Research Article

Corpse-associated odours elicit avoidance in invasive ants

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Abstract

BACKGROUND: Invasive ants, such as *Linepithema humile* (the Argentine ant), pose a global threat, necessitating a better understanding of their behaviour in order to improve management strategies. Traditional eradication methods, including baiting, have had limited success, but the causes of control failure are not always clear. This study aims to investigate whether ants form associations between food odours and corpses, and subsequently avoid areas or food sources with food odours associated with corpses. We propose that ants may learn to avoid toxic baits in part because of their association with ant corpses, which could have implications for pest control strategies.

RESULTS: Ants were tested on a Y-maze after exposure to scented corpses or dummies. 69% ($n = 64$) of ants avoided branches bearing the scent of scented corpses. Colonies neglected food with corpse-associated odours, with only 42% ($n = 273$) of foragers feeding from such sources. However, if corpses were produced by feeding ants scented toxicant, focal ants encountering these corpses did not avoid the corpse-associated scent on a Y-maze (53%, $n = 65$). In dual-feeder tests, ants did not avoid feeding at food sources scented with odours associated with conspecific corpses.

CONCLUSION: Conspecific corpses act as a negative stimulus for ants in a foraging situation, potentially causing avoidance of toxic baits. This study suggests adding odours to baits and cycling them to disrupt the bait–corpse association may be helpful. Interestingly, although avoidance of baits was observed, feeding preferences were not significantly affected.
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Supporting information may be found in the online version of this article.

Keywords: *Linepithema humile*; ant learning; odour association; corpse avoidance; negative stimulus; bait avoidance

1 INTRODUCTION

Invasive ant species can have a significant impact on ecosystems and domestic economies alike, making it crucial to understand their behaviour and find effective management strategies to reduce their consequences.[1–3] *Linepithema humile* (Mayr, 1868), commonly known as the Argentine ant, is one of the most damaging ant species globally,[4] and is the most widely distributed invasive ant species in Europe.[5] The presence of *L. humile* leads to a significant reduction in invertebrate diversity, and also can have an impact on vertebrates.[6–8] In addition, *L. humile* can be an important agricultural pest by enhancing hemipteran populations, which then increases the likelihood of fungal and viral infections.[9]

Effective management of invasive ants can have large beneficial environmental outcomes, especially in terms of restoring arthropod biodiversity.[10,11] Unfortunately, invasive ants are difficult to control: two-thirds of invasive ant eradication attempts have failed.[10] The current gold standard approach for eradication or control of ants is to use baits with a toxicant that can be shared with the entire colony by the foragers before the effects are realized, allowing toxicants to reach the brood and queen(s).[10] Even so, the success rate of eradication attempts is low.[10,12]

We propose that one reason for control failure may be that ants learn to avoid bait-associated cues because they also are associated with ant corpses, created during control efforts. This hypothesis assumes that nestmate corpses act as an *unconditional negative stimulus*. Previous studies have revealed a diverse range of ant behaviours in response to corpses, which are influenced by a multitude of factors.[13] These responses including attraction to corpses (necrophilia) and aversion to corpses (necrophobia).[14] Which, if any, of these responses are shown is influenced by factors such as the social and ecological context, colony activity status, characteristics of the corpse (e.g. origin, age, infection status) and levels of mortality within the nest.[13–18] For instance, when *Formica polyctena* colonies are starved, they collect and consume corpses. When well-fed, though, corpses are disposed of or avoided. If, however, the corpses are infected by a pathogen, they are never consumed, regardless of the ants' starvation level.[19] Corpses also may be used during interspecific conflicts to influence competitors; *Lasius niger* has been reported to carry

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nestmate corpses towards the rival colonies.[20] *Temnothorax lichtensteini* is reported to bury foreign corpses and freshly killed nestmates, but carry away older nestmate corpses out of the nest.[21] The presence of nestmate corpses can cause ants to reject otherwise suitable nesting sites,[22] and refuse dumps can deter ants from foraging.[23,24] Refuse dumps are designated areas where waste materials, debris and ant corpses are collected, managed and segregated, to maintain colony hygiene and prevent disease spread. These examples underscore the complexity of ant responses to corpses and emphasize the need for a comprehensive investigation of ant responses to corpses, particularly in a foraging context where little information is available.

The scent of food consumed by ants can be detected by nestmates that encounter them, even without active food sharing.[25–28] We hypothesize that the scent of a toxicant-laced bait, remaining on ants after they have died, may lead to an aversion to the bait among the living ants that encounter these corpses, causing them to avoid the scent or taste of the bait. Ant corpses are a potential threat to the survival of colonies as they may spread disease.[29] To protect a colony from pathogens, it is crucial for ants to distinguish between living and dead ants. Recognition of corpses is likely to be based on olfactory cues.[30] In *L. humile*, the presence of iridomyrmecin and dolichodial (ant-produced compounds that adhere to the cuticle) on live ants disappears within 40 min of death, which then triggers necrophoresis (corpse removal) behaviour from nestmates.[31]

It is thus reasonable to expect that conspecific corpses may represent an unconditional negative stimulus in a foraging situation. *L. humile* has a formidable olfactory memory and is able to rapidly form long-lasting associations between odours or flavours and a food reward.[32–34] Nevertheless, it is not clear how well *L. humile* learns negative associations. Wagner *et al.*[33] showed in *L. humile*, that using quinine as a negative stimulus does not reinforce learning when a positive stimulus (sucrose reward) was already presented. However, *L. humile* preferred to choose 'low-risk' paths to food sources after they discovered a competitor species or formic acid on a 'high risk' path,[35] and other ant species rapidly form negative olfactory associations, and avoid odours associated with, for example, a bitter quinine solution.[36]

To the best of our knowledge, no prior research has explored the influence of conspecific corpses on the feeding preferences and foraging decisions of ants in a foraging context. Our study is designed to investigate whether ants associate the scent of a food source with the presence of nestmate corpses that may have interacted with the same food source. Specifically, we aim to assess how exposure to scented nestmate corpses affects foraging behaviour and whether it leads to the avoidance of high-quality food sources or similarly scented foraging areas.

2 MATERIALS AND METHODS

2.1 Colony maintenance
Linepithema humile ants were collected in 2021 from Girona, Spain and were all part of the same main European supercolony.[37] Colony fragments (henceforth colonies), consisting of one or more queens and 300–800 workers, were kept in plastic foraging boxes (32.5 × 22.2 × 11.4 cm) with plaster of Paris on the bottom. No aggression between ants from different colony fragments was observed. The walls were coated in fluon to prevent escape. Each box contained several 15-mL plastic tubes, covered in transparent red foil, partly filled with water and plugged with cotton, for use as nests. The ants were maintained on a 12:12 h, light:dark

photoperiod at room temperature (RT, 21–25 °C) and provided with water *ad libitum*. Colonies were fed for 3 days with *ad libitum* 0.5 M sucrose solution and freeze-killed *Drosophila melanogaster*, and deprived of food for 4 days before testing. All experiments were conducted between October 2021 and June 2022.

2.2 Solutions and odours
A 1 M sucrose solution (Südzucker AG, Mannheim, Germany), was used as a reward during training for all experiments. Scented paper overlays, employed to provide environmental odours during experiments, were stored for ≥1 week before the experiments in airtight plastic boxes (19.4 × 13.8 × 6.6 cm). These boxes contained a glass petri dish with 500 μL of either strawberry or apple food flavouring, or cassis and orange food flavouring (Seeger, Springe, Germany). For experiments using flavoured food, 1 μL of the respective flavouring was added per 1 mL of 1 M sucrose solution. The reason for using these odours is to simulate the scent of a bait matrix, as sucrose alone has little or no scent. We have demonstrated in previous work that *L. humile* can readily associate these odours with a reward.[34,38] In a previous study,[38] we also demonstrated that they do not have a strong preference for any of these odours. In the current series of experiments, we employed two distinct odour pairs: orange and cassis in experiments 1–3, and apple and strawberry in the experiment 4. Given the extended 24-h duration of the experiment 4, during which the nest boxes were open, we were concerned that lingering odours from the initial odour pair remained in the room. We thus took the extra precaution of switching to a novel odour pair for this experiment.

2.3 Corpses and dummies
One day before the start of experiments, 24 ants were frozen for 60 min in a −20 °C freezer. These ant corpses were scented by placing them at RT for 24 h into an airtight petri dish (80 × 15 mm, sealed with Parafilm©) containing a small metal plate with 200 μL strawberry or apple food flavouring, or with cassis or orange flavouring. *Linepithema humile* can identify ants as being dead within 40 min of death,[31] so 24 h was considered more than sufficient for corpses to be identified as such by nestmates. Please note that we did not use nestmates from the same colony fragment; instead, we used ants from other fragments. However, all colony fragments were sourced from the same location at the same time, and showed no aggression between each other.

Twenty-four dummies were created by cutting black electrical wire (1 mm diameter, 2.5–3 mm length) to visually simulate corpses, and provide a similar obstacle to movement. The dummies were scented using the same method as for the corpses. Corpses and the dummies had no physical contact with the food flavourings, but were exposed to the scented air in the sealed petri dish.

2.4 Experiment 1—odour preference in a Y-maze test with air-scented corpses
Here we investigated whether ants are capable of forming an association between an odour and dead nestmates (corpses) in a Y-maze test setting. We predicted that ants exposed to scented corpses would avoid the smell associated with the corpses, and hence avoid the corresponding arm of the Y-maze, yet ants exposed to scented dummies would not.

Twelve corpses (treatment) or dummies (control) were placed onto a round plastic arena surrounded by a water moat [arena: 7 cm diameter, inner platform: 4 cm diameter, see Fig. 1(A)] using clean, soft forceps. A colony was connected via a drawbridge to
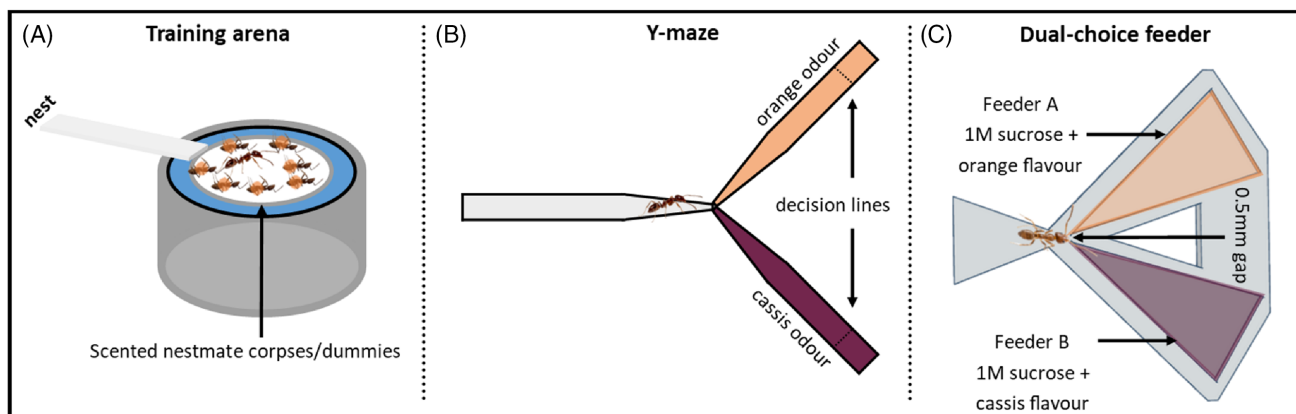
Figure 1. Setup used for experiments 1–3. (A) encounter arena where ants experienced scented corpses or dummy. (B) Preference test on a Y-maze with scented paper overlays (experiments 1 and 2). (C) In experiment 3, ants were tested on a dual-choice feeder containing differently-flavoured sucrose solutions in each well. The two well-tips are so close as to ensure the ant contacts both wells simultaneously (ant image to scale). The colours refer to the two different odours/flavours.

the round arena and a single ant was allowed to enter the platform in the arena via the drawbridge. The drawbridge was then removed, trapping the ant in the arena together with the scented corpses or scented dummies (orange or cassis smelling). After 90 s, or at least ten contacts between the ant and the corpses or dummies, a second drawbridge was added to let the ant leave the arena and enter a Y-maze [see Fig. 1(B)]. This approach was designed to ensure that the ant either had sufficient contact with the corpses or remained in close proximity to the corpses for an extended duration.

The stem of the Y-maze (i.e. the part through which the ant enters the Y-maze) was covered in an unscented paper overlay. One Y-maze arm (e.g. left side) was covered in a scented overlay with the scent matching the scent of the corpses/dummies (e.g. orange), and the other arm (e.g. right side) was covered in a neutral-scented overlay (novel odour cassis). We recorded the ant's final decision [crossing a line 2 cm from an arm end; see Fig. 1(B)]. The position of the odour associated with the corpses or dummies (left or right arm) was systematically varied between ants. The Y-maze was cleaned with ethanol after each ant. Sixteen ants were tested sequentially using the same ant corpses, followed by 16 tested ants on dummies of the same smell as a control. The next day, the same procedure was performed with the opposite odour (e.g. cassis). The associated odour (orange or cassis), and the sequence of corpses/dummies were switched alternately. A total of 128 ants from four colonies were analyzed.

2.5 Experiment 2—odour preference in a Y-maze test with toxicant-scented corpses
Building upon the findings of the first experiment, experiment 2 was designed to investigate whether similar behaviours would be elicited in a more realistic setting. In this experiment, corpses were created by allowing ants to consume a sucrose-toxicants solution with added flavour. As a result, the corpses were expected to acquire the scent of the flavoured toxic food source (orange or cassis).

Two days before the start of the experiment, hydrogel beads[39,40] were placed into a solution containing 7.6 μL Spintor® (containing 44% Spinosad) and 200 μL food flavour (either cassis or orange) in 100 mL 1 M sucrose. One day before the start of the experiment, 24 ants were allowed to feed on these hydrogel beads in a petri dish. After 8 h all ants were dead, and the corpses

were placed in a clean petri dish. The next day (~16 h later) these ants were used as corpses in the behavioural trials. The procedure of the training and testing was identical to experiment 1. A control treatment with dummy ants was not possible in this experiment, as consumption of the flavoured toxicant is required for odour presentation during the experiment. A total of 64 ants from four different colonies were analyzed.

2.6 Experiment 3—assessing the impact of corpse-associated odour on feeding preferences
In this experiment, we explored how ants react to food flavoured to match the scent of nestmate corpses *versus* food with scented dummies. Additionally, we examined whether a shorter period of starvation (2 days) influenced ant responses in comparison to the standard 4-day starvation period. Our hypothesis was that highly hungry ants might exhibit increased motivation to feed on the first available food source, potentially affecting their preference between the two options. Ants encountered corpses or dummies, scented by storing with food flavourings for 24 h, in the round plastic arena as described in experiment 1. After this training, the ant was allowed to enter a drawbridge leading to a 'dual-choice' feeder, made of 3D printed resin [see Fig. 1(C)]. This feeder consisted of two triangular wells, each filled with a different solution (scented 1 M sucrose). The tips of the wells were 0.5 mm apart, smaller than the length of one *L. humile* antenna. This ensured that, regardless of which solution is contacted first, the ant will almost immediately also come into contact with the alternative solution, enabling an informed choice between the two solutions [see Fig. 1(C)]. We noted the time spent feeding from each feeder. Such feeders allow for a sensitive quantification of feeding preference (Wagner *et al.*, in prep).

The feeder side containing the corpse-associated flavoured food was systematically varied between ants. A total of 127 ants from four colonies were analyzed. Tested ants were permanently excluded from the colony, to prevent scented food being returned to their nestmates.

2.7 Experiment 4—influence of scented corpses on collective food choice
The purpose of this experiment was to raise the results of the individual level experiments to the collective level, quantifying collective foraging at a high resolution over the first hour of feeding,

and at a lower resolution over 24 h. Data were split into the first 60 min after food presentation, and the following 23 h. This is because in a laboratory setting, satiation at *ad libitum* feeders often occurs within the first hour.[41]

Hydrogel beads were prepared as in experiment 2, but without the toxicant. A round platform (9 cm diameter, 0.2 cm thick), raised on four plastic pillars (0.3 cm diameter, 5.5 m height), connected to a raised a plastic bridge, was placed into the colony box. The pillars were coated with fluon to ensure that the only access to the platform was via the bridge. Two hydrogel beads, flavoured to match the scent of the corpses, were placed into a 0.1-mm-deep trough on one side of the round platform (see Fig. 2), and two other hydrogel beads with the other flavour were placed on the other side. These beads were cut in half to prevent them rolling.[40] Twelve scented corpses, prepared as in experiment 1, were distributed over the bridge (see Fig. 2). Ants were then allowed free access to the feeders via the bridge. The narrow bridge ensured that all ants encountered most of the corpses. Ants were allowed to recruit and feed on the hydrogel breads for 24 h. Images were recorded automatically by an infrared-sensitive camera with infrared flash (Pi NoIR module 2) connected to a microcomputer (Raspberry Pi 4). An image was taken every 5 min for the first 60 min of the experiment (corresponding to the major feeding and recruitment stage) and thereafter every hour for 24 h. From the images, we counted the number of ants within 5 mm of each feeder. The feeder side and the flavour containing the negative associated hydrogel beads was systematically varied between colonies. A total of 16 colonies were tested.

2.8 Statistical analysis

Data were analyzed using generalized linear mixed-effect models (GLMM)[42] in R v4.1.0 (R Core Team 2021). GLMMs were fitted using the LME4 package.[43] Because the data were binomial in experiments 1 & 2 (novel/corpse odour choice) and in experiment 3 (proportion of drinking time on feeder A *versus* feeder B), a binomial error distribution was used. For experiment 4, a zero-inflated beta model (for proportion data) and a GLMM with a Poisson error distribution (for ant counts on different feeders over time) was used. Because multiple ants were tested per colony, we included colony as random factor. The R package EMMEANS was used for pairwise contrasts, allowing the estimation and comparison of means across different levels of categorical predictors in a model. Model fit was checked using the DHARMa package.[44] Results were plotted using the GGPLOT2 package.[45] The complete code and analysis output is provided in Supporting information Appendix S1.

During the analysis of experiment 4, we excluded four very small nests from the models (≤40 ants foraging over 24 h). For statistical information on all colonies, see Appendix S2. Therein, we provide a complete analysis of the dataset including the four excluded colonies. The results of that analysis show the same patterns as those presented here. We also analyzed the data in terms of proportion of ants on each food type, rather than raw count. Again, the results are similar, and are presented in Appendix S2.

3 RESULTS

3.1 Experiment 1—odour preference in a Y-maze test with air-scented corpses

Ants that had encountered scented corpses chose the Y-maze odour arm associated with the corpses significantly less often than the opposite scented arm [69% chose the novel odour arm, GLMM, z-ratio = 2.92, $p = 0.003$; see Fig. 3(A)]. By contrast, ants in the control group that experienced the odour associated with scented dummies showed no significant preference between the two scented arms [47% chose the novel-odour arm, GLMM, z-ratio = −0.5, $p = 0.62$; see Fig. 3(A)]. The treatment groups differed significantly from each other [EMMEANS, z-ratio = 2.48, $p = 0.013$; see Fig. 3(A)]. The specific corpse/dummy odour (cassis or orange) did not significantly affect choice (GLMM, z-ratio = 0.37, $p = 0.71$).

3.2 Experiment 2—odour preference in a Y-maze test with toxicant-scented corpses

The ants showed no difference in their choice of odour arm [GLMM, z-ratio = 0.58, $p = 0.56$; Fig. 3(B)]: 53% of the ants that
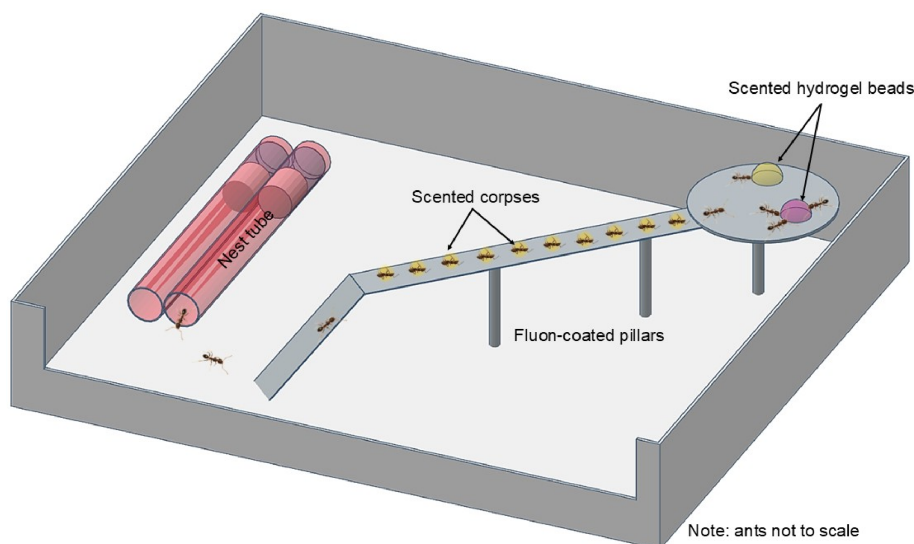
Figure 2. Collective decision-making experiment. A round plastic platform, supported by fluon-coated pillars and reachable by a bridge, was placed in the colony box. Hydrogel beads soaked in flavoured sucrose solution (apple and strawberry) were offered. Twelve scented nestmate corpses were placed on the bridge. Foraging ants had to pass the scented corpses to reach the feeding platform. The number of ants within 0.5 cm of each food type were counted.

were exposed to corpses fed with a flavoured toxicant chose the arm scented with the other odour. The specific odour associated with the corpses did not significantly affect arm choice (GLMM, z-ratio = −0.51, p = 0.61). Unexpectedly, focal ants were commonly observed to lick the mouths of the corpses.

3.3 Experiment 3—assessing the impact of corpse-associated odour on feeding preferences

Ants starved for 2 days did not feed significantly longer on either of the food sources, irrespective of their treatment [GLMM, dummy: z-ratio = 1.74, p = 0.08, 60% fed longer from the novel odour; corpse: z-ratio = 0.51, p = 0.61, 53% fed for longer from the novel feeder; see Fig. 4(A)]. Odour did not significantly affect feeding time (GLMM, feeding time, z-ratio = 0.55, p = 0.58). Similar outcomes were found for ants starved for 4 days feed [GLMM, dummy: z-ratio = −0.56, p = 0.58, 42% fed longer from the novel feeder; corpse: z-ratio = 0.87, p = 0.39, 55% fed longer from the novel feeder; see Fig. 4(B)]. Odour type did not significantly affect feeding time (GLMM, feeding time, z-ratio = −0.25, p = 0.80).

### 3.4 Experiment 4—influence of scented corpses on collective food choice

In the first 60 min, significantly fewer ants (705 of 1769) fed on the hydrogel beads scented to match the corpses [EMMEANS, t-ratio = −7.751 p < 0.0001; Fig. 5(A)/(B)]. Over the following 23 h, foraging levels were extremely low (mean 0.324 ants on each feeder at
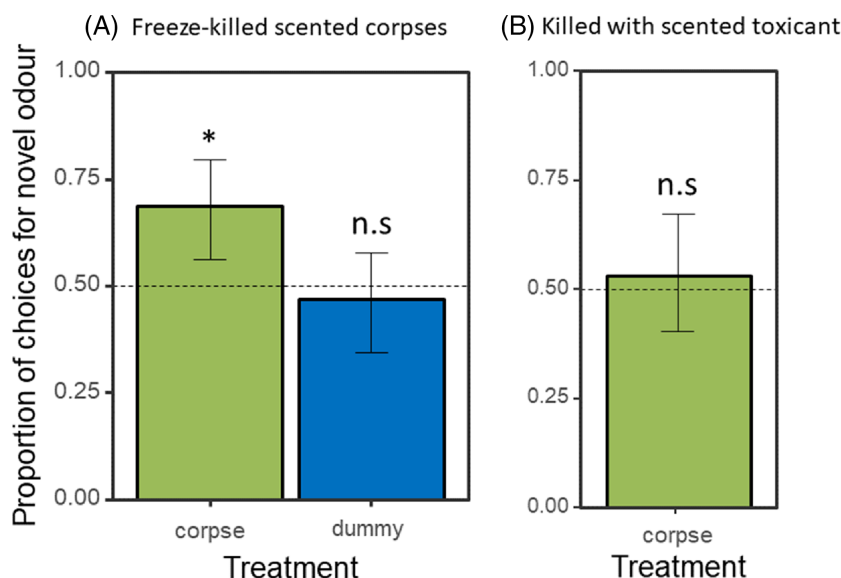
Figure 3. Response of ants to odours associated with nestmate corpses (experiments 1 and 2): (A) Experiment 1: proportion of choices for novel odour, per treatment group, corpses or dummies (control). (B) Experiment 2: proportion of ants which encountered nestmate corpses killed by feeding on scented food choosing the novel scented Y-maze arm. Because experiment 2 relied on ants consuming the scented, toxic bait, no control was possible. Bars depict means, whiskers 95% confidence intervals derived from the fitted GLMM. The dotted horizontal line displays chance level of 50%. Significance: *, p < 0.05.
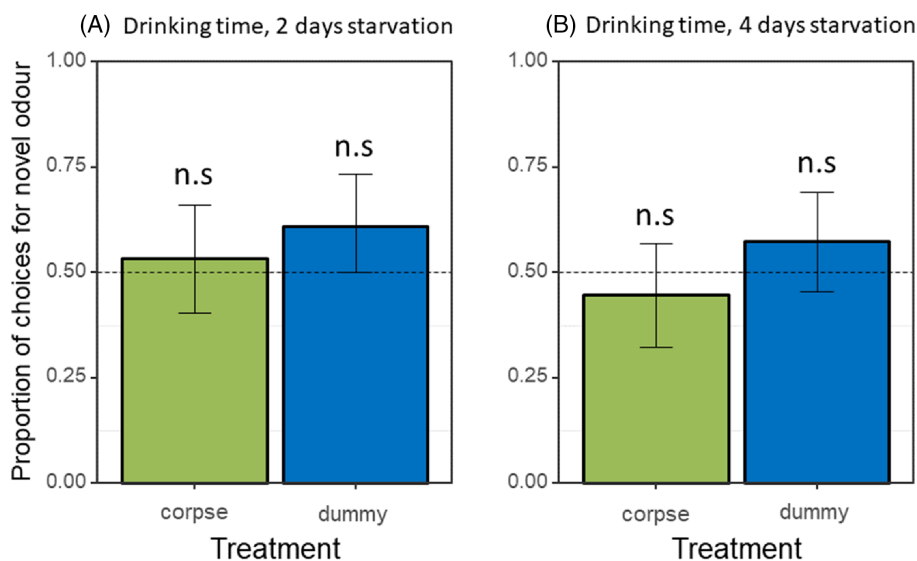
Figure 4. Effect of corpse-associated smell on feeding preference (experiment 3): (A) and (B) show the mean (±95% confidence intervals) proportion of time spent feeding at the feeder not scented to match the corpses or dummies, by ants starved for 2 and 4 days respectively.
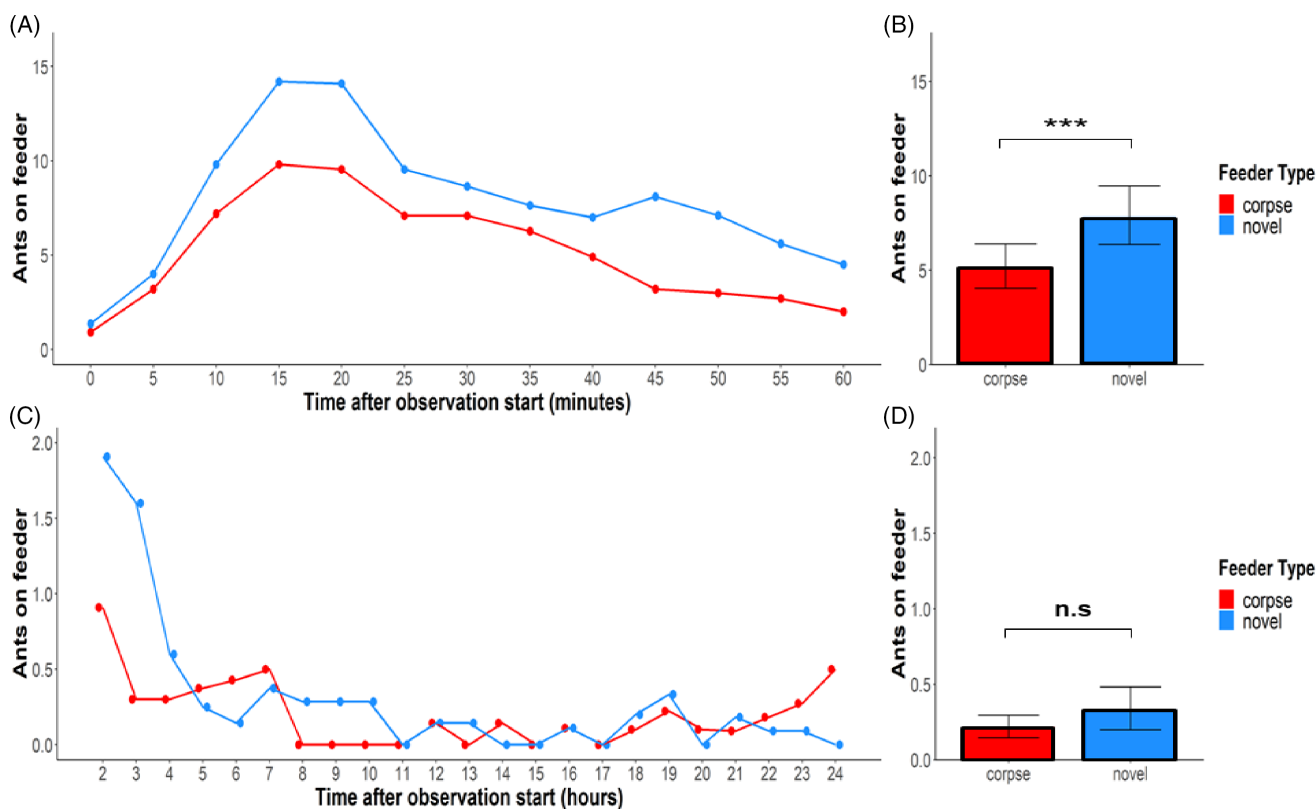
Figure 5. Collective foraging effort on feeders scented to match either nestmate-corpses encountered en-route to the feeder, or scented with a novel odour (experiment 4). (A) Dual-curve plot showing the average number of ants within 0.5 cm of feeder, at 5-min intervals for 60 min after food presentation. (B) Bar plot showing the mean (±SE) number of ants staying ≤1 cm to a feeder over the first 60 min. (C/D) are equivalent to (A/B) but 23 h after food presentation in 1 h intervals. In (A) and (C), the red lines correspond to the corpse treatment and the blue lines correspond to the novel treatment. Bars depict means, whiskers 95% confidence intervals derived from the fitted GLMM or zero-inflated beta model. N counted ants (60 min) = 1769, N counted ants (23 h) = 111. A total of 12 trials were conducted.

each timepoint per colony), with no clear preference for the novel feeder [67 of 111 ants at the novel feeder, EMMEANS, t-ratio = −1.163, p = 0.2456; Fig. 5(C)/(D)]. The specific corpse odour did not significantly affect their choice within 60 min (GLMM, z-ratio = −5.407, p = 0.8669) nor the following 23 h (GLMM, z-ratio = 1.203, p = 0.2291).

4 DISCUSSION

Our study suggests that nestmate corpses are a negative stimulus for the ant *L. humile* in a foraging context. The implication is that corpses may drive toxic bait avoidance at an individual and collective level, by causing the ants to avoid odours associated with them (experiments 1 and 4). However, under conditions more relevant to ant control efforts – the consumption of scented toxicant-laced food (experiment 2)—no avoidance was found. Likewise, in the dual-feeder experiment (experiment 3), ants did not feed less from the solution scented to match the odour of scented corpses.

Avoidance of the scented Y-maze arms associated with nestmate corpses (experiment 1) demonstrates that corpses can act as a negative stimulus for *L. humile* in a foraging situation. Avoidance of cues associated with negative consequences has been demonstrated in ants previously, with ants avoiding Y-maze or T-maze arms scented with an odour associated to a bitter quinine solution.[36,46] Nestmate corpses also have been shown to be avoided by ants, even to the point that potential nest sites

containing foreign ant corpses were avoided.[22] However, ants can discriminate between foreign or nestmate corpses.[21] It is thus unclear if the reason for avoidance in the nest site avoidance study was to avoid a confrontation with a foreign ant colony, or for hygienic reasons, or both. However, given that all the ants in our study stemmed from the same supercolony, and showed no avoidance or aggression between colony fragments, it is unlikely that non-nestmate avoidance plays a large role in the results. Although our study showed a relatively high avoidance rate (69%), it is not clear why avoidance was not higher, such as for avoidance of nests containing corpses.[22] We note that a certain willingness to approach nestmate corpses is required, in order for the corpses to be cleared away, and indeed observed several ants handling, moving and even beginning to dismember corpses. Corpse clearance has been shown to be an important hygienic response, preventing disease spread.[29] It was even shown in Argentine ants that some workers have to apply regular pygidial gland secretions on corpses to prevent growth of pathogenic fungi,[47] which requires the corpses to be approached.

There are other reported cases where ants were attracted to corpses, such as instances of cannibalism, and recruitment to corpse piles in order to clear them away.[19,47,48] This may initially appear to conflict with to our findings. However, these studies primarily focused on intranest responses to corpses. In our study, we investigated ant responses to corpses in a foraging context. Behavioural responses to corpses by ants are diverse and depend on various factors, as mentioned previously. For example, Necrophoretic

behaviours such as burying, carrying, consumption or isolation of corpses heavily depend on the level of pathogenic infection within the corpses.[19] We cannot rule out the possibility that the ant corpses in our study may have been infected, though this seems unlikely given the 24-h decomposition period and our visual inspection of the colony beforehand. Corpses do pose a risk to ant colonies, which necessitates that ants constantly make context-specific decisions regarding whether or not to engage with corpses.[29] Previous studies have shown that covering areas with debris from refuse dumps can delay foraging, and suitable nests were avoided when dead ants were present inside.[22–24] These observations suggest that corpses act as hygiene indicators for specific areas, and ants might avoid these areas. It might be more advantageous from a fitness perspective to respond differently when corpses are found within the nest: An aversion to corpses within the nest context could lead to costly and dangerous nest relocations, or a failure to remove a source of infection. Therefore, it is reasonable to assume that ants respond to corpses in or near the nest differently than in a foraging situation. Another factor could be the relatively short decomposition time of 24 h, which may have influenced the individual ants' decisions. A study in *Myrmica rubra* revealed that only 15% of freshly killed corpses were removed, but this percentage increased to 80% after 6 days.[30] We propose that the avoidance of certain areas is likely to have been triggered by an association with conspecific corpses. In a situation closer to the nest, we might observe different responses. Thus, our findings align with the broader context of behaviour-dependent responses to corpses, which are contingent upon the specific circumstances.

Surprisingly, experiment 2, which used the same setup as the first experiment, found no avoidance of the corpse-associated odour. The key differences between these experiments was how the ants that were used as corpse stimuli were killed, scented, stored. In the first experiment, the ants were freeze-killed then stored for 24 h in a Petri dish containing food flavouring, whereas the ants in the second experiment were allowed to feed on toxic, flavoured hydrogel beads, subsequently died within 8 h, and were then stored for a further 16 h in a Petri dish without any food flavour. It is possible that in this second method the test ants might have not perceived the flavour of the poisoned food on the corpses, because it was too subtle or disappeared after 24 h. Ants can identify nestmates as corpses within 1 h of death,[31] so toxicants with immediate lethal effects may be more detectable, because bait odours may linger sufficiently long on the corpses, whereas toxicants at levels which cause delayed action may be less detectable. Many commercial baits are composed of a matrix with a potentially longer-lasting odour profile, such as fat or protein-rich pastes.[10,49–51] It is possible that such baits imbue ants which feed on them with a long-lasting odour that could drive future rejection when these ants finally die. The water-soluble odour that we used in the current study may not have lasted long enough to be detectable by other ants 16–24 h after feeding.

Interestingly, we observed that most test ants licked the mouths of the dead ants, possibly tasting the food consumed by the poisoned nestmates. They thus might have formed a direct positive association between the odour of the food and its sweetness, which may have interfered with, or counterbalanced, any negative stimulus created by the corpses. Ants have been demonstrated to learn food-related odour cues from returning foragers, even without active food sharing.[25–27,52] It is possible, albeit unlikely, that the varying time of death of individual ants might have resulted in different decomposition durations, potentially leading to varied behavioural responses in ants. A previous study demonstrated that ants tended to carry away freshly deceased nestmates, while they buried older corpses.[21] The results of this experiment thus do not rule out the hypothesis that corpses act as a negative stimulus in these ants, but neither do they support it. The experiment does imply that perhaps this effect will not strongly drive behaviour in a real-world ant control situation using sucrose-filled hydrogels baits. However, other commonly-used bait types based on fat or protein pastes may have elicited an effect (see above).

Experiment 3 showed that ants did not feed less from a food source flavoured to match the scented corpses than from a neighbouring food source offering a novel flavour, irrespective of whether the ants were starved for 2 or 4 days. It is unlikely that the ants were unable to associate the odour impregnated onto the corpses with the taste of the flavoured sucrose feeder because we have previously[33] demonstrated that Argentine ants can learn to associate a flavoured food reward with the corresponding environmental odour after only one exposure. Further experiments with this assay offering 1 M *versus* 0.5 M sucrose, or 1 M *versus* 1 M sucrose + a low level of bitter quinine, have shown that this method is effective at uncovering food preference (unpublished data). A more likely reason for this unexpected result was the overwhelming drive to feed under even moderate starvation conditions: the ants simply continued feeding at the first food source they encountered, and any potential negative stimulus associated with the food flavour was not sufficiently strong to override ongoing feeding. Individual *L. humile* workers are unusually motivated feeders.[53] Alternatively, ants may only show avoidance in terms of orientation, not food rejection once the food is encountered. In other words, the ants might only avoid going towards areas scented to match the scent of corpses, but do not avoid feeding at similarly-flavoured food sources. This fits in well with our understanding of the response to corpses as a nuanced, complex behaviour.

Experiment 4 tested colony-level preference, by examining collective foraging choices. Collectively, over the first hour of foraging where the vast majority of foraging occurred, the ants avoided the corpse-associated hydrogel beads and preferred to feed on the alternative beads. The proximate mechanism driving this pattern is unclear: ants may have avoided approaching the corpse-associated bead, may have left the bead before feeding fully, may have down-modulated their recruitment to the bead, or any combination of these options. Unfortunately, we lack detailed information on the behaviour of the individual ants in the collective foraging assay, especially the first few foragers, which would be needed to distinguish these mechanisms. Observations over the first 10 min suggested that ants accepted the first bead that they contacted, regardless of whether it was the corpse- or control-bead. This fits well with the results from experiment 3, and makes at least the taste-and-reject hypothesis unlikely. As a result of the positive feedback inherent in ant mass-recruitment systems, the behaviour of the first ants in such experiments has a decisive influence on the final collective outcome.[54] Thus, if the first few ants tended to preferentially approach the novel bead, and laid the first pheromone trail in that direction, this would bias all future choices towards that bead. This effect would compound the tendency of the ants to avoid approaching the corpse-associated odour.

Finally, a second positive-feedback process may well be in place: The first ants to feed may have encountered naïve outgoing ants on their return journey, and certainly shared food via trophallaxis with other ants in the nest. Such on-trail and within-nest

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encounters could have created a positive association to the smell of the bead area, causing these newly recruited ants to preferentially choose that area – as has already been shown in this and other species.[25,26,33] In systems characterized by strong positive feedback, such as this one, stochasticity can play a very large role. It is thus not surprising that in a subset of experiments the corpse-associated bead was chosen, as even weaker options, if discovered first, can rapidly outcompete stronger options discovered later.[41,55]

One important aspect to consider is that statistical significance does not always equate to a biologically meaningful effect. Experiment 1 demonstrated a pronounced avoidance response (69%, null hypothesis 50%), which, in our opinion, aligns well with the expected biological logic of a corpse response system, and is certainly a biologically meaningful effect size. The results of experiment 4 (collective choice) are less clear cut. Here only 60% of ants avoided the gel beads scented with the odour associated with corpses: a statistically significant effect, but a lower effect size. While a 10% change from the null hypothesis is often considered a strong effect size, it is up to individual researchers to decide if they consider this biologically meaningful. Considering the very close proximity of the two food sources, we consider this effect meaningful because a preference was nonetheless detected. One crucial question is when the ants perceived the odour – was it upon entering the arena or after they started to feed? If ants detected the scent too late, they might have already began feeding, and as seen in experiment 3, once they begin feeding this outweighs other considerations. Therefore, we maintain that our results from experiments 1 and 4 hold biological significance.

Our study demonstrates that *L. humile* can form negative associations, in a foraging context, with a scent associated with nest-mate corpses, which leads them to avoid the corresponding odour. However, this aversion may not be sufficiently strong to override positive stimuli, such as the presence of a desired food. These findings have important implications for ant control strategies, as they suggest that the presence of nestmate corpses may reduce bait efficacy. The formation of a negative association between a bait and affected nestmates may negatively impact the efficacy of control efforts. However, given the lack of effect found in experiment 2, which used more field-realistic methods, it is unclear whether this effect plays a major role in real-world control efforts. Nevertheless, if this association-driven avoidance of baits is indeed impacting control efforts, a straightforward intervention suggests itself: baits can be scented with distinct odours, and odours can be either cycled between treatments, or multiple odours can be deployed simultaneously. Both interventions should reduce the ability of ants to form firm associations between the bait odour and the odour of killed ants. This approach may well have other benefits as well, such as making the baits easier for ants to find, because they can be more easily detected at a distance. The commercial availability of cheap and environmentally benign food flavourings makes such an intervention easily and broadly deployable.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

AUTHOR CONTRIBUTIONS

T.Wagner and T. J. Czaczkes designed the study. T. Wagner collected the data. T. Wagner analyzed the data and wrote the manuscript. T. J. Czaczkes edited the manuscript. All authors read and approved the manuscript.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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