



Original Article

# Threat detection: contextual recognition and response to parasites by ants

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The ability of an organism to detect threats is fundamental to mounting a successful defense and this is particularly important when resisting parasites. Early detection of parasites allows for initiation of defense mechanisms, which are vital in mitigating the cost of infection and are likely to be especially important in social species, particularly those whose life history makes parasite pressure more significant. However, understanding the relative strength of behavioral responses in different species and situations is still limited. Here, we test the response of individual ants to fungal parasites in 3 different contexts, for 4 ant species with differing life histories. We found that ants from all 4 species were able to detect fungi on their food, environment, and nest mates and initiate avoidance or upregulate grooming behaviors accordingly to minimize the threat to themselves and the colony. Individuals avoided fungal-contaminated surfaces and increased grooming levels in response to fungal-contaminated nest mates. Ants from all species responded qualitatively in a similar way although the species differed quantitatively in some respects that may relate to life-history differences. The results show that ants of multiple species are capable of recognizing fungal threats in various contexts. The recognition of parasite threats may play an important role in enabling ant colonies to deal with the ever-present threat from disease.

**Key words:** disease resistance, harvester ant, host–parasite interaction, leaf-cutting ant, *Metarhizium*, parasite detection, social insect, weaver ant, wood ant.

## INTRODUCTION

Organisms possess an array of defenses to help combat potential threats from predators and parasites. Organisms can increase their fitness by monitoring predator abundances, parasite levels and habitat stability, and acting accordingly (Hart 1990; Blaustein et al. 2004; de Roode and Lefèvre 2012). In each case, detection of the threat is a fundamental prerequisite for the launch of any targeted response or decision. This is particularly key for resisting parasites, whose coevolutionary arms race with their host can select for better defended and more vigilant hosts, and also for more exploitative and harder to detect parasites (Ebert and Hamilton 1996; Decaestecker et al. 2007). In insects, microparasites such as entomopathogenic fungi are often lethal to the host, and proactive avoidance of exposure is invariably a better strategy than relying on resistance postexposure (Shah and Pell 2003). Early and accurate detection potentially allows for avoidance of the threat altogether, or at least the initiation of early defense mechanisms, which may be vital in mitigating the cost of the infection (Hart 1990; Schmid-Hempel and Ebert 2003; Wisenden et al. 2009). Although there

has been substantial work on the detection and triggering of physiological immune responses (Hoffmann et al. 1996; Medzhitov and Janeway 2000; Siva-Jothy et al. 2005), our understanding of the ability of insects to detect the threat of parasites prior to infection is less well developed, despite its probable importance (Hart 1990).

Eusocial insects are thought to be particularly at risk from the threat of disease due to living in dense groups, with homeostatic nest environments, and high levels of relatedness within a colony (Schmid-Hempel 1998). However, evidence of this increased parasite pressure is often lacking and this is thought to be because social insects have reduced the cost of group living through the development of effective group-level defenses, termed “social immunity” (Cremer et al. 2007). These include behavioral adaptations such as undertaking, waste management, and grooming behavior, which are effective at removing parasites from individuals and the colony as a whole (Boomsma et al. 2005; Wilson-Rich et al. 2009). As many of these behaviors rely on the ability to target a contaminated item or individual, parasite detection is likely to be particularly important in eusocial insects. Food sources, such as flowers or leaves (Durrer and Schmid-Hempel 1994; Griffiths and Hughes 2010; Parker et al. 2010; Fouks and Lattorff 2011), can become a dangerous hub for the horizontal transmission of parasites to visiting individuals, and consequently, insects can detect and

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avoid contaminated food (de Roode and Lefèvre 2012). Similarly, termites, crickets, and ladybirds will preferentially avoid environments heavily contaminated with entomopathogenic fungi (Staples and Milner 2000; Thompson and Brandenburg 2007; Ormond et al. 2011), and ants and termites will increase self-grooming or allogrooming in the presence of fungal conidia (Rosengaus et al. 1998; Yanagawa and Shimizu 2006; Yanagawa et al. 2008; Walker and Hughes 2009; Morelos-Juárez et al. 2010; Reber et al. 2011). Responses have been shown to scale with the severity of the threat in termites, whose antennae play an important role in the detection of fungal conidia (Rosengaus et al. 1999; Myles 2002; Yanagawa et al. 2009). Additionally, the ability to detect an external parasite threat may vary between species with different evolutionary parasite pressures and life histories or between different parasites. Host species that are exposed to greater parasite pressure, for example, because they have vulnerable food stores, feed on more contaminated food or in more contaminated environments may be more adept at detecting and recognizing threats (Boomsma et al. 2005; Walker and Hughes 2011). Similarly, hosts will be selected to be better at detecting more dangerous threats, such as from virulent entomopathogens (Dieckmann 2002; Poulin 2007; Mburu et al. 2011). Both host life-history and parasite virulence have been shown to affect the evolution of host disease resistance (Currie 2001; Sumner et al. 2003; Fernández-Marín et al. 2006 2009; Hughes et al. 2008), but their effects on disease avoidance are less clear.

Although there has, therefore, been much progress made in understanding the resistance to parasites of insects in general, and of social insects in particular, our understanding of the behavioral recognition phase of resistance is still limited. In particular, it is not clear how the recognition and response of social insects to parasites may vary depending on the context of exposure, for example, whether the parasite is encountered in food, the general environment, or on a nest mate. It is also not clear the extent to which the ability to behaviorally recognize and respond to parasites is present across taxa or indeed if the ability may differ between social insect species. The behavioral response of social insects to parasites can sometimes be counterintuitive (Brütsch et al. 2014), so knowledge of this stage is important for a full understanding of the complete process that takes place from encountering a parasite to resistance or infection.

Here, we test the ability of individual ants to detect and respond to the presence of 2 fungal pathogens. To determine how the response was affected by context, we presented the fungal pathogens under controlled laboratory conditions, without the confounding effects of other environmental cues, and mimicked exposure via the 3 key routes of ingress for a parasite into a colony: through contaminated food, environment, and nest mates (Schmid-Hempel 1998; Boomsma et al. 2005). Studies typically focus on a single species, but interspecific variation in parasite resistance is likely, so we here tested the response of ants from 4 species with similar colony sizes but different life histories: 1) *Polyrhachis dives*, an omnivorous Southeast Asian weaver ant, which may frequently encounter contaminated food but which only has to protect its own nest mates from disease (Hung 1967); 2) *Messor barbarus*, a granivorous, European seed-harvesting ant that has, in addition to its nest mates, to protect its food store of seeds from microbial contaminants (Plowes et al. 2013); 3) *Acromyrmex echinator*, a mycophagous Panamanian leaf-cutting ant that also has to protect a food store, but in which this is in the form of a fungal crop that is highly vulnerable to foreign microbes (Currie et al. 1999); 4) *Formica rufa*, a European wood ant that build large nests out of conifer needles

and plant debris and feeds on a mix of insect honeydew and scavenged carcasses. Harvester ants, leaf-cutting ants, and wood ants possess antimicrobial producing metapleural and venom glands, but weaver ants lack the metapleural gland, and the venom of wood ants and weaver ants may have particularly strong antimicrobial properties because it consists largely of formic acid (Attygalle and Morgan 1984; Hölldobler and Wilson 1990; Billen 2009; Yek and Mueller 2011; Tragust et al. 2013). As leaf-cutting ants and harvester ants have vulnerable food stores to protect, we would predict that they may be more vigilant at preventing the ingress of pathogens into the colony, but we would also predict that all ant species will show effective behaviors to minimize their risk from parasites in the environment and on themselves.

## METHODS

The experiments were conducted using randomly selected individual foragers. We tested individuals from 4 colonies each of leaf-cutting ants (Ae396, Ae398, Ae399, and Ae088), harvester ants (Mb0801, Mb1201, Mb1301, and Mb1302), and wood ants (Fr1301, Fr1302, Fr1303, and Fr1304) and 2 colonies of weaver ants (Pd0701 and Pd0704). The colonies had been kept at 27 °C, 80% relative humidity, 12:12h photoperiod, on species-specific diets provided twice a week (*Tenebrio* larvae and 20% sucrose solution for weaver ants and wood ants, the same supplemented by grass seeds for harvester ants, and privet leaves for leaf-cutting ants). All leaf-cutting ant and weaver ant colonies and harvester ant colonies Mb0801 and Mb1201 had been kept as above for at least 12 months prior to the experiments, whereas all the wood ant colonies and the harvester ant colonies Mb1301 and Mb1301 had been kept for 1 month, and all appeared in good health (no signs of parasite infections or excessive mortality). The colonies were given ad libitum water and 20% sucrose solution throughout the experiment, but were starved of solid food during, and for 3 days prior to, Experiment 1: looking at the response of ants to contaminated food. Four treatments were tested: 1) conidia of the specialist entomopathogenic fungus *Metarhizium anisopliae* (strain ARSEF 144467, isolated from the soil of a Canadian maize field), 2) conidia of the facultative entomopathogenic fungus *Aspergillus flavus* (GU172440.1, isolated from bees in an experimental apiary West Yorkshire, UK; Foley et al. 2012), 3) talcum powder control (to control for the presence of a physical particulate; talcum particles were  $5.2 \pm 6.6 \mu\text{m} \times 5.3 \pm 7.7 \mu\text{m}$  compared with  $5.5 \pm 0.2 \mu\text{m} \times 3.3 \pm 0.2 \mu\text{m}$  for the *M. anisopliae* conidia and  $3.6 \pm 0.1 \mu\text{m} \times 3.6 \pm 0.2 \mu\text{m}$  for the *A. flavus* conidia), and 4) blank control (to control for the Triton-X used as a surfactant in delivery of fungal conidia). Both *M. anisopliae* and *A. flavus* are very common in the soil environment of ants at many locations and have been reported as natural parasites of ants on numerous occasions (Jouvenaz et al. 1972; Allen and Buren 1974; Alves and Sosa-Gómez 1983; Lofgren and Vander Meer 1986; Gilliam et al. 1990; Diehl-Fleig et al. 1992; Humber 1992; Sanchez-Pena and Thorvilson 1992; Quiroz et al. 1996; Schmid-Hempel 1998; Hughes et al. 2004; Poulsen et al. 2006; de Zarzuela et al. 2007 2012; Castilho et al. 2010; Lacerda et al. 2010; Rodrigues et al. 2010; Ribeiro et al. 2012). Multiple species of *Aspergillus* have also been reported growing on the fungal garden of leaf-cutting ants or nest material of weaver ants and will, given the opportunity, quickly overgrow them (Fountain and Hughes 2011; Tranter et al. 2013). Additionally, many opportunistic fungal species are found on the seeds harvested and stored within the colonies of the granivorous harvester ants, and *Aspergillus* can be a common

and important threat to seed stores (Klich et al. 1984; Crist and Friese 1993; Satish et al. 2007). Although the generalist nature of both parasites makes coevolution with ant hosts unlikely, we used exotic strains of both parasites to avoid any potential for the parasites to have evolved to avoid recognition by any of the ant species used here. *Metarhizium anisopliae* is a more virulent entomopathogen than *A. flavus* (Zimmermann 1993; Glare et al. 1996; Frazzon et al. 2000; Hughes and Boomsma 2004; Scully and Bidochka 2005) and thus would be expected to stimulate a more extreme behavioral response from ants. In Experiments 1 and 2 below, the conidia and talcum particles were made up as suspensions of  $1.5 \times 10^8$  conidia or particles per milliliter in 0.05% Triton-X surfactant using a blank hemocytometer, with the control being pure 0.05% Triton-X solution. In Experiment 3, looking at the response of ants to contaminated nest mates, the conidia and talcum powder were applied dry to avoid grooming being stimulated by the presence of a liquid on the cuticle. Fungal conidia were harvested from freshly sporulating media plates, and viability was confirmed to be >90% throughout the experiments by plating the conidia solutions onto Sabouraud dextrose agar, incubating for 24 h and quantifying successful conidia germination, defined as the production of a germ tube longer than the conidia diameter (Siegel 2012).

### Experiment 1: response to contaminated food

In order to test the ability of the individual ants to detect and avoid contaminated food, ants from each species were presented with food treated with either *Metarhizium* or *Aspergillus* conidia, talcum powder control, or Triton-X control solution. Weaver ant and wood ant workers were provided with a section of *Tenebrio molitor* larvae (length: 8 mm, diameter: 2.5 mm), harvester ant workers with 2 grains of rice (length: 7 mm, diameter: 1.8 mm), and leaf-cutting ant workers with a section of fresh privet leaf (*Lingustrum* sp. length: 8 mm, width: 8 mm), with the surface area of the food ( $\sim 64 \text{ mm}^2$ ) being the same in each case. Shortly prior to the experiment, an even coating of 8  $\mu\text{L}$  of the treatment solution was pipetted over the surface of the food and allowed to dry, resulting in a treatment density of approximately 1875 conidia/ $\text{mm}^2$  (the number of conidia adhering to the different food sources was very similar, see Supplementary Table S1). A Fluon-lined 90-mm Petri dish was placed in the foraging arena of each colony on a Fluon-coated tripod so that ants could only enter via a removable bridge (Supplementary Figure S1). The ants were allowed to acclimatize to the general apparatus over several days. For each trial, a piece of filter paper was placed in the dish and a single foraging worker was allowed to enter the dish, with the ant then confined within an inverted transparent pot (25 mm diameter) for 5 min to allow it to acclimatize and ensure a consistent starting position within the dish to avoid biasing. The food was then placed in the center of the Petri dish, the pot removed, and interactions between the ant and the food recorded by eye for 15 min. The behaviors recorded were 1) whether the ants appeared to attempted to harvest the food, that is, transported the food from the center of the Petri dish to the edge closest to their nest where the bridge was previously located, 2) the length of time spent interacting with the food (i.e., direct antennation, cutting or feeding, picking up without moving), and 3) the length of time spent self-grooming. For leaf-cutting ants and wood ants, this was repeated with  $n = 64$  ants from each species for each of the 4 treatments (16 ants for each of the 4 colonies per species, per treatment). For harvester ants, this was repeated with  $n = 48$  ants per treatment (16 ants from 2 colonies, 8 ants from 2 colonies), and with  $n = 32$  ants per treatment for weaver ants (16 ants from 2

colonies). The filter paper was replaced after each trial to remove any cues potentially left on the paper from the previous trial.

### Experiment 2: response to contaminated environment

A 90-mm diameter filter paper of 2  $\mu\text{m}$  porosity, sufficient to prevent the passage of fungal conidia (see dimensions above), was divided into two, with one half infused evenly with 0.4 mL of 1 of the 4 treatments, resulting in an approximate treatment density of 1875 conidia/ $\text{mm}^2$  for the fungal treatments or 1875 particles/ $\text{mm}^2$  for the talcum powder treatment, and the other half infused with 0.4 mL of 0.05% Triton-X control solution. Once dry, the two halves were placed in a Petri dish to provide a simple choice setup, with one half of the Petri dish treated and the other untreated. The Petri dish arena was placed in an enclosure formed of blank white surrounding walls with diffuse lighting in order to remove visual orientation cues. For each trial, an ant was placed in the Petri dish and confined within a transparent pot (25 mm diameter) at the center of the dish for 5 min to allow the ants to calm down after their initial alarm and ensure a consistent starting position. The pot was then removed and the ant video recorded (Logitech B910 HD) from a fixed and consistent position above the dishes for 15 min. Lighting was provided by strip lighting on the ceiling 170 cm above dish, and trials were performed at a room temperature of 21 °C. For leaf-cutting ants and wood ants, this was repeated with  $n = 64$  ants from each species for each of the 4 treatments (16 ants for each of the 4 colonies per species, per treatment). For harvester ants, this was repeated with  $n = 48$  ants per treatment (16 ants from 2 colonies, 8 ants from 2 colonies) and with  $n = 32$  ants per treatment for weaver ants (16 ants from 2 colonies). The filter paper was replaced between each trial, and the Petri dish replaced and reoriented by 180° every fourth trial, to remove any chemical cues and control for any visual cues that may have influenced the results. The videos were analyzed to obtain the length of time spent, speed travelled, distance covered, and time spent stopped, by the ant on either half of the Petri dish. Results from the video analysis were outputted from AntTrak (Supplementary Table S2), a path analysis program designed for this task, as data for analysis and as images of the tracks for visual inspection.

### Experiment 3: response to contaminated nest mates

The ability of individual ants to recognize contaminated nest mates was tested by applying dry conidia or talcum powder evenly to the gaster of a treatment ant with a cotton bud, before placing the treated ant with an untreated nest mate in a Petri dish. Application was performed so as to provide a constant treatment layer between trials while accounting for species and body size. A clean blank cotton bud was brushed onto the ant in the same manner as above for the control treatment. Instances of contact (defined as any non-grooming interaction, e.g., antennation) between the 2 ants, self-grooming by the treatment ant, and allogrooming were tallied by eye during 10 min. For leaf-cutting ants and wood ants, this was repeated with  $n = 64$  ants from each species for each of the 4 treatments (16 ants for each of the 4 colonies per species, per treatment). For harvester ants, this was repeated with  $n = 48$  ants per treatment (16 ants from 2 colonies, 8 ants from 2 colonies) and with  $n = 32$  ants per treatment for weaver ants (16 ants from 2 colonies). None of the ants were used more than once and none were returned to the colony after use to avoid influencing the independence of other workers.

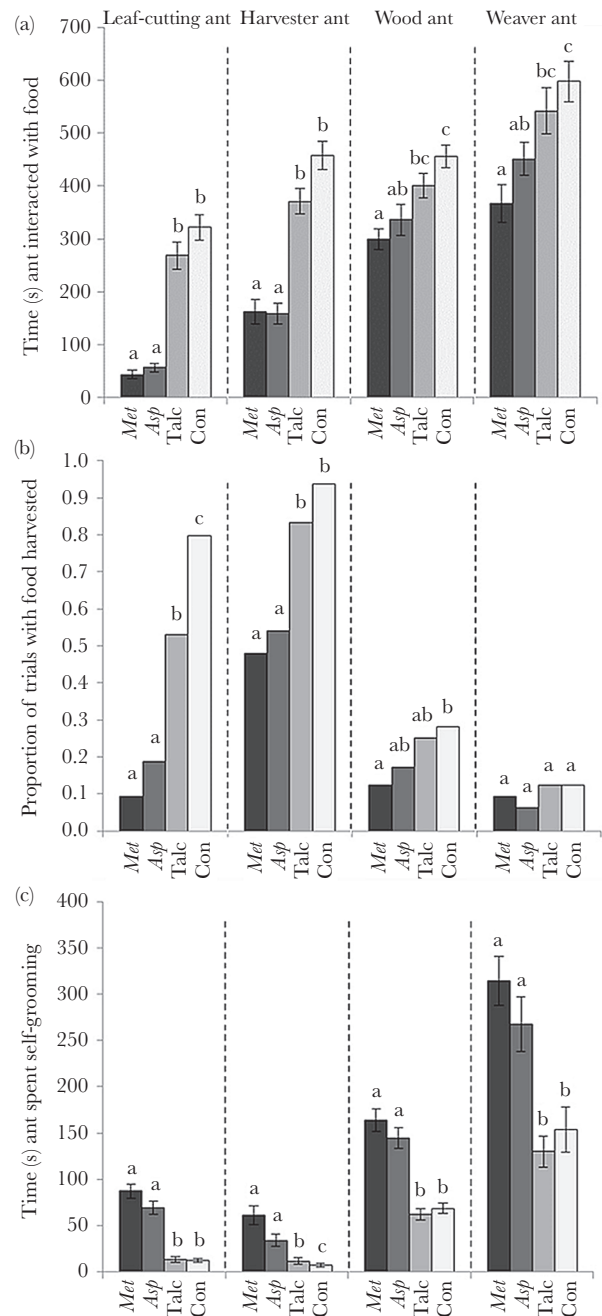
## Statistical analysis

Data from all experiments were nonnormal, so generalized linear mixed models were used throughout with model distribution determined based on Akaike information criterion scores and the structure of the nonnormal data. No overdispersion was observed based on model deviance/degrees of freedom values. Nonsignificant interaction terms within the models were removed based on likelihood-ratio tests to achieve the minimum adequate models. The effect of the colony from which the individual ants tested were obtained was included in all Generalized linear mixed model (GLMM) analyses as a random factor but was nonsignificant ( $P > 0.05$ ) in all cases except when comparing contact rates in Experiment 3. For all experiments, overall tests were run on total data with species and treatment as factors, but the effect of treatment was additionally analyzed for each species individually. In Experiment 1, the effects of treatment and species on the length of time individual ants spent engaged in each of the activities were analyzed using a GLMM with gamma distribution and log link function. The proportion of trials in which ants harvested food was analyzed using a GLMM with binomial distribution and probit link function. For Experiment 2, a GLMM with gamma distribution and log link function was used to analyze the effect of treatment and species on the relative proportions of length of time, speed, and distance travelled (see Supplementary Figure S3 for distance travelled). A GLMM with negative-binomial distribution and log link function was used to analyze the effect of time spent inactive on the treated side. For Experiment 3, the number of contacts, allogrooming, and self-grooming were tested between the 4 different treatments and 4 different species using a GLMM with gamma distribution and log link function. Post hoc comparisons between individual treatment groups were conducted using a pairwise sequential Bonferroni comparisons for all 3 experiments. All analyses were conducted using SPSS 20.

## RESULTS

### Experiment 1: response to contaminated food

There was a significant overall interaction between ant species and treatment, indicating that ants from different species were responding differently to the various treatments, on the length of time ants spent interacting with food ( $F_{9,816} = 17.04$ ,  $P < 0.001$ ), the proportion of trials in which ants harvested food ( $F_{9,816} = 3.45$ ,  $P = 0.002$ ), and the length of time ants spent self-grooming ( $F_{9,816} = 4.49$ ,  $P < 0.001$ ; Supplementary Table S3). When analyzed individually, all species showed a significant effect of treatment on the length of time spent interacting with, and in the time spent grooming after exposure to, contaminated food (Supplementary Table S4). Both leaf-cutting and harvester ants also showed a significant difference in their propensity to harvest food (i.e., transporting the food toward the position where the bridge leading back to the nest had been located) depending on whether it was contaminated or uncontaminated, but the wood ants and weaver ants did not. Weaver ants interacted with both contaminated and uncontaminated food for the longest time, and leaf-cutting ants the least (Figure 1a; Supplementary Figure S2). In all species, there was no significant difference in interaction time with food between the 2 fungal treatments or between the 2 control treatments. Leaf-cutting ants and harvester ants showed a much greater difference in the time spent interacting with fungal-treated opposed to control-treated food, when compared with wood ants and weaver ants. In both wood ants and weaver ants, there was no significant difference in food-interaction time between the *Aspergillus* and talcum powder treatments.



**Figure 1**

The (a) mean  $\pm$  SE time spent interacting with food, (b) proportion of food harvested (transported the food to the position of the bridge leading back to the colony), and (c) mean  $\pm$  SE time spent self-grooming by leaf-cutting ants ( $n = 64$ ), harvester ants ( $n = 48$ ), wood ants ( $n = 64$ ), and weaver ants ( $n = 32$ ) in Experiment 1. Food was treated with either the *Metarhizium* (*Met.*) or *Aspergillus* (*Asp.*) fungal pathogens, talcum powder control (Talc.), or control solution (Con.). Within each graph, treatments within each species group with different letters differed significantly from one another at  $P < 0.05$ .

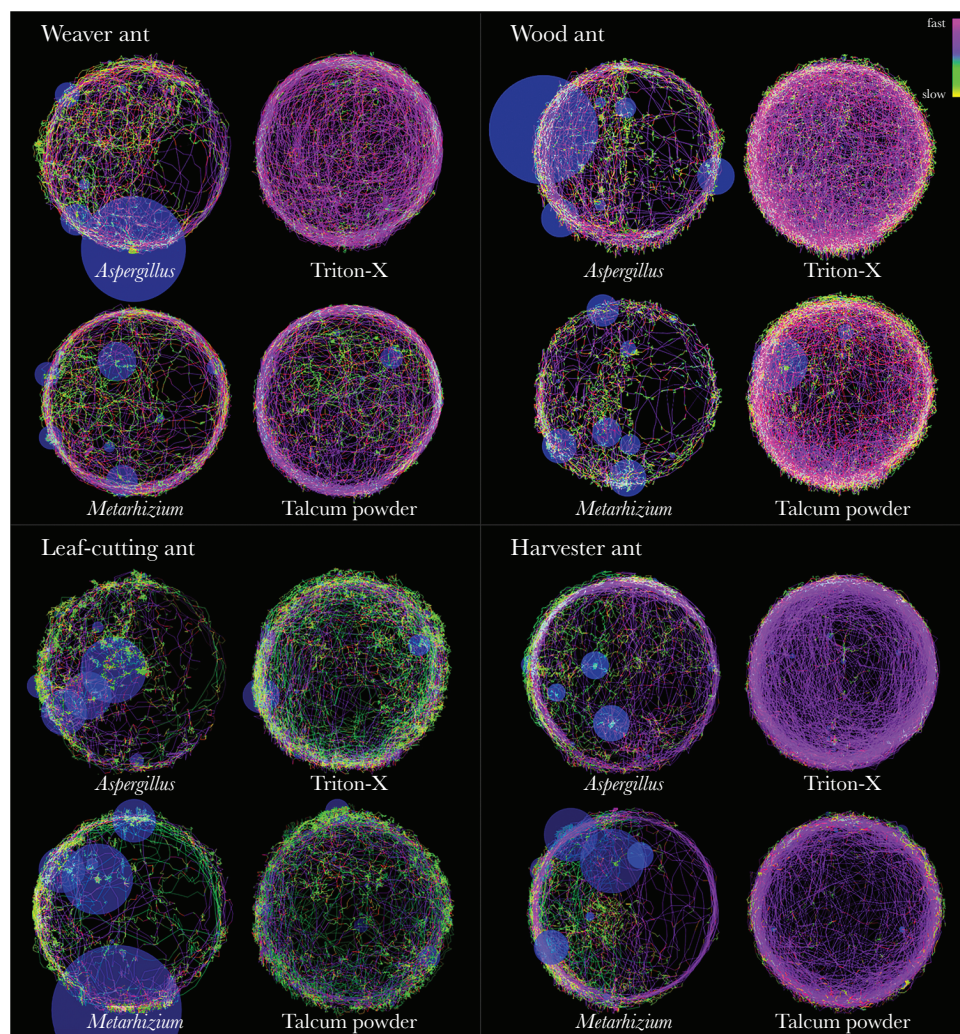
Harvester ants harvested the food in most trials, whereas weaver ants very rarely harvested the food offered (Figure 1b). Less food was harvested by leaf-cutting ants and harvester ants when it was contaminated with *Metarhizium* or *Aspergillus* conidia than when it had been treated with the blank control or talcum powder. Wood ants

only showed a difference in the proportion of trials where food was harvested between the *Metarhizium* and blank control treatments, and weaver ants showed no significant difference between any of the treatments. Leaf-cutting ants also were significantly more likely to harvest food treated with the blank control compared with food treated with talcum powder. Harvester ants groomed in the fewest trials, but at a similar level to leaf-cutting ants, whereas wood ants and weaver ants self-groomed for much longer (Figure 1c). All species of ants groomed significantly more after interacting with *Metarhizium*- and *Aspergillus*-treated food compared with the 2 control treatments, and harvester ants groomed less after interacting with on blank control-treated food compared with food treated with a talcum powder control.

## Experiment 2: detection of contaminated environment

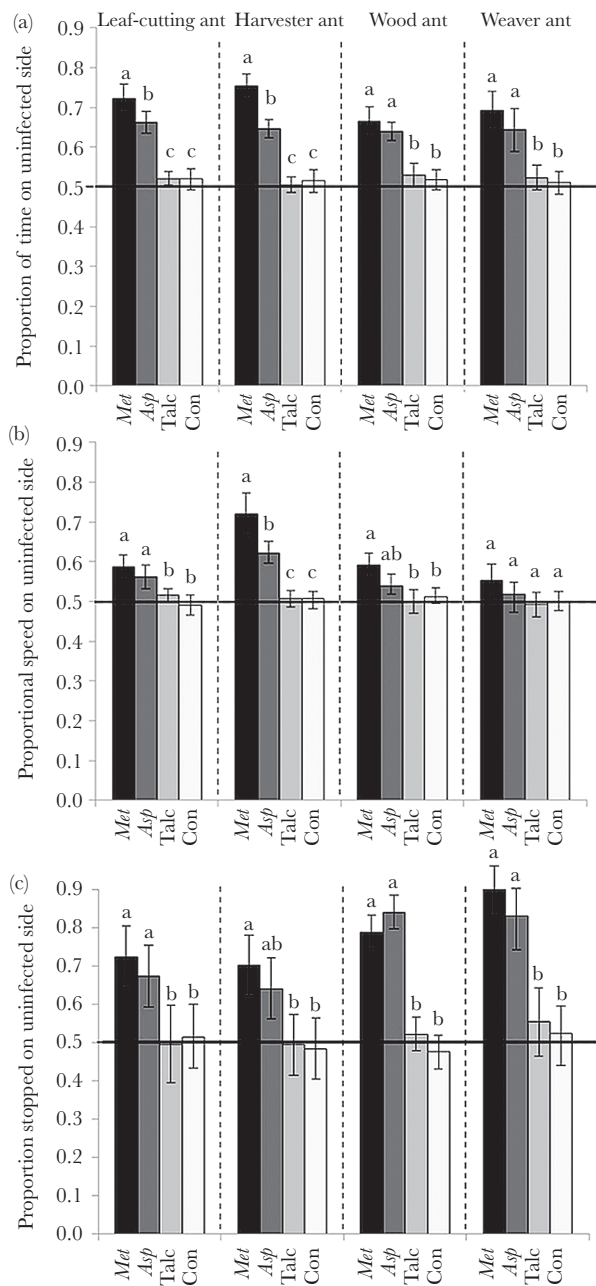
Data from the video analysis of ant tracks (Figure 2) showed that there was overall a significant interaction between the effect of species and treatment on the proportion of time spent ( $F_{3,816} = 5.98$ ,

$P = 0.001$ ), speed travelled at ( $F_{3,816} = 4.24$ ,  $P = 0.006$ ), and length of time spent inactive ( $F_{3,816} = 16.65$ ,  $P < 0.001$ ) by ants on the untreated side of the enclosure (Supplementary Table S5). All 4 species, when analyzed individually, showed a significant effect of treatment on the total time spent, and also the time spent inactive, on the treatment side. Harvester ants and wood ants also moved at significantly different speeds on the treated half depending on the treatment applied (Supplementary Table S6). All species spent significantly longer on the uncontaminated half when the other side had a fungal treatment applied compared with the blank and talcum powder control treatments where ants showed no preference for either side (Figure 3a; Supplementary Figure S3). Additionally, leaf-cutting ants and harvester ants spent longer on the uncontaminated side when the other half had been treated with *Metarhizium* compared with when it had been treated with *Aspergillus*. Leaf-cutting ants, harvester ants, and wood ants moved significantly faster on surfaces treated with either *Metarhizium* or *Aspergillus* compared with either control treatment, where the ants travelled at the



**Figure 2**

Composite tracks from Experiment 2 for 4 environmental treatments produced from the video analysis of choice trials in 4 ant species (top left: weaver ant, top right: wood ant, bottom left: leaf-cutting ant, and bottom right: harvester ant). Each of the 4 graphics within each quarter represents an overlay of 10 individual paths. Labels below each graphic show the treatment applied to the right side of the circle compared with a control treatment on the left side. The track is color coded from pink and purple, where the ant travelled fastest, to green and yellow, where the ant travelled more slowly. A blue circle is present to represent the point at which an ant stopped, and the larger the circle, the longer the time spent stationary.



**Figure 3**

The (a) proportion  $\pm$  95% confidence interval (CI) of time spent, (b) speed travelled, and (c) time spent inactive, on the treatment side of a choice arena treated with either the *Metarhizium* (*Met.*) or *Aspergillus* (*Asp.*) fungal pathogens, talcum powder control (Talc.), or control solution (Con.) in Experiment 2, for leaf-cutting ants ( $n = 64$ ), harvester ants ( $n = 48$ ), wood ants ( $n = 64$ ), and weaver ants ( $n = 32$ ). Proportions with 95% CI error bars that do not overlap 0.5 line show a significant difference between treated and untreated sides. Within each graph, treatments within each species group with different letters differed significantly from one another at  $P < 0.05$ .

same speed on either half of the Petri dish (Figure 3b). This difference was greatest in harvester ants, with the largest difference in speed observed in the *Metarhizium* treatment. Weaver ants did not alter their speed depending on whether they were on treated or untreated halves in any of the treatments. All 4 species of ants stopped for significantly longer on the untreated side in the fungal

trials but not in the control trials (Figure 3c). This difference was greatest in the wood ants and weaver ants, which spent around 80% of their inactive time on the untreated sides when fungal conidia were present on the alternative.

### Experiment 3: detection of contaminated nest mates

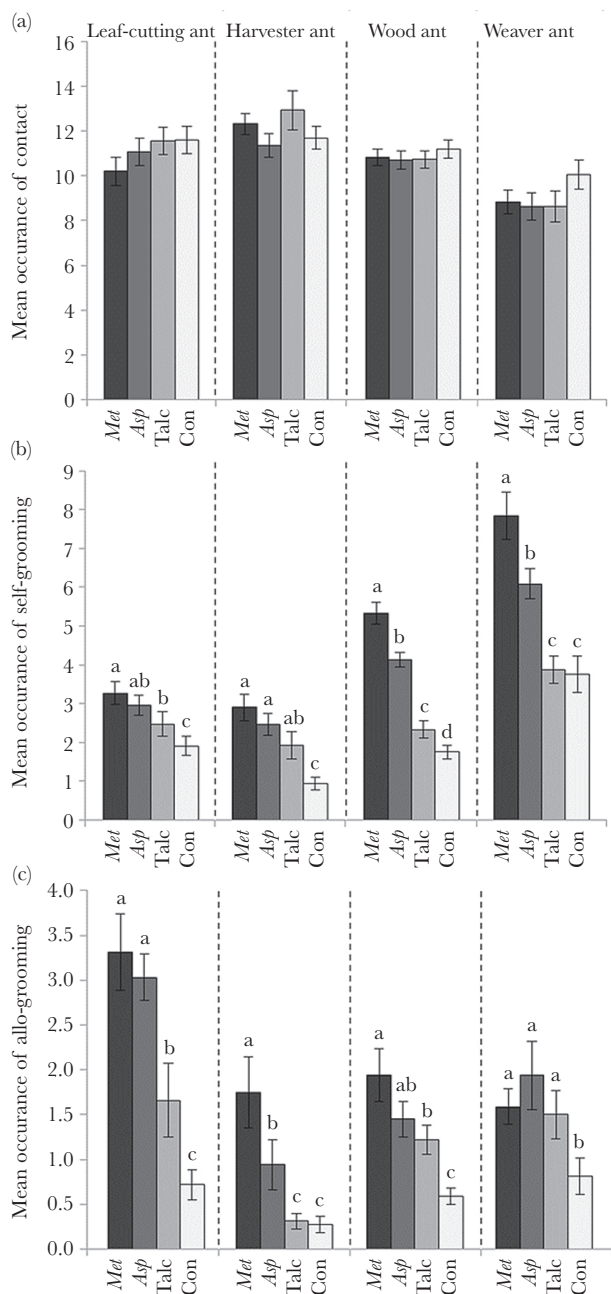
Contact rates between the contaminated ant and its uncontaminated nest mate showed a significant difference between the 4 species ( $F_{3,816} = 10.82$ ,  $P < 0.001$ ), with weaver ants showing slightly lower baseline levels of contact compared with the other species, but there was no overall difference between treatments or evidence of interaction ( $F_{3,816} = 2.21$ ,  $P = 0.085$ , and  $F_{9,816} = 1.80$ ,  $P = 0.06$ , respectively; Supplementary Table S7). When analyzed individually, only weaver ants showed a significant effect of treatment on contact rates (Supplementary Table S8). There was a significant effect of colony on overall contact rates with Mb1 showing consistently higher rates of contact than the other harvester ant colonies ( $\zeta = 5.95$ ,  $P < 0.001$ ). Harvester ants exhibited the highest contact rates, significantly higher than weaver ants, which had the lowest contact rate (Figure 4a; Supplementary Figure S4).

There was a significant interaction between the effect of species and treatment on both self-grooming ( $F_{9,816} = 2.77$ ,  $P = 0.003$ ) and allogrooming rates ( $F_{9,816} = 2.03$ ,  $P = 0.03$ ). Weaver ants had higher baselines levels of self-grooming than any of the other species, which showed similar levels of self-grooming (Figure 4b). Ants from all species showed higher frequencies of self-grooming after interaction with nest mates treated with a fungal pathogen. This was significantly different to control treatments for harvester ants and weaver ants in the *Aspergillus* treatment and in the *Metarhizium* treatment for leaf-cutting ants. Harvester ants allogroomed the least, less than weaver ants, who in turn allogroomed less than leaf-cutting ants (Figure 4c). When analyzed individually, each species showed a significant effect of treatment on allogrooming and self-grooming responses, except allogrooming in weaver ants. In all species, the control treatment resulted in the lowest incidence of allogrooming, with this being significantly lower than all other treatment groups in leaf-cutting ants and significantly lower than the *Metarhizium* treatment in harvester ants. Harvester ants allogroomed *Metarhizium*-treated nest mates more than those treated with talcum powder or untreated nest mates, but in leaf-cutting ants, the talcum powder treatment also resulted in significantly higher rates of allogrooming than the blank control. This result is likely due to colony Ae396, where talcum powder produced dramatically high allogrooming rates (Figure 4c; Supplementary Figure S4c).

## DISCUSSION

The results demonstrate the ability of ants to detect fungal pathogens on their food, environment, and nest mates. Ants from all the species tested avoided fungal-contaminated surfaces and increased either allo or self-grooming behaviors when they detected contaminants on a nest mate. Treatments of the obligate entomopathogen *Metarhizium* generally resulted in the strongest recognition responses compared with the facultatively entomopathogenic *Aspergillus* and the control treatments. Individual ants from the 4 species showed different responses depending on the source of contamination.

When presented with contaminated food, individual ants were highly discriminatory between food treated with the controls and that treated with either *Metarhizium* or *Aspergillus* fungal parasites. Avoidance of parasites when feeding may be particularly important.



**Figure 4**

The (a) mean  $\pm$  SE occurrence of contact and (b) self-grooming by the test ant and of (c) allogrooming between treated and test ant in Experiment 3 of leaf-cutting ants ( $n = 64$ ), harvester ants ( $n = 48$ ), wood ants ( $n = 64$ ), and weaver ants ( $n = 32$ ) to nest mates that had been treated with either the *Metarhizium* (*Met.*) or *Aspergillus* (*Asp.*) fungal pathogens, talcum powder control (*Talc.*), or control solution (*Con.*). Within each graph, treatments within each species group with different letters differed significantly from one another at  $P < 0.05$ .

Although fungal conidia can be deactivated in the guts of adult insects, any ingestion will carry a risk of infection if deactivation is not completely effective and the larvae, for which proteinaceous food is primarily collected, may lack the deactivation capabilities of adults (Broome et al. 1976; Dillon and Charnley 1988; Siva-Jothy et al. 2005; Chouvenec et al. 2010). Additionally, food stored in the crop which is transferred by trophallaxis may still pose a risk of

horizontal transmission to other ants, as the fungistatic activity may only be sufficient to retard germination and not completely sterilize conidia (Shah and Pell 2003; Chouvenec et al. 2010).

Ants that did interact with food with fungi present showed significantly higher rates of self-grooming. Similarly, the individual ants from all species tested upregulated self-grooming, and 3 out of the 4 species also allogroomed more frequently, in response to fungal-contaminated nest mates. Grooming is an important defense against parasites and is an adaptive behavior that ants and other social insects can use on encountering fungal pathogens in various contexts, both to protect themselves, and nest mates (Cremer et al. 2007; Yanagawa et al. 2008; Reber et al. 2011). As well as directly removing parasites from the cuticle, grooming also transfers antimicrobial secretions from the metapleural gland and venom glands (Fernández-Marín et al. 2006; Tragust et al. 2013). The relative investment into these different forms of grooming may vary based on the nature of the threat and ant species (Okuno et al. 2011). For example, weaver ants have relatively high levels of self-grooming and low levels of allogrooming, whereas leaf-cutting ants have the opposite pattern (Figure 4b,c). Allogrooming as a defense may require a greater investment from the colony as it involves the time and activities of 2 or more individuals, but it may also be more effective (Hughes et al. 2002; Yanagawa and Shimizu 2006). Although preliminary observations of ants encountering uncontaminated food in the Experiment 1 arena when the bridge was left in place (Supplementary Figure S1) confirmed that ants picking up the food then transported it back to the nest, we cannot be certain that this would have been the case for the ants in all our trials although it seems likely.

It is likely that ants in these experiments were using chemical receptors to detect the presence of contaminants (Yanagawa et al. 2009), though physical detection may have also played a part in the trials where the treatments were applied dry. Ants possess a well-developed ability to detect and communicate information via chemical signals, which is fundamental to nest mate recognition and recruitment, trail building, and alarm behaviors (Hölldobler 1978; Hölldobler and Wilson 1990; Hughes and Goulson 2001). Fungi produce small size volatile organic compounds (Morath et al. 2012), which are detectable by insects and can act as signaling molecules (Rohlfis et al. 2005). Beetles are attracted to food through detection of volatiles produced by wood-rotting fungi (Drilling and Dettner 2009), pollinators can be deceived by flower-mimic fungi, which produce volatiles similar to the real flower (Ngugi and Scherm 2006), and ant queens may, unusually, be attracted to nest sites with entomopathogenic fungi (Brütsch et al. 2014). Conversely, invertebrates may be repelled by, or show alarm behavior in response to, chemical cues from fungi, which may indicate a potential threat (Rosengaus et al. 1999; Staples and Milner 2000; Wood et al. 2001; Hussain et al. 2010; Fouks and Lattorff 2011).

Although the results show that ants can detect the various parasite threats, they do not reveal whether the differences are due to differences in detection ability or in behavioral response after detection. Further work will be needed to establish this. Additionally, ants may alter their response threshold to a detectable threat based on the costs of avoidance or defense. For social insects in particular, this trade-off may be complicated to assess as any benefits and costs need to be considered at both individual and colony levels (Wilson-Rich et al. 2009). In natural conditions, avoidance of contaminated food or reduced exploration of unhygienic environments may protect the individual ant from infection (Wisenden et al. 2009), but this benefit may carry a colony-level cost by reducing

food harvesting. Avoidance of contaminated nest mates may result in a reduced individual hazard, but overall a much greater threat to the colony as a whole, should a parasitized ant be allowed into the nest without intervention (Wilson-Rich et al. 2009, but see Hughes et al. 2002; Konrad et al. 2012).

Although all 4 ant species responded to parasites in a qualitatively similar way, there were some interesting quantitative differences in their responses. In particular, leaf-cutting ants and, to a lesser extent, harvester ants were more strongly discriminatory of contaminated food than weaver ants and wood ants. The leaf fragments that leaf-cutting ants retrieve are used as a substrate for their mutualistic fungal crop, which is very vulnerable to other fungi, including *Aspergillus* (Luciano et al. 1995; Ortiz and Orduz 2001; Little et al. 2006; Pagnocca 2012; Tranter et al. 2013), and leaf-cutting ants are well known to scrupulously clean material to protect their fungal crop (Currie and Stuart 2001; Van Bael et al. 2009; Griffiths and Hughes 2010; Morelos-Juárez et al. 2010). Harvester ants store their seed food in granaries, which may also be vulnerable to fungal growth, whereas weaver ants and wood ants possess no equivalent, long-term, within-colony food store to protect. In addition, leaf-cutting ants showed relatively high levels of allogrooming, whereas weaver ants showed relatively high levels of self-grooming. It may be that weaver ants have evolved high rates of self-grooming to compensate for their lack of antibiotic-producing metapleural glands by greater mechanical removal of parasites or because self-grooming is needed to spread their antimicrobial venom actively over their cuticle (Hölldobler and Engel-Siegel 1984; Yek and Mueller 2011; Graystock and Hughes 2011; Tragust et al. 2013). Future comparative studies with more species will be important to establish whether such life-history differences do indeed drive variation in parasite response behavior, and we may expect the response of these species to be even stronger when presented with fungi that are more dangerous parasites of food stores, such as *Escovopsis* (Crist and Friese 1993; Currie 2001).

In conclusion, the results show that individual ants are capable of recognizing fungal threats in various contexts. Host–parasite interaction studies are often conducted on a single host species, but here, we use 4 different ant species to better investigate how individual ants respond to the threat. Ant societies are well known for their organized division of labor and task partitioning, and it will be interesting to see whether ants vary in their ability to detect parasites according to their role in the colony (Anderson and Ratnieks 1999; Vitikainen and Sundström 2011). It will also be interesting to see whether species differences are due to differences in detection ability or behavioral response, whether detection thresholds relate to infectivity thresholds (Rosengaus et al. 1999; Mburu et al. 2009), and if ants are able to recognize and respond to parasites when they are at much lower doses or masked by other environmental cues. There has been much progress in our understanding of the individual and group-level defenses of social insects against parasites, and further comparative studies of different species will be valuable to elucidate the selection pressures that have shaped their evolution.

## SUPPLEMENTARY MATERIAL

Supplementary material can be found at <http://www.beheco.oxfordjournals.org/>

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