# A systematic examination of learning in the invasive ant *Linepithema humile* reveals very rapid development of short and long-term memories

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## Abstract

The Argentine ant (*Linepithema humile*) is one of the most damaging and widespread invasive ant species worldwide. However, control attempts often fail due to insufficient bait uptake, or bait abandonment. Increasing preference for, and consumption of, is thus an important requirement for successful control. Associative learning and within-nest information transfer might be a potential tool for achieving this goal. Here, we conducted a detailed and systematic investigation of olfactory learning and side learning in Argentine ants. The ants showed very strong and rapid side learning, choosing the correct arm in a Y-maze 65% of time after just one visit, and 84% correct after two. Odour learning was even more rapid, with just one visit to a flavoured food source, reached by a scented runway, leading to 85% choices for the corresponding scent on a Y-maze. Further experiments demonstrated that having two cues (runway odour and food flavour) does not improve learning significantly over just one cue. This rapid learning is long-lasting, with one exposure to a runway odour associated with a reward resulting in a strong preference (73%) for this odour even after 48 hours. Food flavour information is transferred efficiently between nestmates in the nest, driving preference: naïve ants housed with ants fed on flavoured food show a strong preference (77%) for that odour after 24 hours. Our results demonstrate the impressive learning abilities of *Linepithema humile*, which coupled with efficient intranidal information transfer and strong use of pheromonal recruitment may help explain their ability to discover and then dominate resources. However, these strengths could potentially be used against them, by exploiting learning and information transfer to increase toxic bait uptake during control efforts. Steering ant preference by leveraging learning might be an underappreciated tool in invasive alien species control.

**Keywords**: Associative learning, memory, Argentine ants, invasive species, odour

# Introduction

With increasing trade, and the concomitant breakdown of biogeographic barriers, invasive species are spreading globally (Mack and Lonsdale 2001; Mooney and Cleland 2001; Perrings et al. 2005). Invasive species are often economically damaging and ecologically devastating (Marbuah et al. 2014; Escobar et al. 2018), with invertebrates being amongst the most damaging invasive groups (Ricciardi 2015). Ants are especially effective at invading habitats outside their native range (Tsutsui and Suarez 2003), where they displace native species through competition and predation (Ness and Bronstein 2004; Abbott 2006; Matsui et al. 2009).

The Argentine ant (*Linepithema humile* (Mayr, 1868)) is one of the most damaging ant species worldwide, and the most widespread invasive ant in Europe (Human and Gordon 1999; Trigos-Peral et al. 2021). The presence of *L. humile* in invaded areas causes a massive decrease in invertebrate diversity and even affects vertebrates (Wetterer et al. 2001; Suarez et al. 2005; Alvarez-Blanco et al. 2021). 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 economic damage through invasive species costs the EU an estimated €12.5 billion a year (European Commission, 2011). Especially combatting invasive ants has become a top priority for conservation programs (Hoffmann et al. 2016). Unfortunatly, in addition to being ecologically and economically damaging, invasive ants are also difficult to control. Two-thirds of *L. humile* eradication attempts fail (Hoffmann et al. 2016). Insect control methods usually rely on the use of insecticide spraying, which is known to be ineffective against ants. The reason behind this ineffectiveness is primarily the strongly sheltered reproductive part of a colony, the queens (Williams et al. 2001). The queens and the brood are not only physically protected, but may also be protected by tuned interaction networks within the colony, which regulate the exposure of vulnerable individuals, at least to pathogens (Stroeymeyt et al. 2018). The current gold standard approach for eradication of ants is the use of baits with a slow-acting poison, which allows workers to bring the food-poison mixture back to the nest to distribute it to the brood and the queen (Hoffmann et al. 2016). Even so, the success rate of such eradication attempts is still low (Souza et al. 2008; Hoffmann 2011). A big issue is the availability of high-quality natural food, which is often preferred by ants and acts as a competitor for the poisoned bait, leading to low bait consumption rates (Rust et al. 2003;

Silverman and Brightwell 2008). Driving bait preference and increasing consumption is thus a critical step towards successful ant control.

One approach to increasing bait attractiveness, beyond a change in bait formulation, is to steer ant behaviour. Studies in leaf-cutting ants showed that alarm pheromones can be used to increase bait consumption and foraging activity (Hughes et al. 2001, 2002). A study in Argentine ants showed that synthetic (Z)-9-hexadecenal, the main component of the trail pheromones in this species, increased bait (liquid sucrose) consumption (Greenberg and Klotz 2000). Pheromones can not only be used to lure ants to a bait, but also to disrupt their trail-following behaviour (Tatsuki et al. 2005; Tanaka et al. 2009). An open field experiment demonstrated that the combination of a highly-concentrated synthetic pheromone and insecticidal baits may provide effective control of Argentine ant populations (Sunamura et al. 2011).

However, preference and consumption can also be manipulated by exposing individuals to tailored information. Insights from behavioural economics and cognition research are regularly used to drive preference and consumption behaviour in humans. A widespread example is the exploitation of the endowment effect, where the perceived value of a good is increased by the subject already owning it. For example, someone who would be willing to pay €5 for a mug may, if given the mug for free, demand €8 to sell it (Kahneman et al. 1991). Companies and advertisers exploit this by providing a service at a discount for a limited period, to cause endowment, before raising the price. This demonstrates that insights from behavioural economics and cognition research can be used to effectively drive preferences and consumption behaviour. Driving preference using such behavioural economic effects has been shown also in animals. Primates and birds are known to be sensitive to irrational behaviours like the endowment effect (Shafir et al. 2002; Bateson 2002; Lakshminaryanan et al. 2008). A related concept to an endowment effect was shown in *Lasius niger* ants, where foragers were trained to expect one food flavour, and then provided another food source of equal value but with a different flavour. These ants undervalued the novel flavoured food, as demonstrated by reduced food and recruitment (Oberhauser and Czaczkes 2018). However, we are still far from a sophisticated understanding of insect value perception.

Possibly the easiest way of changing the perceived value of a cue is via associative learning. Associative learning is perhaps the most fundamental form of learning, and strongly impacts perceived value. Classical association experiments involve the pairing of an

97 unconditioned stimulus (e.g. food), which innately produces a response, with a neutral
98 stimulus (e.g. odour), which usually triggers no response. After training, exposure to the
99 conditioned stimulus can trigger the unconditional response which the unconditional stimulus
100 usually elicits, or allows the subject to predict that the unconditional stimulus is imminent
101 (Fanselow 1998; Clark 2004).

102 Ants have been shown to be good associative learners. Ants can form impressive route
103 memories which allow them to associate a direction, panorama, or a route with a food reward,
104 nest (Aron et al. 1988; Harrison et al. 1989; Graham and Collett 2006; Grüter et al. 2011;
105 Knaden and Graham 2016) or even with a negative outcome (i.e. getting trapped) (Wystrach
106 et al. 2020). Many ant species have been shown to associate an odour with a food reward
107 (Roces 1990; Helmy and Jander 2003; Dupuy et al. 2006; Czaczkes et al. 2014; Oberhauser et
108 al. 2019; Czaczkes and Kumar 2020). Food-odour associations can be rapidly formed and may
109 last for days (Josens et al. 2009; Arenas and Roces 2018; Piqueret et al. 2019).

110 However, very little is known about the associative learning ability of invasive ants. In
111 one study it was shown that Argentine ants (*L. humile*) can use visual and spatial cues to find
112 a food source, but the experimental setting was a binary choice test between visual cues and
113 a pheromone trail (Aron et al. 1988, 1993). A recent study in *L. humile* showed that they are
114 also able to associate an odour spot in a circular arena with a sucrose solution (Rossi et al.
115 2020). Preexposure to trail pheromone increased food acceptance rates, but had no effect on
116 associative learning. Rossi et al. (2020) is a very important paper for us, as it is the only
117 currently available investigation of associative learning in free-running Argentine ants. In that
118 study, ants were allowed to visit the sucrose reward three times. It is thus unclear how many
119 exposures to the reward would be required in order for the individual to form a short-term
120 association. Furthermore, no long-term memory tests were conducted, nor has information
121 transfer of food-associated odours between nestmates been investigated, as previously
122 shown in bees, wasps, and other ants (Farina et al. 2005; Provecho and Josens 2009; Schueller
123 et al. 2010).

124 Here, we conduct a comprehensive in-depth investigation of learning in the
125 ecologically important ant *L. humile*. We study how rapidly *L. humile* form short- and long-
126 term memories, which types of cues best support this, and whether food-related cues are
127 transferred between nestmates intranidally. Such information is critical if we hope to develop
128 cognition-based control strategies.

# 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 300-1000 workers, were kept in plastic foraging boxes (32.5 x 22.2 x 11.4 cm) with a plaster of Paris on the bottom. The walls were coated in fluon to prevent escape. Each box contained several 15mL red plastic tubes partly filled with water, plugged with cotton, for use asas 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 *Drosophila melanogaster*, and deprived of food for four days prior to testing. For all experiments using odours and/or flavours, donor and recipient colony pairs were used. Such colonies were collected in the same location at the same time, however the donor colonies had never experienced any of the chemicals used, whilst the recipient colonies received individuals that had experienced these.

Solutions and odours

1M sucrose solutions, (Südzucker AG, Mannheim, Germany), were used as a reward during training for all experiments. Where a negative reinforcement was also presented, 0.6mM quinine (Merck KGaA, Darmstadt, Germany) solutions were used. Paper overlays, used during odour experiments, were stored for at least 1 week prior to the experiments in airtight plastic boxes (19.4 x 13.8 x 6.6 cm) containing a glass plate with 500µl of either strawberry or apple food flavouring (Seeger, Springe, Germany). For experiments were flavoured food was used, 1µL of the respective flavouring was added per mL of 1M sucrose solution. Pilot studies showed *L. humile* workers having a slight preference for strawberry over apple flavour (58% of the ants prefered strawberry, N = 158, see supplement 3).

Experiment 1 – Short-term spatial memory

Can *L. humile* ants learn to associate a location, one arm of a Y-maze, with the presence of a reward? Here we investigate this by offering a reward, sucrose, on one of the Y-maze arms and a punishment, quinine, on the opposing arm. 6-8 ants per day were tested, testing in total 46 ants from 7 colonies

Y-maze setup and training methods follow Czaczkes (2018). A colony was connected via a drawbridge to a Y-maze (arms 10cm long, 1cm wide, tapering to 2mm at the bifurcation, see fig. 1a) covered in unscented disposable paper overlays. A c. 20µl drop of sucrose solution was placed at the end of one arm of the maze, and a drop of quinine solution on the other.

The first 1-3 ants to initially chose the arm leading to the punishment was marked with acrylic paint after it subsequently found the sucrose reward. This protocol ensures that the focal ants do not have an innate preference for the rewarded side. From this point on, only one by one of the marked ants were selectively allowed to move onto the setup. Upon satiation, ants ran back over the bridge to the nest and unloaded their collected food to their nestmates. While unloading, the Y-maze paper overlays were replaced with fresh overlays, to remove any pheromone trails or cuticular hydrocarbons left by the ant. After unloading, the ants were allowed back onto the Y-maze via the bridge. We recorded the ant's initial decision (defined as the antennae crossing a line 2cm from the bifurcation) and final decision (crossing a line 8cm from the bifurcation, 2cm from the arm end). We then allowed the ant to carry out 3 more such visits (5 visits to the sucrose, 4 trained decisions in total). Half the ants were rewarded on the left, the other half rewarded on the right.

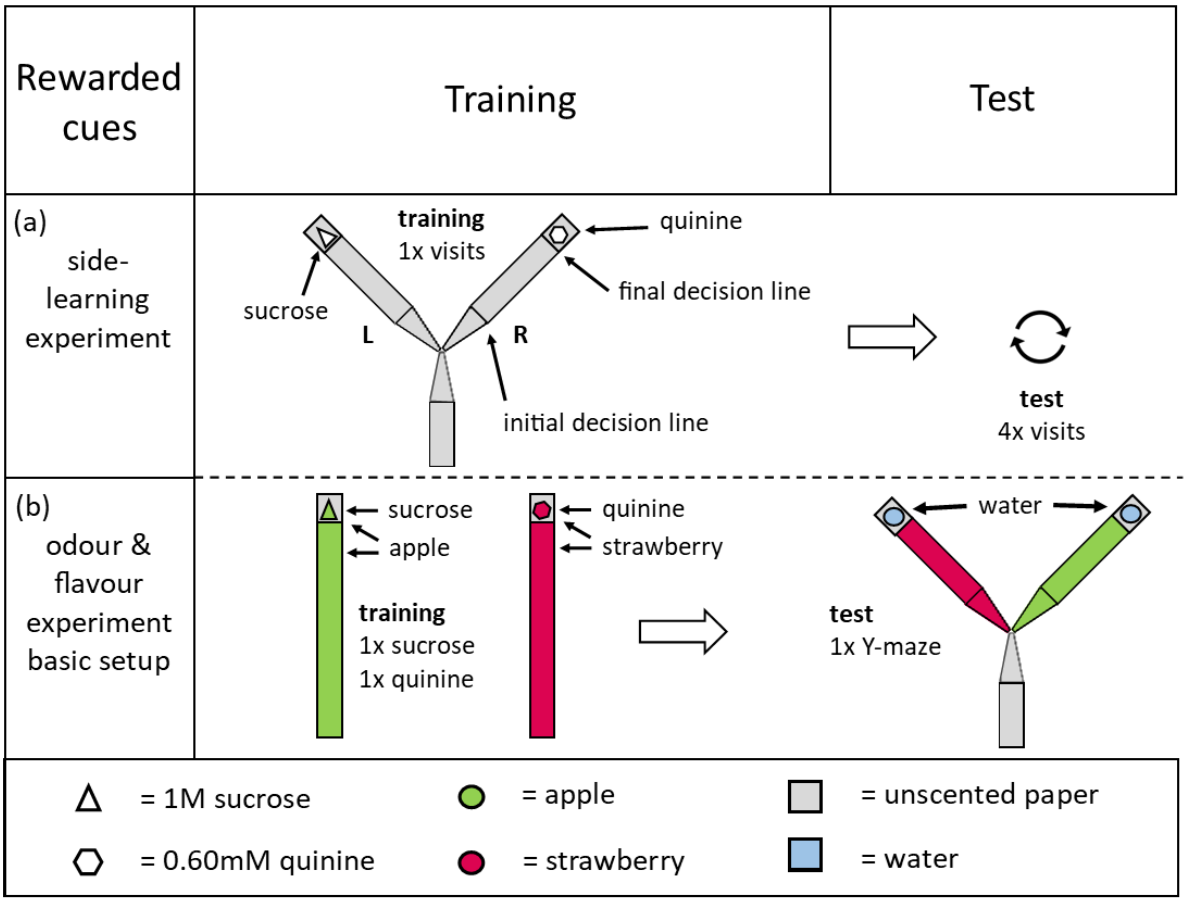


Figure 1: Basic setup used for all experiments: a) Ants were trained to associate a side of the Y-maze with a 1M sucrose reward, while the other side contained 0.60mM quinine or water (exp 1). In all other experiments (b), ants were trained to associate an odour (apple or strawberry) or/and a flavour (apple or strawberry) with a 1M sucrose reward on a linear runway and were then tested on a Y-maze. In experiments where quinine was used as negative reinforcer (2,4,6,8) the flavour and odour of the quinine was the converse of that of the reward. In the experiments (3,5,8,9) the quinine punishment training was removed. In the test trials the arms of the Y-maze contained a water drop. The ants' decision was noted when they crossed the initial decision line (2cm after arm start) and a final decision line (8cm after arm start, 2 cm before end).

| Experiment | Runway odour | Food flavour | Punishment visit (quinine) | N | Set | Short or long-term memory | % correct choices |
|---|---|---|---|---|---|---|---|
| *2) Scented & flavoured reward vs. punishment* | X | x | X | 72 | 1 | short | **84%** |
| *3) scented & flavoured reward vs. neutral stimulus* | X | X | | 48 | 1 | Short | **85%** |
| *4) scented & unflavoured reward vs. punishment* | X | - | X | 72 | 1 | Short | **72%** |
| *5) unscented & flavoured reward vs. neutral stimulus* | | X | | 48 | 1 | Short | 65.5% |
| *6) unscented & flavoured reward vs. punishment* | | X | X | 44 | 2 | Short | **86%** |
| *7) scented & unflavoured reward vs. punishment* | X | - | X | 48 | 2 | short | **78.5%** |
| *8) scented & flavoured reward vs. neutral stimulus* | X | X | | 70 | 1 | Long (8, 24, 48 hours) | 8h = **85%**<br>24h = **81%**<br>48h = **80%**<br><br>Nestmates:<br>24h = **77%,**<br>48h = **67%** |
| *9) scented & unflavoured reward vs. neutral stimulus vs. neutral stimulus* | x | - | X | 56 | 1 | Long (8, 24, 48 hours) | 8h = **67%,**<br>24h = **78%**<br>48h = **73%**<br><br>Nestmates:<br>24h = 40%<br>48h = **30%** |
| *1) Side learning: Training on a Y-maze, reward on one arm, punishment on the other* | | | X | | 1 | short | Visit #<br>2 = **65%,**<br>3 = **84%**<br>4 = **84%**<br>5 = **88%** |

Table 1) Experimental overview. Experiments 2-9 involved training on a linear runway and testing on a Y-maze with scented arms. Experiment 1 involved training over multiple visits on a Y-maze. Runway odour: was the runway scented during training? Food flavour: was the food (and punishment where present) flavoured during training? Experiments in set 1 were carried out between April and August. Experiments in set 2 were carried out between November and December. In short-term learning experiments training occurred immediately after testing, c. 1-5 minutes. In long-term memory experiments testing occurred the stated number of hours after training. % correct choices are printed in **bold** if significantly different from random choice. In experiments 8 and 9 untrained nestmates, housed with the trained nestmates, were also tested.

197 Experiment 2 – Short-term memory: scented and flavoured reward versus punishment
198 (experimental set 1)
199 Experiments conducted in summer are considered as experimental set 1.
200 The aim of this experiment was to examine if ants can associate two environmental cues
201 (odour and a flavour) with a sucrose reward. An ant was allowed access to a 10cm-long straight
202 runway covered by a scented paper overlay, which had a drop of flavoured sucrose solution
203 at the end (apple or strawberry). After the ant offloaded the collected food in a recipient sub-
204 colony nest, created to prevent them from sharing odour or flavour information with future
205 tested ants, it was allowed to visit the runway again. The reward odours and flavours used
206 were alternated between different ants, where if an ant experienced strawberry as a reward
207 then it would experience apple as a punishment and vice-versa. The training phase was
208 concluded once the ant experienced both the reward and punishment in a straight runway
209 and was allowed back into the recipient colony. The ant was then allowed onto a Y-maze (see
210 above), in which one arm was covered in a reward-associated scented overlay and the other
211 in the punishment-scented overlay, and the ant's preference was tested as in experiment 1
212 (see "test" in fig. 1b & table 1). 72 ants from three donor colonies were tested, ensuring an
213 equal number of individuals was tested for each odour/flavour and Y-maze side combination
214 daily.
215
216 Experiment 3 – Short-term memory: scented and flavoured reward versus neutral stimulus
217 (experimental set 1)
218 The aim of this experiment was to test whether ants need a positive and a negative stimulus
219 for successful learning, or if only a positive stimulus is sufficient. Ants were trained as in
220 experiment 2 (runway odour and flavoured food), but no second visit to a punished runway
221 was performed. The test was carried out as in experiment 2 (see also fig. 1 & table. 1). 48 ants
222 from two donor colonies were tested, and again both odours/flavours and Y-maze sides were
223 alternated in every combination possible to remove any potential bias.
224
225 Experiment 4 – Short-term memory: scented and unflavoured reward versus punishment
226 (experimental set 1)
227 The aim of this experiment was to determine whether ants can associate runway odour cues
228 alone with a reward or punishment. Ants were trained as in Experiment 2 (see fig. 1 & table

229 1), except this time, the food reward and the quinine were not flavoured. 72 ants from 6
230 colonies were tested and both odours and Y-maze sides were alternated daily.
231
232 Experiment 5 – Short-term memory: unscented and flavoured reward versus neutral stimulus
233 (experimental set 1)
234 The aim of this experiment was to test whether flavoured food, without runway odour or
235 negative reinforcement, is sufficient to form an association between flavoured food and an
236 runway odour presented on a Y-maze arm. This is especially important for potential future
237 applications in pest control. Ants were trained as in Experiment 3, except that the runway
238 overlay was unscented (see fig. 1 & table.1 ). In the test, ants had the choice between a
239 scented arm and an unscented one. 48 ants from two colonies were tested.
240
241 Experiment 6 – Short-term memory: unscented and flavoured reward versus punishment
242 (experimental set 2)
243 We had the oppertunity to collect additional data in winter, which we considered as
244 experimental set 2.
245 The aim of this experiment was to test whether flavoured food, without runway odour, is
246 sufficient to form an association between flavoured food and an runway odour presented on
247 a Y-maze arm when we add a quinine visit as punishment (contrary to experiment 5). Ants
248 were trained as in experiment 5 except that a quinine visit was added. The test was carried
249 out as in experiment 2 (see fig. 1 & table 1). 44 ants from three colonies were tested.
250
251 Experiment 7 – **Short-term memory: scented and unflavoured reward versus punishment**
252 (experimental set 2)
253 The results of Experiment 6 were unexpected, considering the ones from Experiment 3.
254 Therefore Experiment 4 was repeated to ensure these surprising results were not caused by
255 seasonal effects or some other systematic change between experimental sets (see fig. 1 &
256 table 1). 48 ants from three colonies were tested.
257 Experiment 8 – Long-term memory: scented and flavoured reward versus neutral stimulus
258 (experimental set 1)
259 In this experiment, ants were trained as in experiment 3 (see fig. 1 & table 1) with the
260 exception that every colony was only conditioned to one reward odour (strawberry or

apple). Two colonies were conditioned to strawberry and two to apple. 50 ants were trained and, after training, housed in a small sub-colony with 60 naïve ants from their nest. Trained ants were tested in a Y-maze, as in experiment 3, after 6 hours, 24 hours and 48 hours (4 colonies, 14 ants per period). Each individual ant was only tested once and was then removed. After the trained ants were tested we also tested the untrained nestmates (4 colonies, 20 ants per period, 24h and 48h), to test whether contact with the trained ants, which had fed on flavoured food, allowed them to learn the food flavour and thus follow this odour cue. The colony test order was alternately between a colony conditioned to apple or strawberry. In total, we tested 70 trained and 80 untrained ants per time period (trained = 6h, 24h and 48; untrained = 24h and 48h).

Experiment 9 – Long-term memory: scented and unflavoured reward versus neutral stimulus

The aim of this experiment was to exclude the possibility that the ants in experiment 8 were not remembering their association for the whole time period, but rather were refreshing their memory by repeatedly sampling the flavoured food from other nestmates. To this end, this experiment was identical to experiment 8 but with an unflavoured reward - only a scented runway was used (see fig. 1 & table 1). From the previous experiments, we expected that the trained ants would form a strong association even without food flavour, but that the untrained ants would not be able to gain any relevant odour information from their sisters. In total, we tested 56 trained and 80 untrained ants from 4 colonies per time period.

**Statistical analysis**

Only 3.6% – 10.9% of initial and final choices differed, depending on experiment, so we focused our analysis on the final choices. 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 distribution was used. Since multiple ants were tested per colony, we included colony as random factor. Each model was validated using the DHARMa package (Hartig 2018). Results were plotted using the gglot2 package (Wickham 2016). The complete code and analysis output is provided in supplement 1.

# Results

The complete dataset is provided in supplement 2.

Experiment 1 - Side-learning

This experiment tested whether Argentine ants can associate a side of the Y-maze with the reward (food), and how this memory develops over subsequent visits.

Over all visits, significantly more choices were made for the arm leading to the food (GLMM, n = 46, z-ratio = 2.513 p = 0.045, see fig. 2). In visit 2, after only one visit to the food, 65% (30/46) of ants chose the arm leading to the food (n =46, z-ratio = 2.581, p = 0.042). This rose to 84% (39/46) in visit 3 (n = 46, z-ratio = 1.860, p = 0.026), 84% in visit 4 (38/45) (n = 45, z-ratio = 1.469, p = 0.032) and 88% (39/44) in visit 5 (n = 44, z-ratio = 2.245, p = 0.009). For a pairwise comparison between visits, see online supplement 3

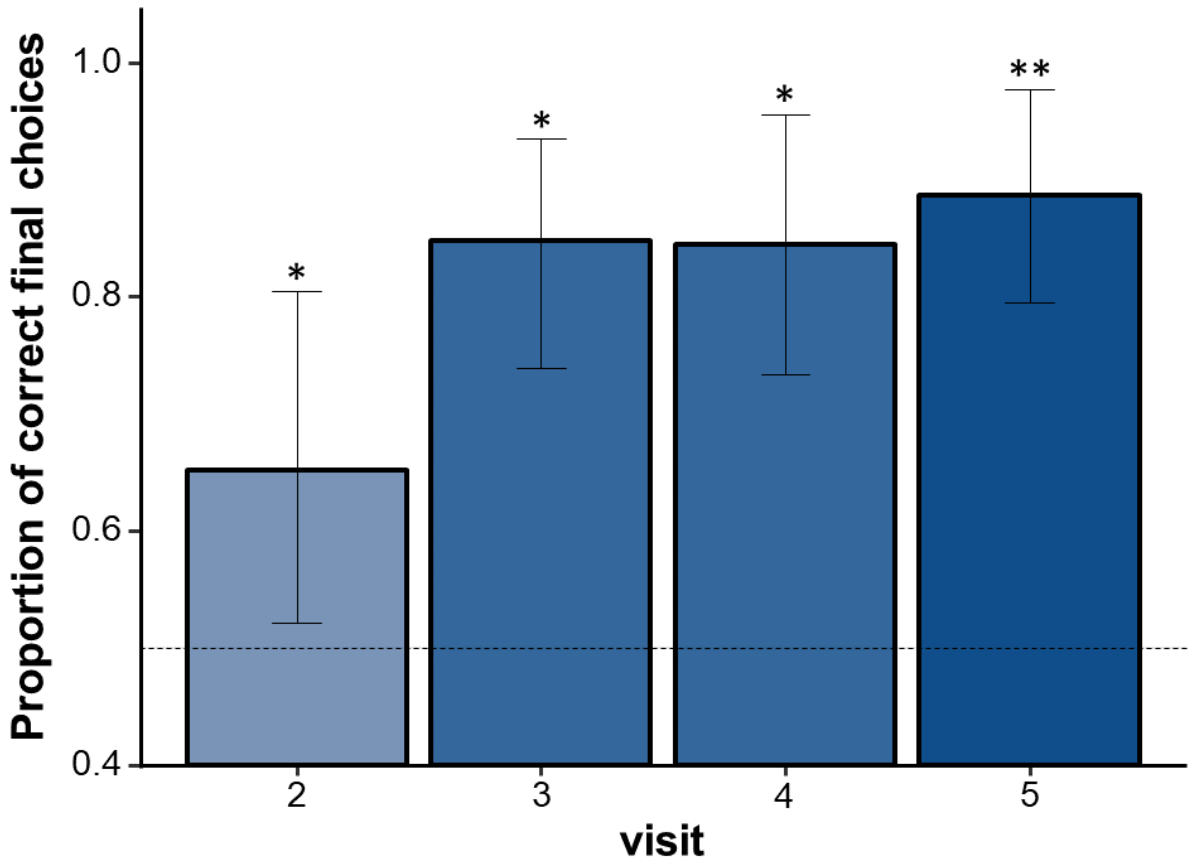


Figure 2: Side-learning: Proportion of ants choosing the side associated with reward (food) per visit. Ants chose the side associated with reward significantly more often than chance after all visits. Bars depict mean, whiskers 95% confidence intervals derived from the fitted GLMM. The dotted horizontal line displays chance level of 50%. *$p < 0.05$; **$p < 0.01$; ***p < 0.001.

**Odour association: Short-term memory**

The aim of experiments 2 – 7 was to understand if and how well Argentine ants can associate odours and food flavours with food rewards, and whether punishment improves learning (as has been shown in honeybees, Avargues-Weber et al. 2010).

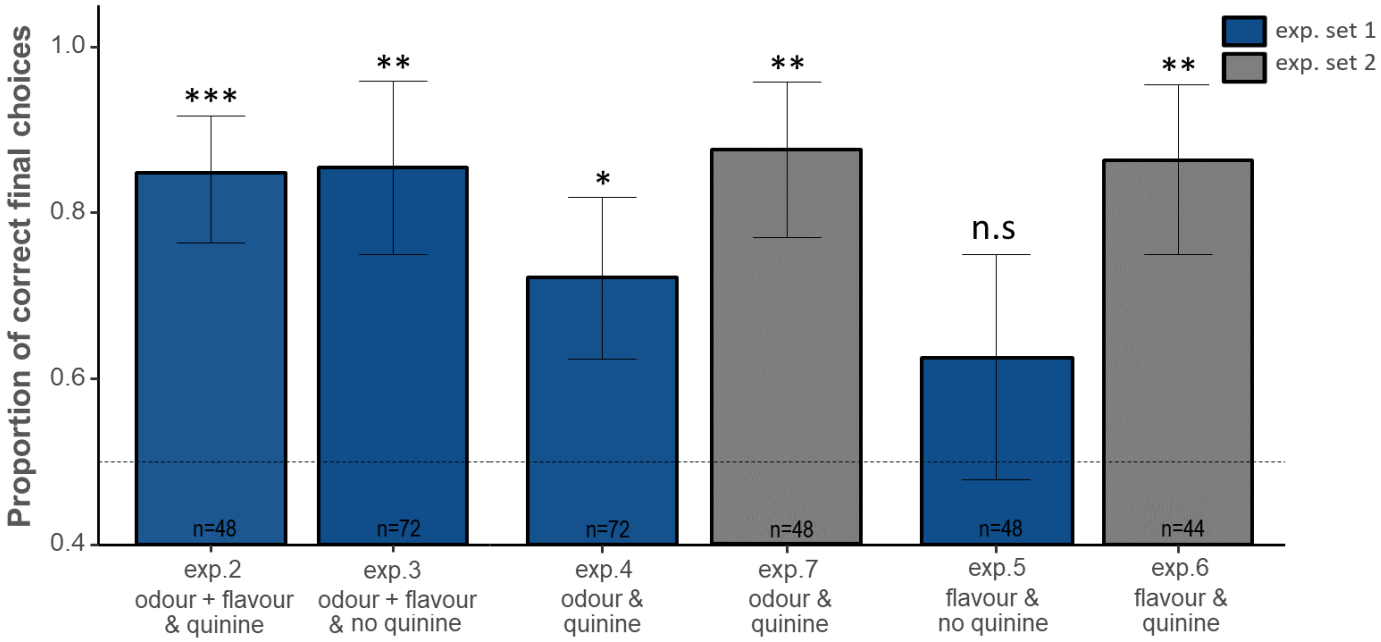


Figure 3: Short-term odour associative learning: Proportion of ants reaching the end of a Y maze arm scented with an odour associated with a sucrose reward. Ants were able to form a strong association to both training runway odour and food flavour. Punishment of the contrasting odour using quinine did not improve learning. Ants learned better in experimental set 2 (conducted in the winter) – this is especially noteable when ants were trained to flavoured food without a scented runway. Offering both odour and flavour cues did not increase learning compared to offering only one cue. Bars depict means, whiskers 95% confidence intervals derived from the fitted GLMM. The dotted horizontal line displays chance level of 50%. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Experiment 2 - Runway odour and flavoured reward (experimental set 1)

This experiment tested if ants learn to associate the sucrose reward with both environmental cues, a food flavour and the corresponding runway odour (apple or strawberry). 84% (61/72) of the ants chose the food-associated odour arm (GLMM, z-ratio = 3.375, $p < 0.001$, see fig. 3). The specific odour rewarded did not significantly affect choice accuracy (apple 80% and strawberry 88%, z-ratio = -0.972, $p = 0.3312$).

Experiment 3 – Runway odour and flavoured reward, no quinine visit (experimental set 1)

This experiment tested whether a reward alone, and no punishment (quinine), results in an equally high proportion of choices for the food-associated odour. 85% (41/48) of the ants

chose food-associated odour arm (GLMM, z-ratio = 2.938, p = 0.003, see fig. 3). Again, the specific odour rewarded did not significantly affect choice accuracy (apple 83% and strawberry 87%, z-ratio = -0.408, p = 0.683).

Experiment 4 - Runway odour, unflavoured reward (experimental set 1)

This experiment tested if Argentine ants can associate a runway odour (apple or strawberry) with an unflavoured reward. 72% (52/72) of the ants chose the side of the Y-maze with a scent associated with a reward (GLMM, z-ratio = 2.568, p = 0.010). Ants performed identically on both rewarded odours (72% correct decisions for both odours, z-ratio = 0.00, p = 1.0000, see fig. 3).

Experiment 5 - Flavoured reward, no runway odour, no quinine (experimental set 1)

This experiment was run to test the ant´s learning ability without negative reinforcement and using only one cue, the flavoured food. 62.5% (30/48) of ants chose the food-associated odour arm, which does not differ significantly from random choice (GLMM, z-ratio = 1.212, p = 0.226, see fig. 3). Again, there was no significant difference between performance with the two odours (62.5% correct decisions for both odours, z-ratio = 0.000, p = 1.000).

Experiment 6 - Flavoured reward, no runway odour (experimental set 2)

This experiment was similar as the 5$^{th}$ experiment except that a quinine visit was added and with only one cue (flavour). Again, the purpose of this study was to test for the need of a punishment visit. Unlike in summer (experimental set 1), the winter experiment (experimental set 2) showed that ants were able to learn to associate just one cue (flavour) with a sucrose reward. 86% (38/44) of the ants chose the side of the Y-maze with a scent associated with reward (GLMM, z-ratio = 2.721, p = 0.0065, see fig. 3. There was no significant difference between the two odours (apple 81% and strawberry 90%, z-ratio = -0.863, p = 0.388)

Experiment 7 - Runway odour, unflavoured reward (experimental set 2)

This experiment, conducted in winter, was the same as the 4$^{th}$ experiment. The purpose was to test if ants show a systematic difference in learning between the two experimental sets. The ants again formed a strong association between just one cue (odour) and the sucrose

reward. 87.5% (42/48) of the ants chose the side of the Y-maze with a scent associated with reward a reward (GLMM, , z-ratio = 2.656, p = 0.007, see fig. 3). Again, the specific odour rewarded did not significantly affect choice accuracy (apple 79% and strawberry 95%, z-ratio = -1.582, p = 0.1138).

The roles of runway odour and food flavour in learning

In order to characterise the relative roles of runway odour and food flavour in driving learning, we compared the behaviour of ants in experiments 2 (runway odour and food flavour during training) with the results from experiment 4 (only runway odour) and experiment 5 (only food flavour). Note, however, that experiment 5 did not include a negative reinforcement, while experiments 2 and 4 did. We consider this appropriate, since negative reinforcement did not improve learning (compare experiments 2 & 3).

Combined runway odour and flavoured food led to a significantly higher proportion of correct choices than only having flavoured food (z-ratio = -2.714, p = 0.018). However, runway odour alone was not significantly weaker than the combined cues (z-ratio = -1.118, p = 0.502). Runway odour alone did not result in significantly more correct choices than food flavour alone (z-ratio = 1.803, p = 0.168).

**Long-term odour and flavour association**

The aim of experiments 8 and9 was to examine if Argentine ants also form a long-term (6h, 24h, 48h) association between runway odours and food rewards (experiment 9) and with flavoured food rewards (experiment 8).

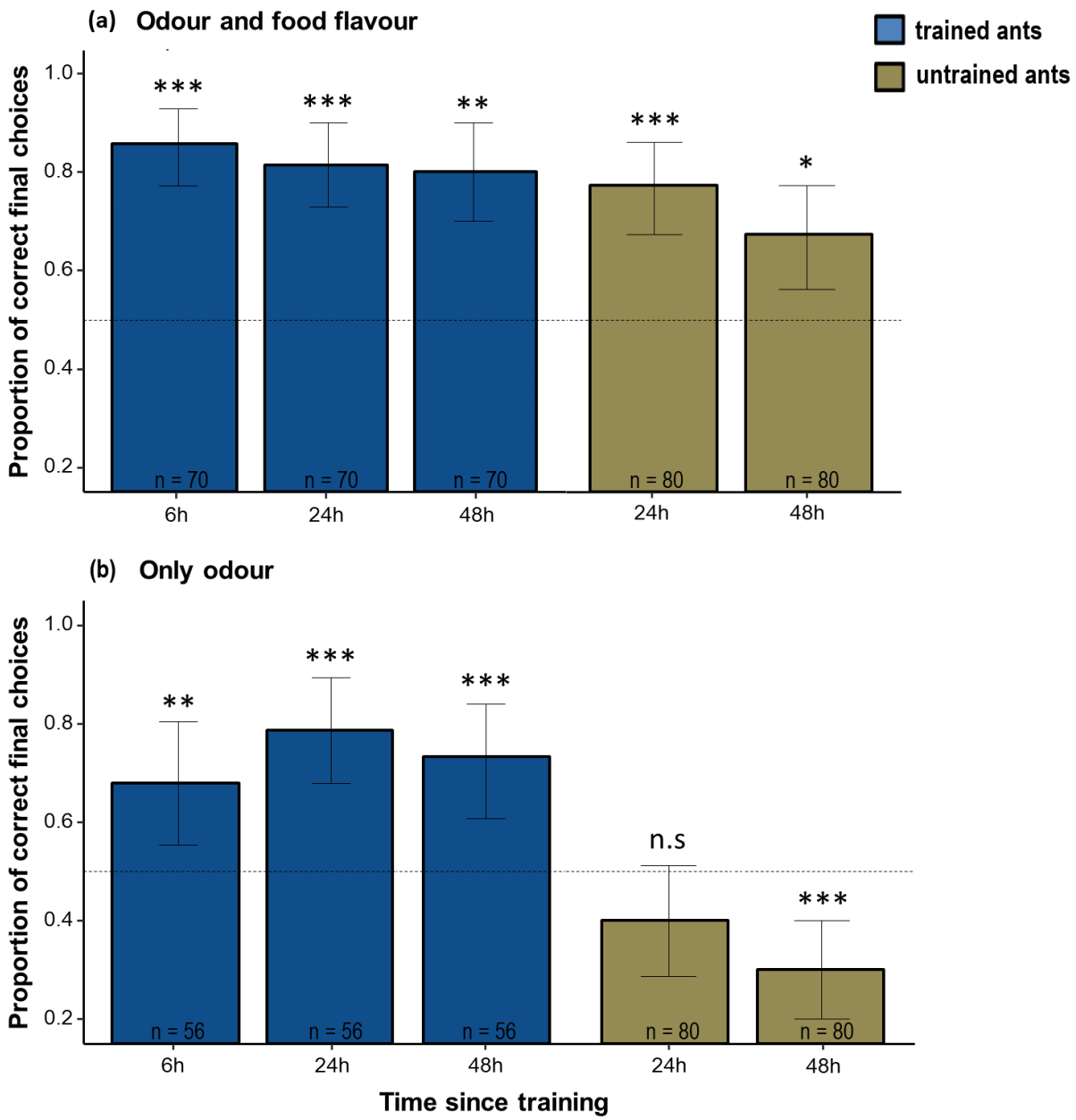


Figure 4: Long-term odour associative learning: Proportion of ants choosing the odour associated with a sucrose reward. Trained ants were tested after 6h, 24h, and 48h. **4a:** Untrained nestmates also showed a preference for the rewarded odour when food was flavoured, which was not significantly weaker than that of trained ants. **4b:** Trained ants were able to associate a runway odour alone with an unflavoured sucrose reward after 6h, 24h and 48h. By contrast, untrained nestmates did not show any odour preference after 24 hours, and showed an aversion for the food odour after 48 hour. Bars depict means, whiskers 95% confidence intervals derived from the fitted GLMM. The dotted horizontal line displays chance level of 50%. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Experiment 8 - Long-term memory, runway odour and flavoured food

*Trained ants and their ability to build a long-term association*

Ants trained on combined runway odour and food flavour significantly preferred the odour-associated Y-maze arm after 6 hours, 24 hours and 48 hours (GLMM, z-ratio = 4.204, p <

0.0001, see fig. 4a), with 85% (60/70), 81% (57/70), and 80% (56/70) correct choices after 6, 24, and 48 hours respectively (6 hours: GLMM, z-ratio = 5.246 , $p < 0.0001$, 24 hours: GLMM, z-ratio = 4.809, $p < 0.0001$ , 48 hours: GLMM, z-ratio = 8.515, $p < 0.0001$).

*Odour preference in untrained ants housed with flavoured-food fed nestmates (trained)*

Untrained ants housed with ants trained with flavoured food showed a strong preference for that food flavour after 24 and 48 hours (z-ratio = 3.830, $p < 0.0001$, see fig. 4a). 77% (62/80) and 67% (54/80) of ants chose food-associated odour after 24 and 48 hours respectively (24 hours: GLMM, z-ratio = 4.619, $p < 0.0001$, 48 hours: GLMM, n = 80, z-ratio = 2.511, $p = 0.012$).

Experiment 9 - Long-term memory, only runway odour, unflavoured reward

This experiment was run to quantify long term learning without access to informative flavoured food, since this could refresh the ants' memories.

*Trained ants and their ability to build a long-term association with unflavoured food*

Ants trained with runway odour and unflavoured food nonetheless significantly preferred the odour-associated Y-maze arm after 6, 24 and 48 hours (GLMM, z-ratio = 3.990, $p < 0.0001$, see fig. 4b), with 67% (38/56), 78% (44/56), 73% (41/56) correct choices after 6, 24, and 48 hours respectively (6 hours: GLMM, z-ratio = 2.611 , $p = 0.009$, 24 hours: GLMM, z-ratio = 3.990, $p < 0.0001$ , 48 hours: GLMM, z-ratio = 3.332, $p < 0.0001$.

*Odour preference in <u>untrained</u> ants housed with <u>unflavoured-food</u> fed nestmates (trained)*

Unlike naïve ants housed with nestmates trained on flavoured food, naïve ants housed with nestmates trained on unflavoured food showed no preference for the food-associated odour (z-ratio = -1.777, $p = 0.075$, see fig. 4b). 40% (32/80) and 30% (24/80) of ants chose the food-odour covered Y-maze arm after 24 and 48 hours, respectively. Indeed, while the choice of naïve ants after 24 hours did not differ from chance (GLMM, z-ratio = -1.381, $p = 0.268$), after 48 hours ants significantly avoided the scented arm (z-ratio = -3.473, $p < 0.0001$).

*The effect of food flavour presence on the long-term memory of trained ants*

Here we compared how well the Argentine ants performed on the long-term memory test (6h, 24h, 48h) when they had a flavoured reward vs. an unflavoured reward. Do they form a significantly better memories when they had a flavoured reward?

There was no significant difference between trained ants who had an unflavoured reward treatment (Experiment 9) and those trained on flavoured food (experiment 8) after 6 hours, 24 hours and 48 hours (6 hours: GLMM, n =56[exp.8] and n=70[exp.9] , z-ratio = 2.344 , p = 0.176 ,24 hours: GLMM, n =56[exp.8] and n=70[exp.9] , z-ratio = 0.399 , p = 0.998, 48 hours: GLMM, n =56[exp.8] and n=70[exp.9] , z-ratio = 0.897, p = 0.947).

However, untrained ants who were treated with an unflavoured reward (9. Experiment) performed significantly worse than ants with a flavoured reward treatment (8. Experiment) after 24 hours and 48 hours (24 hours: GLMM, n =80[exp.8] and n=80[exp.9], z-ratio = 4.709, $p < 0.001$, 48 hours: GLMM, n =80[exp.8] and n=80[exp.9], z-ratio = 4.631, $p < 0.001$).

# Discussion

*Linepithema humile* are adept learners: The side-learning results show that Argentine ants are capable of learning to associate a direction or an arm of a Y-maze with a food reward after just one visit, and accuracy improves with further visits (see fig. 2). Such rapid and reliable learning is surprising, given the fact that *L. humile* relies strongly on social information (pheromone trails), while other recruiting social insects which learn equally fast, such as the ants *Lasius niger* and *Paraponera clavata* ants, and the honeybee *Apis melifera*, prioritize memories over recruitment signals when the two conflict (Harrison et al. 1989; Aron et al. 1993; Grüter et al. 2008, 2011; Czaczkes et al. 2013; von Thienen et al. 2016). *L. humile's* strong individual memory may allow them to reliably recruit even to distant resources, while their strong reliance on social information, and ability to recruit from active trails (Flanagan et al. 2013), may allow them to rapidly dominate these resources once found. Indeed, the rapid discovery of new resources and massive, rapid recruitment might be an important contribution to making Argentine ants competitive against native ants (Holway 1999), but see (Cordonnier et al. 2020). Route learning and navigation is hindered by higher complexity, at least in *La. niger* ants, but when trail pheromones are present, error rates decrease (Czaczkes et al. 2013). The combination of social information and a strong individual side-learning memory could hence be extremely beneficial and explain why Argentine ants developed such a strong individual route learning ability in parallel to their pheromone recruitment system. However, a systematic exploration of learning in (social) insects has not been carried out, so it is also possible that many or most ants are equally good learners.

Alongside their impressive route learning, Argentine ants also show even more rapid learning of olfactory cues. Ants were able to form a strong association after only one rewarded visit (see fig. 3 & table 1). These findings are again comparable to results from *Lasius niger* (Czaczkes et al. 2014; Czaczkes and Kumar 2020). Somewhat surprisingly, results from both the short-term and long-term learning experiments showed that punishing the alternative odour with quinine does not improve response accuracy. While some studies report ants (*Camponotus fellah*) failing to form a negative association to a scent (Josens et al. 2009), studies on *Las. niger* ants and honeybees do (Avarguès-Weber et al. 2010; Wenig et al. 2021). Also remarkable is that *L. humile* is able to form an association with a food source given only one cue: a food flavour or a runway odour. Although the difference between choice accuracy

477 when trained on only one cue or both was not significantly, the gain of 10-16 (see fig. 3 & table
478 1) percentage points when both are available, and the fact that both single cues were
479 sufficient to form an association, suggests to us that cue effects could have an additive or sub-
480 additive effect on learning.

481 To our knowledge, the learning ability of *Linepithema humile* has never before been
482 described in such detail, and their very rapid learning of olfactory cues never fully appreciated.
483 Rossi et al. (2020) previously demonstrated that *L. humile* could associate an odour with a
484 food reward. Our results support their findings using a different experimental approach, and
485 with a higher resolution: the Rossi study allowed 3 visits to a circular open foraging arena, so
486 did not explore the limits of ant learning. Our study controlled the experiences of the
487 individual ants more tightly, by reducing the number of rewarded visits to one, and separating
488 rewarded and unrewarded learning events. While other studies have also demonstrated that
489 ants can associate an odour alone with a reward, in combination with a quinine negative trial,
490 these studies required extensive training, with 6-24 visits to the reward, and the odour was
491 blown by an air stream instead of using scented paper overlays (Dupuy et al. 2006; Guerrieri
492 and d'Ettorre 2010). To our knowledge, our study is also the first study to demonstrated that
493 *Linepithema humile* can learn to associate the flavour of the food alone with the food reward.

494 Alongside the strong short-term olfactory memory, our study demonstrates that
495 Argentine ants also possess a strong long-term memory, which lasts for at least 48 hours (see
496 fig. 4 & table 1). As in our short-term memory tests, an runway odour alone, without food
497 flavour, was sufficient to drive strong and stable learning. This is in line with studies on other
498 species (*Formica fusca* and *Camponotus fellah)*, which show stable memory for at least 72
499 hours, and a decay in learning after a week (Josens et al. 2009; Piqueret et al. 2019). However,
500 the study on *Camponotus fellah* again used very extensive training (16 training visits). We
501 found that one exposure alone, without a punishment visit, was all that was required to elicit
502 strong and stable learning. One similar study showed, that *Cataglyphis fortis* desert ants can
503 also build after only visit a strong association between a food source and an odour (Huber and
504 Knaden 2018). This again suggests that *L. humile* are an extraordinarily good olfactory
505 learners, as well as strong route learners, although differences in experimental protocol may
506 also have contributed to the impressive *L. humile* results.

507 It is also important to note that, in the long-term memory experiments, tested ants
508 were housed in a small sub-nest where they fed their nestmates after their training. As a

509 result, flavoured food was likely distributed amongst the workers via trophallaxis. This could
510 have acted to 'refresh' the memory of the tested ants for some time. However, learning
511 remained strong even if ants were trained only with runway odours, and without food odours.
512 These impressive learning skills are not limited to trained ants. Tellingly, inclusion of food
513 flavour resulted in the untrained nestmates also developing a preference for that odour,
514 demonstrating a very effective distribution of food-related information within the nest. Such
515 intranidal information spread affecting future foraging decision has been reported in both
516 other ants (Roces 1990; Provecho and Josens 2009; Arenas and Roces 2018), bees (Farina et
517 al. 2005) and wasps (Jeanne and Taylor 2009).

518 Finally, our results indicate that there might be seasonal variation in learning, although
519 this was not tested systematically. While our summer experiments showed that flavour alone
520 is not sufficient for ants to form a strong association with the food reward, repeating the
521 experiment in the winter resulted in very strong learning. Furthermore, our only runway odour
522 experiment conducted in winter revealed an even stronger learning than in summer (see. fig.
523 3 & table 1). However, it has to be mentioned that the flavour experiment in winter had a
524 quinine visit, which summer experiments did not. Nevertheless, as mentioned above, quinine
525 does not seem to improve learning within a season, which makes a seasonal effect more likely.
526 The apparent improvement in learning in the winter may be ecologically relevant, but may
527 also be a (to us) inexplicable laboratory artefact. We note that the opposite pattern was found
528 in *Lasius niger* in a route-learning paradigm (Oberhauser et al. 2018). Season and temperature
529 effects on foraging and recruitment behaviour are commonly reported in social insects (Porter
530 and Tschinkel 1987; Stuble et al. 2013; Stroeymeyt et al. 2014; Park and Nieh 2017)

531 *Linepithema humile* are adept learners and rapidly disseminate food-related
532 information in the nest – abilities which might help them to adapt to new environments and
533 dominate resources, and could be one reason for their success as invaders. However, their
534 strength could potentially become a weakness, if their memory can be used against them. One
535 possibility would be to use associative learning and intranidal information transfer to steer
536 foraging preference towards poisoned baits, much as honeybee preference can be steered for
537 pollination purposes (Farina et al. 2020). Similarly, foraging preference of leaf-cutter ant
538 colonies can be steered to a specific food target by exploiting their preference for foraging on
539 resources which smell or taste like those already being collected (Arenas and Roces 2018).
540 While leveraging learning to control animal behaviour is commonly used in behavioural

conservation of vertebrates, it is mostly used to reduce crop damage or to minimize negative interactions with humans (Matsuzawa et al. 1983; Webb et al. 2015; Valenta et al. 2021). Leveraging learning to improve the control of invasive animals may be an underappreciated tool in the conservation toolbox.

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