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Disease defences across levels of biological organization: individual and social immunity in acorn ants

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Eusocial insect societies possess complex multilevel disease defences, including individual level protection conferred by physical (e.g. cuticle) and immunological obstacles and colony level protection mediated by collective behaviours (social immunity). It remains unclear whether and how these two levels of disease protection are related to one another in jointly driving colonies' susceptibility to disease. Here, we examine whether a relationship exists between individual worker survival after exposure to a fungal pathogen (a proxy for immunity) and corpse removal (a colony level social immunity metric) in the acorn ant *Temnothorax curvispinosus*. Since behavioural avoidance is the first line of defence against infection, we also tested whether individual ants exhibited parasite avoidance behaviour during exploration and whether colonies exhibit avoidance behaviour during foraging. We found that individual level and colony level immunity were negatively correlated: colonies that removed corpses more rapidly contained workers with weaker individual defences. We did not detect parasite avoidance behaviour by individual workers or whole colonies, nor were these two factors related. These data suggest that individual immunity and social immunity may trade off, regulating overall parasite protection. Alternatively, optimized social immunity at the colony level may compensate for disease vulnerability to infection at the individual level, and thus provide a protective benefit in overall colony defence in the absence of pathogen avoidance.

Protection against parasites is critical to survival in virtually all organisms (see Hawley & Altizer, 2011), but the investment strategies into these defences can be highly variable and context dependent. The first line of defence for animals are avoidance behaviours or prevention of situations that increase the risk of parasite exposure, such as preferring less risky food environments or behaving more hygienically within domiciles (Weinstein, Buck et al., 2018). Once exposed to parasites, animals exhibit individual protections including anatomical barriers (e.g. exoskeletons), physiological protection (e.g. immune activation) and behavioural modifications (e.g. sickness behaviours) (Hite, Pfenning, & Cressler, 2020; Moret & Moreau, 2012; Otti, Tragust, & Feldhaar, 2014).

Many facets of disease defences are energetically costly and may cause organisms to adjust behavioural defences to utilize the least costly trait, or experience a trade-off that hinders optimality. Across many systems, certain immune activation traits and reproductive capacity reciprocally diminish one another, which causes investment in one trait to decrease as the other increases (e.g. increased immunity reduces reproductive output; Schwenke, Lazzaro, & Wolfner, 2016). Immune responses also trade off with each other, where increased internal immunity (e.g. melanisation) leads to a reduction in external immunity (e.g. antimicrobial excretions; Otti et al., 2014). Trade-offs between internal and external immunity are best understood in flour beetles, where artificial selection for increased antimicrobial excretion (external immunity) reduces the production of phenoloxidase (internal immunity) (Joop, Roth, Schmid-Hempel, & Kurtz, 2014). It is unclear how widespread trade-offs are between internal and external immunity because in some systems there is no apparent trade-off between traits across these immune factors (see Baeuerle, Feldhaar, & Otti, 2020).

Given how costly disease defences can be, animals commonly avoid potentially infectious agents in the environment as a first line of defence. Parasite avoidance has recently received ample attention and draws inspiration from the well-established 'landscape of

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fear' that arises from avoidance of predation risk (Behringer, Butler, & Shields, 2006; Brown, Laundre, & Gurung, 1999; Buck, Weinstein, & Young, 2018; Sarabian, Curtis, & McMullan, 2018; Weinstein, Buck et al., 2018). The 'landscape of disgust' has received support from many systems but again highlights potential trade-offs, for example between resource acquisition and parasite avoidance. Increased foraging need reduces parasite avoidance when parasites are close to food sources (Lafferty & Morris, 1996; Merrill, Hall, Merrill, & Caceres, 2019; Weinstein, Moura et al., 2018). Trade-offs like this one prevents total avoidance of parasites since organisms must search for food even if it puts them at risk. Furthermore, parasite avoidance in group-foraging animals can be expressed at the individual and the group level.

Once exposed to parasites, individuals can express a diverse set of responses that can be broadly separated into external and internal immunity. External immunity includes both immunological defences, such as antimicrobial excretions, and nonimmunological defences, like sickness behaviours (Otti et al., 2014; Parker, Barribeau, Laughton, de Roode, & Gerardo, 2011). Internal immunity is the classic disease protection provided by innate immune defences like antimicrobial peptides (Hernández López, Riessberger-Gallé, Crailsheim, & Schuehly, 2017) and parasite encapsulation (Cerenius, Lee, & Söderhäll, 2008). Internal and external immunity that protects individuals is analogous to groups' individual and social protection against infectious agents. Determining how different components of disease protection unite to influence the overall immune protection of groups is important for discovering how social groups protect themselves against infection.

Eusocial insects, unlike many animals, possess a complex highly regulated 'social immune system' in addition to the internal and external immunity possessed by individual group members (Cremer, Armitage, & Schmid-Hempel, 2007; Cremer & Sixt, 2009; Pull & McMahon, 2020). Social immunity is colony level protection against disease achieved by emergent collective behaviours built on individual actions of group members. Social immunity can result from emergent properties (e.g. social network modulation), task differentiation (e.g. corpse removal), or via reciprocal action (e.g. allogrooming) (Cotter & Kilner, 2010; Cremer, 2019; Cremer et al., 2007, 2018). The social immune systems, an emergent property of individuals' external immunity, in eusocial insects have a care–kill dichotomy where workers care for individuals until the threat to the colony is too great, at which point infectious individuals are destructively targeted (Cremer, 2019). For example, workers utilize nest hygiene and sanitary care behaviours (Scharf, Modlmeier, Beros, & Foitzik, 2012) but switch to protecting against the proliferation and transmission of parasites by sacrificing individuals (Pull et al., 2018) and modulating social interaction patterns (Stroeymeyt et al., 2018).

Behaviours that contribute to social immunity may depend on the expression of individual immune defences (Pull & McMahon, 2020). For example, social immunity could potentially interact with individual immunity (1) positively, where highly defended colonies contain highly defended individuals, (2) negatively, where highly defended colonies contain weakly defended individuals, or (3) neither, where there is no correlation between levels of immune defence. A negative correlation may occur via a trade-off or if colonies compensate for weak individual immunity by optimizing collective defences accordingly. Social defences can also be up- or downregulated depending on immediate parasite exposure (Hernández López et al., 2017). Corpse removal is a common behaviour that contributes to social immune protection in ants, bees and termites that serves as an important preventative measure against colony contamination (Bordoni et al., 2019; Sun & Zhou, 2013). Colonies' investment into removing dead nestmates is a plastic trait regulated by factors like corpse infection status or worker–corpse relatedness (Sun & Zhou, 2013). Thus, the relative expression of individual level and group level immunity will impact colonies' overall protection against disease.

Here, we assess the relationship between individual level and colony level disease defences in the acorn ant *Temnothorax curvispinosus*. This system is well suited to quantify factors of social immunity because colonies are small and relatively easy to collect. Workers have divergent mortality risks between foragers (food collection specialists) and nurses (brood care specialists) that result in younger, more valuable workers performing nurse tasks (Kohlmeier et al., 2017). To assess the interaction between the individual and social disease protection, we conducted two separate assays. We inferred individual immunity by tracking survivorship (i.e. time to death) after exposure to a fungal pathogen since survivorship during infection is generally correlated with immunity across species (Lee, Wikelski, Robinson, Robinson, & Klasing, 2008; Schmid-Hempel, 2003). We assumed that the mortality we observed in exposed individuals was due to their lack of immune protection rather than an overcompensating immune response. We quantified social immunity by measuring colonies' propensity to maintain nest hygiene (i.e. rate of corpse removal) when presented with dead uninfected nestmates. We did not measure whether individual or social immunity provides more effective protection, but rather tested whether there is a relationship between our measures at both levels of defence. We also tested avoidance behaviour at each level (i.e. individual and collective) when exposed to the presence of *Metarhizium robertsii* in the environment. Understanding the relationship between social and individual immunity, coupled with responses to environmental parasite presence, allows us to identify how colonies regulate defences against parasites across two levels of biological organization.

METHODS

Ant and Fungal Collection and Maintenance

We collected 41 acorn ant (*T. curvispinosus*) colonies in a single patch of forest in Geneva, Ohio, U.S.A. in August 2018. We collected a total of 1819 female ants with 1510 brood in a forest on either side of a paved road over a total collection area of about 2 ha. *Temnothorax* species have shown some flexibility with their dispersal but seem to favour independent colony founding (i.e. start new colonies rather than join existing colonies), which requires dispersal out of the population (Howard, 2006). Therefore, the area we sampled was presumed to be sufficient to include the natural variation in relatedness for these ants. We transported them to the laboratory at the University of Florida under permit number 2018-045 from the Florida Department of Agriculture and Consumer Services/Division of Plant Industry. We maintained them at ambient temperatures and light cycles and housed colonies in artificial nests constructed from Plexiglas and glass microscope slides (described in supplemental materials in Keiser et al., 2018). We provided colonies with ad libitum water and a maintenance diet of a sugar cube and a rotation between cat food and domestic cricket pieces. Colonies contained 19–121 workers, 0–12 queens and 0–227 brood. We used the entomopathogenic fungus *M. robertsii*, a model generalist parasite (ARSEF no. 2576) to measure survival after exposure. This strain was originally obtained from the U.S. Department of Agriculture (USDA)-Agriculture Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF) in Ithaca, New York, U.S.A. and has been maintained on Sabouraud dextrose agar (SDA) plates in the laboratory. This strain is virulent to *T. curvispinosus* and was isolated and maintained following methods in Keiser et al. (2018). For this experiment, we regrew *M. robertsii* on SDA plates seeded by a previously infected

T. curvispinosus cadaver to reduce the likelihood the strain lost pathogenicity in the laboratory environment. Live animals were maintained for the duration of the experiment and frozen at the conclusion.

Individual Immunity: Experimental Parasite Exposure

We measured survival as a proxy for individual immunity given the correlation between these outcomes across ant species. In other words, we assumed the primary mortality of exposed individuals would be lack of immune protection rather than an over-compensating immune response. We created *M. robertsii* conidiospore suspension by pouring a 0.05% solution of Triton X-100 (Sigma-Aldrich, St Louis, MO, U.S.A.) onto the surface of a sporulating *M. robertsii* culture grown on SDA, agitating the conidia and collecting the suspension with a micropipette. Then, we vortexed the suspension for 30–40 s to homogenize the concentration and let it sit for 30 min to remove larger particulate matter. We collected 3–12 workers from each colony, alternating between foragers ($N = 45$) and nurses ($N = 135$). We identified these individuals by observing individuals that were completing a specific task and collecting them (foragers: visiting food source outside colony; nurses: handling brood within the colony). We collected both foragers and nurses from 27 colonies and only foragers from 10 colonies. We collected workers that focused on different tasks to account for ontogenic differences between individuals (foragers are older; see Kohlmeier et al., 2017). Collecting individuals across the task allocation of the colony allowed us to obtain an accurate measure of individual immunity within and between colonies. We submerged individuals into a dish of spore suspension (2.64×106 conidiospores/ml) using sterile forceps for 3 s and placed them onto sterile filter paper to dry (Keiser et al., 2018) ($N = 67$ nurses, $N = 15$ foragers; $N = 82$ total). Although we do not know whether this method exposed each individual to a consistent numbers of spores, we thoroughly mixed the spore suspension before submerging each ant to keep it as consistent as possible. As a control, we submerged workers in a sterile 0.05% solution of Triton X-100 ($N = 66$ nurses, $N = 22$ foragers; $N = 88$ total). Then, we maintained individuals in groups of three from the same colony in a 3 ml plastic vial with a sterile cotton ball for water and recorded time to death for each individual. Although maintaining individuals in groups of three allows for allogrooming, a behaviour that contributes to social immunity, we chose to maintain individuals in groups to account for isolation-associated mortality found in many eusocial insects (Koto, Mersch, Hollis, & Keller, 2015). Exposing ants in groups has been employed previously for determining susceptibility to *Metarhizium* (Abonyo et al., 2016; Bos, Kankaanpaa-Kukkonen, Freitak, Stucki, & Sundstrom, 2019). Following death, individuals were surface-sterilized with 1% bleach and 70% ethanol and placed in a petri dish with filter paper and a cotton ball to maintain a humid environment (following methods in Keiser et al., 2018). Corpses were monitored daily and scored for fungal growth and sporulation. We found 3/88 control ants with evidence of fungal growth, likely from contamination, and 8/82 ants in the exposed treatment with no evidence of fungal growth after death. We found 8/86 control ants that did not die within 30 days after experimental treatments, so we censored these in the data analysis.

Social Immunity: Corpse-removal Assay

Before starting behavioural assays, we removed five worker ants from each colony during foraging and placed them in a −20 °C freezer overnight. This may impact colonies' corpse removal, given that foragers have the most experience carrying objects out of the nest. However, we removed foragers consistently across all colonies and we included colony size as a fixed effect in our analysis to account for how removing individuals may have unequally affected smaller colonies. We used corpses that were killed by freezing rather than using infected or sporulating corpses to assess baseline nest hygiene behaviour rather than a response to parasitized individuals. Additionally, we wanted to avoid parasite transmission within our experimental colonies, which would confound future assays. We initiated corpse-removal assays by removing nests ($N = 41$) from their housing containers and individually placing artificial nests into novel petri dish arenas (diameter = 14 cm). After giving the colonies a 5 min acclimation period, we placed the uninfected corpse of a dead nestmate at the entrance to the colony. Length of acclimation to novel arenas can have an impact on how the animals behave, so we followed previously published methods on this species and chose a 5 min period (see Keiser et al., 2018). We measured the latency between the time the first ant encountered the carcass and the time that workers removed the corpse from the nest. We conducted three repeated measures of corpse-removal measurements to detect consistent colony level differences in this collective behaviour. This allowed us to determine whether a component of social immunity was repeatable at the group level (i.e. collective personality) and whether colonies responded to corpses more quickly over repeated exposures. Assays where the colony never removed the corpse within 1 h ($N = 18/114$ instances) were assigned the maximum value of 3600 s and censored (see Statistical Analyses section).

Pathogen Avoidance Assays

We tested whether individual workers and whole colonies avoided infectious fungal spores in their environment using two preference tests. For both individuals and colonies, we tested for avoidance using two concentrations of conidiospores in case avoidance is parasite density dependent (Weinstein, Buck et al., 2018).

To test individual workers' avoidance of conidiospores during exploration of the environment, we constructed open field exploration chambers using 100 mm petri dishes containing two halves of 100 mm circular filter paper (Appendix, Fig. A1a). One half had been exposed to 150 µl of conidiospores solution 1–3 h prior to testing while the other half was exposed to a sterile solution of 0.05% Triton-X and left to air-dry. Filter papers were still damp at the time of study, allowing the paper to better adhere to the plastic petri dish. We placed an individual worker in the centre of the petri dish, gave it a 5 min acclimation period under a small plastic lid, and then measured the amount of time the worker spent on either side of the chamber over a 5 min period. Between each replicate, we rotated whether the spores were on the left or the right of the chamber relative to the entrance to the nest observer (Appendix, Fig. A1b), and observers were naïve to the location of spores during testing. We tested three separate workers from each of 35 colonies: two tested with a spore concentration of 1×10^6 spores/ml and one tested at 1×10^7 spores/ml.

We tested colony level pathogen avoidance by starving the ants for 1 week and then moving the entire nest into a 150 mm arena containing two food sources (100 µl droplet of 20% sugar water) equidistant from the colony entrance. Each droplet sat atop a sterile plastic circle atop a piece of filter paper – one filter paper containing 150 µl of conidiospores solution and the other containing only a sterile 0.05% Triton-X solution. Thus, workers could only reach the sugar water by crossing over a strip of the exposed or control filter paper (Appendix, Fig. A1b). We then recorded the number of workers feeding at each food site every 5 min for 2 h. We tested each colony ($N = 35$) three times: twice with a spore concentration of 1×10^6 spores/ml and once with a concentration of 1×10^7 spores/ml.

Statistical Analyses

Survivorship

We quantified time to death after exposure to infectious spores using a Cox proportional hazards model from the coxme package (Therneau, 2020) in the statistical programing language R (R Core Team, 2018) with treatment (spores versus control) and worker type (nurse versus forager), as well as their interaction term as independent variables. We also included colony identity (ID) and vial ID as random effects. Ants that died within 48 h of exposure were excluded from analysis, as they likely died from the experimental procedure rather than infection ($N = 8$). Ants that did not die within 30 days ($N = 7$) were censored. We performed all survivorship analyses in R v.3.6.0.

Corpse removal

To analyse colony corpse-removal behaviour, we calculated the latency from the time the first worker interacted with the corpse to the time they removed it from the colony. Latency values were log-transformed to meet model assumptions, and we used a Cox proportional hazards model from the coxme package (Therneau, 2020) in the statistical programing language R (R Core Team, 2018) with colony size and assay number as independent variables and colony ID as a random effect (Falconer & Mackay, 1996). Assays in which the corpse was never removed ($N = 18$) were censored. We used Tukey's HSD for post hoc tests. Finally, we estimated the repeatability (i.e. among-colony variation) in corpse-removal behaviour by calculating the intraclass correlation coefficient (ICC; Hayes & Jenkins, 1997). Higher ICC values indicate differences between colonies and consistency within colonies through time (Bell, Hankison, & Laskowski, 2009). We performed all corpse-removal analyses in R v.3.6.0.

Pathogen avoidance

To test whether individual workers avoided spores in the environment, we measured the proportion of time each worker spent on both sides of the arena (control and exposed) and calculated a preference index by subtracting the proportion of time spent on the exposed side from the proportion of time spent on the control side. Positive values denote workers that spent more time on the control side and negative values denote workers that spent more time on the spore side. To test whether colonies avoided spores when foraging, we calculated the sum of all workers observed to visit the exposed and control food patches. We then calculated a preference index by subtracting the total number of ants foraging on the exposed side from the total number of ants foraging on the control patch and dividing that value by colony size. Thus, positive values denote colonies with more foragers on the control patch and negative values represent colonies with more foragers on the exposed patch. We tested for a relationship between worker spore avoidance during exploration in an open field and colony avoidance of spores during foraging using a general linear mixed model with colony level preference index as a dependent variable, individual worker preference index, spore concentration, and their interaction term as independent variables, and colony ID as a random effect.

Relationship between individual and social immunity

Lastly, we tested the relationship between the average time to death for infected workers and the corpse-removal latency from colonies' first exposure to a corpse. To test for this relationship, we calculated a linear regression between corpse-removal latency from the first assay and the average time to death for infected workers from each of colony. We used only the first corpse-removal observation in this analysis to account for colonies' improvement in corpse removal over time. We used the initial value rather than the average corpse removal because the average value included both the intercept of innate corpse removal and the slope relating to the learning component, which could also differ across colonies. By using just the initial value in this analysis, we were able to compare the baseline corpse-removal measure for the colony with their average time to death. We present an additional analysis using the average corpse-removal latency from all three assays in the Appendix. We used Akaike's information criterion for small samples (AICc) to compare a linear and nonlinear (quadratic) fit to this relationship. A Shapiro–Wilk test was used to verify the normality of residuals ($W = 0.95$, $P = 0.41$). For the relationship between individual and social immunity, we combined nurses and foragers. We created an additional plot splitting nurses and foragers (Appendix, Fig. A3), but uneven sample sizes between the two worker types reduced our confidence in any statistical comparisons.

RESULTS

The survival of *T. curvispinosus* workers was reduced by fungal exposure, where workers exposed to infectious spores died twice as quickly compared to workers exposed to the control solution ($z = 3.42$, $P < 0.001$; Fig. 1a). Foragers, which were collected outside

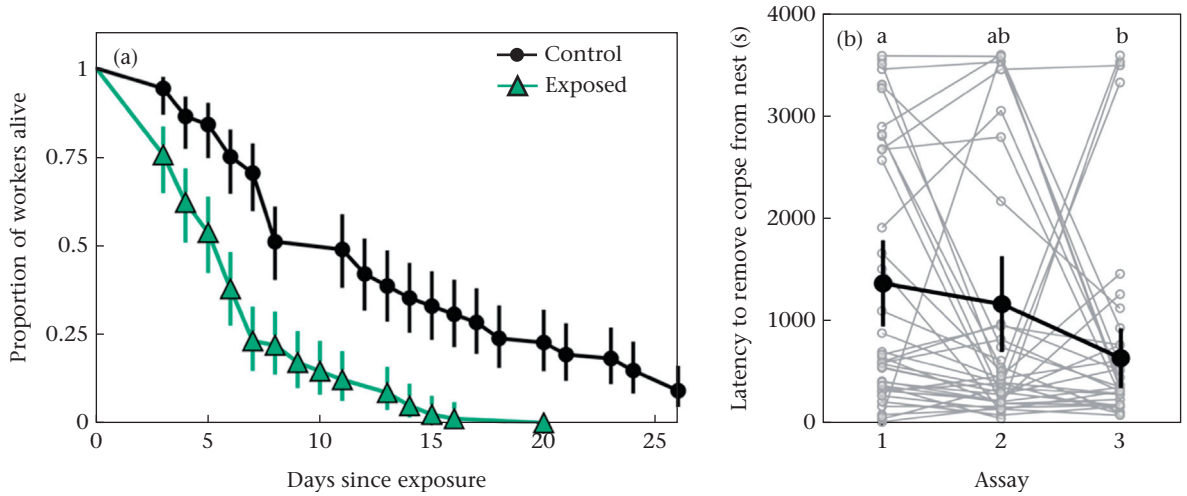

Figure 1. (a) Proportion of *T. curvispinosus* workers alive following pathogen exposure. (b) Colonies' latency to remove a corpse from nest over three assays. Different letters denote significant differences ($P < 0.05$) between assays. Grey lines represent individual colonies; the bold black line represents the average of all colonies.

the nest, also died twice as rapidly compared to nurses, which were collected while interacting with brood ($z = -5.09$, $P < 0.001$). The interaction term between treatment and worker type was not significant ($z = 0.52$, $P = 0.6$), indicating that infection affected different worker types similarly.

Colonies generally removed corpses more quickly over the three observations ($z = 2.83$, $P = 0.005$; Fig. 1b). On average, colonies removed the corpse over twice as rapidly during the third assay compared to the first assay (Tukey's post hoc test: $Q = 2.39$, $P < 0.05$). Despite an overall increase in the speed with which colonies removed the corpse, the intraclass correlation coefficient indicated a considerable amount of variation among colonies in this behaviour (ICC = 0.48), where some colonies removed the corpse consistently more rapidly than other colonies. We did not detect an effect of colony size on corpse-removal behaviour ($z = -1.87$, $P = 0.06$).

Model comparison using AICc suggested that a quadratic regression was a preferable fit to the relationship between colonies' corpse-removal behaviour and individual survivorship compared to a linear regression (ΔAICc = 11.06, $df = 18$, $R^2 = 0.70$; Fig. 2). We found that colony level and individual level disease defences were negatively correlated: colonies that removed corpses more quickly (i.e. greater social immunity) contained workers that died more rapidly when exposed to a generalist fungal pathogen (i.e. worse individual immunity) (Fig. 2).

We found no evidence for avoidance behaviour when individual workers were exploring an open field arena when half was exposed to 1×10^6 spores/ml (95% CI: −0.1354, 0.1499) or 1×10^7 spores/ml (95% CI: −0.2817, 0.1546), nor did we find evidence for avoidance behaviour when colonies were confronted with a choice between a control food patch and a food patch exposed to 1×10^6 spores/ml (95% CI: −0.0513, 0.0548) or 1×10^7 spores/ml (95% CI: −0.1523, 0.0999). Colony preferences during foraging were not related to worker preferences during exploration in an open field assay ($F_{1,56.4} = 0.07$, $P = 0.80$) and were unaffected by environmental spore concentration ($F_{1,33.8} = 0.02$, $P = 0.89$; Fig. 3). The interaction term between worker preference index and spore concentration was not significant ($F_{1,59.6} = 2.64$, $P = 0.11$).

DISCUSSION

Collective behavioural defences can mitigate the risk of disease outbreaks associated with group living. We set out to investigate how these collective defences relate to the defences possessed by individual group members by measuring collective hygiene behaviour and survivorship in acorn ants. We also explored whether immunity is coupled with avoidance of parasites at the individual and colony level. Here, we found that colonies with a greater propensity for hygienic behaviour (social immunity) were composed of workers that died faster when exposed to a generalist parasite (individual immunity). We found no evidence for pathogen avoidance at the individual or colony level for this parasite. These results suggest that the execution of colonies' social immunity may be regulated depending on the individual level disease protection of workers therein.

Disease protection at the individual level is known to vary across worker types within eusocial insect colonies, determined by age or caste (Armitage & Boomsma, 2010; Ribeiro, de Souza, Gandra, & Della Lucia, 2011). This could indicate differential allocation of resources across workers completing different tasks, but most likely is a function of the ontogeny of task switching, where individuals change tasks as they age (age polyethism) (Robinson, 1992). Indeed, immunosenescence, or the decrease in immune function of older individuals, has been found in social insects (Amdam et al., 2004). With older workers at a higher risk of succumbing to infection, young individuals generally perform less risky tasks in close proximity to queens and/or brood and then switch to tasks with higher parasite exposure as they age and become more expendable (Robinson, 1992). In our study, we found that workers collected while engaging in brood care (nurses) survived twice as long as workers collected while gathering food (foragers) whether exposed or unexposed to a generalist fungal parasite. Foragers are likely older individuals with weaker individual immunity, so they experienced faster mortality when exposed to parasites. However, behavioural interventions via social immunity can additively increase immune protection when combined with individuals' physiological immune system (Daly & Johnson, 2011; Johnson &

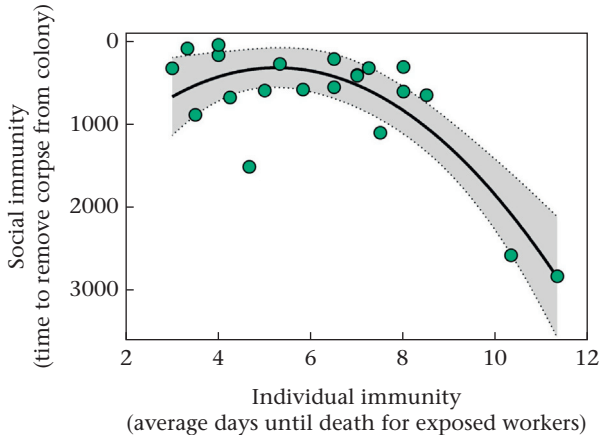

Figure 2. Correlation between individual immunity (time to death in days after pathogen exposure) and colony social immunity (latency from corpse discovery to removal in seconds). Because longer latencies for corpse removal indicate weaker social immunity, we plotted those values as an inverse on the *Y* axis to present the relationship more intuitively.

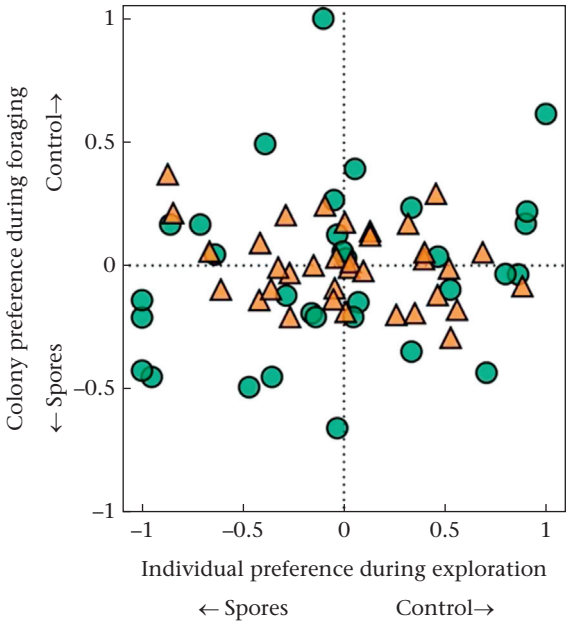

Figure 3. Pathogen avoidance for individual workers and whole colonies. We tested three separate workers from each of 35 colonies: two tested with a spore concentration of 1×10^6 spores/ml (triangles) and one tested at 1×10^7 spores/ml (circles). Positive values suggest pathogen avoidance, while negative values suggest attraction to the pathogen patch. Each point represents the mean colony and individual preference for a single colony.

Hoverman, 2014), potentially protecting workers at greater risk of infection.

Corpse removal is an important behaviour that reduces infection risk to the colony across many insect societies (Sun & Zhou, 2013). We use the broader definition of hygienic behaviour to include removing noninfected individuals rather than the stricter definition that only includes removal of diseased individuals (see Rothenbuhler, 1964). This broad definition allowed us to identify infection prevention mechanisms not limited by the worker's ability to detect parasite infection or presence. Previous research has shown corpse removal in eusocial societies is managed by group members with similar task specialization and influenced by group size and number of queens (Walton, Jandt, & Dornhaus, 2019). In our study, we observed repeatable differences in colonies' speed of corpse removal. This suggests that components of social immunity may represent a facet of 'behavioural syndromes' at the colony level (Jandt et al., 2014), where colonies' collective phenotypes represent a suite of correlated collective traits. Investigating the repeatability of this collective behaviour allowed us to conduct a more robust comparison of within- and among-colony variation in immune defences across scales. Future studies should identify the degree to which different forms of collective immunity (e.g. inside and outside the nest) are correlated.

Parasite avoidance has been shown to be broadly applicable to many animal systems (Buck et al., 2018; Weinstein, Buck et al., 2018) but its benefits can be negligible under certain conditions (Lafferty & Morris, 1996; Merrill et al., 2019). In our study, we attempted to control for hunger to reduce the confounding effect of the need for food and the preference to avoid parasites. We did this by depriving our ants of food, but given their starvation resistance and cannibalism of brood (Rueppell & Kirkman, 2005), we may have been unable to disentangle this conflict in our avoidance assays. Our results may also indicate *T. curvispinosus*' lack of ability to detect *M. robertsii* spore presence or that the selective pressure to recognize and avoid this generalist pathogen is relatively weak. This would be despite its virulent pathogenicity and coexistence in soils proximately located around ant colonies (Angelone & Bidochka, 2018). In fact, some ant species prefer nests that contain an individual infected with *Metarhizium* over nests with an uninfected individual (Pontieri, Vojvodic, Graham, Pedersen, & Linksvayer, 2014). Another mechanism that might contribute to the lack of avoidance is the motivation to mitigate infection risk within versus outside the nest. Inside the nest, the valuable queens and brood are present, whereas outside the nest, workers with the highest mortality risk are foraging (Kohlmeier et al., 2017). Since there are many mechanisms to prevent outbreaks within the nest (e.g. grooming, corpse removal, network modulation, etc.), the pressure for less valuable older ants (i.e. foragers) to avoid parasites outside the nest may be minimal.

To our knowledge, few studies have investigated the relationship between social insects' collective behaviours and worker immunity. These results have yielded conflicting patterns across taxa. Individual and social immunity have been shown to trade off in subsocial burying beetles, where increased wound-healing investment leads to decreased antimicrobial excretion in the group (Cotter, Littlefair, Grantham, & Kilner, 2013). This trade-off can become more pronounced as the degree of sociality increases, where eusocial societies can contain individuals with weaker encapsulation responses compared to solitary species (López-Uribe, Sconiers, Frank, Dunn, & Tarpy, 2016). However, trade-offs do not always emerge. In honey bees (*Apis mellifera*), worker bee hygienic behaviour is not negatively correlated to an individual's innate immunity (Harpur et al., 2014). Scharf, Modlmeier, Fries, Tirard, and Foitzik (2012) used phenoloxidase (PO), an important component of invertebrate immune function (Cerenius et al., 2008), as a proxy measurement for immune defence in *Temnothorax nylanderi*. They found that innate immune defence positively correlated with collective nest relocation (Scharf, Modlmeier, Fries, et al., 2012). They hypothesized that nest relocation may be a response to microparasite exposure, as *Temnothorax* ants have been shown to relocate within acorns to avoid microbial growth (Karlik, Epps, Dunn, & Penick, 2016). Interestingly, Scharf, Modlmeier, Fries, et al. (2012) did not find a relationship between worker immunocompetence and colony corpse removal. In our study we did not measure a specific component of immunocompetence as Scharf, Modlmeier, Fries, et al. (2012) did, which could indicate a species-specific or trait-specific trade-off.

Disease defences are presumably energetically costly at both the individual and social level, so colonies may not be able to manage increased protection at both levels. The optimal expression of individual and social immunity may differ across ecological contexts, providing a source of variation in multilevel immune function. Any potential trade-off between levels of disease protection could contribute to the mechanisms that maintain the variation individually and collectively. We were unable to differentiate whether there is a trade-off between individual and social immunity (i.e. due to resource limitation) or whether colonies adaptively regulate the expression of social immunity depending on the physiological defences of workers therein. Incorporating behavioural and molecular tools in colonies from different environments could differentiate between these two hypotheses. Nevertheless, our results indicate that colonies consistently vary in both individual and social immunity, and that defences at these two levels are negatively correlated. Future studies should focus on the underlying mechanisms driving this relationship and to further integrate immune allocation with variable parasite risk across environments to better understand how colonies allocate resources across levels of biological organization to defend against parasites.

Data Accessibility

The raw data associated with this manuscript are available from the Figshare Digital Repository (https://doi.org/10.6084/m9.figshare.14848026).

Author Contributions

S.T.C., T.T., N.D., C.G., G.J., A.L. and S.S. conducted behavioural assays. J.C. conducted measurements on individual ant mortality. C.M.W. collected and provided ant colonies. C.N.K. conducted analyses and S.T.C. and C.N.K. prepared the manuscript.

Declaration of Interest

We declare that we have no known competing financial interests or personal relationships that influenced the work reported in this paper.

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Appendix

Additional Analysis

Colony size

We verified that colony size ($F_{1,27} = 0.02$, $P = 0.88$) and queen number ($F_{1,27} = 0.21$, $P = 0.21$) did not predict the average time to corpse removal using a general linear model. The average latency to remove the corpse was log-transformed to meet model assumptions.

Correlation with average corpse removal

We found that colony level and individual level immunity were inversely correlated: colonies that removed corpses more rapidly contained workers that died more rapidly when exposed to a generalist fungal pathogen (linear model: $F_{1,19} = 6.67$, $P = 0.018$). However, AICc suggested that a quadratic regression was a preferable fit to these data (ΔAICc = 2.427, $R^2 = 0.45$; Fig. A2). This pattern may have been driven by nurse ants, which exhibited the same correlation, whereas foragers showed no correlation (Fig. A2).

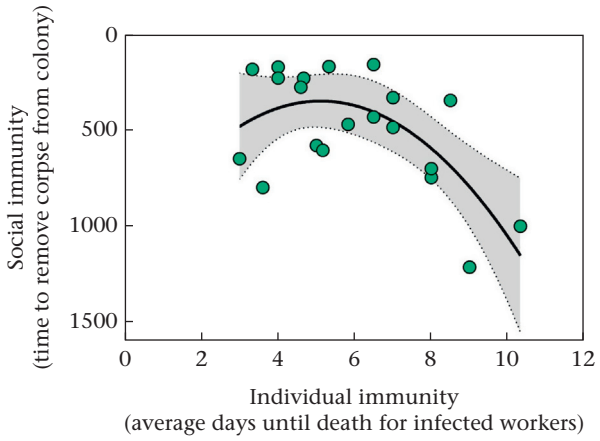

Figure A2. Correlation between individual immunity (time to death in days after pathogen exposure) and 'average' colony social immunity (latency from corpse discovery to removal in seconds). Because longer latencies for corpse removal indicate weaker social immunity, we plotted those values as an inverse on the *Y* axis to present the relationship more intuitively.

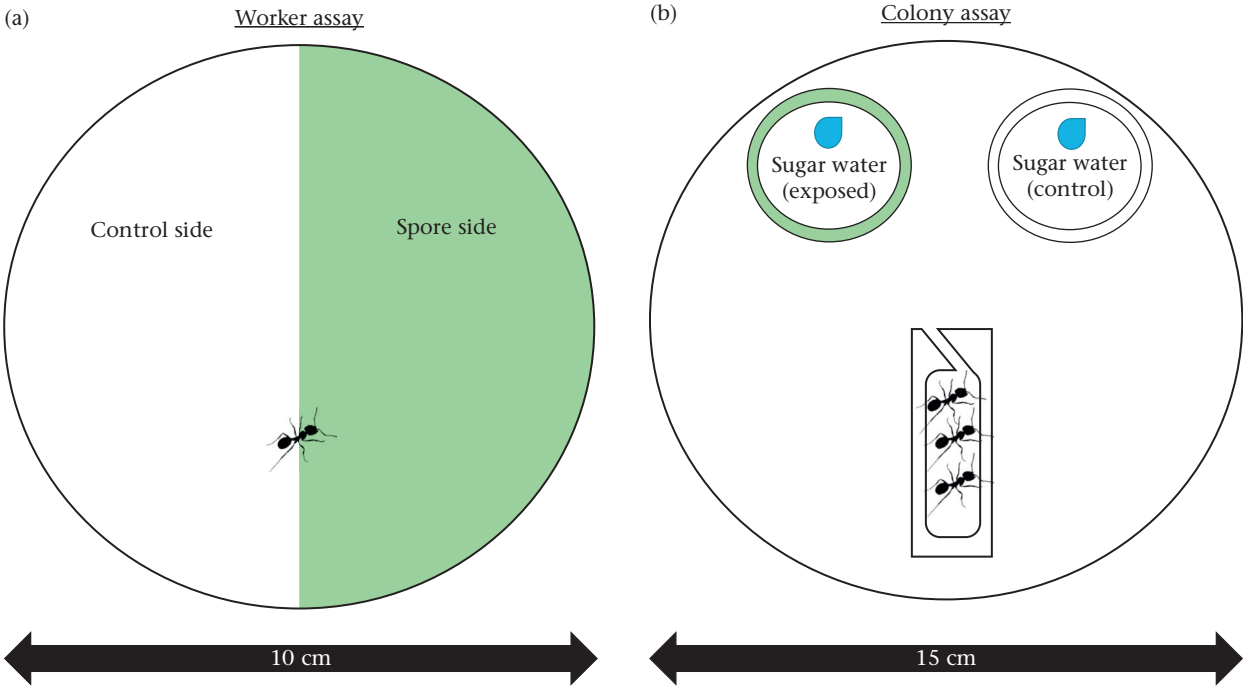

Figure A1. Parasite-exposed filter paper is shown in green; all other sections represent sterilized environments. (a) Exploration chambers using petri dishes containing two halves of 100 mm circular filter paper. (b) Entire nest into a 150 mm arena containing two food sources. Workers could only reach the sugar water by crossing over a strip of the exposed or control filter paper.

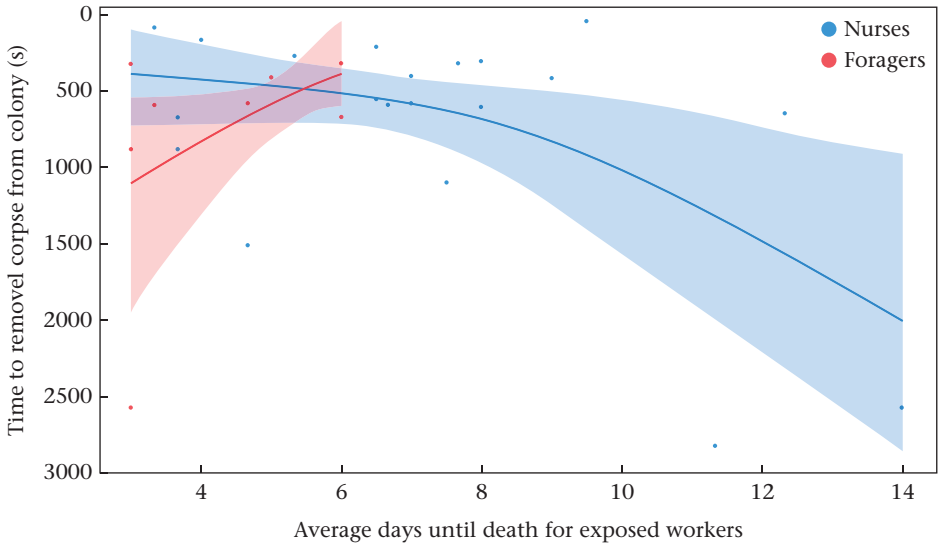

Figure A3. Relationship between colonies' average corpse-removal time and the time to death for the different worker types.