preparation and gene expression analyses were performed as detailed in the electronic supplementary material. Expression levels of the immune genes were normalized to the housekeeping gene (*GAPDH*) before further statistical analysis (for primer sequences see Table S22).

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AUTHOR CONTRIBUTIONS

A.D. and E.C. conceived the study. Experiments were designed by E.C., A.P.E., S.C. and A.D., and performed by E.C., E.L. and H.L., using resources of the laboratories of A.D., S.C. and J.H. Data were curated by E.C., and statistically analyzed and designed as figures by E.C. and A.D. with the input of S.C. and S.J.S. The first draft of the manuscript was written by E.C., S.C., S.J.S. and A.D. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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Figure 1. Spore production and fungal growth on different nutrient compositions (artificial diets, Table S1 and S2). Figure A, C-E. Performance responses. **A.** Number of spores produced by *M. brunneum* per μL (volume = $0.1\mu\text{L}$) when the fungus was confined for 30 days to one out of 18 diets varying in both the ratio and total amount of amino acids and carbohydrates. The response surface regression analyses yielded a significant relationship: $p < 0.001$. **B.** Photograph of fungal growth on a 1:4 artificial diet after 30 days. **C and D.** Fungal growth (mm^2) was determined for fungus grown for 14 and 30 days on one of 18 diets. The response surface regression analyses showed significant relationships for day 14 ($p < 0.001$) and day 30 ($p < 0.001$). The number of replicates per diet is between 11 and 20. **E.** Total number of spores calculated for fungi confined for 30 days to one of 18 diets. For each replicate, the number of spores harvested by the surface unit was multiplied by the total surface covered by the fungus. The response surface regression analyses yielded a significant relationship ($p < 0.001$). **F.** Effect of past diet on spore production. Mean number of spores harvested (per $0.1\mu\text{L}$) on a standard medium (secondary spores) as a function of the diet (AA:C ratio, 1:4, 1:199 and 2:1) used to produce the inoculated spores (primary spores) ($N = 5$ to 6 replicates per diet). Linear model $F_{2,13} = 4.57$, $p = 0.031$ followed by all-pairwise post-hoc comparisons (Tukey method; 1:199 vs 1:4 $p = 0.696$, 1:199 vs 2:1 $p = 0.139$, 1:4 vs 2:1 $p = 0.027$). Error bars indicate the 95%CI.

Figure 2. Spore production and fungal growth measured during choice experiments (artificial diets, Table S1). A. Number of spores per μL (volume = $0.1\mu\text{L}$) produced by *M. brunneum* grown for 30 days on 6 different diet pairings offering one of six carbohydrate-biased diets (1:8, 1:12, 1:16, 1:24, 1:49, or 1:199) versus an amino acid biased diet (2:1). For the experiment schematic design see Figure S2. For each Petri dish, the spores were

counted separately for the two diets. **B.** Area covered by the fungus on each diet recorded after 30 days for the 6 diet pairings. In **A, B** the black line corresponds to the results from the no-choice experiments for the optimal fungal diet 1:4. The grey area represents the 95% confidence interval for the 1:4 diet.

Figure 3. A. The effect of diet composition and infection on ant lifespan. Individual *L. humile* ants were exposed to 0.5 μl of 1×10^7 spores ml^{-1} suspension of *M. brunneum* (N = 100), while the ants of the control group received Triton X solution (N = 100). Infected and uninfected ants were confined to either a 1:199 or a 1:4 diet. Estimated functions for Cox regression of survival time based on the infection status and diet of *L. humile* ants. **B. Foraging choice of foragers of infected and uninfected colonies.** The number of foraging workers from uninfected or infected colonies visiting the two diets (open circle = 1:199 and filled circle = 1:4) offered simultaneously in the food choice experiment throughout the 5 weeks (duration of each feeding assay 1 h). **C. Amino acids (AA) and carbohydrates (C) collection during choice experiment.** For each assay, the approximate nutrient collection for the colony was calculated using the following equations $\text{AA-collection} = \text{FA}_{1:4} * [\text{AA}]_{1:4} + \text{FA}_{1:199} * [\text{AA}]_{1:199}$ and $\text{C-collection} = \text{FA}_{1:4} * [\text{C}]_{1:4} + \text{FA}_{1:199} * [\text{C}]_{1:199}$ where FA is the foraging activity (i.e., the number of ants visiting each diet) and [AA] and [C] are the concentrations in amino acids and carbohydrates. Points along each trajectory represent the average intake of AA and C per ant (AA-collection and C-collection divided by the number of ants) over the weeks per colony. Black lines show the diets 1:199 and 1:4. **D. Proportion of infected and uninfected ants alive over the 5 weeks of the experiment.** Dots and error bars represent the mean and confidence intervals respectively (N = 10 and 12 colonies respectively for uninfected and infected colonies). In each graph, the blue color represents uninfected ants and the red *Metarhizium*-infected ants.

Figure 4. Foraging choice of infected and only immune-stimulated ants. A. Immune gene expression (tested by a candidate gene approach) of ants infected by spores of the fungal pathogen *Metarhizium* (N = 16; Triton-X control, N = 16) and ants having received an immune stimulation with fungal cell wall material (β -1,3-glucan) in the absence of a live infection (N = 21; control-injected with physiological saline solution, N = 15). The expression level of the immune gene *Hymenoptaecin* (an antimicrobial polypeptide) was measured by droplet digital PCR (ddPCR) and was normalized to the housekeeping gene (*GAPDH*) before further statistical analysis. **B.** The ants treated above were offered two

diets 1:199 and 1:4 for 1 h and we estimated diet composition (AA:C) from foraging activity (see methods section). Boxplots show median (black line), 25–75% quartiles, maximum/minimum range, and outliers.







