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## Research

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# A carbohydrate-rich diet increases social immunity in ants

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Increased potential for disease transmission among nest-mates means living in groups has inherent costs. This increased potential is predicted to select for disease resistance mechanisms that are enhanced by cooperative exchanges among group members, a phenomenon known as social immunity. One potential mediator of social immunity is diet nutritional balance because traits underlying immunity can require different nutritional mixtures. Here, we show how dietary protein-carbohydrate balance affects social immunity in ants. When challenged with a parasitic fungus *Metarhizium anisopliae*, workers reared on a high-carbohydrate diet survived approximately 2.8× longer in worker groups than in solitary conditions, whereas workers reared on an isocaloric, high-protein diet survived only approximately 1.3× longer in worker groups versus solitary conditions. Nutrition had little effect on social grooming, a potential mechanism for social immunity. However, experimentally blocking metapleural glands, which secrete antibiotics, completely eliminated effects of social grouping and nutrition on immunity, suggesting a causal role for secretion exchange. A carbohydrate-rich diet also reduced worker mortality rates when whole colonies were challenged with *Metarhizium*. These results provide a novel mechanism by which carbohydrate exploitation could contribute to the ecological dominance of ants and other social groups.

## 1. Introduction

Social insects make up more than half of global insect biomass [1], an ecological dominance that has arisen in spite of inherent costs associated with group living. One such cost is the increased risk of parasite transmission among closely packed and closely related nest-mates [2]. It may be offset by increased *per capita* investment in immune function [3] and by group-level defences termed 'social immunity' [4,5]. Food availability is a likely mediator of social immunity. It is generally considered an important constraint on immune function [6] owing to the high maintenance costs of immune functions relative to other physiological costs [7]. Many studies have shown trade-offs between individual immunity and other fitness-enhancing functions that become more acute under starvation and caloric restriction treatments [8–10].

Both the quantity of food components and their relative availability can affect the nature and extent of immune responses [11–14], as well as a range of physiological and life-history traits [15]. If specific traits promoting immunity require different nutritional mixtures, then scarcity of particular dietary components should influence the relative costs of those different mechanisms promoting an organism's immune function [15,16]. Dietary protein (P) and carbohydrate (C), both their distinct and interactive effects, have shaped immune function [12,13,16] (reviewed in [17]) for a variety of solitary organisms. While protein nutrition can constrain immune response in honeybees [18], we know of no study investigating how diet macronutrient composition affects social immunity.

Here, we tested how dietary protein : carbohydrate (P : C) ratio affects social immunity in a tropical ant, *Ectatomma ruidum*, combatting the parasitic fungus

*Metarhizium anisopliae*, a model pathogen for studying social immunity [19–22]. Ants have two prominent mechanisms for social immunity (for additional mechanisms, see [23,24]). Allogrooming is a time-intensive [25] activity where nest-mates clean each other's cuticle, reducing the number of fungal spores [20,26,27]. The metapleural gland, a structure unique to ants [28], secretes antibiotics that reduce spore viability [20,29–31]. Both allogrooming and metapleural gland production can thus lower infection rates of ant workers exposed to *Metarhizium*. As dietary P:C constrains many worker- and colony-level traits [32], including storage biochemistry [33], worker longevity [32] and possibly worker activity rates [34] (but see [35]), we predicted that lower dietary P:C ratios would increase nest-mate interaction frequency and, in turn, the benefits of social grouping after exposure to *Metarhizium*. Our results provide new insight into the social value of carbohydrate resource exploitation.

## 2. Material and methods

We conducted all work in June–July 2011, January 2012 and June–July 2012 on Barro Colorado Island, a lowland, seasonally wet forest in Lake Gatun of the Panama Canal. *Ectatomma ruidum* (subfamily Ectatomminae) is a ground-nesting ant with zero to four queens and approximately 10–150 workers per nest at this site; queenless nests are common. Colonies commonly consist of multiple nests. We excavated nests (58 in June 2011, 43 in January 2012 and 24 in June 2012) and maintained them in circular containers (18 × 8 cm) lined with Fluon and covered with mesh to prevent escape. In each container, we placed three nest chambers, which were 20 × 100 mm glass test tubes half-full of water at the base and stopped with cotton. We covered nest chambers with foil. We maintained colonies in a covered shelter at ambient understorey light, temperature and humidity.

### (a) Nutrition effects on worker social immunity

In June 2011 and January 2012, we tested whether nutrition affects social immunity in worker groups. We reared colonies (all workers, pupae and larvae excavated from a nest; any queens were excluded) on isocaloric, semi-synthetic diets that differed only in P:C ratio (diet modified slightly from [36]; electronic supplementary material, table S1). We used two P:C ratios (1P:3C and 3P:1C) that are readily consumed by *E. ruidum* [37], differentially affect colony growth and worker biochemistry [33], and bracket the P:C ratio collected by *E. ruidum* in feeding trials in the field [37]. We assigned 52 colonies (26 in June 2011, 26 in January 2012) to diet treatment after ordering them by size (sum of principal components from analysis of worker, pupa and larva numbers); we split five nests with more than 60 workers (three in June 2011, two in January 2012) and assigned halves to different diets. We reared colonies for 18 days by providing approximately 1 g blocks of food every second day (*ad lib* feeding), and quantified food dry mass loss as in [33]. We also counted and removed dead workers every 2 days.

For survival assays, we measured responses of solitary and social ant groupings to treatment with *M. anisopliae* (strain KVL02-73 collected at this site [38]). After the 18-day diet manipulation, we created one-ant (solitary ants) or five-ant (worker groups) sets from each colony. In June 2011, we assigned 10 solitary ants and two worker groups per colony to a *Metarhizium* treatment; in January 2012, we assigned five solitary ants and one worker group per colony to a *Metarhizium* treatment, and the same number to a control treatment. The *Metarhizium* treatment consisted of applying to the thorax of each ant 0.5  $\mu$ l of an LD<sub>50</sub> concentration ( $6.92 \times 10^{-6}$ , determined at the

outset) of *Metarhizium* spores suspended in 0.05% Triton-X. Control ants received 0.5  $\mu$ l of 0.05% Triton-X. For treatment, we held ants with sterilized forceps and applied the dose to the dorsal surface of the thorax. We housed solitary ants and worker groups in Petri dishes (3 cm diameter) with moistened cotton (re-moistened every 2 days) and checked daily for mortality over 14 days (in June 2011) or 22 days (in January 2012). We removed dead ants, sterilized them (with 1% NaClO), placed them on filter paper and monitored them for 7 days for signs of *Metarhizium* infection (characteristic conidia growth that occurs within 2–3 days).

### (b) Allogrooming effects on nutrition-related social immunity

In June 2012, we assessed whether diet affects allogrooming, an established mechanism of social immunity in ants [20]. We assigned 24 colonies to either the 1P:3C or 3P:1C diet, as above. After an 18-day rearing period, we created solitary ant and worker group sets, and treated them with either *Metarhizium* or the control Triton-X solution. We then immediately began assaying grooming behaviour after Walker & Hughes [39]. We observed behaviour for 30 s periods at every 10 min for 60 min, as most grooming occurs immediately after solution application (A.D.K. & A.J.B. 2011, personal observation; see also [26]). We recorded the number of antennal self-grooming and allogrooming events. Behaviours that occurred for more than a 5 s period were split into multiple events.

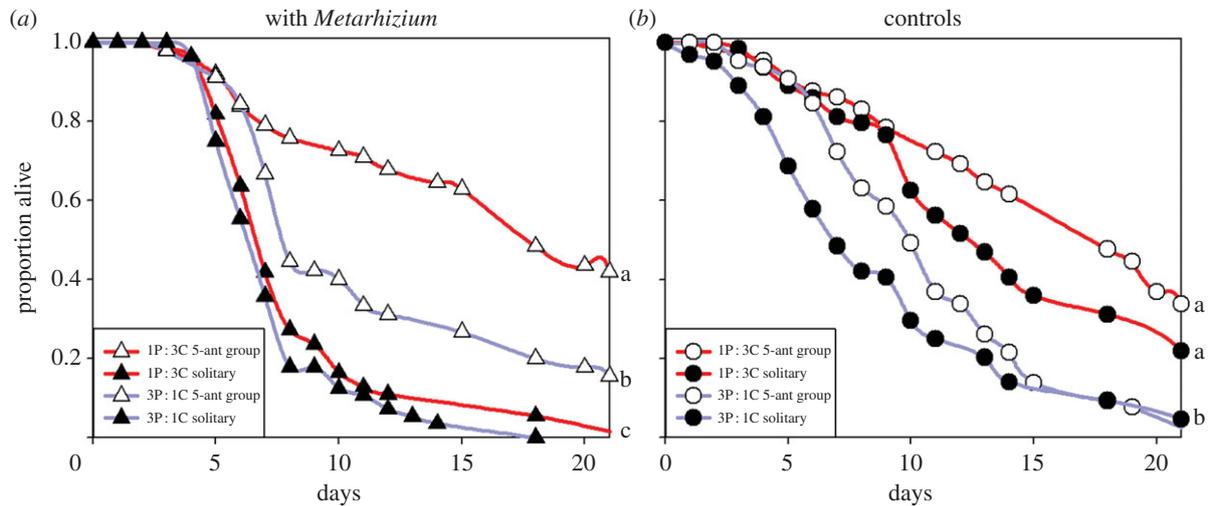
### (c) Metapleural gland effects on nutrition-related social immunity

In January 2012, we tested whether secretions from the metapleural gland (a specialized gland on the thorax of ants that produces antibiotic secretions) influences diet-related immunity. To do this, we reared 12 colonies on either the 1P:3C or 3P:1C diet for 18 days. We then blocked the metapleural gland (using nail polish) on 10 ants per colony, created five solitary ant or one worker group sets and treated all ants with *Metarhizium* as described above. We assessed survivorship as above. We used workers sampled from field colonies to show that blocking metapleural glands *per se* did not affect survivorship (Cox proportional hazards: L-R  $\chi^2 = 0.087$ , d.f. = 1,  $p = 0.762$ ,  $n = 40$ ).

### (d) Nutrition effects on colony-level immunity

In June 2011 and January 2012, we tested how nutrition affects colony-level immunity, and whether nutritional immunity depended on the presence of larvae in colonies. Given that nutritional feedbacks between workers and larvae influence P:C regulation in ant colonies [32], we predicted that larval presence would enhance nutritional effects on colony immunity.

For this test, we used 16 colonies in June 2011 and 10 in January 2012. We assigned colonies (blocked by size as above) to the 1P:3C or 3P:1C diet and removed larvae from half of the colonies in each treatment; we removed and returned larvae for control colonies. We reared colonies for 21 days. During the rearing period, we challenged colonies with *Metarhizium* by placing headless ant corpses covered in *Metarhizium* spores in each nest every 2 days. We used this approach to challenge colonies (rather than direct application to workers of spores in solution, as above) because we wanted to sufficiently challenge colonies to test for differences among diet treatments; spore-covered corpses could be added at regular intervals, whereas periodic solution application to workers would have considerably disturbed colonies. We counted the number of dead ants in each colony (easily distinguishable from headless ant additions) every 2 days and monitored each for signs of infection (as above).



**Figure 1.** The effects of diet composition, social grouping and challenge from the *Metarhizium* fungal parasite on ant worker survivorship. Ants are from colonies maintained on either a 1 protein (P) : 3 carbohydrate (C) ratio diet (red lines) or a 3P : 1C (blue lines) diet. Data in panel (a) are from 2011 and 2012. Data in panel (b) are from 2012 only. (a) Survivorship for *Metarhizium*-challenged ants when alone (solitary) or in 5-ant worker groups. (b) Survivorship for ants not facing *Metarhizium* challenge. Different lower-case letters indicate survival curve differences at  $p < 0.01$  in pairwise comparisons using Kaplan–Meier survival tests.

### (e) Statistical analyses

We analysed total carbohydrate and protein intake using the restricted maximum-likelihood (REML) method, with year of the study as a random factor and diet as a fixed factor. We analysed effects of diet and social grouping (fixed effects) on worker survivorship using the Cox proportional hazards model (year was included as a random factor). We analysed colony-level effects of nutrition on *Metarhizium* resistance using REML. The response variable was number of dead workers divided by number of initial workers in the colony per day; explanatory variables were diet and *Metarhizium* treatment (fixed effects), and year of study was included as a random effect. We analysed grooming behaviour using a repeated-measure ANOVA with diet and *Metarhizium* treatment as fixed factors. We did not include colony as a random factor in this analysis because some colonies had too few workers to allocate to multiple treatments. For these small colonies, worker groups were randomly assigned to treatments. We tested whether ant mortality was associated with evidence of *Metarhizium* infection using a likelihood ratio test. We conducted all analyses using JMP v. 8.0.1.

## 3. Results

### (a) Nutrition effects on worker social immunity

Colonies consumed similar amounts of food in the two diet treatments, resulting in approximately  $3\times$  higher carbohydrate intake and approximately  $3\times$  lower protein intake for colonies on the lower-P : C diet versus those on the higher-P : C diet (see electronic supplementary material, figure S1).

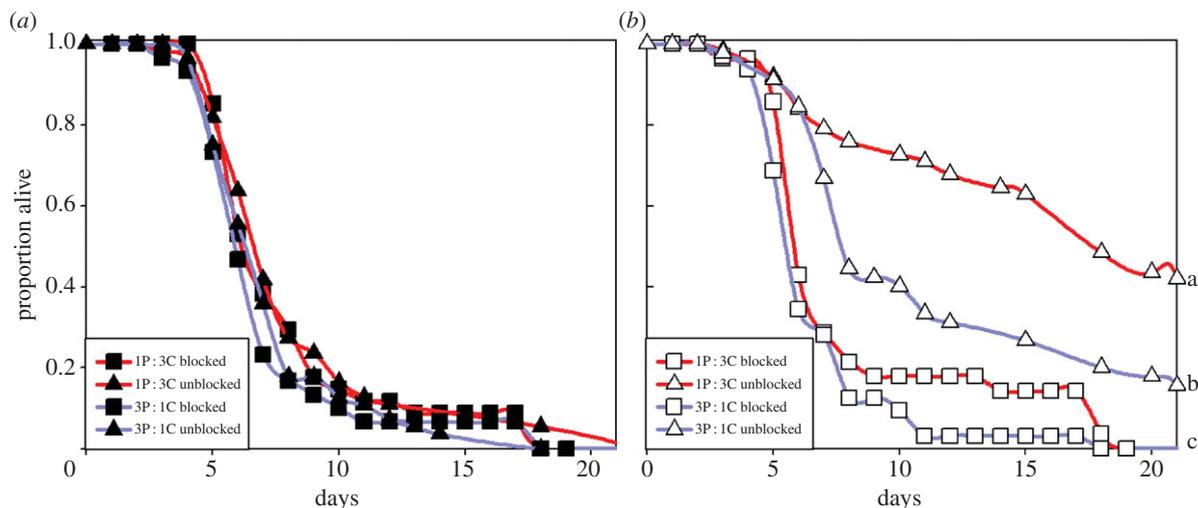
A high-carbohydrate diet enhanced social immunity in workers (figure 1; electronic supplementary material, table S2). When workers were challenged with *Metarhizium* (figure 1a), both the lower-P : C diet (Cox proportional hazards: L-R  $\chi^2 = 15.886$ , d.f. = 1,  $p < 0.001$ ,  $n = 745$ ) and social grouping (L-R  $\chi^2 = 86.871$ , d.f. = 1,  $p < 0.001$ ) increased worker survivorship. Importantly, there was a significant diet-by-social grouping interaction (L-R  $\chi^2 = 10.499$ , d.f. = 1,  $p = 0.001$ ), as the 1P : 3C diet increased worker survivorship in worker groups more than for solitary ants. We conducted *Metarhizium* challenge in both 2011 and 2012; survivorship differed between the two replicates of the experiment (main effect

of year: L-R  $\chi^2 = 24.748$ , d.f. = 1,  $p < 0.001$ ), but there was no significant year-by-diet-by-social grouping interaction (L-R  $\chi^2 = 0.689$ , d.f. = 1,  $p = 0.407$ ) as the 1P : 3C diet enhanced social immunity in workers in both years. We included ants that were not challenged with *Metarhizium* (controls) in 2012. With these data, we found that in the absence of *Metarhizium* challenge (figure 1b), the lower-P : C diet (L-R  $\chi^2 = 36.143$ , d.f. = 1,  $p < 0.001$ ,  $n = 258$ ) and social grouping (L-R  $\chi^2 = 5.541$ , d.f. = 1,  $p = 0.019$ ) increased worker survivorship, but there was no significant diet-by-social grouping interaction (L-R  $\chi^2 = 0.081$ , d.f. = 1,  $p = 0.777$ ).

In *Metarhizium* treatments, fewer dead ants showed signs of *Metarhizium* infection in worker groups (approx. 44%) than in the solitary ant setting (approx. 93%;  $\chi^2 = 66.4$ , d.f. = 1,  $p < 0.001$ ), and evidence of infection did not differ significantly with diet ( $\chi^2 = 1.06$ , d.f. = 1,  $p = 0.303$ ). About 5% of ants showed signs of *Metarhizium* infection in control groups. This probably represents a natural infection level owing to our use of ants recently collected from the field. It does not affect our conclusions because our experiments explicitly compared these control ants with ants treated with *Metarhizium*.

### (b) Metapleural gland effects on nutrition-related social immunity

Blocking metapleural glands eliminated effects of diet composition and worker grouping on survivorship for ants facing *Metarhizium* challenge (figure 2). Survivorship for solitary ants with blocked metapleural glands was similar to levels in solitary ants with unblocked glands, regardless of diet (figure 2a; Cox proportional hazards: blocking: L-R  $\chi^2 = 0.682$ , d.f. = 1,  $p = 0.409$ ,  $n = 175$ ; diet: L-R  $\chi^2 = 1.632$ , d.f. = 1,  $p = 0.201$ ; blocking-by-diet: L-R  $\chi^2 = 0.007$ , d.f. = 1,  $p = 0.933$ ). By contrast, blocking metapleural glands significantly reduced survivorship in worker groups, particularly for ants reared on the 1P : 3C diet (figure 2b; blocking: L-R  $\chi^2 = 40.004$ , d.f. = 1,  $p < 0.001$ ,  $n = 167$ ; diet: L-R  $\chi^2 = 13.682$ , d.f. = 1,  $p < 0.001$ ; blocking-by-diet: L-R  $\chi^2 = 3.862$ , d.f. = 1,  $p = 0.049$ ). An analysis restricted to ants with blocked metapleural glands showed that neither diet nor worker grouping affected survivorship under these conditions (diet: L-R



**Figure 2.** Effect of blocking metapleural glands on survivorship of workers exposed to the *Metarhizium* parasite and reared on either a 1 protein (P) : 3 carbohydrate (C) diet (red) or a 3P : 1C (blue) diet in either (a) solitary or (b) five-ant worker groups. Different lower-case letters indicate survival curves differences at  $p < 0.01$  in pairwise comparisons using Kaplan–Meier survival tests.

$\chi^2 = 2.249$ , d.f. = 1,  $p = 0.134$ ; grouping: L-R  $\chi^2 = 0.060$ , d.f. = 1,  $p = 0.807$ ). Survivorship for *Metarhizium*-challenged ants in solitary conditions with blocked glands (figure 2a) did not differ significantly from survivorship for challenged, solitary ants with unblocked glands (blockage: L-R  $\chi^2 = 0.682$ , d.f. = 1,  $p = 0.409$ ), indicating that gland blockage itself did not affect survivorship.

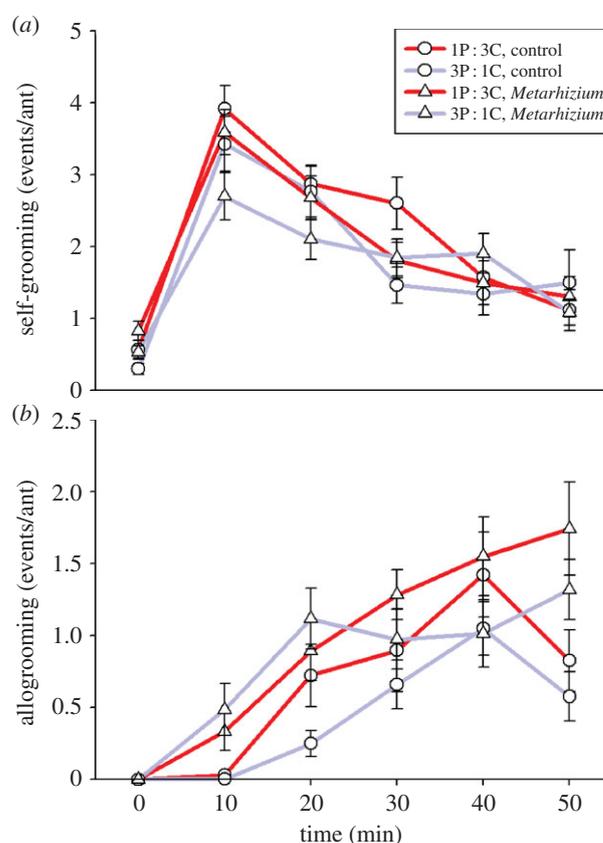
Almost all *Metarhizium*-treated ants with blocked metapleural glands showed evidence of *Metarhizium* infection (solitary 87.4%, social 86.6%), and evidence of infection did not differ significantly with diet ( $p > 0.75$ ). Evidence of *Metarhizium* infection did not depend on metapleural gland blockage for solitary ants ( $\chi^2 = 0.010$ , d.f. = 1,  $p = 0.920$ ); in worker groups, evidence of *Metarhizium* infection was significantly higher for ants with blocked metapleural glands than it was for ants without blocked glands ( $\chi^2 = 86.428$ , d.f. = 1,  $p < 0.001$ ).

### (c) Allogrooming effects on nutrition-related social immunity

We found little evidence for diet effects on grooming behaviour (figure 3). Allogrooming frequency was significantly higher in *Metarhizium*-challenged ant groups than in control groups (repeated-measures ANOVA:  $F_{5,66} = 3.128$ ,  $p = 0.014$ ), but it did not depend on rearing diet ( $F_{5,66} = 0.762$ ,  $p = 0.580$ ). Self-grooming frequency was not significantly affected by either *Metarhizium* challenge ( $F_{5,71} = 2.32$ ,  $p = 0.052$ ) or rearing diet ( $F_{5,71} = 1.053$ ,  $p = 0.393$ ), and there was no significant *Metarhizium*-by-diet interaction ( $F_{5,71} = 1.803$ ,  $p = 0.123$ ). Allogrooming frequency was significantly higher in *Metarhizium*-challenged ant groups ( $F_{5,66} = 3.128$ ,  $p = 0.014$ ) but did not depend on rearing diet ( $F_{5,66} = 0.762$ ,  $p = 0.580$ ) and there was no significant *Metarhizium*-by-diet interaction ( $F_{5,66} = 0.796$ ,  $p = 0.556$ ).

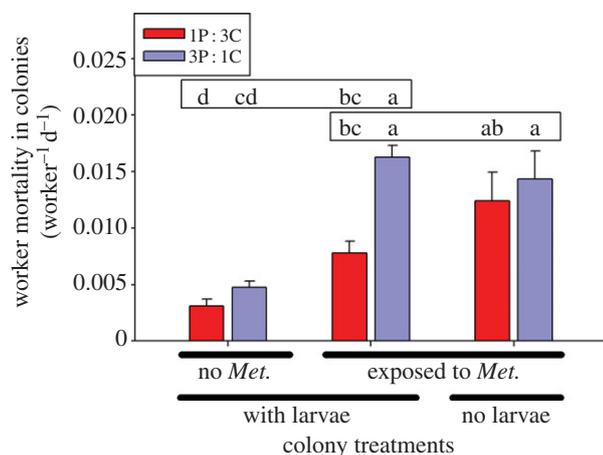
### (d) Nutrition effects on colony-level immunity

A high-carbohydrate diet also enhanced immunity in whole colonies (figure 4). Regular addition of spore-covered corpses to colonies (*Metarhizium* treatment) increased worker mortality more in the 3P : 1C diet than in the 1P : 3C diet



**Figure 3.** Frequency of grooming behaviours in groups of five ants that were reared on either a 1 protein (P) : 3 carbohydrate (C) diet or a 3P : 1C diet and either were or were not challenged with the parasitic fungus *M. anisopliae*. Response variables are (a) antennal self-grooming or (b) nest-mate grooming (allogrooming) events during 30 s observation periods. Behaviours that occurred for more than a 5 s period were split into multiple events. Neither self-grooming nor allogrooming was significantly affected by rearing diet.

(figure 4; diet-by-*Metarhizium*:  $F_{1,71} = 11.339$ ,  $p = 0.001$ ). There was also a significant effect of larval presence on diet-related immunity (figure 4; diet-by-larvae:  $F_{1,41} = 4.167$ ,  $p = 0.047$ ). Most (77.4%) workers that died during the experiment showed signs of *Metarhizium* infection, and the likelihood of infection in dead workers did not depend on



**Figure 4.** Effect of *M. anisopliae* challenge and larval presence on worker mortality in colonies fed either a 1 protein (P) : 3 carbohydrate (C) diet (red bars) or a 3P : 1C (blue bars) diet. Worker mortality (number of dead workers divided by number of initial workers in the colony per day) values are least-square means with random variable year included in the model. Two targeted comparisons were made: among colonies with larvae (exposed or not exposed to *Metarhizium*) and among colonies exposed to *Metarhizium* (with or without larvae). Colonies with larvae exposed to *Metarhizium* were used in both comparisons. Different letters indicate  $p < 0.05$  in Tukey's HSD post hoc test for each comparison (upper box,  $\pm$  *Metarhizium* comparison; lower box,  $\pm$  larvae comparison).

diet treatment or larval presence ( $p > 0.56$ ). *Metarhizium*-challenged colonies had higher food consumption than did control colonies, and food consumption was particularly high for *Metarhizium*-challenged colonies with larvae on the high-protein diet (figure S1).

## 4. Discussion

We show for the first time that diet macronutrient composition affects immunity gains that arise from social grouping. When challenged with a pathogenic fungus (*M. anisopliae*), *E. ruidum* ants in worker groups survived longer when reared on a high-carbohydrate (1P:3C) diet than did those reared on a high-protein (3P:1C) diet, while solitary ants had similarly low survival rates when reared on either diet. These results do not simply reflect a general response to a low-quality diet because benefits from the high-carbohydrate diet only emerged in worker groups; there was no significant effect of diet in solitary conditions. In addition, a carbohydrate-rich diet reduced worker mortality rates when whole colonies were challenged with *Metarhizium*. Together, these results provide a novel mechanism by which nutrient balance can affect social groups and suggest an evolutionary advantage of carbohydrate exploitation by social insects.

As implied by the adage 'feed a cold, starve a fever', bulk food availability is generally considered an important mediator of immunity [6]. Immune functions have high metabolic costs relative to other physiological systems [7], and many studies have used starvation or caloric restriction treatments to show how bulk energy availability impairs individual disease resistance [8–10]. However, traits underlying immunity can require different nutritional mixtures and, as a result, the extent and nature of immune responses can depend on the relative availability of dietary components [12,18], including P : C balance [16,17]. Our results provide the first evidence that the immune consequences of nutrient balance can extend to

social groups and may emerge as a consequence of nest-mate interactions.

Antibiotics played a key role in this nutrition-related social immunity. Blocking metapleural glands, the main source of antibiotic secretions, eliminated both diet and social grouping effects on immunity (figure 2), and increased the likelihood that cadavers showed signs of *Metarhizium* infection. Metapleural gland function can account for 13–20% of basal metabolic rate [40], so carbohydrate scarcity in a high-protein diet could reduce metabolite allocation to metapleural glands or exact trade-offs on other metabolic functions. Note that *Metarhizium* resistance does not depend on the metapleural gland in some ant species [23,24], and thus constraints on metapleural secretions will not explain nutrition-related social immunity in all ants.

By contrast, our results do not support the hypothesis that increases in energetically expensive allogrooming result from a higher-carbohydrate diet. Even though carbohydrate-rich diets can lead to higher metabolic rates [41], and possibly higher activity rates in insects, we found no evidence for nutritional effects on grooming frequency. Note, however, that we only observed grooming behaviour for 50 min after treatment (as in [26]); it is possible that significant grooming frequency differences among treatments could emerge with a longer observation period.

Results from the whole-colony experiment (figure 4) also revealed the benefits of a high-carbohydrate diet to immune function. These results suggest that colonies can buffer isolated effects of high dietary protein or *Metarhizium* challenge, but the effect of these factors in combination can overwhelm colony resistance and lead to higher worker mortality rates. We also found that removing larvae from colonies eliminated diet effects on worker mortality rates when colonies were challenged with *Metarhizium* (figure 4). Larvae, which (unlike workers) can digest protein, can serve to dampen effects of nutritionally imbalanced diets for ants [32]. Our results suggest that these protein digestion benefits may improve worker immunity in colonies on high-carbohydrate diets.

Our results identify a neglected component to the story of the rise of sociality as represented by eusocial insects [42]. The diversification of the ants is closely tied to the rise of angiosperms [43], which gave ants access to a particular nutrient (carbohydrates) and a resource ('honeydew') that they could exploit, farm and dominate [44]. Carbohydrates have been suggested as a mechanism for maintaining the particularly high ant abundances in tropical canopies [45] owing to changes in territorial behaviour [44,34], worker longevity [32,35,33] and colony growth [46]. Our results suggest that readily available carbohydrates, by fuelling the expensive metapleural glands, also enhanced *per capita* immune function. Such an added benefit may have been crucial in allowing more ants to live together in tightly confined spaces. More generally, enhanced social immunity on a high-carbohydrate diet provides a novel mechanism by which carbohydrate exploitation could facilitate the evolution of social behaviour.

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**Data accessibility.** Data are available through the Dryad Digital Repository.

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