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 corpses 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. This would disrupt the association between bait odour and corpses.

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

Introduction

Invasive ant species can have a significant impact on ecosystems and 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 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 toxicant that can be shared with the entire colony by the foragers before the effects are realised, allowing toxicants to reach the brood and queen(s) (10). Even so, the success rate of eradication attempts is low (10,11).

We propose that one reason for control failure may be 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* (12,13). It has been shown that the scent of food that ants have consumed can be detected by nestmates that encounter them, even without active food sharing (14–17). 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 due to the possibility of infection (18). 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 (19). 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 (20). 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 (21,22) and refuse dumps can deter ants from foraging (23,24).

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 (25–27). Nevertheless, it is not clear how well *L. humile* learns negative associations. Wagner et al. (26) 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 (28), and other ant species rapidly form negative olfactory associations, and avoid odours associated with, for example, a bitter quinine solution (29).

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

<u>Colony maintenance</u>

Linepithema humile ants were collected in 2021 from Girona, Spain and were all part of the same main European supercolony (30). 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 colony fragments was observed. The walls were coated in fluon to prevent escape. Each box

95 contained several 15mL plastic tubes, covered in transparent red foil, partly filled with water
96 and plugged with cotton, for use as nests. The ants were maintained on a 12:12 light:dark
97 cycle at room temperature (21-25 °C) and provided with water *ad libitum*. Colonies were fed
98 for three days with *ad libitum* 0.5M sucrose solution and freeze-killed *Drosophila*
99 *melanogaster*, and deprived of food for four days prior to testing. All experiments were
100 conducted between October 2021 and June 2022.
101
102 Solutions and odours
103 1M sucrose solutions (Südzucker AG, Mannheim, Germany), were used as a reward during
104 training for all experiments. Scented paper overlays, used during experiments to provide
105 environmental odours, were stored for at least 1 week prior to the experiments in airtight
106 plastic boxes (19.4 x 13.8 x 6.6cm) containing a glass petri dish with 500µL of either
107 strawberry or apple food flavouring, or blackcurrent and orange food flavouring (Seeger,
108 Springe, Germany). For experiments where flavoured food was used, 1µL of the respective
109 flavouring was added per 1mL of 1M sucrose solution.
110
111 Corpses and dummies
112 One day prior to the start of experiments, 24 ants were frozen for 60 min in a -20°C freezer.
113 These ant corpses were scented by placing them at air temperature for 24 hours into an
114 airtight petri dish (80x15mm, sealed with Parafilm©) containing a small metal plate with
115 200µL of strawberry or apple food flavouring, or with blackcurrent or orange flavouring. *L.*
116 *humile* can identify ants as being dead within 40 minutes of death (20), so 24 hours was
117 considered more than sufficient for corpses to be identified as such by nestmates. Note that
118 we did not attempt to test ants only with corpses from their own fragments. However, all
119 colony fragments were sourced from the same location at the same time, and showed no
120 aggression between each other.
121 24 dummies were created by cutting black electrical wire (mm diameter and 2.0-2.5mm
122 length) to visually simulate corpses, and provide a similar obstacle to movement. Dummies
123 were scented identically to the corpses. We chose to use plastic-coated wire, rather than
124 glass beads, as we considered plastic to be more similar to the ants' surface in terms of how
125 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.

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

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, 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), and the other arm was covered in a neutral-scented overlay (novel odour e.g. blackcurrent). We recorded the ant's final decision (crossing a line 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 with ethanol after each ant. 16 ants were tested sequentially using the same ant corpses, followed by 16 tested with dummies of the same smell. The next day, the same procedure was performed with the opposite odour (e.g. blackcurrent). The negative-associated odour, and the sequence of corpses/dummies were switched alternately. 32 ants were tested per day, testing in total 128 ants from 4 colonies.

156 Experiment 2 – Testing the effect of corpse-scent association on odour preference using
157 corpses killed with a scented toxicant
158 Experiment 1 found that the ants did indeed avoid the corpse-associated odour, but not the
159 dummy associated odour. The aim of this experiment was to test whether the same applied
160 when corpses were infused with scent from just feeding on a flavoured toxic food source.
161 Two days prior to start of the experiment hydrogel beads (31,32) were placed into a solution
162 containing 7.6µl Spintor® (containing 44% Spinosad) and 200µl food flavour (either
163 blackcurrent or orange) in 100ml 1M sucrose. One day prior the start of the experiment, 24
164 ants were fed on these hydrogel beads in a petri dish. After 8h all ants were dead, and the
165 corpses were placed in a clean petri dish. The next day (~16h later) these ants were used as
166 corpses in the behavioural trials. The procedure of the training and testing was identical to
167 experiment 1. Note that a control treatment with dummy ants was not possible in this
168 experiment, as consumption of the flavoured toxicant is required for odour presentation
169 during the experiment. 16 ants were tested per day, testing in total 64 ants from 4 colonies
170 over 4 days on corpses.
171
172 Experiment 3 – Testing the effect of corpse-associated odour on feeding preference
173 Here we asked whether ants respond differently to food flavoured to match the scented
174 nestmates corpses or scented dummies. Furthermore, we tested if a lower starvation level (2
175 days) changed the response of the ants compared to the standard 4 days. The idea here was
176 that extremely hungry ants may be too motivated to feed at the first food encountered to
177 show preference between the two options.
178 Ants encountered corpses or dummies, scented by storing with food flavourings for 24
179 hours, in the round plastic arena as described in experiment 1. After this training the ant was
180 allowed to enter a drawbridge leading to a 'dual-choice' feeder, made of 3D printed resin
181 (see fig. 1C). This feeder consisted of two triangular wells, each filled with a different
182 solution (scented 1M sucrose). The tips of the wells were 0.5mm apart, smaller than the
183 length of one *L. humile* antenna. This ensured that, regardless of which solution is contacted
184 first, the ant will almost immediately also come into contact with the alternative solution,
185 enabling an informed choice between the two solutions (see figure 1C). We noted the time
186 spent feeding from each feeder.

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

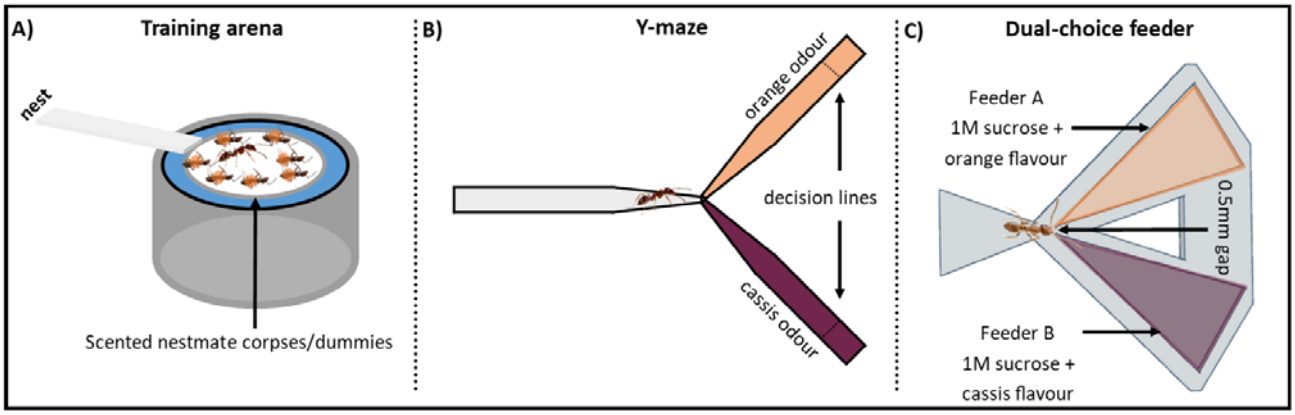

Figure 1: Setup used for experiments 1-3: A) Ants were confined to an encounter arena, containing scented dummies or nestmate corpses (experiments 1 & 3), or corpses of ants that had fed on scented toxicant (experiment 2). After 90s or 10 contacts, in experiments 1 & 2, the ants' odour preference was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

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

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 hours. Data were split into the first 60min after food presentation, and the following 23h. This is because in a laboratory setting, satiation at ad libitum feeders often occurs within the first hour (33).

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 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 0.1mm 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 (32). 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.

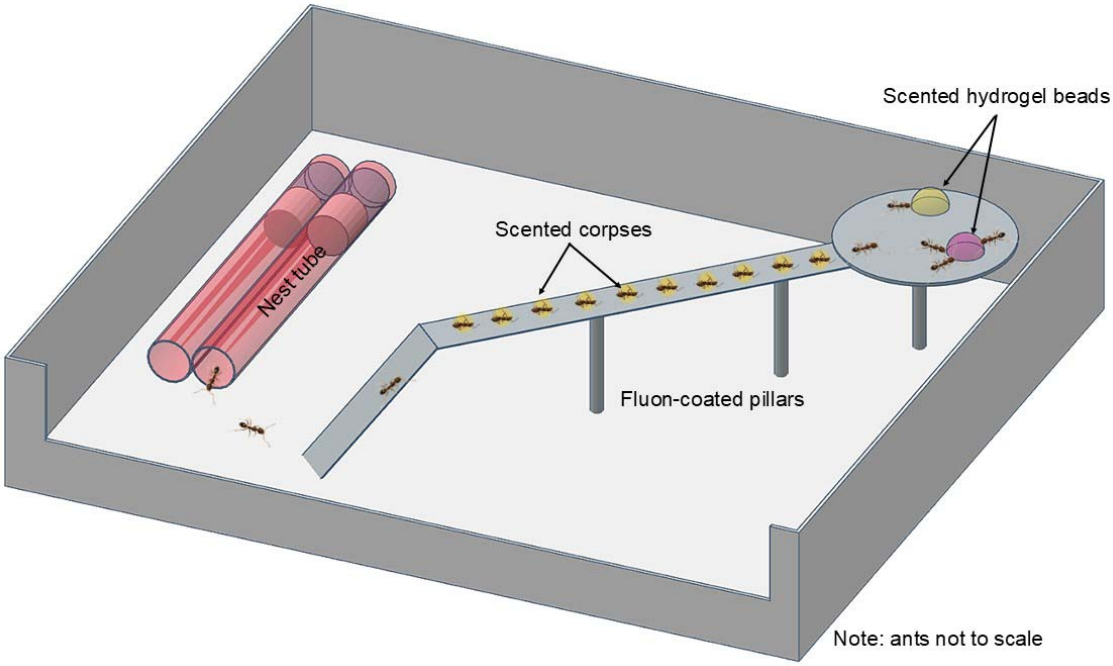

Figure 2: Collective decision-making experiment. A round plastic platform, supported by fluon-coated pillars and reachable by a bridge, was placed in the colony box. Hydrogel beads

232 soaked in flavoured sucrose solution (apple and strawberry) were offered. 12 scented
233 nestmate corpses were placed on the bridge. Foraging ants had to pass the scented corpses
234 to reach the feeding platform. The number of ants within 0.5 cm of each food type were
235 counted.
236
237 Statistical analysis
238 Data were analysed using generalized linear mixed-effect models (GLMM) (34) in R version
239 4.1.0 (R Core Team 2021). GLMMs were fitted using the lme4 package (35). Because the data
240 were binomial in experiments 1 & 2 (novel/corpse odour choice) and in experiment 3
241 (proportion drinking time on feeder A vs feeder B), a binomial error distribution was used.
242 For experiment 4, a Zero-Inflated Beta Model (for proportion data) and a glmm with a
243 poisson error distribution (for ant counts on different feeders over time) was used. Because
244 multiple ants were tested per colony, we included colony as random factor. The R package
245 emmeans was used for pairwise contrasts. It allows for the estimation and comparison of
246 means across different levels of categorical predictors in a model. Model fit was checked
247 using the DHARMa package (36). Results were plotted using the gglot2 package (37). The
248 complete code and analysis output is provided in supplements 1.
249 Note that in our analysis, we excluded four very small nests from the models (40 ants or less
250 foraging over 24 hours). For statistical information on all colonies, see supplement 2. Therein
251 we also provide a complete analysis of the dataset including the four excluded colonies. The
252 results of that analysis show the same patterns as those presented here. We also analyse the
253 data in terms of proportion of ants on each food type, rather than raw count. Again, the
254 results are similar, and are presented in supplement 2.
255
256
257 # Results
258 Experiment 1 – Testing the effect of corpse-scent association on odour preference using
259 scented corpses
260 Ants that had encountered scented corpses chose the Y-maze odour arm associated with the
261 corpses significantly less often than the opposite scented arm (69% chose the novel odour
262 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).

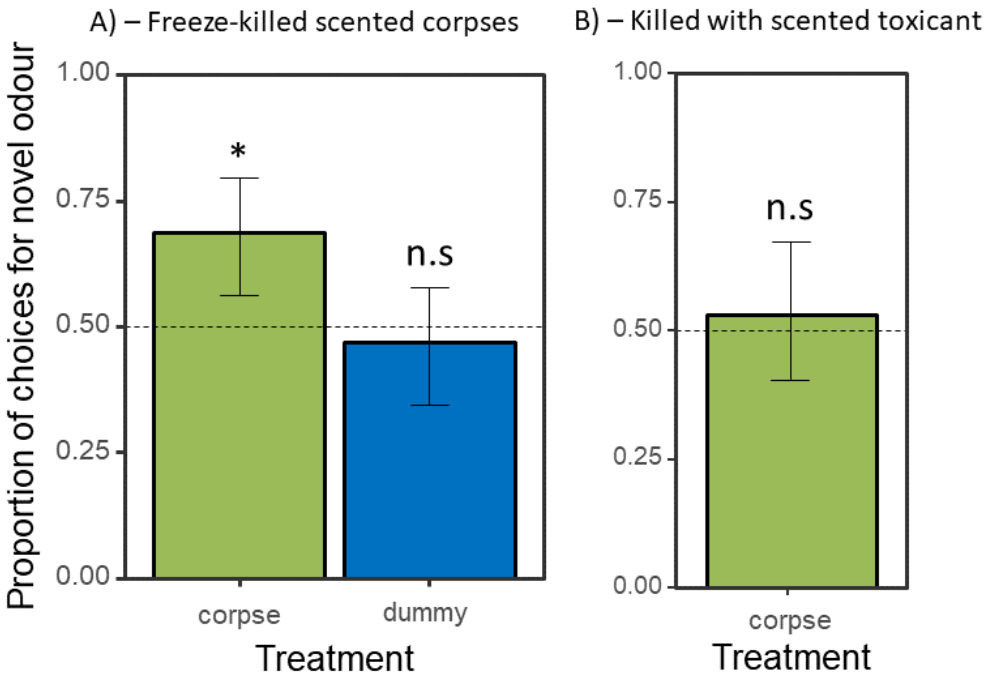

Figure 3: The response of ants to odours associated with nestmate corpses (experiments 1 & 2): A) Experiment 1: Proportion of choices for novel odour, per treatment group, corpses or dummies (control). B) Proportion of ants which encountered nestmate corpses killed by feeding on scented food choosing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

278 Experiment 2 – Testing the effect of corpse-scent association on odour preference using ants
279 killed with scented toxicant
280 The ants showed no difference in their choice of odour arm (GLMM, z-ratio = 0.58, p = 0.56,
281 fig.3B): 53% of the ants that were exposed to corpses fed with a flavoured toxicant chose the
282 arm scented with the other odour. The specific odour associated with the corpses did not
283 significantly affect arm choice (GLMM: z-ratio = -0.51, p = 0.61). Unexpectedly, focal ants
284 were commonly observed to lick the mouths of the corpses.
285
286 Experiment 3 – Testing the effect of corpse-associated odour on feeding preference
287 Ants starved for two days did not feed significantly longer on either of the food sources,
288 irrespective of their treatment (GLMM, dummy: z-ratio = 1.74, p = 0.08, 60% fed longer from
289 the novel odour. Corpse: z-ratio = 0.51, p = 0.61, 53% fed for longer from the novel feeder,
290 see fig. 4A). Odour did not significantly affect these measures (GLMM, feeding time, z-ratio =
291 0.55, p = 0.58). Similar outcomes were found for ants starved for 4 days feed (GLMM,
292 dummy, z-ratio = -0.56, p = 0.58, 42% fed longer from the novel feeder, corpse, z-ratio =
293 0.87, p = 0.39, 55% fed longer from the novel feeder, see fig. 4B). Odour type did not
294 significantly affect choice (GLMM, feeding time, z-ratio = -0.25, p = 0.80).

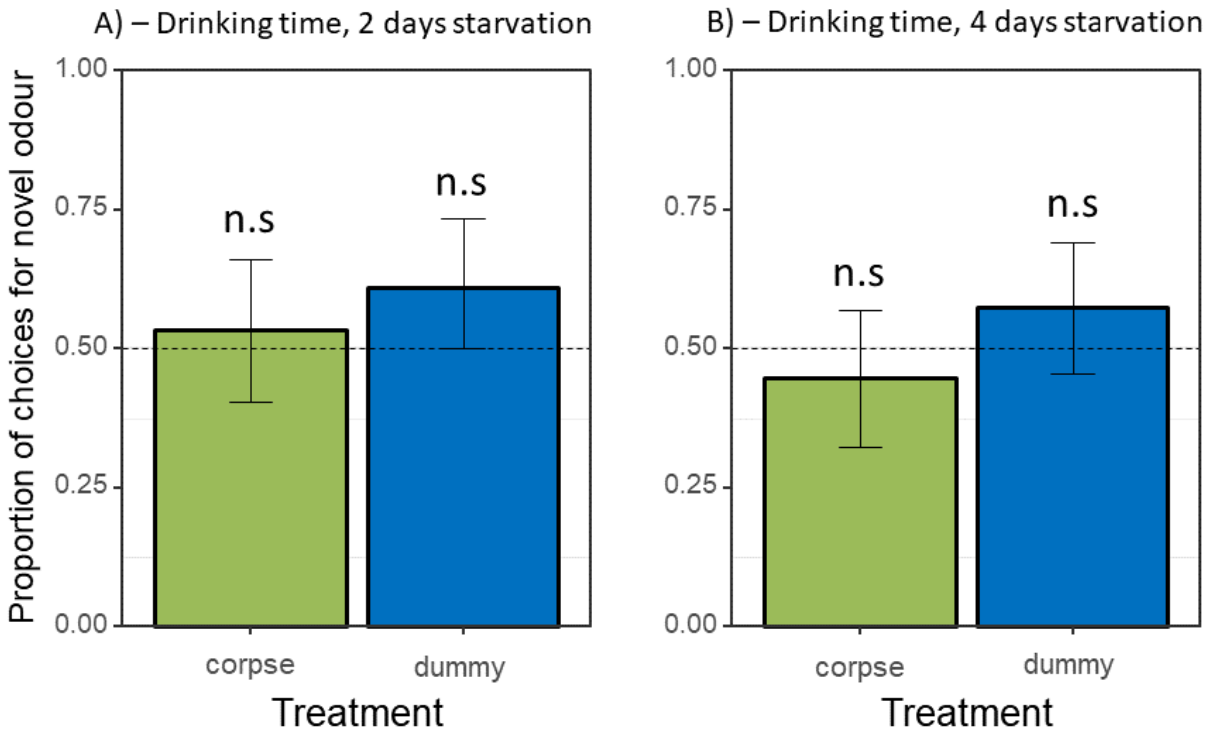

Figure 4: Effect of corpse-associated smell on feeding preference (exp 3): A) and B) show the mean (±95% confidence intervals) proportion of time spent feeding at the feeder not scented to match the corpses or dummies, by ants starved for 2 and 4 days respectively.

<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), with no clear preference for the novel feeder (67/111 ants at the novel feeder, emmeans: t-ratio = -1.163, $p = 0.2456$, fig. 5C/D. 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, $p = 0.2291$).

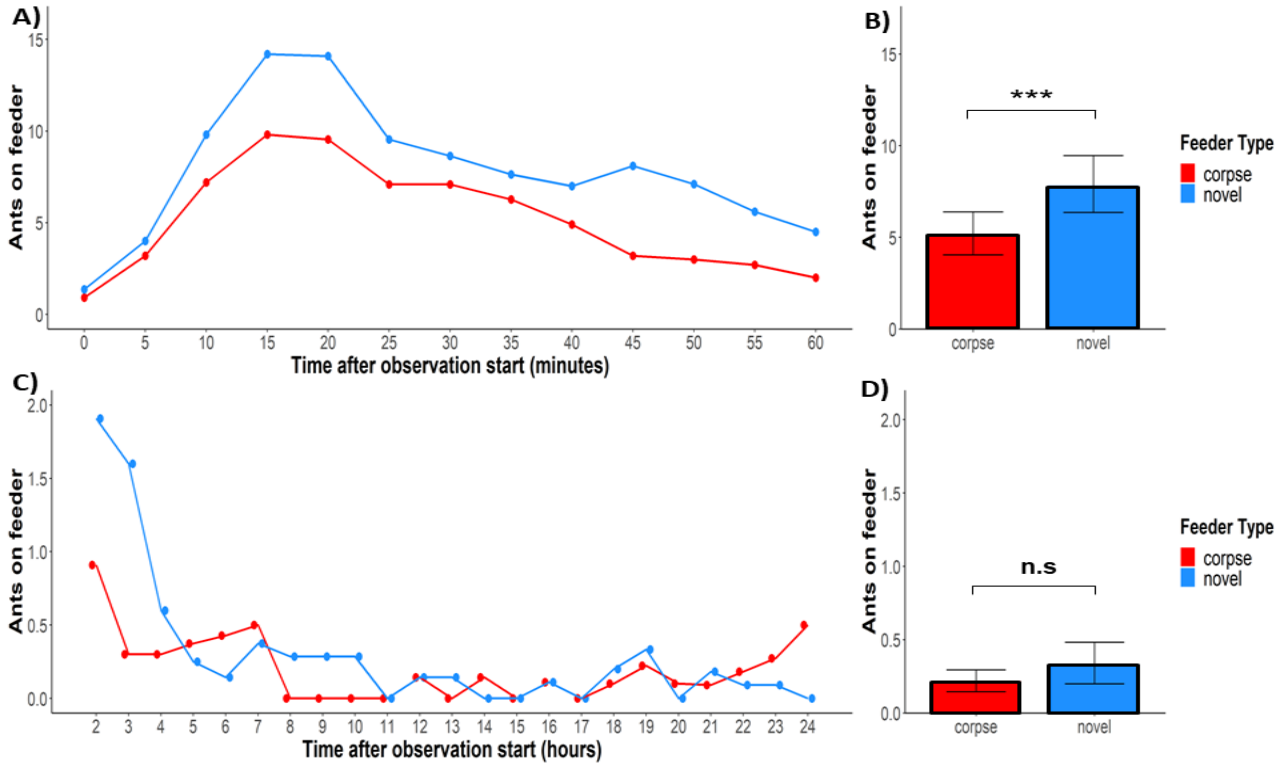

Figure 5: Collective foraging effort on feeders scented to match either nestmate-corpses encountered en-route to the feeder, or scented with a novel odour (exp 4): A) Dual-curve plot, showing average number of ants within 1 cm of feeder, at 5 minute intervals for 60 minutes post food presentation. B) Bar plot, Mean (±SE) 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. N counted ants (60min) = 1769, N counted ants (23hours) =111. A total of 12 trials were conducted.

Discussion

Our study strongly suggests that nestmate corpses are a negative stimulus for the ant *Linepithema humile.* 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.

328 Similarly, in the dual-feeder experiment (experiment 3), ants did not feed less from the
329 solution scented to match the odour of scented corpses.
330
331 Avoidance of the scented Y-maze arms associated with nestmate corpses
332 (experiment 1) demonstrates that corpses can act as a negative stimulus for *L. humile*.
333 Avoidance of cues associated with negative consequences has been demonstrated in ants
334 previously, with ants avoiding Y-maze or T-maze arms scented with a bitter quinine solution
335 (29,38). Nestmate corpses have also been shown to be avoided by ants, even to the point
336 that potential nest sites containing foreign ant corpses were avoided by *Temnothorax*
337 *albipennis* (21). However, we note that ants can discriminate between foreign or nestmate
338 corpses (22). It is thus unclear if the reason for avoidance in the nest site avoidance study
339 was to avoid a confrontation with a foreign ant colony, or for hygienic reasons, or both.
340 However, given that all the ants in our study stemmed from the same supercolony, and
341 showed no avoidance or aggression between colony fragments, it is unlikely that non-
342 nestmate avoidance plays a large role in the results. Although our study showed a relatively
343 high avoidance rate (69%), it is not clear why avoidance was not higher, such as for
344 avoidance of nests containing corpses (21). We note that a certain willingness to approach
345 nestmate corpses is required, in order for the corpses to be cleared away, and indeed
346 observed several ants handling, moving, and even beginning to dismember corpses. Corpse
347 clearance has been shown to be an important hygienic response, preventing disease spread
348 (18). It was even shown in Argentine ants that some workers have to apply regular pygidial
349 gland secretions on corpses to prevent growth of pathogenic fungi (39), which requires the
350 corpses to be approached.
351
352 Surprisingly, our second experiment, which had the same setup as the first
353 experiment, found no avoidance of the corpse-associated odour. The key difference
354 between these experiments was how the ants that were used as corpse stimuli, died and
355 were stored. In the first experiment the ants were freeze-killed then stored for 24h in a Petri
356 dish containing food flavouring, whereas the ants in the second experiment were allowed to
357 feed on toxic, flavoured hydrogel beads, subsequently died within 8h, and were then stored
358 for a further 16h in a Petri dish without any food flavour. It is possible that in this second
359 method the test ants might have not perceived the flavour of the poisoned food on the

360 corpses, because it was too subtle or disappeared after 24 hours. Ants can identify
361 nestmates as corpses within 1 hour of death (20), so toxicants with immediate lethal effects
362 may be more detectible, because bait odours may linger long enough on the corpses,
363 whereas toxicants at levels which cause delayed action may be less detectible. We also note
364 that many commercial baits are composed of a matrix with a potentially longer-lasting odour
365 profile e.g. fat or protein-rich pastes (10,40–42). It is possible that such baits imbue ants
366 which fed on them with a long-lasting odour that could drive future rejection when these
367 ants finally die. The water-soluble odour we used in the current study may not have lasted
368 long enough to be detectable by other ants 16-24 hours after feeding.

369 Interestingly, we observed that most test ants licked the mouths of the dead ants,
370 possibly tasting the food consumed by the poisoned nestmates. They thus might have
371 formed a direct positive association between the odour of the food and it's sweetness,
372 which may have interfered with, or counterbalanced, any negative stimulus created by the
373 corpses. Ants have been demonstrated to learn food-related odour cues from returning
374 foragers, even without active food sharing (14–16,43). The results of this experiment thus do
375 not rule out that corpses act as a negative stimulus in these ants, but imply that perhaps this
376 effect will not strongly drive behaviour in a real-world ant control situation using sucrose-
377 based baits. However, other commonly-used bait types based on fat or protein pastes may
378 have elicited an effect (see below).

379 The third experiment showed that ants did not feed less from a food source
380 flavoured to match the scented corpses than from a neighbouring food source offering a
381 novel flavour, irrespective of whether the ants were starved for 2 or 4 days. It is unlikely that
382 the ants were unable to associate the odour impregnated onto the corpses with the taste of
383 the flavoured sucrose feeder because we have previously (26) demonstrated that Argentine
384 ants can learn to associate a flavoured food reward with the corresponding environmental
385 odour after only one exposure. Further experiments with this assay offering 1M vs 0.5M
386 sucrose, or 1M vs 1M sucrose + a low level of bitter quinine, have shown that this method is
387 effective at uncovering food preference (unpublished data). A more likely reason for this
388 unexpected result was the overwhelming drive to feed under even moderate starvation
389 conditions: The ants simply continued feeding at the first food source they encountered, and
390 any potential negative stimulus associated with the food flavour was not sufficiently strong

391 to override ongoing feeding. Individual *L. humile* workers are unusually motivated feeders
392 (44). Alternatively, ants may only show avoidance in terms of orientation, not food rejection
393 once the food is encountered. In other words, the ants might only avoid going towards areas
394 scented to match the scent of corpses, but do not avoid feeding at similarly-flavoured food
395 sources.
396
397 The fourth experiment tested colony-level preference, by examining collective
398 foraging choices. Collectively, over the first hour of foraging where the vast majority of
399 foraging occurred, the ants avoided the corpse-associated hydrogel beads and preferred to
400 feed on the alternative beads. The proximate mechanism driving this pattern is unclear: ants
401 may have avoided approaching the corpse-associated bead, may have left the bead before
402 feeding fully, may have down-modulated their recruitment to the bead, or any combination
403 of these options. Unfortunately, we lack detailed information on the behaviour of the
404 individual ants in the collective foraging assay, especially the first few foragers, which would
405 be needed to distinguish these mechanisms. Observations over the first 10 minutes
406 suggested that ants accepted the first bead they contacted, regardless of whether it was the
407 corpse- or control bead. This fits well with the results from experiment 3, and makes at least
408 the taste-and-reject hypothesis unlikely. Due to the positive feedback inherent in ant mass-
409 recruitment systems, the behaviour of the first ants in such experiments has a decisive
410 influence on the final collective outcome (45). Thus, if the first few ants tended to
411 preferentially approach the novel bead, and laid the first pheromone trail in that direction,
412 this would bias all future choices towards that bead. This effect would compound the
413 tendency of the ants to avoid approaching the corpse-associated odour.
414 Finally, a second positive-feedback process may well be in place: The first ants to
415 feed may have encountered naïve outgoing ants on their return journey, and certainly
416 shared food via trophallaxis with other ants in the nest. Such on-trail and within-nest
417 encounters could have created a positive association to the smell of the bead area, causing
418 these newly recruited ants to preferentially choose that area – as has already been shown in
419 this and other species (14,15,26). Note that in systems characterised by strong positive
420 feedback, such as this one, stochasticity can play a very large role. It is thus not surprising
421 that in a subset of experiments the corpse-associated bead was chosen, as even weaker
422 options, if discovered first, can rapidly outcompete stronger options discovered later (33,46).

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 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. The commercial availability of cheap and environmentally benign food flavourings makes such an 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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