Food odours associated with conspecific corpses cause food avoidance in an invasive ant

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Abstract

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. Here we propose that ants may learn to avoid toxic baits in part due to their association with ant corpses. 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. At a collective level, colonies neglected food sources associated with scented ant corpses in favour of a food source with a novel odour, with only 42% (n = 273) of foragers feeding from the corpse-scent associated food source. However, if corpses were produced by feeding ants scented toxicant, focal ants encountering these ants did not avoid the corpse-associated scent on a Y maze (53%, n = 65). Moreover, in a dual-feeder test, ants did not avoid feeding at food sources scented with an odour associated with conspecific corpses. The study demonstrates that conspecific corpses can act as a negative stimulus for *Linepithema humile*, leading to avoidance of odours associated with corpses, which can lead to potential avoidance of toxic baits. Why the more realistic Y-maze trial with corpses of ants that had ingested the toxicant elicited no avoidance is unclear: it may be due to weaker odour cues from ingested food, or a counterbalancing of the negative corpse stimulus by the positive presence of food remains on the corpse. Nonetheless, this study demonstrates that conspecific corpses act as a negative stimulus for ants, and this should be kept in mind when planning control efforts. A simple solution to this issue would be adding odours to baits, and cycling baits between treatments, or including multiple bait odours in one treatment round.

Keywords:
Bait avoidance – learning – negative stimulus – odour association – corpse avoidance

Introduction

Invasive ant species can wreak havoc on ecosystems and economies alike, making it crucial to understand their behaviour and find effective management strategies for them (1–3). *Linepithema humile* (Mayr, 1868), commonly known as the Argentine ant, is a highly destructive ant species that causes significant ecological and economic damage globally (4), and is the most widely distributed invasive ant species in Europe (5). Invasion by *L. humile* leads to a significant reduction in invertebrate diversity, and can also have an impact on vertebrates (6–8). In addition, *L. humile* can be an important agriculture 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). Unfortunately, invasive ants are difficult to control: two-thirds of *L. humile* eradication attempts have failed (10). The current gold standard approach for eradication or control of ants is to use baits with a slow-acting toxicant, which workers return to the brood and the queen (10). Even so, the success rate of eradication attempts is low (10,11). It has been shown in Argentine ant that foraging choices reflect the nutritional need within the colony over the year, leading to a higher protein-rich diet intake in summer and a reduction of it in winter (12,13). Consequently, if the food used for baits doesn't match the colony's nutritional needs during the current season and when high-quality natural food is readily available, ants may ignore the bait (14), resulting in low bait consumption rates and challenges in achieving eradication. While poor bait uptake is often suspected, the specific reasons behind such poor uptake are still poorly explored in the literature. In laboratory settings, ants have been observed to switch foraging preferences after satiating on one type of favoured food, leading them to seek alternative food sources (13). The presence of other ant species can also cause a swift abandonment of food sources (15,16). However, *L. humile* is an aggressive and dominant species, and is rarely reported to be successfully driven away from resources (5,16), but see also (17).

We propose an alternative hypothesis, not mutually exclusive to those discussed above: that ants learn to avoid bait-associated cues because they are also associated with ant

corpses, created during control efforts. This hypothesis assumes that nestmate corpses act as an *unconditional negative stimulus* (18,19). It has been shown that the scent of food which ants have consumed can be detected by nestmates which encounter them, even without active food sharing (20–23). 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 which 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 due to the possibility of infection (24). To protect a colony from pathogens, it is crucial for ants to distinguish between living and dead ants. Recognition of corpses is likely based on olfactory cues (25). In *L. humile*, the presence of iridomyrmecin and dolichodial (ant-produced compounds that adhere to the cuticle) on live ants disappears within 40 minutes of death, which then triggers necrophoresis behaviour from nestmates (26). Studies have demonstrated that ant corpses can inhibit foraging or nesting. For example, it was shown that the presence of nestmate corpses can cause ants to reject otherwise suitable nesting sites (27,28) and refuse dumps can deter ants from foraging (29,30).

It is thus reasonable to expect that conspecific corpses may represent an unconditional negative stimulus. *L. humile* has a formidable olfactory memory and is able to rapidly form long-lasting associations between odours or flavours and a food reward (31–33). Nevertheless, it is not clear how well *L. humile* learns negative associations. Wagner et al. (32) showed 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 (34), and other ant species rapidly form negative olfactory associations, and avoid odours associated with, for example, a bitter quinine solution (35).

To the best of our knowledge, no previous studies have investigated whether exposure to conspecific corpses affects the feeding preferences and decision-making of foraging ants. We hypothesize that *L. humile* workers can form a negative association between the odour of a food source and the presence of conspecific corpses which fed on the same food source, or died in the food source´s vicinity, and hence have the food scent on their bodies. Specifically, we investigated the impact of exposure to scented nestmate corpses on foraging behavior,

and to determine whether this exposure leads to avoidance of high-quality food sources or areas.

Materials and Methods

Colony maintenance

Linepithema humile ants were collected in 2021 from Girona, Spain and were all part of the same main European supercolony (36). Colony fragments (henceforth colonies), consisting of one or more queens and 300-800 workers, were kept in plastic foraging boxes (32.5 x 22.2 x 11.4cm) with plaster of Paris on the bottom. No aggression between ants from different colonies was observed. The walls were coated in fluon to prevent escape. Each box contained several 15mL 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 light:dark cycle at room temperature (21-25 °C) and provided with water *ad libitum*. Colonies were fed for three days with *ad libitum* 0.5M sucrose solution and freeze-killed *Drosophila melanogaster*, and deprived of food for four days prior to testing. All experiments were conducted between October 2021 and June 2022.

Solutions and odours

1M sucrose solutions (Südzucker AG, Mannheim, Germany), were used as a reward during training for all experiments. Scented paper overlays, used during experiments to provide environmental odours, were stored for at least 1 week prior to the experiments in airtight plastic boxes (19.4 x 13.8 x 6.6cm) containing a glass petri dish with 500µL of either strawberry or apple food flavouring, or blackcurrent and orange food flavouring (Seeger, Springe, Germany). For experiments where flavoured food was used, 1µL of the respective flavouring was added per 1mL of 1M sucrose solution.

Corpses and dummies

One day prior to the start of experiments, 24 ants were frozen for 60 min in a -20°C freezer. These ant corpses were scented by placing them into an airtight petri dish (80x15mm, sealed with Parafilm©) containing a small metal plate with 200µL of strawberry or apple food flavouring, or with blackcurrent or orange flavouring. The container was stored at room temperature for 24 hours. *L. humile* can identify dead ants as being dead within 40 minutes of

death (26). 24 hours is more than sufficient for corpses to be identified as such by nestmates. Note that we did not attempt to test ants only with corpses from their own fragments. However, all colony fragments were sourced from the same location at the same time, and showed no aggression between each other.

24 dummies were created by cutting black electrical wire (mm diameter and 2.0-2.5mm length) to visually simulate corpses, and provide a similar obstacle to movement. Dummies were scented identically to the corpses. We chose to use plastic-coated wire, rather than glass beads, as we considered plastic to be more similar to the ants' surface in terms of how readily it would absorb odours. Note that the corpses and the dummies had no physical contact with the food flavourings but were exposed to the scented air in the sealed petri dish.

<u>Experiment 1 – Testing the effect of corpse-scent association on odour preference using scented corpses</u>

Here we investigate 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, but ants exposed to scented dummies would not.

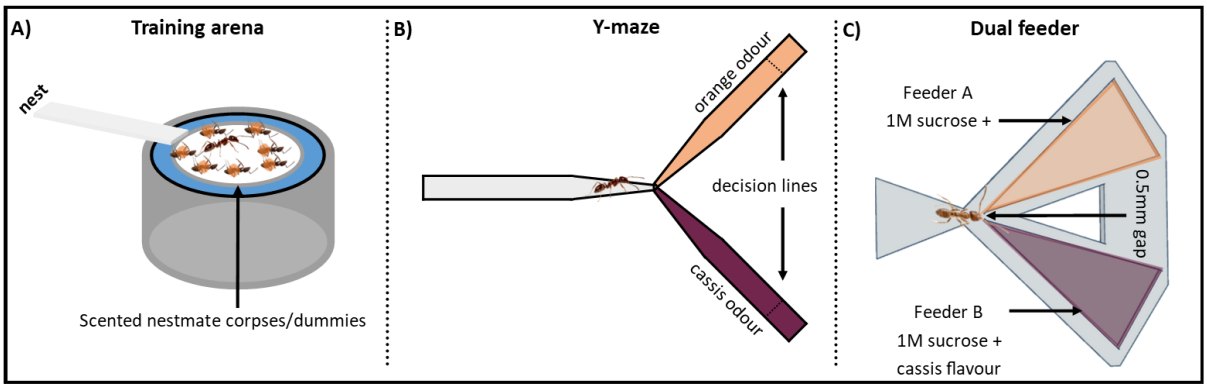
12 corpses (treatment) or dummies (control) were placed onto a round plastic arena surrounded by a water moat (arena: 7cm diameter, inner platform: 4cm diameter, see figure 1A) using clean soft forceps. A colony was connected via a drawbridge to the round arena and a single ant was allowed to enter the 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 (e.g. orange). After 90 seconds, or at least 10 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. 1B).

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 while one arm (e.g. left past the bifurcation point) was covered in a scented overlay, with the scent matching the scent of the corpses/dummies (e.g. orange), while the other arm was covered in a neutral-scented overlay (novel associated odour e.g. blackcurrent). We recorded the ant's initial and final decision (crossing a line 8cm from the bifurcation, 2cm from the arm end, see fig. 1B). The position of the odour associated with the corpses or dummies was systematically varied between ants. The Y-maze was cleaned

158 with ethanol after each ant. 16 ants were tested sequentially using the same ant corpses,
159 followed by 16 tested with dummies of the same smell. The next day, the same procedure was
160 performed with the opposite odour (e.g. blackcurrent). The negative-associated odour, and
161 the sequence of corpses/dummies were switched alternately. 32 ants were tested per day,
162 testing in total 128 ants from 4 colonies.
163
164 Experiment 2 – Testing the effect of corpse-scent association on odour preference using
165 corpses killed with a scented toxicant
166 Experiment 1 found that the ants did indeed avoid the corpse-associated odour, but not the
167 dummy associated odour. The aim of this experiment was to test whether the same applied
168 when corpses were infused with scent from just feeding on a flavoured toxic food source.
169 Two days prior to start of the experiment hydrogel beads (16,37) were placed into a solution
170 containing 7.6µl Spintor © (containing 44% Spinosad) and 200µl food flavour (either
171 blackcurrent or orange) in 100ml 1M sucrose. One day prior the start of the experiment, 24
172 ants were fed on these hydrogel beads in a petri dish. After 8h all ants were dead, and the
173 corpses were placed in a clean petri dish. The next day (~16h later) these ants were used as
174 corpses in the behavioural trials. The procedure of the training and testing was otherwise
175 identical to experiment 1. Note that a control treatment with dummy ants was not possible in
176 this experiment, as consumption of the flavoured toxicant is required for odour presentation
177 during the experiment. 16 ants were tested per day, testing in total 64 ants from 4 colonies
178 over 4 days on corpses.
179
180 Experiment 3 – Testing the effect of corpse-associated odour on feeding preference
181 Here we asked whether ants respond differently to food flavoured to match the scented
182 nestmates corpses or scented dummies. Furthermore, we tested if a lower starvation level (2
183 days) changed the response of the ants compared to the standard 4 days. The idea here was
184 that extremely hungry ants may be too motivated to feed at the first food encountered to
185 show preference between the two options.
186 Ants encountered corpses or dummies, scented by storing with food flavourings for 24 hours,
187 in the round plastic arena as described in experiment 1. After this training the ant was allowed
188 to enter a drawbridge leading to a two-choice 'dual-choice' feeder, made of 3D printed resin
189 (see fig. 1C). This feeder consisted of two triangular wells, each filled with a different solution

190 (scented 1M sucrose). The tips of the wells were 0.5mm apart, smaller than the length of one
191 *L. humile* antenna. This ensured that, regardless of which solution is contacted first, the ant
192 will almost immediately also come into contact with the alternative solution, enabling an
193 informed choice between the two solutions (see figure 1C). We noted the time spent feeding
194 from each feeder.

195 The feeder side containing the corpse-associated flavoured food was systematically
196 varied between ants. 32 ants were tested per day, testing in total 127 ants from 4 colonies. 64
197 ants were trained on dummies and 63 on corpses. Tested ants were permanently excluded
198 from the colony, to prevent scented food being returned to their nestmates.



199

200 **Figure 1:** Setup used for experiments 1-3: A) Ants were confined to an encounter arena,
201 containing scented dummies or nestmate corpses (experiments 1 & 3), or corpses of ants that
202 had fed on scented toxicant (experiment 2). After 90s or 10 contacts, in experiments 1 & 2,
203 the ants' odour preference was then tested on a Y-maze with scented paper overlays, the
204 colour referring to the two different odours. We noted which decision line was crossed. In
205 experiment 3 ants were tested on a dual-choice feeder (C): Ants enter via a constriction,
206 encountering two adjacent wells, containing differently-flavoured sucrose solution. The ants
207 first choice, time to first feeding interruption, and total feeding time from each well was
208 tested. Note that the two well tips are so close as to ensure the ant contacts both wells (ant
209 image to scale).

210

211 <u>Experiment 4 – Can the presence of scented corpses drive collective food choice?</u>

212 The purpose of this experiment was to raise the results of the individual level experiments to
213 the collective level, quantifying collective foraging at a high resolution over the first hour of
214 feeding, and at a lower resolution over 24 hours. Data were split into the first 60min after food

presentation, and the following 23h. This is because in lab setting, satiation at ad libitum feeders often occurs within the first hour (38).

Hydrogel beads were prepared as in experiment 2, but without the toxicant.

A round platform (9cm diameter, 0.2cm thick), raised on 4 plastic pillars (0.3cm diameter, 5.5m height), connected to a raised a plastic bridge, was placed into the colony box. The pillars were coated in 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 0.1mm 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 (16). 12 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 24h. 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 minutes for the first 60 minutes of the experiment (corresponding to the major feeding and recruitment stage) and thereafter every hour for 24h. From the images, we counted the number of ants within 5mm of each feeder.

The feeder side and the flavour containing the negative associated hydrogel beads was systematically varied between colonies. We tested 16 colonies, and each colony was only tested once.

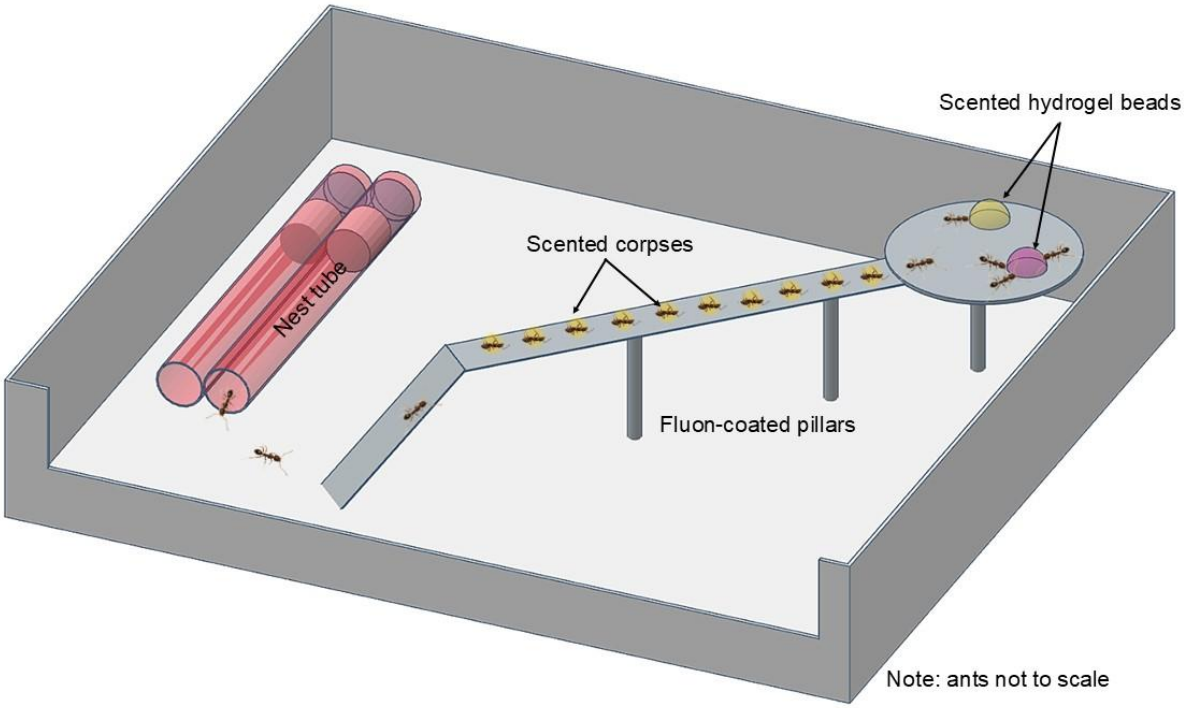


Figure 2: Collective decision-making experiment. A round plastic platform, supported by fluon-coated pillars and reachable by a bridge, was placed in the colony box. Hydrogel beads soaked in flavoured sucrose solution (apple and strawberry) were offered. 12 scented nestmate corpses were placed on the 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.

Statistical analysis

Data were analysed using generalized linear mixed-effect models (GLMM) (39) in R version 4.1.0 (R Core Team 2021). GLMMs were fitted using the lme4 package (40). Because the data were binomial in experiments 1 & 2 (novel/corpse odour choice) and in experiment 3 (proportion drinking time on feeder A vs 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. Since multiple ants were tested per colony, we included colony as random factor. The R package emmeans was used for pairwise contrasts. It allows for the estimation and comparison of means across different levels of categorical predictors in a model. Model fit was checked using the DHARMa package (41). Results were plotted using the gglot2 package (42). The complete code and analysis output is provided in supplements 1.

Results

Experiment 1 – Testing the effect of corpse-scent association on odour preference using 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. 3A). 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.3A). The treatment groups differed significantly from each other (emmeans, z-ratio = 2.48, p = 0.013, see fig. 3A). The specific corpse/dummy odour (blackcurrent or orange) did not significantly affect choice (GLMM: z-ratio = 0.37, p = 0.71). Several focal ants were observed handling the corpses, or even beginning to dismember them. Ants, including *L. humile*, commonly handle and dismember nestmate corpses found in their colony boxes (TW & TJC, pers. obs.).

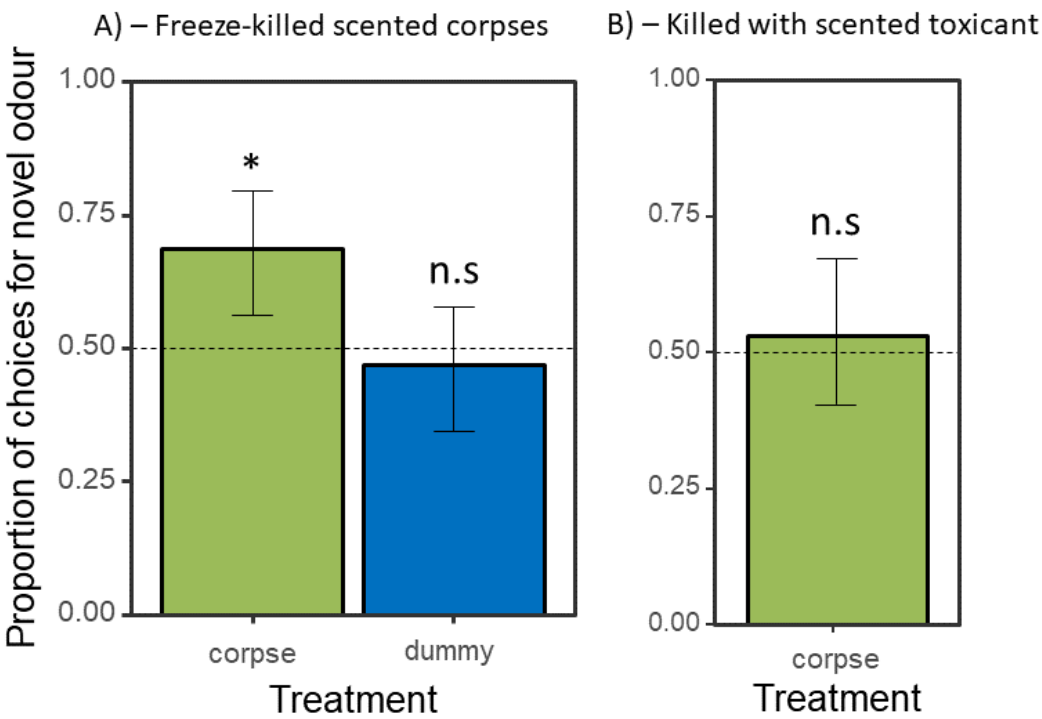


Figure 3: The response of ants to odours associated with nestmate corpses (experiments 1 & 2): A) Experiment 1: Proportion of choices for novel odour, per treatment group, corpses or dummies (control). B) Proportion of ants which encountered nestmate corpses killed by feeding on scented food choosing the novel scented Y-maze arm. Note that since 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%. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

<u>Experiment 2 – Testing the effect of corpse-scent association on odour preference using ants killed with scented toxicant</u>

The ants showed no difference in their choice of odour arm (GLMM, z-ratio = 0.58, p = 0.56, fig.3B): 53% of the ants that 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). Interestingly, focal ants were commonly observed to licks the mouths of the corpses.

<u>Experiment 3 – Testing the effect of corpse-associated odour on feeding preference</u>

Ants starved for two 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. 4A). Odour did not significantly affect these measures (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. 4B). Odour type did not significantly affect choice (GLMM, feeding time, z-ratio = -0.25, p = 0.80).

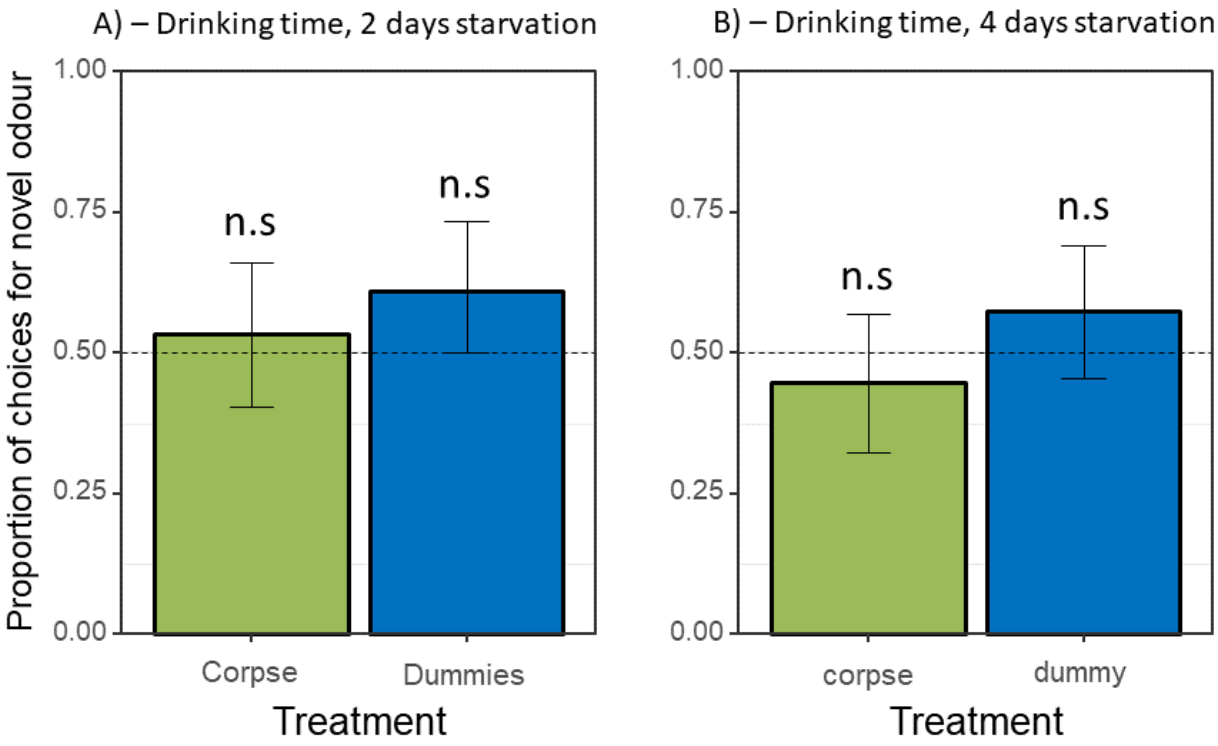


Figure 4: Effect of corpse-associated smell on feeding preference (exp 3): A) and B) show the mean (±95% confidence intervals) proportion of time spent feeding at the feeder not scented to match the corpses or dummies, by ants starved for 2 and 4 days respectively.

<u>Experiment 4 – Can the presence of scented corpses drive collective food choice?</u>

In the first 60 minutes significantly fewer ants (705/1769) fed on the hydrogel beads scented to match the corpses (emmeans: t-ratio = -7.751 $p < 0.0001$, fig. 5A/B). Over the following 23h, foraging levels were extremely low (mean 0.324 ants on each feeder at each timepoint per colony). Over this 23 h period ants showed only a weak and non-significant trend towards the novel feeder (67/111 (emmeans: t-ratio = -1.163, $p = 0.2456$, fig. 5D/E). The specific corpse odour did not significantly affect their choice within 60 minute (GLMM: z-ratio = -5.407, $p = 0.8669$) nor the following 23 hours (GLMM: z-ratio = 1.203, $p = 0.2291$). Note that in our analysis, we excluded four very small nests from the models (40 ants or less over 24 hours). For statistical information on all colonies, see supplement 2. Therein we also 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 analyse 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 supplement 2.

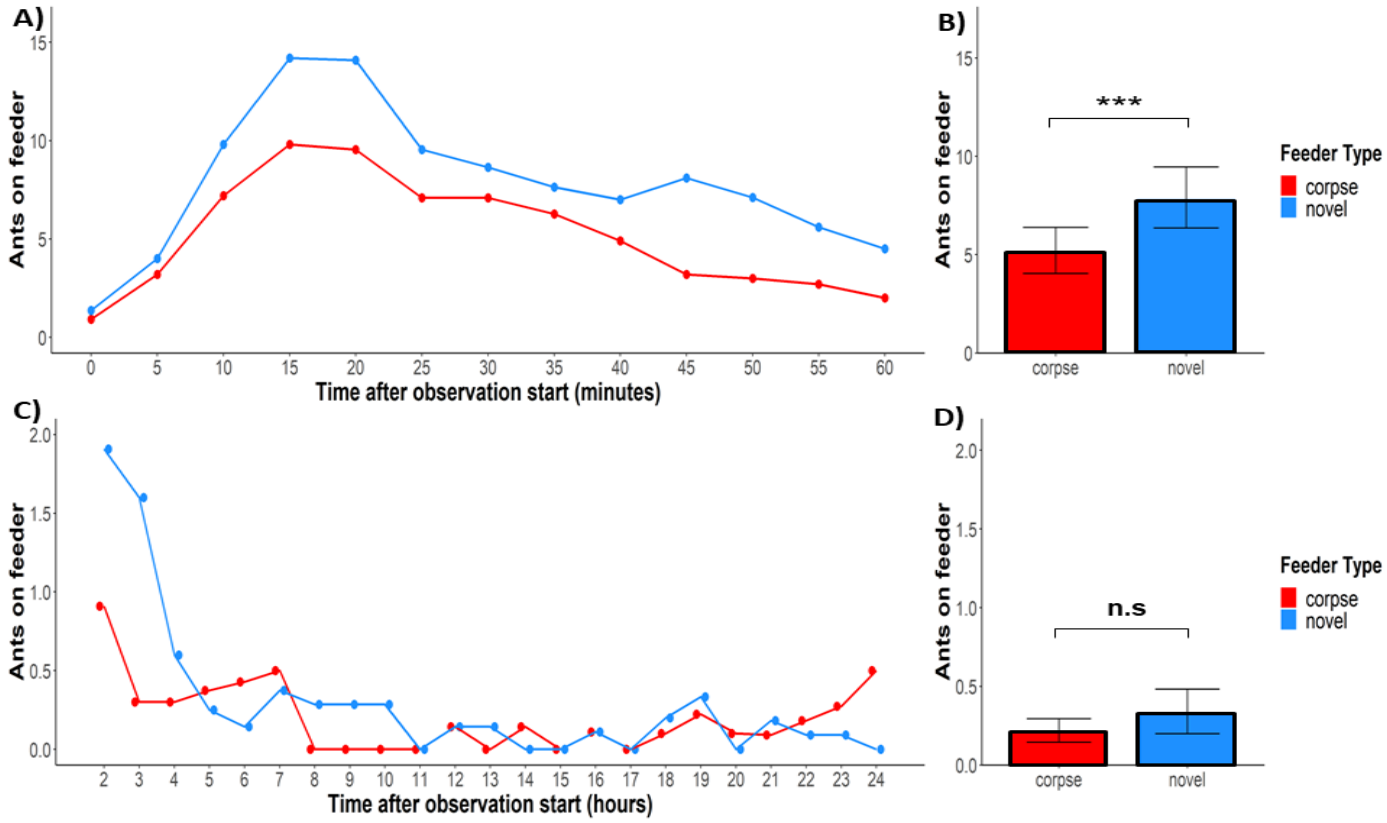


Figure 5: Collective foraging effort on feeders scented to match either nestmate-corpses encountered en-route to the feeder, or scented with a novel odour (exp 4): A) Dual-curve plot, average number of ants sticking 1 cm or closer to a feeder, 60 minutes post food presentation per 5 minutes intervals. B) Bar plot, average number of ants sticking 1 cm or closer to a feeder over the first 60 minutes. C/D) corresponding to A/B but 23 hours post food presentation in 1 hour intervals. Bars depict means, whiskers 95% confidence intervals derived from the fitted GLMM or Zero-Inflated Beta Model. Note the different scales of figures A/B and C/D. N counted ants (60min) = 1769, N counted ants (23hours) =111. All in all 12 trials were conducted.

329 # Discussion

330 Our study strongly suggests that nestmate corpses are a negative stimulus for the ant
331 *Linepithema humile.* The implication is that corpses may drive toxic bait avoidance at an
332 individual and collective level, by causing the ants to avoid odours associated with them
333 (experiments 1 and 4). However, under conditions more relevant to ant control efforts – the
334 consumption of scented toxicant-laced food (experiment 2) no avoidance was found.
335 Similarly, in the dual-feeder experiment (experiment 3), ants did not feed less from the
336 solution scented to match the odour of scented corpses.
337
338 Avoidance of the scented Y-maze arms associated with nestmate corpses (experiment
339 1) demonstrates that corpses can act as a negative stimulus for *L. humile*. Avoidance of cues
340 associated with negative consequences has been demonstrated in ants previously: Ants avoid
341 Y-maze or T-maze arms scented with an odour associated with a bitter quinine solution
342 (35,43). Nestmate corpses have also been shown to be avoided by ants: potential nest sites
343 containing foreign ant corpses were avoided by *Temnothorax albipennis* (27). However, we
344 note that ants can discriminate between foreign or nestmate corpses (28). It is thus unclear if
345 the reason for avoidance in the nest site avoidance study was to avoid a confrontation with a
346 foreign ant colony, or for hygienic reasons, or both. However, given that all the ants in our
347 study stemmed from the same supercolony, and showed no avoidance or aggression between
348 colony fragments, it is unlikely that non-nestmate avoidance plays a large role in the results.
349 While our study showed a relatively high avoidance rate (69%), it is not clear why avoidance
350 was not higher, such as for avoidance of nests containing corpses (27). We note that a certain
351 willingness to approach nestmate corpses is required, in order for the corpses to be cleared
352 away. Corpse clearance has been shown to be an important hygienic response, preventing
353 disease spread (24). It was even shown in Argentine ants that some workers have to apply
354 regular pygidial gland secretions on corpses to prevent growth of pathogenic fungi (44), which
355 requires the corpses to be approached.
356
357 Surprisingly, our second experiment, which had the same setup as the first experiment,
358 found no avoidance of the corpse-associated odour. The key difference between these
359 experiments was how the ant, used as corpse stimuli, died and were stored. While in the first
360 experiment the ants were freeze-killed then stored for 24h in a Petri dish containing food

361 flavouring, the ants in the second experiment were allowed to feed on poisoned, flavoured
362 hydrogel beads, subsequently died within 8h, and were then stored for a further 16h in a Petri
363 dish without any food flavour. It is possible that in this second method the focal ants might
364 have not perceived the flavour of the poisoned food on the corpses, because it was too subtle
365 or disappeared after 24 hours. Ants can identify nestmates as corpses within 1 hour of death
366 (26), so toxicants with immediate lethal effects may be more affected by this association
367 mechanism, as bait odours may linger long enough on the corpses, while toxicants with
368 delayed action may be less impacted. We also note that many commercial baits are composed
369 of a matrix with a potentially longer-lasting odour profile e.g. fat or protein-rich pastes (10,45–
370 47). It is possible that such baits imbue ants which fed on them with a long-lasting odour which
371 could drive future rejection when these ants finally die. The water-soluble odour we used in
372 the current study may not have lasted long enough to be detectable by other ants 16-24 hours
373 after feeding.

374 Interestingly, we observed that most test ants licked the mouths of the dead ants,
375 possibly tasting the food consumed by the poisoned nestmates. They thus might have formed
376 a direct positive association between the odour of the food and it's sweetness, which may
377 have interfered with, or counterbalanced, any negative stimulus created by the corpses. Ants
378 have been demonstrated to learn food-related odour cues from returning foragers, even
379 without active food sharing (20–22,48). The results of this experiment thus do not rule out
380 that corpses act as a negative stimulus in these ants, but imply that perhaps this effect will not
381 strongly drive behaviour in a real-world ant control situation using sucrose-based baits.
382 However, other commonly-used bait types based on fat or protein pastes may have elicited
383 an effect (see below).

384 The third experiment showed that ants did not feed less from a food source flavoured
385 to match the scented corpses than from a neighbouring food source offering a novel flavour,
386 irrespective of whether the ants were starved for 2 or 4 days. It is unlikely that the ants were
387 unable to associate the odour impregnated onto the corpses with the taste of the flavoured
388 sucrose feeder: we previously (32) demonstrated that Argentine ants can learn to associate a
389 flavoured food reward with the corresponding environmental odour after only one exposure.
390 Further experiments with this assay offering 1M vs 0.5M sucrose, or 1M vs 1M sucrose + a low
391 level of bitter quinine, show that this method is effective at uncovering food preference

392 (unpublished data). A more likely reason for this unexpected result was the overwhelming
393 drive to feed under even moderate starvation conditions: The ants simply continued feeding
394 at the first food source they encountered, and any potential negative stimulus associated with
395 the food flavour was not sufficiently strong to override ongoing feeding. Individual *L. humile*
396 workers are unusually motivated feeders (49). Alternatively, ants may only show avoidance in
397 terms of orientation, not food rejection once the food is encountered. In other words, the ants
398 might only avoid going towards areas scented to match the scent of corpses, but do not avoid
399 feeding at similarly-flavoured food sources.
400
401 The fourth experiment tested colony-level preference, by examining collective
402 foraging choices. Collectively, over the first hour of foraging where the vast majority of
403 foraging occurred, the ants avoided the hydrogel beads containing flavoured sucrose
404 corresponding to the corpses scent and preferred to feed on the alternative beads. The
405 proximate mechanism driving this pattern is unclear: ants may have avoided approaching the
406 corpse-associated bead, may have left the bead before feeding fully, may have down-
407 modulated their recruitment from the bead, or any combination of these options.
408 Unfortunately, we lack detailed information on the behaviour of the individual ants in the
409 collective foraging assay, especially the first few foragers, which would be needed to
410 distinguish these mechanisms. Casual observations over the first 10 minutes suggested that
411 ants accepted the first bead they contacted, regardless of whether it was the corpse- or
412 control bead. This fits well with the results from experiment 3, and makes at least the taste-
413 and-reject hypothesis unlikely. Due to the positive feedback inherent in ant mass-recruitment
414 systems, the behaviour of the first ants in such experiments has a decisive influence on the
415 final collective outcome (50). Thus, if the first few ants tended to preferentially approach the
416 novel bead, and laid the first pheromone trail in that direction, this would bias all future
417 choices towards that bead. This effect would compound the tendency of the ants to avoid
418 approaching the corpse-associated odour. Finally, a second positive-feedback process may
419 well be in place: The first ants to feed may have encountered naïve outgoing ants on their
420 return journey, and certainly shared food via trophallaxis with other ants in the nest. Such on-
421 trail and within-nest encounters could have created a positive association to the smell of the
422 bead area, causing these newly recruited ants to preferentially choose that area – as has
423 already been shown in this and other species (20,21,32). Note that in systems characterised

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 (38,51).

Our study demonstrates that *Linepithema humile* can form negative associations with a scent associated with "nestmate" corpses, which leads them to avoid areas with the corresponding odour. However, this aversion may not be strong enough 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 poison bait efficiency. 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 solution 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 location. The commercial availability of cheap and environmentally benign food flavourings, makes this intervention easily and broadly deployable.

Declaration of Interest

None.

Author contribution:

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

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