



Social Parasite Exposure and Nest Hygiene in Acorn Ants

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Abstract Antagonistic interactions impose pressures that can trigger shifts in defensive phenotypes. For instance, one natural enemy may activate defensive phenotypes that influence defenses that protect against other enemies. Socially parasitic ants (*Temnothorax americanus*) are both parasites and predators of other coevolved *Temnothorax* species, whose brood they either consume as prey or steal during raids to utilize as a work force in their own colonies. Since these social parasites impose a significant threat to host colonies, we explored whether exposing a *T. americanus* worker to *T. curvispinosus* host colonies could impact nest hygiene behavior, a component of collective disease defense. Specifically, we measured the latency to remove colony-mate corpses from the nest. We measured corpse removal twice before and twice after exposure to a *T. americanus* worker collected in sympatry to the focal host colonies. We found that simulating the initial stage of a scout raid had no effect on this measure of collective nest hygiene. These results indicate that some measures of social immunity may remain robust after a potentially stressful antagonistic interaction from a coevolved heterospecific.

Keywords Brood parasite · corpse removal · slave-maker ant · social immunity · *Temnothorax americanus* · *Temnothorax curvispinosus*

Introduction

Frequent social encounters between closely related individuals can increase disease risk, and as such highly related social insects frequently use behavior to decrease risk to individuals and groups, a phenomenon known as social immunity (Cremer et al. 2007). Behavioral avoidance is the first line of defense against disease in which individuals and groups prevent exposure to parasites, such as preferring food less likely to be contaminated or nesting in areas with lower infection risk (Weinstein et al. 2018a, b). If avoidance is not possible, then groups can employ social immunity, such as nest hygiene or allogrooming, to mitigate the consequences of exposure to prevent further contamination, infection, replication, and transmission (Cremer et al. 2007; Cremer 2019). Some of these behaviors are not constitutively expressed but rely on cues of infection risk. For example, pathogen presence in dampwood termite colonies induces a vibratory alarm response which causes nestmates to socially distance themselves (Rosengaus et al. 1999). Disease defenses could even increase antagonistic interactions with heterospecifics, as theory shows colonies that are more territorial around their nest have greater protection against disease epidemics (Lemanski et al. 2021). Corpse removal is one component of nest hygiene, which is a vital element of social immunity within social insect

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colonies (Cremer et al. 2007; Sun and Zhou 2013). Removing corpses from the nest protects against contaminants that could lead to disease-associated mortality (Sun and Zhou 2013; Cremer 2019).

In addition to defenses against disease risk, social insects must also defend against predators and social parasites, interactions that can induce defensive responses affecting a myriad of behaviors such as communication, aggression, and social immunity. During army ant raids, some social wasp species perform vibrational alarm calling which induces a synchronous evacuation of the nest (Chadab 1979). If exposure to social parasites alters colony activity budgets, like investing more time in defense or aggression (Jongepier et al. 2014), then colonies may not be able to perform other behaviors as effectively, like hygiene. In other words, if colonies are focused on protecting themselves from social parasites, they may alter their activity budgets to compensate. In response to the presence of conspecific enemies, *Pheidole pallidula* ants will increase their investment in soldier production, to collectively increase aggressive responses to enemies (Passera et al. 1996). Similarly, ant populations that contain socially parasitic ants become more aggressive to prevent future raids, whereas unparasitized populations flee rather than fight off the raid (Jongepier et al. 2014). How cues of antagonistic interactions influence colonies' defensive phenotypes against other stressors (e.g., social hygiene) remains relatively untested.

Acorn ants (*Temnothorax curvispinosus*) live in colonies with relatively small sizes, ranging from a few individuals to a few hundred individuals, which reside in hollow acorns, sticks, galls, and other plant materials (Hölldobler and Wilson 1990). Throughout their range in Eastern North America, social parasites like *T. americanus* (formally *Protomognathus americanus*) raid *T. curvispinosus* colonies by stealing/eating their brood (Johnson 2008). The larvae they steal are brought back to the *T. americanus* nest and, once they mature, perform tasks that benefit their captor's nest (Hölldobler and Wilson 1990). *Temnothorax americanus* social parasites are closely coevolved with their host species in the genus *Temnothorax* (Brandt et al. 2005). These social parasites exert intense selective pressure that forces host colonies to prioritize defense against raids by implementing defensive behavioral strategies (Foitzik et al. 2001), which could affect behaviors that defend against infectious diseases.

Here, we measured one component of nest hygiene (corpse removal), an important social immunity trait, in *T. curvispinosus* colonies before and after exposure to an individual worker from a *T. americanus* colony to detect any induced changes in disease protection. We exposed half of the host colonies to a single *T. americanus* worker and assessed corpse removal speed before and after the experimental exposure compared to unexposed colonies (Fig. 1). We also assessed how corpse removal speed correlated with number of workers attacking the social parasite to investigate if colonies' aggression may be associated with hygienic behavior. Understanding how this important antagonistic interaction affects a critical hygienic trait could elucidate the types of consequences that may arise when a closely coevolved natural enemy threatens a host colony.

Methods

Ant Collection and Maintenance

We collected 16 acorn ant (*T. curvispinosus*) colonies (containing a total of 874 workers with 746 brood) and one social parasite (*T. americanus*) colony along a paved road across a 2-ha forest patch in Geneva, Ohio, U.S.A. in July 2019. *Temnothorax curvispinosus* colony sizes ranged from 17–172 workers, 0–1 males, 1–8 queens and 9–151 brood. We transported colonies to the laboratory at the University of Florida (Division of Plant Industry permit number: 2018–045; FDACS). We maintained colonies at ambient lab temperature and light conditions and housed them in artificial nests that we constructed using glass slides covering a clear plastic inlay (described in Keiser et al. 2018). We provided colonies with *ad libitum* water, sugar cubes, and dry cat food. We maintained live ants for the duration of the experiment and froze all colonies at the experiment's conclusion.

Nest Hygiene: Corpse-Removal Assay

We assessed social immunity by measuring a nest hygiene trait (corpse removal) using the same methods as Cassidy et al. (2021). We briefly summarize these methods here. Prior to behavioral assays, we removed 12 workers from each host colony and placed them in

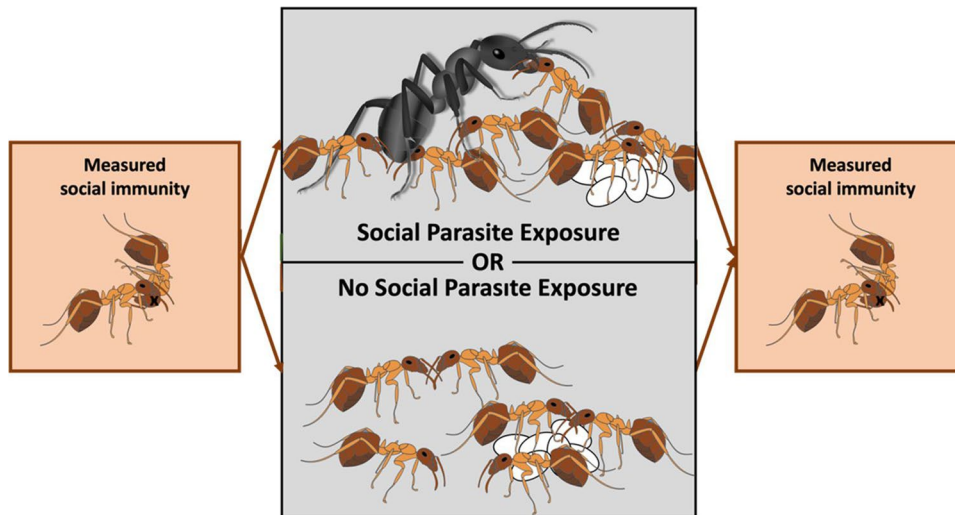


Fig. 1 Experimental design. We measured social immunity (i.e., corpse removal) in *T. curvispinosus* colonies before and after social parasite exposure. We exposed half of our colonies to social parasites and compared social immunity to unexposed colonies

a $-20\text{ }^{\circ}\text{C}$ freezer to use in corpse removal assays. We removed workers three days before the first behavioral assay. We initiated corpse-removal by acclimating entire colonies ($N=16$) to a novel arena in a 150 mm petri dish and placing a corpse at the entrance to their nest. We then measured the latency for workers to remove the corpse from the nest measured as time from first interaction with corpse until it was fully removed from the colony. We considered a corpse to be fully removed from the colony when no part of its body remained in the glass slide nest we constructed (described in Keiser et al. 2018). We conducted two corpse removal assays before and two after social parasite exposure for a total of four measures of corpse-removal over the course of the experiment. Slower corpse removal speed should indicate weaker social immunity because the longer a corpse remains in the colony, the greater the risk of contamination and infection. Assays where the colony never removed the corpse within 30 min ($N=27/63$ instances) were assigned the maximum value of 1800s and censored (see *Statistical Analyses* section). We acknowledge that this is a high proportion censored of data points. A previous study that observed colonies for 60 min, found 15% of colonies did not remove a corpse (Cassidy et al. 2021). In our study, we only observed colonies for 30 min. We would have likely found a much higher rate of removal if we had observed for at least an hour. Nonetheless, we believe that censoring

these data correctly represents these colonies as slower corpse removers.

Social Parasite Exposure

To assess the impact of exposure to a biotic stressor on disease defenses, we exposed half of the *T. curvispinosus* colonies ($N=8$) to a single live *T. americanus* worker ant. We did this by placing one *T. americanus* worker 1–2 cm into the nest entrance and sealing the nest with a cotton ball for 10 min. We sealed the nest entrance to ensure interaction between the *T. americanus* worker and the *T. curvispinosus* colony. This was intended to induce a stress response in the host colony by simulating the prolonged presence of a social parasite within the nest. During exposure, we observed the colony once per minute for 10 min and counted the number of *T. curvispinosus* ants attacking the foreign *T. americanus* worker. After 10 min, we removed the *T. americanus* worker from the colony and unsealed the entrance. We then averaged the number of attackers by adding up the total number of attackers observed at each minute and divided by 10 (i.e., total number of observations). We sealed the other half of the colonies ($N=8$) with a cotton ball for 10 min without the introduction of a *T. americanus* worker. Social parasite exposure occurred 10 days after the first round of corpse removal assays. The second round of assays began 1 day after we exposed colonies to the social parasite.

Statistical Analyses

We used a Cox proportional hazards model from the `coxme` package (Therneau 2020) in R (R Core Team 2018) to detect any differences in corpse removal before and after social parasite exposure. We chose a proportional hazards model because our response variable was a latency value with censoring (i.e., some colonies never removed the corpse), which has previously been used to analyze corpse removal speed (Cassidy et al. 2021). As a response variable, we averaged the two corpse removal speeds before and the two after to account for variation among single measurements. We used social parasite treatment, timepoint (i.e., before/after social parasite exposure), and timepoint x social parasite treatment interaction term as fixed effect independent variables. We used colony ID as a random effect. Assays in which the corpse was not removed after 30 min ($N=27$ out of 63) were censored. We acknowledge that this is a relatively high proportion of data censored and is likely due to us observing colonies for only 30 min (e.g., relative to Cassidy et al. 2021 where over 80% of *T. curvispinosus* colonies removed corpses within 60 min). We also conducted a linear regression to determine if there was a correlation between host colonies' corpse removal speed and average number of ants used to attack the social parasite. We performed analyses in R version 3.6.0.

Results

Colonies did not remove corpses at different speeds over time ($z=-0.61$, $P=0.54$; Fig. 2), nor did social parasite exposure alter colonies' corpse removal speeds ($z=-0.06$, $P=0.96$; Fig. 2). Additionally, the interaction between social parasite exposure and timepoint was not significant ($z=0.57$, $P=0.57$). This indicates that exposure to a social parasite worker (*T. americanus*) did not affect the corpse removal speed of *T. curvispinosus* colonies. Results were qualitatively similar when replacing before/after exposure with trial number with the same analysis, which can be found in the Supplemental Information (Fig. S2). During the exposure to the social parasite to the host colony, we found that *T. curvispinosus* workers from all colonies attacked the single *T. americanus* worker in their nest during the 10 min exposure period. Colonies attacked

with an average of 3.7 workers over the 10 min observation period (averaged from counting attackers every minute for 10 min). Queens also participated in the attack in 37.5% of colonies (3/8 exposed colonies). We did not find any correlation between corpse removal speed and number of *T. curvispinosus* ants that attacked the *T. americanus* worker in exposed colonies ($R^2=0.0605$, $F_{1,6}=1.451$, $p=0.274$; Fig. S1). Lastly, our colony sizes ranged considerably (17–172 workers), resulting in a large reduction in number of workers for the smaller colonies when removing ants for corpse removal. However, variation in corpse removal speed did not appear drastically different across colony sizes (i.e., small and large colonies removed corpses at both slow and fast speeds across trials; Fig S3).

Discussion

Antagonistic interactions with heterospecifics can affect both individual and collective behavior in social insects (Chadab 1979; Passera et al. 1996; Jongepier et al. 2014; Grüter et al. 2018; Lemanski et al. 2021). In group-living animals, social parasites take advantage of host behavior to further their own survival and/or reproduction (Grüter et al. 2018). Here, we explored how an interaction with a social parasite may affect a trait that is vital to protect against pathogen contamination, though is unlikely to protect against future social parasite raids. We found that social parasite exposure does not affect one measure of nest hygiene (i.e., corpse removal speed). Many studies studying corpse removal observe colonies for a relatively short period of time (i.e., 60–90 min), so shorter differences in removal speeds are considered important for corpse management in these systems (e.g., Diez et al. 2013; Walton et al. 2019; Cassidy et al. 2021). However, we only observed colonies' corpse removal for 30 min, which seemed inefficient given the proportion of censored data and that most studies observe colonies for at least 60 min. Nonetheless, our study suggests that host colonies do not compromise this component of their hygienic behavior in response to exposure to socially parasitic ants, which is a major selective force for *T. curvispinosus*. Host colonies may have evolved unique ways to mitigate stress caused by this interaction, thereby maintaining hygienic behavior essential to protection against disease outbreaks.

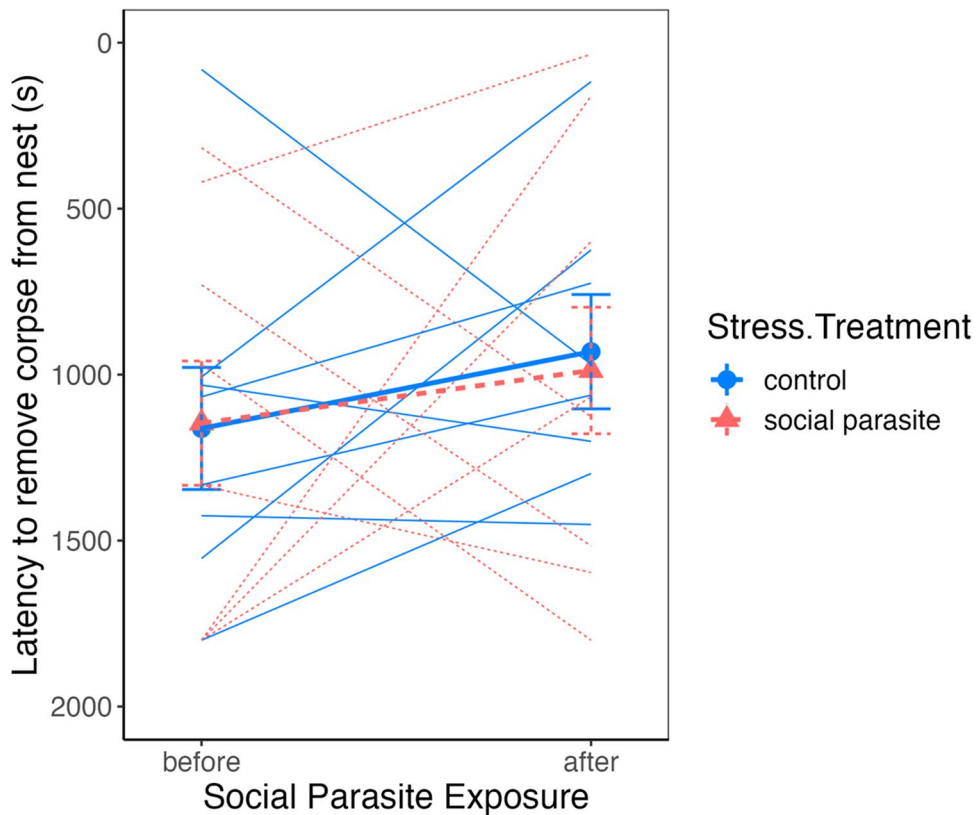


Fig. 2 Corpse removal latency for *T. curvispinosus* colonies before and after exposure to a single *T. americanus* worker. Each line represents the average corpse removal speed for each individual colony, in which we found no effect of treatment on corpse removal. The points connected by thicker lines represent the average corpse removal latency for each treatment group before and after exposure. The longer it takes for colonies to remove a corpse, the weaker their protection against infection (i.e., longer latency = weaker social immunity). This

is because the longer a corpse remains in the colony, the higher probability that any parasites that corpse contains will be transmitted to living colony mates. We flipped the y-axis to reflect this so that a positive slope indicates an increase in social immune protection. Red triangles with the dotted lines represent colonies exposed to social parasites, whereas blue circles with solid lines represent control colonies left unexposed to social parasites; error bars around the mean represent standard error

Disease defenses are important traits in natural systems due to the intense selective pressures imposed by pathogens (e.g., Hamilton and Zuk 1982). Exposure to biotic stressors like social parasites may shift activity budgets towards defenses that may leave colonies at risk for pathogen invasion. We found that exposure to a social parasite did not alter nest hygiene (via corpse removal) in *T. curvispinosus*. Maintenance of disease protection could be vital to social insect colonies and therefore robust against interactions with other enemies like predators or social parasites. Many host colonies respond to social parasites by activating defenses that are intended to limit costs and prevent future exploitation (Grüter

et al. 2018). For instance, raids by *T. americanus* can alter host colony aggressiveness to protect colonies in future raids (Kleeberg et al. 2014). These induced defenses could indirectly affect other traits if host colonies prioritize social parasite protection over other tasks, like disease defense. The colonies collected for our experiment may exhibit flexible task participation since they live sympatrically with social parasites in the wild, which has been shown to favor generalist workers over specialists (Jongepier and Foitzik 2016). Having higher proportions of generalist workers could mitigate indirect effects any induced defenses have on other host colony traits. Workers in generalist colonies may readily switch

between social parasite defense and hygienic tasks to maintain protection against both enemies.

We used laboratory experiments to determine if an aggressive social parasite affected one component of nest hygiene in acorn ants. Future studies should investigate the responses of different social immunity traits, such as allogrooming, secretion of anti-microbial peptides, and social network modulation after social parasite exposure. Future observations of *T. curvispinosus* corpse removal should be longer than 30 min, as that will likely yield clearer results since it will reduce the proportion of censored data. Social immunity traits, such as corpse removal, appear to trade-off with individual immunity in certain contexts (Cassidy et al. 2021). Thus, measuring the effect these raids have on the immunocompetence of individuals could be particularly interesting relative to impacts social parasite exposure has on social immunity. Determining if social immunity is affected by social parasite exposure, and the intensity of these responses, will elucidate how these coevolved natural enemies can indirectly affect host colonies' pathogen defenses.

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Author Contribution CNK and STC designed the experiment. STC, KS, CG, YZ and AG conducted behavioral assays. STC conducted statistical analyses, and STC and CNK prepared the manuscript.

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Data Availability The data, associated metadata, and R code required to reproduce all the analyses are available on Figshare via the private link below. Upon manuscript acceptance, the data and R code will be made publicly available with no access restrictions at the DOI below:

Private link for peer review: <https://figshare.com/s/ff5aa-dca802b6d29628b>
<https://doi.org/10.6084/m9.figshare.20032238>

Declarations

Ethics These experiments were conducted on invertebrate animals and thus are not under the governance of the US National Research Council, though we adhered to the guidelines of the ASAB/ABS Guidelines for the care and research of animals wherever possible.

Competing Interests The authors declare that we have no competing interests.

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