Can nestmate corpses cause bait neglect in invasive ants?

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Abstract

Invasive ants like *Linepithema humile* (the Argentine ant) pose a significant threat to ecosystems and economies worldwide, making it imperative to understand their behaviour and find effective management strategies. However, traditional eradication methods such as baiting have had limited success due to low bait consumption rates over time, driven in part by the abandonment of toxic baits. We propose that ants learn to avoid the odour of toxic baits because they are associated with the corpses of ants which fed on them. We investigated the effect of corpse-scent association on the odour and flavour preferences of ants by using scented corpses and corpses killed by feeding on scented toxicant-laden baits. Ants were tested in a Y-maze or a dual-choice feeder after exposure to scented corpses or dummies. When focal ants encountered scented freeze-killed ants, 69% (n = 64) avoided a branch of a Y-maze bearing the same scent. At a collective level, when colonies were given access to two differently scented food sources via a path laden with scented ant corpses, the matching food source was neglected compared to an alternative food source. However, ants exposed to scented corpses did not feed for less time on food scented to match the corpse compared to a control food in a dual-choice feeder. Moreover, when ants encountered ants killed by ingesting scented poison, as opposed to artificially-scented corpses, no avoidance was found. This study demonstrated that nestmate corpses act as a negative stimulus for *Linepithema humile* ants, leading to avoidance of odours associated with the corpses and potential avoidance of toxic bait at both individual and collective levels, but not a reduction in feeding once the food source is encountered. Why the more realistic trial with poisoned ants elicited no avoidance is unclear: it may be due to weaker odour cues from ingested food, a counterbalancing of the negative corpse stimulus by the positive presence of food remains on the corpse, or some other reason. 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.

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 (Escobar et al., 2018; Marbuah et al., 2014) *Linepithema humile* (Mayr, 1868), commonly known as the Argentine ant, is a highly destructive ant species that causes significant ecological and economic damage globally (Angulo et al., 2022), and is the most widely distributed invasive ant species in Europe (Rowles & O'Dowd, 2006). Invasion by *L. humile* leads to a significant reduction in invertebrate diversity, and can also have an impact on vertebrates (Alvarez-Blanco et al., 2021; Suarez et al., 2005; Wetterer et al., 2009). In addition, *L. humile* can act as an important agriculture pest by enhancing Hemipteran populations, which then increase the likelihood of fungal and viral infections (Wetterer et al., 2009).

The management of invasive ants has become an important part of conservation programs (Hoffmann et al., 2016a). Unfortunately, in addition to being ecologically and economically damaging, invasive ants are also difficult to control: two-thirds of *L. humile* eradication attempts have failed (Hoffmann et al., 2016a). The current gold standard approach for eradication of ants is to use baits with a slow-acting poison, which workers return to the brood and the queen (Hoffmann et al., 2016a). Even so, the success rate of such eradication attempts is low (Hoffmann, 2011; Souza et al., 2008). The availability of high-quality natural food, which is often preferred by ants and acts as a competitor for the poisoned bait, is a major issue in pest control. This can lead to low bait consumption rates, which further decrease over time, making it challenging to achieve successful eradication (Rust et al., 2003a; Silverman & Brightwell, 2008).

The reason for this gradual decrease in bait consumption is not clear. In a laboratory setting, ants can satiation on one type of favoured food (e.g. honey), leading them to begin foraging on an alternative food source (e.g. protein-rich fishmeal) (Edwards & Abraham, 1990). The presence of other ant species can also cause a swift abandonment of food sources (Fellers, 1987). However, *L. humile* is an aggressive and dominant species, and are rarely reported to be successfully driven away from resources (Rowles & O'Dowd, 2006), but see also (Cordonnier et al., 2020).

While all the above-mentioned factors might affect bait consumption, we propose an alternative, not mutually exclusive, hypothesis: that ants learn to avoid bait-associated cues

because they are also associated with ant corpses. This hypothesis assumes that nestmate corpses act as an *unconditional negative stimulus* (Pavlov, 1927; Rescola & Wagner, 1972) 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 (Czaczkes et al., 2019; Josens et al., 2016; Roces, 1990; Sorvari et al., 2008). The scent of the bait, remaining on ants after they have died, may lead to an aversion to the bait among the living ants which have contacted these corpses, causing them to avoid the scent or taste of the bait, and potentially stay away from the area where the corpses or the bait were located. Ant corpses are a potential threat to the survival of colonies due to the possibility of infection (Diez et al., 2014). A few studies have demonstrated that ant corpses can inhibiting their foraging behavior or causing them to avoid an area. For example, it was shown that the presence of nestmate corpses can cause ants to reject otherwise suitable nesting sites (Franks et al., 2005; Renucci et al., 2011) and refuse dumps can deter ants from foraging (Ballari & Farji-Brener, 2006; Farji-Brener & Sasal, 2003).

To protect a colony from disease and negative effects, it is crucial for ants to distinguish between living and dead ants, as well as between nestmates and foreign ants. Recognition of corpses is likely based on olfactory cues (Diez et al., 2013). 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 (Choe et al., 2009). Additionally, it has been shown that ants can discriminate between alien and nestmate corpses for up to 2 days (Choe et al., 2009)

It is thus reasonable to expect that nestmate corpses may represent an unconditional negative stimulus. *L. humile* has a formidable olfactory memory and is able to rapidly form long-lasting association between odours or flavours and a food reward (Galante & Czaczkes, 2023; Rossi et al., 2020; Wagner et al., 2022). Nevertheless, it is not clear how well *L. humile* learn negative associations. Wagner et al. (2022) showed that using quinine as 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 (Lessig & Nonacs, 2021), and other ant species rapidly form negative olfactory associations, and avoid odours associated with, for example, a bitter quinine solution (Wenig et al., 2021)

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

This study might help provide an alternative hypothesis for why baits in nature are rejected over time. To overcome these rejections, a simple, cost-effective solution could be to rotate the bait's odour or flavour. This approach could potentially enhance the efficacy of ant pest control.

Materials and Methods

Colony maintenance

Linepithema humile ants were collected in 2021 from Girona, Spain and Proença-a-Nova, Portugal, and were all part of the same European supercolony. 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. 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

130 strawberry or apple food flavouring, or cassis and orange food flavouring (Seeger, Springe,
131 Germany). For experiments where flavoured food was used, 1µL of the respective flavouring
132 was added per 1mL of 1M sucrose solution.
133
134 Corpses and dummies
135 One day prior the experiment's start, 24 ants were frozen for 60 min in a -20°C freezer.
136 These ant corpses were scented by placing them into an airtight petri dish (80x15mm, sealed
137 with Parafilm©) containing a small metal plate with 200µL of strawberry or apple food
138 flavouring, or with cassis or orange flavouring (Seeger, Springe, Germany). The container was
139 stored at room temperature for 24 hours. 24 hours is more than sufficient for corpses to be
140 identified as such by nestmates (Choe et al., 2009)
141 24 dummies were created by cutting black electrical wire (mm diameter and 2.0-2.5mm
142 length) to visually simulate corpses, and provide a similar obstacle to movement. Dummies
143 were scented identically to the corpses. We chose to use plastic-coated wire, rather than
144 glass beads, as we considered plastic to me more similar to the ants' surface in terms of how
145 readily it would absorb odours. Note that the corpses and the dummies had no physical
146 contact with the food flavourings but were exposed to the scented air in the sealed petri
147 dish.
148
149 Experiment 1 – Testing the effect of corpse-scent association on odour preference using
150 scented corpses
151 Here we investigate whether ants are capable of forming an association between an odour
152 and dead nestmates (corpses) in a Y-maze test setting. We predicted that ants exposed to
153 scented corpses would avoid the smell associated with the corpses, while ants exposed to
154 scented dummies would not.
155 12 corpses (treatment) or dummies (control) were placed onto a round plastic arena
156 surrounded by a water moat (arena: 7cm diameter, inner platform: 4cm diameter, see figure
157 1A) using clean soft forceps. A colony was connected via a drawbridge to the round arena
158 and a single ant was allowed to enter the platform in the arena via the drawbridge. The
159 drawbridge was then removed, trapping the ant in the arena together with the scented
160 corpses or scented dummies (e.g. orange). After 90 seconds, or at least 10 contacts between

161 the ant and the corpses/dummies, a second drawbridge was added to let the ant leave the
162 arena and enter a Y-maze (see fig. 1B).
163 The stem of the Y-maze was covered in an unscented paper overlay while one arm
164 (e.g. left) was covered in a scented overlay, with the scent matching the scent of the
165 corpses/dummies (e.g. orange), while the other arm was covered in a neutral-scented
166 overlay (opposite odour e.g. cassis). We recorded the ant's initial decision (defined as the
167 antennae crossing a line 2cm from the bifurcation) and final decision (crossing a line 8cm
168 from the bifurcation, 2cm from the arm end, see fig. 1B). The position of the odour
169 associated with the corpses/dummies was systematically varied between ants. The Y-maze
170 was cleaned with ethanol after each ant. 16 ants were tested sequentially using the same
171 scented ant corpses, followed by 16 tested with dummies of the same smell. The next day,
172 the same procedure was performed with the opposite odour (e.g. cassis). The negative-
173 associated odour, and the sequence of corpses/dummies were switched alternately. 32 ants
174 were tested per day, testing in total 128 ants from 4 colonies.
175
176 Experiment 2 – Testing the effect of corpse-scent association on odour preference using
177 corpses killed with scented poison
178 Experiment 1 found that the ants did indeed avoid the corpse-associated odour, but not the
179 dummy associated odour. The aim of this study was to test this effect in a more field-
180 realistic, and challenging, assay. We thus used corpses created by poisoning with a flavoured
181 food laced with toxicant, rather than scented corpses.
182 Two days prior to start of the experiment hydrogel beads were placed into a solution
183 containing 7.6µl Spintor © (containing 44% Spinosad) and 200µl food flavour in 100ml 1M
184 sucrose. One day prior the start of the experiment, 24 ants were fed on these soaked
185 hydrogel beads in a petri dish. After 8h all ants were dead, and were added to a clean petri
186 dish without any flavoured food. The next day (~16h later) these ants were used as corpses.
187 The procedure of the training and testing was otherwise identical to experiment 1.
188 16 ants were tested per day, testing in total 64 ants from 4 colonies over 4 days on corpses.
189
190 Experiment 3 – Testing the effect of corpse-associated odour on feeding preference
191 Here we asked whether ants avoid food which tastes is flavoured to match the one the scent
192 of scented nestmates corpses, or feed less from it. Furthermore, we tested if a lower

starvation level (2 days) changed the response of the ants compared to the standard 4 days. The idea here was that extremely hungry ants may be too motivated to feed at the first food encountered to show preference.

Ants encountered scented corpses or dummies in the round plastic arena as described in experiment 1. After this training the ant was allowed to enter a drawbridge leading to a two-choice 'delta' feeder, made of 3D printed resin (see fig. 1C). This feeder consisted of two triangular wells, each filled with a different solution (scented 1M sucrose). The tips of the wells were 0.5mm 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 figure 1C). We noted the first feeder drinking choice (defined as the mandibles entering one well), the time until first drinking interruption (mandibles withdrawn from solution and head movement) and the time spent drinking from each feeder. The feeder side containing the negative 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.

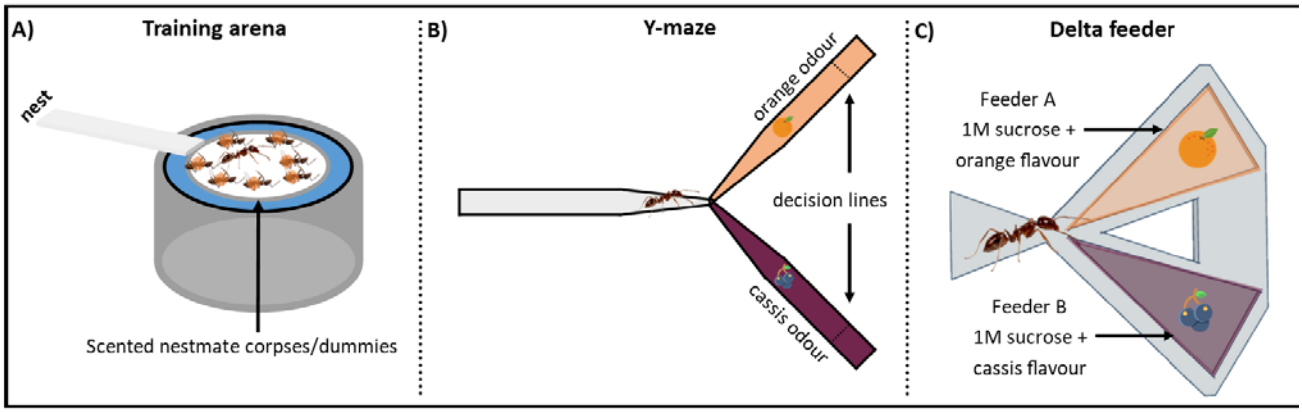

Figure 1: Setup used for the 1-3 experiment: A) Ants were confined to a round platform arena, containing scented dummies or nestmate corpses (1st experiment), or corpses fed with scented poison (2nd experiment). After 90s or 10 contacts, the ants' odour preference was tested on a Y-maze with scented paper overlays B). Results were noted when the ants crossed the decision line. C) Delta feeder for testing feeding preference. Ants enter via a constriction, encountering two adjacent wells, containing differently-flavoured sucrose solution. Their initial choice, time to first drinking interruption, and total drinking time from each well was tested.

Experiment 4 – Can the presence of scented corpses drive 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 hours. Data was 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 (Czaczkes et al., 2016)

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 prevent unwanted foraging shortcuts, ensuring 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 opposite flavour were placed on the other side. These beads were halved to prevent them rolling about. 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 gel 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.

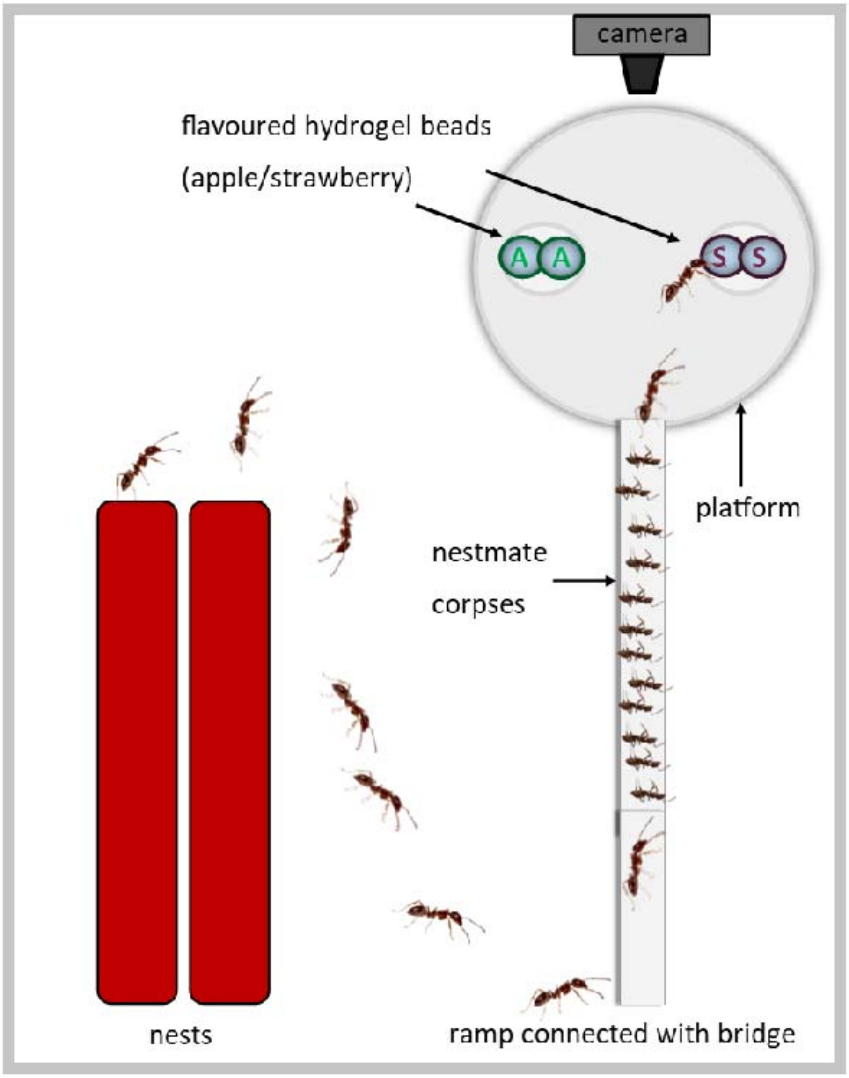

Figure 2: Collective decision-making experiment. A round plastic platform, supported by fluon-coated pillars and reachable by a bridge, is placed in the colony box. Hydrogel beads soaked in flavoured sucrose solution (apple and strawberry) are offered 12 scented nestmate corpses are placed on the bridge the bridge. Foraging ants must pass the scented corpses to reach the feeding platform. The number of ants within 0.5 cm of each food type are counted.

Statistical analysis

Data were analysed using generalized linear mixed-effect models (GLMM) (Bolker et al. 2009) in R version 4.1.0 (R Core Team 2021). GLMMs were fitted using the lme4 package (Bates et al. 2015). As the data were binomial (correct / incorrect), a binomial error

257 distribution was used. Since multiple ants were tested per colony, we included colony as
258 random factor. Model fit was checked using the DHARMa package (Hartig 2018). Results
259 were plotted using the gglot2 package (Wickham 2016). The complete code and analysis
260 output is provided in supplements 1.

261

262 # Results

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

265 This experiment tested if encountering scented conspecific corpses influenced the
266 preference of ants between a neutral odour and an odour associated with the corpses. Ants
267 which had encountered scented corpses chose the Y-maze odour arm associated with the
268 corpses significantly less often than the opposite scented arm (69% chose the opposite arm,
269 GLMM, z-ratio = 2.92, p = 0.003, see fig. 3A). By contrast, ants in the control group which
270 experienced the odour associated with scented dummies showed no significant preference
271 between the two scented arms (47% chose the opposite arm, GLMM, z-ratio = -0.5, p = 0.62,
272 see fig.3A). The treatment groups differed significantly from each other (emmeans, z-ratio =
273 2.48, p = 0.013, see fig. 3A). The specific corpse/dummy odour (cassis or orange) did not
274 significantly affect choice accuracy (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 ants, per treatment group, avoiding the Y-maze arm scented with an odour associated with the surface scent of corpses (treatment) or dummies (control). B) Proportion of ants which encountered nestmate corpses killed by feeding on scented food avoiding the corpse-associated scent on a Y-maze arm, split by scent type. 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 poison</u>

The procedure of this experiment was similar to the first experiment except that instead of killing the ants by freezing they were killed by feeding them poisoned scented sugar water via hydrogel beads. The ants did not choose the Y-maze odour arm associated with the corpses significantly less often than the opposite scented arm (GLMM, z-ratio = 0.58, p = 0.56, see fig.3B): 56% of the ants which were exposed to corpses fed with orange flavoured poison and 50% of the ants exposed to corpses fed with cassis chose the opposite arm. The

292 specific corpse poison odour or flavour did not significantly affect arm choice (GLMM: z-ratio
293 = -0.51, p = 0.61).
294
295 Experiment 3 – Testing the effect of corpse-associated odour on feeding preference
296 In this experiment, we tested whether ants' drinking preference was affected by pre-
297 exposure to an odour clinging to ant corpses. Half the ants were tested after 2 days of
298 starvation, the other after 4. The 2 days starved ant groups (dummy and corpse) did not
299 choose as a first choice one food source significantly more often than the other (GLMM,
300 dummy: z-ratio = -0.26, p = 0.79, 48% chose the novel odour. Corpse: z-ratio = -0.76, p =
301 0.45, 45% chose the novel odour, see fig. 4A). Likewise, both groups did not drink
302 significantly longer on either of the food sources (GLMM, dummy: z-ratio = 1.74, p = 0.08,
303 60% drank longer from the novel odour. Corpse: z-ratio = 0.51, p = 0.61, 53% drank longer
304 from the novel feeder, see fig. 4C). The specific corpse/flavour odour did not significantly
305 affect these measures (GLMM, first feeder choice, z-ratio = 0.72, p = 0.43, drinking time, z-
306 ratio = 0.55, p = 0.58). A similar pattern can be seen for the 4 days starved ants' first choice
307 (GLMM, dummy: z-ratio = 1.25, p = 0.21, 58% chose the novel odour. Corpse: z-ratio = -0.12,
308 p = 0.90, 49% chose the novel odour, see fig. 4B) and for the drinking time (GLMM, dummy,
309 z-ratio = -0.56, p = 0.58, 42% drank longer from the novel feeder. Corpse, z-ratio = 0.87, p =
310 0.39, 55% drank longer from the novel feeder, see fig. 4D). Again, the specific corpse/flavour
311 odour did not significantly affect choice (GLMM, first feeder choice, z-ratio = -1.17, p = 0.24,
312 drinking time, z-ratio = -0.25, p = 0.80).

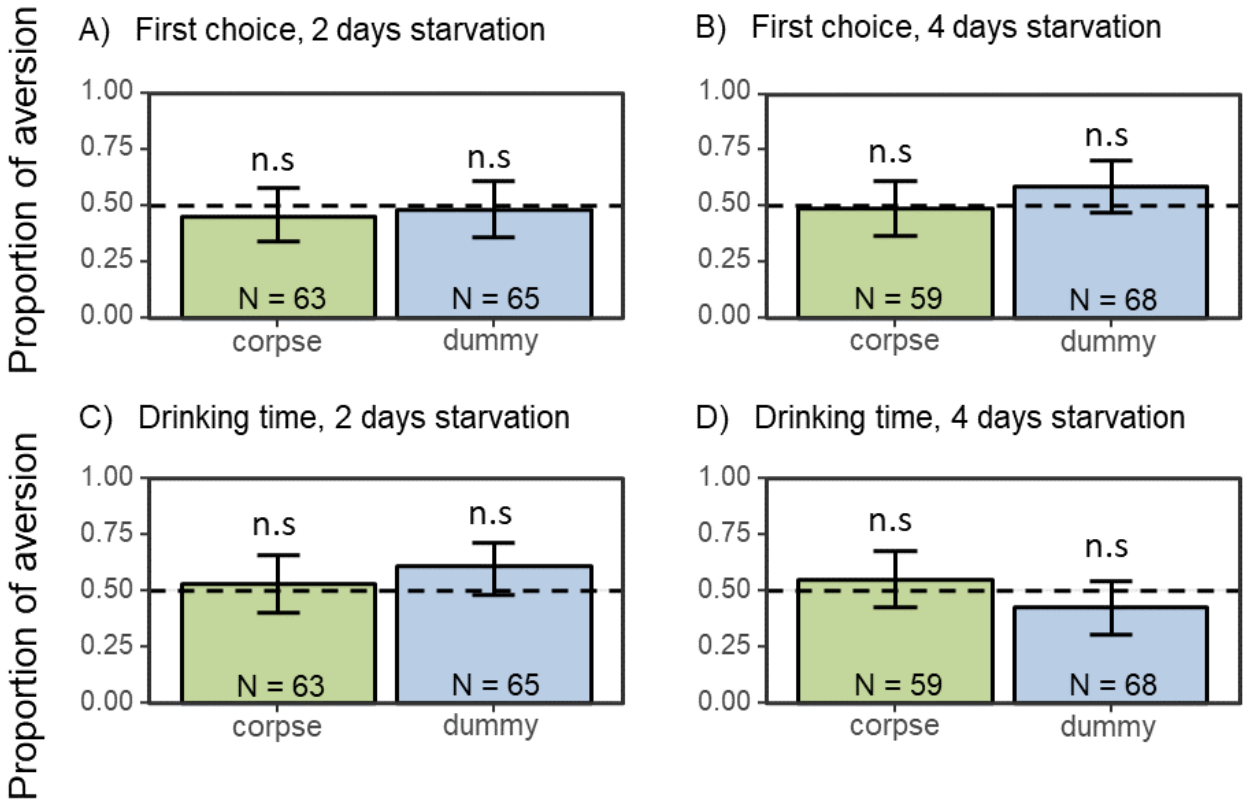

Figure 4: Effect of corpse-associated smell on feeding preference (exp 3): (A&B) Proportion of ants avoiding the feeder (first choice) associated with a corpse scent, per treatment group after 2 (A) or 4 (B) days of starvation. C) and D) show the proportion of time spent drinking at the feeder not scented to match the corpses or dummies i.e. avoidance, for 2 and 4 days respectively. Bars depict means, whiskers 95% confidence intervals derived from the fitted GLMM. The dotted horizontal line displays chance level of 50%.

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

The aim of this experiment was to test whether corpse-odour avoidance can drive collective foraging behaviour. In the first 60 minutes significantly fewer ants (784/1881) fed on the hydrogel beads scented to match the corpses (GLMM: z-ratio = 7.44, $p < 0.0001$, see fig. 5A). Over the following 23h observation ants showed only a trend towards avoiding the feeder (78/135) associated with the dead ants (GLMM: z-ratio = 1.80, $p = 0.072$, see fig. 5B). The specific corpse odour did not significantly affect choice accuracy within 60 minute (GLMM: z-ratio = -5.407, $p = 0.8669$) nor the following 23 hours (GLMM: z-ratio = 1.203, $p = 0.2291$).

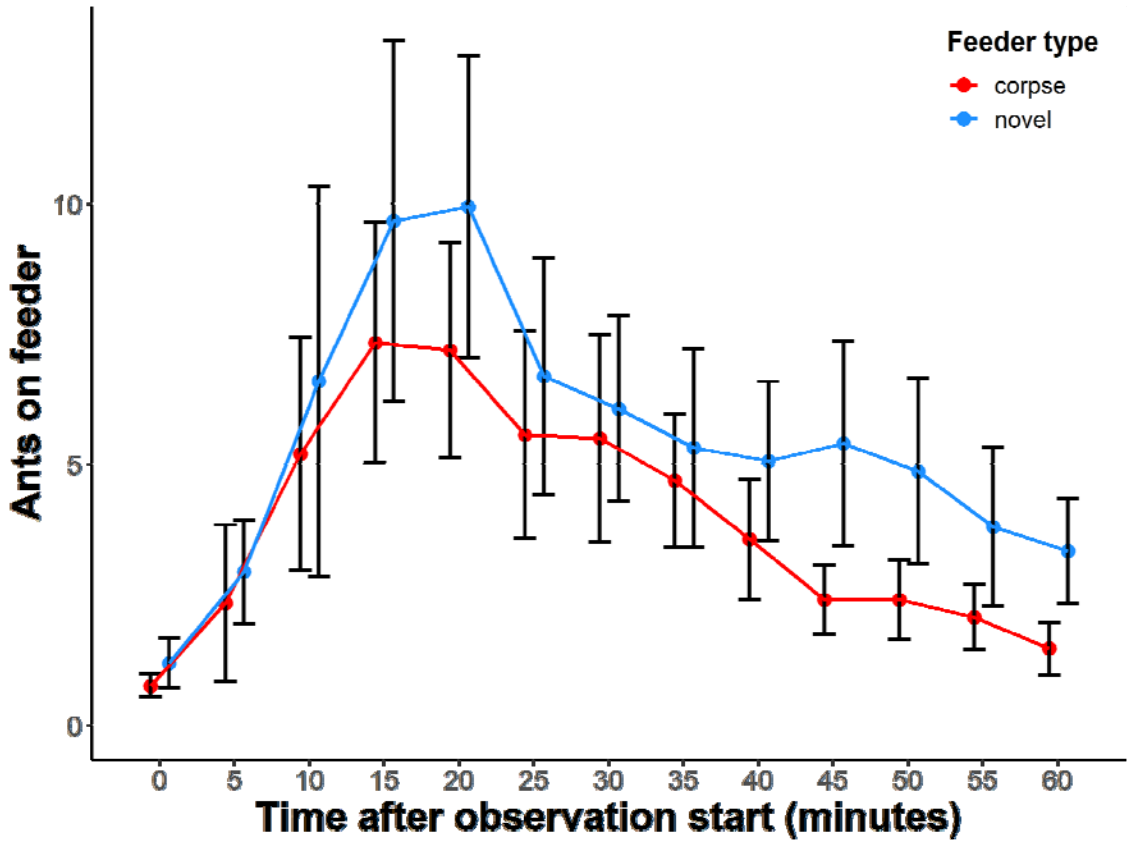

Figure 5: Collective foraging effort on feeders scented to match either nestmate-corpses encountered en-route to the feeder, or scented with a novel odour (exp 4): A) First 60 minutes post food presentation. Bars depict means, whiskers 95% confidence intervals derived from the fitted GLMM.

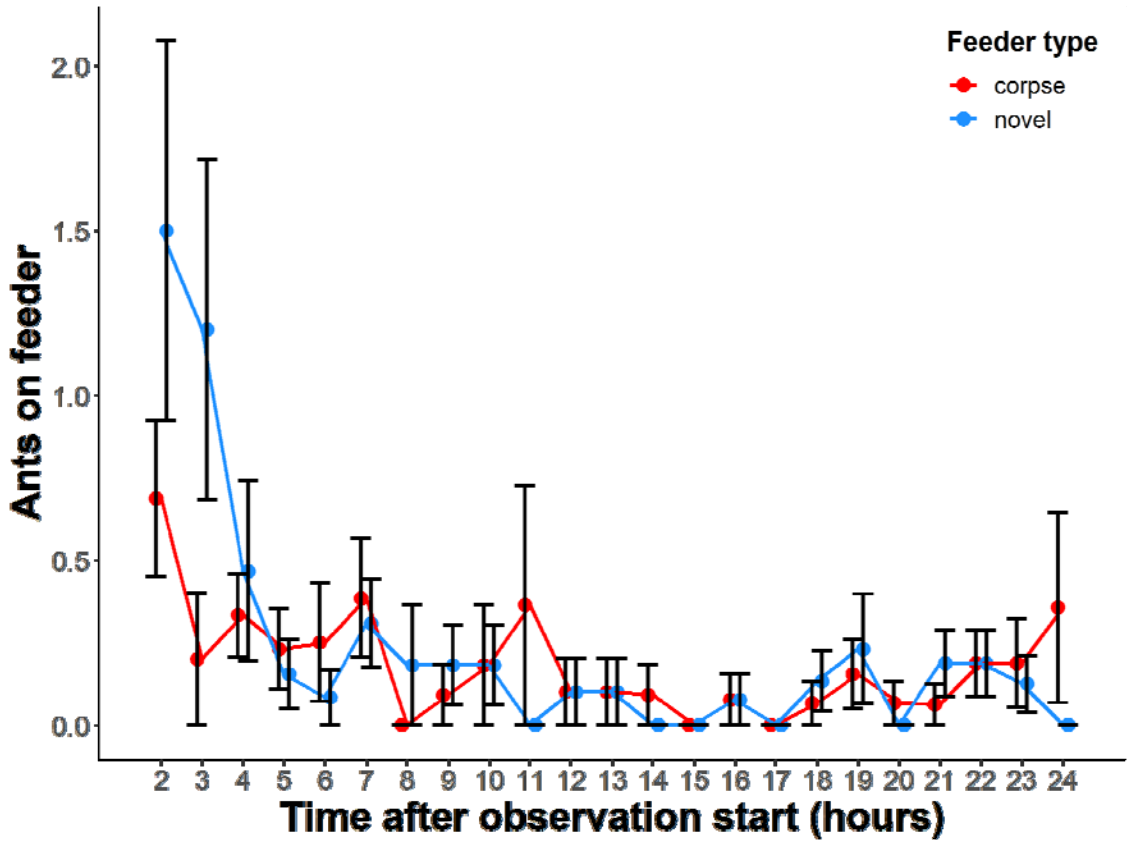

Figure 6: Collective foraging effort on feeders scented to match either nestmate-corpses encountered en-route to the feeder, or scented with a novel odour (exp 4): A) 2-24 hours post food presentation. Bars depict means, whiskers 95% confidence intervals derived from the fitted GLMM.

Discussion

Our study demonstrated that nestmate corpses are a negative stimulus for the ant *Linepithema humile.* 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 a more natural condition (experiment 2, poison-killed ants) no avoidance was found. The negative association might have been counterbalanced by a positive association, such as the sugary taste that sticks to the mouth of ants after they fed on the corpses. The lack of avoidance of the corpse-associated food in experiment 3 may either be due to the motivation to continue feeding overriding any aversion present, or be due to aversion only being shown in terms of orientation or recruitment, but not feeding.

353

354 Avoidance of the scented Y-maze arms associated with nestmate corpses
355 (experiment 1) demonstrates that corpses do act as a negative stimulus for *L. humile*.
356 Avoidance of cues associated with negative consequences has been demonstrated in ants
357 previously: Ants avoid Y-maze or T-maze arms scented with an odour associated with a bitter
358 quinine solution (Czaczkes, 2018; Wenig et al., 2021). Nestmate corpses have also been
359 shown to be avoided by ants: potential nest sites containing foreign ant corpses were
360 avoided by *Temnothorax albipennis* (Franks et al., 2005). However, we note that ants can
361 discriminate between foreign or nestmate corpses (Renucci et al., 2011). It is thus unclear if
362 the reason for avoidance in the nest site avoidance study was the fear of a confrontation
363 with a foreign ant colony, or for hygienic reasons, or both. However, given that all the ants in
364 our study stemmed from the same supercolony, and showed no avoidance or aggression
365 between colony fragments, it is unlikely that non-nestmate avoidance plays a large role in
366 the results. While our study showed a respectable avoidance rate (69%), comparable to the
367 results of corpse-containing nest avoidance (Franks et al., 2005), one might ask why
368 avoidance was not higher. We propose that a certain willingness to approach nestmate
369 corpses is required, in order for the corpses to be cleared away. Corpse clearance has been
370 shown to be an important hygienic response, preventing disease spread (Diez et al., 2014). It
371 was even shown in Argentine ants that some workers have to apply regular pygidial gland
372 secretions on corpses to prevent growth of pathogenic fungi (Kesäniemi et al., 2019), which
373 requires the corpses to be approached.

374

375 Surprisingly, our second experiment, which had the same setup as the first
376 experiment, found no avoidance of the corpse-associated odour. The key difference
377 between these experiments was how the ant, used as corpse stimuli, died and were stored.
378 While in the first experiment the ants were freeze-killed then stored for 24h in a Petri dish
379 containing food flavour to be scented, the ants in the second experiment were allowed to
380 feed on poisoned, flavoured hydrogel beads, subsequently died within 8h, and were then
381 stored for a further 16h in a Petri dish without any food flavour. It is possible that in this
382 second method the ants might have not perceived the flavour of the poisoned food, because
383 it was too subtle or disappeared after 24 hours. Interestingly, we observed that most test
384 ants licked the mouths of the dead ants, possibly tasting the food consumed by the poisoned

385 nestmates. They thus might have formed a positive association between the odour of the
386 food remains and the food remains themselves, which may have interfered with, or
387 counterbalanced, any negative stimulus created by the corpses. Ants have been
388 demonstrated to learn food-related odour cues from returning foragers, even without active
389 food sharing (Czaczkes et al., 2019; Josens et al., 2016; Roces, 1990). The results of this
390 experiment thus do not rule out that corpses act as a negative stimulus in these ants, but
391 imply that perhaps this effect will not strongly drive behaviour in a real-world ant control
392 situation using sucrose-based baits. However, other commonly-used bait types based on fat
393 or protein pastes may have elicited an effect (see below).
394
395 The third experiment showed that ants did not drink less from a food sources
396 flavoured to match the scented corpses. It did not matter if the ants were heavily (4 days) or
397 moderately (2 days) starved. It is unlikely that the ants were unable to associate the odour
398 impregnated onto the corpses with the taste of the flavoured sucrose feeder: we previously
399 (Wagner et al., 2022) demonstrated that Argentine ants can learn to associate a flavoured
400 food reward with the corresponding environmental odour. Further experiments with this
401 assay offering 1M vs 0.5M sucrose, or 1M vs 1M sucrose + a low level of bitter quinine, show
402 that this method is effective at uncovering food preference (manuscript in preparation). A
403 more likely reason for this unexpected result was the overwhelming drive to feed under
404 even moderate starvation conditions: The ants simply continued feeding at the first food
405 source they encountered, and any potential negative stimulus associated with the food
406 flavour was not sufficiently strong to override ongoing feeding. Alternatively, ants may only
407 show avoidance in terms of orientation, not food rejection once the food is encountered.
408
409 The fourth experiment tested colony-level preference, by examining collective
410 foraging choices. Collectively, the ants avoided the hydrogel beads containing flavoured
411 sucrose corresponding to the corpses scent and preferred to feed on the alternative beads.
412 The proximate mechanism driving this pattern is unclear: ants may have avoided
413 approaching the corpse-associated bead, may have left the bead before feeding fully, may
414 have down-modulated their recruitment from the bead, or any combination of these
415 options. Unfortunately, we lack detailed information on the behaviour of the individual ants,
416 especially the first few foragers, which would be needed to distinguish these mechanisms.

417 Casual observations over the first 10 minutes suggested that ants accepted the first bead
418 they contacted, regardless of whether it was the corpse- or control bead. This fits well with
419 the results from experiment 3, and makes at least the taste-and-reject hypothesis unlikely.
420 Due to the positive feedback inherent in ant mass-recruitment systems, the behaviour of the
421 first ants in such experiments has a decisive influence on the final collective outcome
422 (Sumpter & Beekman, 2003). Thus, if the first few ants tended to preferentially approach the
423 non-corpse bead, and laid the first pheromone trail in that direction, this would bias all
424 future choices heavily towards that bead. This effect would strongly compound the tendency
425 of the ants to avoid approaching the corpse-associated odour. Finally, a second positive-
426 feedback process may well be in place: The first ants to feed may have encountered naïve
427 outgoing ants on their return journey, and certainly food via trophallaxis with other ants in
428 the nest. Such on-trail and within-nest encounters could have created a positive association
429 to the smell of the bead area, causing these newly recruited ants to preferentially choose
430 that area (Czaczkes et al., 2019; Josens et al., 2016; Wagner et al., 2022). Note that in
431 systems characterised by strong positive feedback, such as this one, stochasticity can play a
432 very large role. It is thus not surprising that in a subset of experiments the corpse-associated
433 bead was chosen as even weaker options, if discovered first, can rapidly outcompete
434 stronger options discovered later (Beckers et al., 1990; Czaczkes et al., 2016).
435
436 Our study demonstrates that *Linepithema humile* can form negative associations with a
437 scent associated with "nestmate" corpses, which leads them to avoid areas with the
438 corresponding odour. However, this aversion may not be strong enough to override positive
439 stimuli, such as the presence of a desired food. These findings have important implications
440 for ant control strategies, as they suggest that the nestmate corpses may reduce poison bait
441 efficiency. Increasing bait rejection is a major problem for ant control efforts, especially for *L.*
442 *humile* (Rust et al., 2003b; Silverman & Brightwell, 2008). The formation of a negative
443 association between the bait and poisoned nestmates is a reasonable explanation for this
444 behaviour. However, given the lack of effect found in experiment 2, which used more field-
445 realistic methods, it is unclear whether this effect plays a major role in real-world control
446 efforts. The lack of effect in experiment 2 may have been driven either by a
447 counterbalancing caused by the presence of rewarding sugar on the nestmate corpses, or by
448 the disappearance of the bait-associated odour by 24 hours after feeding. However, ants can

identify nestmates as corpses within 1 hour of death (Choe et al., 2009), and toxic baits can cause death within 1-4 days (LT_{50}) with commonly used slow-acting poisoned baits. We note that many commercial baits are composed of a matrix with a potentially longer-lasting odour profile e.g. fat or protein-rich pastes (Hoffmann et al., 2016b; Klotz, 2008; Lofgren et al., 1961; Terayama et al., 2021). It is possible that such baits imbue ants which fed on them with a long-lasting odour which could drive future rejection when these ants finally die. The water-soluble odour we used in the current study may not have lasted long enough to be detectable by other ants 16-24 hours after feeding.

The results of this study demonstrate that delayed bait rejection could be driven, at least in part, by negative associations derived from the corpses of poisoned nestmates. Important future steps would include tests with commercial baits, ideally in field-realistic situations. This effect should be kept in mind when considering control strategies, especially re-treatment of infested areas, where cycling food flavours and odours might be a very cheap and easy method of overcoming this potential issue.

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