

# Invasive ant learning is not affected by seven potential neuroactive chemicals

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## Abstract

Argentine ants *Linepithema humile* are one of the most damaging invasive alien species worldwide. Enhancing or disrupting cognitive abilities, such as learning, has the potential to improve management efforts, for example by increasing preference for a bait, or improving ants' ability to learn its characteristics or location. Nectar-feeding insects are often the victims of psychoactive manipulation, with plants lacing their nectar with secondary metabolites such as alkaloids and non-protein amino acids which often alter learning, foraging, or recruitment. However, the effect of neuroactive chemicals has seldomly been explored in ants. Here, we test the effects of seven potential neuroactive chemicals—two alkaloids: caffeine and nicotine; two biogenic amines: dopamine and octopamine, and three nonprotein amino acids:  $\beta$ -alanine, GABA and taurine—on the cognitive abilities of invasive *L. humile* using bifurcation mazes. Our results confirm that these ants are strong associative learners, requiring as little as one experience to develop an association. However, we show no short-term effect of any of the chemicals tested on spatial learning, and in addition no effect of caffeine on short-term olfactory learning. This lack of effect is surprising, given the extensive reports of the tested chemicals affecting learning and foraging in bees. This mismatch could be due to the heavy bias towards bees in the literature, a positive result publication bias, or differences in methodology.

**Key words:** associative learning, caffeine, memory, neuroactive chemicals

Thus far, global invasive ant control attempts have cost over 10 billion euros (Angulo et al. 2022). *Linepithema humile* (Mayr, 1868) is one of the most damaging invasive alien species worldwide (Lowe et al. 2000), and the fourth most costly invasive ant species (Angulo et al. 2022). Being both ecologically and economically damaging, these ants have become a top priority for conservation programs (Hoffmann et al. 2016). Nevertheless, eradication attempts have often met with failure (Souza et al. 2008; Hoffmann 2011), as competition with natural food sources leads to a lack of sustained bait consumption (Rust et al. 2003; Silverman and Brightwell 2008; Nyamukondiwa and Addison 2011). Enhancing or disrupting cognitive abilities could be a key step towards improving invasive species control.

One way of increasing preference for, and consumption of, target foods is to target learning, a critical cognitive ability which, if exploited, can be used to steer preference (Farina et al. 2020). Associative learning, one of the most important types of learning, links an unconditional stimulus (any stimulus which, without learning, causes a response) with a conditional stimulus (one which can be perceived, but does not by itself result in a response). Once linked, sensing the conditional stimulus results in a similar response to the one caused by the unconditional stimulus (Pavlov 1927; Rescorla and Wagner 1972; Dickinson 2012).

Ants use chemical, olfactory, and visual cues when foraging (Aron et al. 1988; Roces 1990; Czaczkes et al. 2014; Arenas and Roces 2018), acquiring landmark information and building

complex navigational routes (Helmy and Jander 2003; Graham and Collett 2006; Knaden and Graham 2016; Wystrach et al. 2020). They are strong associative learners, requiring as little as one experience to form a memory which may last for up to three days (Dupuy et al. 2006; Josens et al. 2009; Huber and Knaden 2018; Oberhauser et al. 2019; Piqueret et al. 2019; Czaczkes and Kumar 2020). Specifically, *L. humile* have been shown to be incredibly fast learners, requiring as little as two experiences for 84% of the studied individuals to successfully associate a side of a Y-maze with the presence of a reward (Wagner et al. 2022). Similar results were observed in olfactory learning, in this case with a single experience, and long-term memories were shown to last up to two days (Rossi et al. 2020; Wagner et al. 2022).

Plants are known to lace nectar with bioactive secondary metabolites, some of which act as neurotransmitters, binding with neuron receptor proteins, thus influencing neural activity and pollinator behavior (Mustard 2020). For example, caffeine and nicotine are thought to modulate cholinergic neuron activity, which is strongly implicated in multiple sensory functions, by interfering with acetylcholine receptors (Mustard 2014; Baracchi et al. 2017). Non-protein amino acids, such as  $\beta$ -alanine and taurine, neuromodulators involved in muscle performance, are abundant in the nervous system and likely regulate neuronal excitability acting in tandem with GABA, the principal inhibitory neurotransmitter in invertebrates (Nepi 2014). Interfering with insect neuronal signal transduction is thought to increase pollination

and seed dispersal (Wink 2018). For example, caffeine causes bees to form stronger, longer-lasting associations between odours and rewards, although such effects tend to be short-lived (Wright et al. 2013; Arnold et al. 2021). Additionally, it leads to bees overestimating resource quality, increasing foraging frequency and recruitment (Singaravelan et al. 2005; Couvillon et al. 2015; Thomson et al. 2015).

Similarly, lacing food with  $\beta$ -alanine and GABA has been reported to improve associative learning and memory retention in bees. However, when ingested prior to conditioning, GABA,  $\beta$ -alanine and taurine hindered learning, but not memory retention, which was surprisingly improved by  $\beta$ -alanine and taurine (Carlesso et al. 2021). Dopamine and octopamine, neuromodulators in the central nervous system of invertebrates, are involved in information flow regarding food source quality, with octopamine showing an increased use of private information in bees (Linn et al. 2020). Octopamine and dopamine receptors have been linked to appetitive learning, with artificial increases of dopamine increasing the value of sucrose solution and improving olfactory learning and memory retrieval in both wasps and bees (Lenschow et al. 2018; Huang et al. 2022). Furthermore, dopamine is positively correlated with foraging activity in ants and likely modulates their sensitivity to olfactory cues (Seid and Traniello 2005; Friedman et al. 2018).

The effect of secondary metabolites and neurotransmitters in modulating foraging and learning in insects is currently a very active field of research. Table 1 provides examples of the effects of seven potential neuroactive chemicals on learning and memory across the Hymenoptera, whilst highlighting the significant bias towards honeybees and bumblebees as model organisms. In fact, upon extensive search, to our knowledge only six studies investigated the effects of these chemicals on ants, three of which focusing exclusively on whether the chemical elicited preference or aversion. Caffeine was shown to act as an attractant or repellent, depending on the extracts and concentrations used, likely altering food value perception (Majid et al. 2018; Yeoh et al. 2018; Madsen and Offenberg 2019). Furthermore, both caffeine and nicotine have been reported to improve conditioning and memory, albeit while decreasing food consumption (Cammaerts et al. 2014a, 2014b). More recently, dopamine has been linked to long-term memory consolidation and octopamine to appetitive learning of olfactory cues (Wissink and Nehring 2021).

Here, we test the effects of seven potential neuroactive chemicals (two alkaloids: caffeine and nicotine; two biogenic amines: dopamine and octopamine; three non-protein amino acids:  $\beta$ -alanine, GABA, and taurine) on the cognitive abilities of invasive *L. humile* in a laboratory setting. We mainly focus on short-term effects on spatial associative learning, as previous work suggests there is little room for improvement when it comes to olfactory associative learning in a laboratory setting (Wagner et al. 2022). Improving ant navigational skills could lead to sustained bait consumption by improving both foraging and recruiting of toxicant-laced baits. The motivation for this study was potential future application in an invasive species management setting. We thus focused on effects which manifest directly after consumption, without the need for pre-treatment or topical application.

## Materials and Methods

### Colony maintenance

*Linepithema humile* (Mayr, 1868) were collected from Portugal (Proença-a-Nova and Alcácer do Sal) and Spain (Girona) between April 2021 and April 2022. Ants were split into

colony fragments (henceforth colonies), containing three or more queens and 200–1000 workers, kept in non-airtight plastic boxes (32.5 × 22.2 × 11.4 cm) with a plaster of Paris floor and PTFE coated walls. 15mL red transparent plastic tubes, partly filled with water, plugged with cotton, were provided as nests. Ants were maintained on a 12:12 light:dark cycle at room temperature (21–26 °C) with *ad libitum* access to water. Between experiments, ants were fed *ad libitum* 0.5M sucrose solution and *Drosophila melanogaster* twice a week. During experiments, ants were fed once a week and deprived of carbohydrates for four to five days prior to testing, ensuring high foraging motivation. Experiments were conducted between March 2022 and September 2022 using 18 colonies divided into donor/recipient pairs. Donor colonies were kept naïve, never exposed to any of the chemicals used. During testing, focal ants left the donor colony, but returned to the recipient colony, where they unloaded the contents of their crop.

### Chemicals and solutions

Caffeine (CAS 58-08-2), nicotine (CAS 65-30-5), dopamine (CAS 62-31-7), octopamine (CAS 770-05-8),  $\beta$ -alanine (CAS 107-95-9), GABA (CAS 56-12-2), taurine (CAS 107-35-7) and ascorbic acid (CAS 50-81-7) were obtained from Sigma-Aldrich (Taufkirchen, Germany). 1M sucrose solutions (Südzucker AG, Mannheim, Germany) mixed with a single chemical were used as treatments. Identical 1M sucrose solutions were used as controls across all experiments. Chemical concentrations were chosen based on previous reports of their effects on Hymenoptera. When multiple concentrations were reported, intermediate ones were often used. Caffeine has shown neuroactive effects at a wide range of concentrations (Mustard 2014). Therefore, 1.29  $\mu\text{mol mL}^{-1}$ , a moderately high concentration, ten-fold the naturally occurring one, was used (Singaravelan et al. 2005; Mustard et al. 2012). Nicotine was used at 0.02  $\mu\text{mol mL}^{-1}$  (Thany and Gauthier 2005; Cammaerts et al. 2014b; Baracchi et al. 2017). 10.55  $\mu\text{mol mL}^{-1}$  of dopamine or octopamine were mixed with 9.94  $\mu\text{mol mL}^{-1}$  of ascorbic acid to reduce oxidation of the biogenic amines (Scheiner et al. 2002; Linn et al. 2020).  $\beta$ -Alanine, GABA and taurine were used at 0.27  $\mu\text{mol mL}^{-1}$ , 0.73  $\mu\text{mol mL}^{-1}$ , and 0.32  $\mu\text{mol mL}^{-1}$ , respectively (Carlesso et al. 2021). A double-blind procedure was applied to all solutions used to minimize experimenter bias.

### Y-maze experimental setup: Spatial learning

Y-mazes (three 10 cm long, 1 cm wide arms, tapering to 2 mm at the bifurcation) were used to assess the effects of each chemical on spatial memory and learning (Czaczkes 2018). Each donor colony was connected to a Y-maze via a drawbridge, both covered in unscented disposable paper overlays. A drop of sucrose solution (positive stimulus), either the control or the treatment, was placed at the end of one of the maze arms, and a drop of water (neutral stimulus) on the opposing arm. The first two ants willing to walk up the drawbridge were allowed onto the Y-maze and marked with differently colored acrylic paint while drinking the sucrose solution. Upon satiation, ants were not allowed back into their original donor colony. Rather, they were allowed to return to the paired recipient colony, where they offloaded the content of their crop. Meanwhile, the Y-maze paper overlays were replaced, to remove any pheromone trails left behind, and the solution drops reapplied to their original maze arm. Following trophallaxis,

**Table 1.** Overview of the effects of neuroactive chemicals on learning and memory in Hymenoptera.

	Chemical	Species	Effect		
Alkaloids	Caffeine (see <a href="#">Mustard 2014</a> for a review)	<i>Apis mellifera</i>	Increased foraging frequency and waggle dancing, quadrupling colony-level recruitment ( <a href="#">Ishay and Paniry 1979</a> ; <a href="#">Couvillon et al. 2015</a> ). Elicited feeding preference ( <a href="#">Singaravelan et al. 2005</a> ). Enhanced motivation and cognitive performance in complex learning tasks ( <a href="#">Si et al. 2005</a> ). Affects memory formation but not early long-term memory ( <a href="#">Mustard et al. 2012</a> ). Longer lasting olfactory memory associations which can last several days ( <a href="#">Wright et al. 2013</a> ). Increases learning performance. Memory retention increases when caffeine is mixed with arginine ( <a href="#">Marchi et al. 2021</a> ).		
		<i>Bombus impatiens</i>	Increased pollination of flowers offering moderate concentrations of caffeine in nectar ( <a href="#">Thomson et al. 2015</a> ). Interaction of octopamine and tyramine with caffeine eliminated aversion to caffeine while enhancing visitation rate ( <a href="#">Muth et al. 2022</a> ).		
		<i>Bombus terrestris</i>	Lowered overall food consumption ( <a href="#">Tiedeken et al. 2014</a> ). Short-lived decrease in handling times and improvement in odour associations ( <a href="#">Arnold et al. 2021</a> ).		
		<i>Vespa orientalis</i>	Enhanced motor activity, appetite for proteins and exaggerated response to optic and acoustic stimuli ( <a href="#">Ishay and Paniry 1979</a> ).		
		<i>Myrmica sabuleti</i>	Increased linear speed, conditioning ability, and memory. Decreased food consumption ( <a href="#">Cammaerts et al. 2014a</a> ).		
		Other ant species	Can act as a repellent or an attractant depending on the extract and concentration used ( <a href="#">Majid et al. 2018</a> ; <a href="#">Yeoh et al. 2018</a> ; <a href="#">Madsen and Offenberg 2019</a> ).		
		Biogenic amines	Nicotine	<i>Apis mellifera</i>	Elicited feeding preference ( <a href="#">Singaravelan et al. 2005</a> ). Increased sucrose sensitivity and improved olfactory learning retention ( <a href="#">Thany and Gauthier 2005</a> ). Partial repellent potentially enhancing cross-pollination ( <a href="#">Köhler et al. 2012</a> ).
				<i>Bombus terrestris</i>	Lowered overall food consumption ( <a href="#">Tiedeken et al. 2014</a> ). Enhanced memory for floral traits and reduced ability to reverse learn ( <a href="#">Baracchi et al. 2017</a> ).
				<i>Myrmica sabuleti</i>	Enhanced cognitive abilities and increased locomotion. Decreased food consumption ( <a href="#">Cammaerts et al. 2014b</a> ).
				<i>Apis mellifera</i>	Decreased sucrose responsiveness ( <a href="#">Scheiner et al. 2002</a> ). Blocking of dopaminergic receptors suppresses aversive learning ( <a href="#">Vergoz et al. 2007</a> ). Regulates motor behaviour ( <a href="#">Mustard et al. 2010</a> ). Reduced punishment perception ( <a href="#">Agarwal et al. 2011</a> ). Impairs appetitive memory consolidation ( <a href="#">Klappenbach et al. 2013</a> ). Increased likelihood of visiting training feeder ( <a href="#">Linn et al. 2020</a> ). Increased perceived value of sucrose solution and improved olfactory learning and memory retrieval ( <a href="#">Huang et al. 2022</a> ). Improved learning success and might regulate optimal motivational or attentional levels ( <a href="#">Raza et al. 2022</a> ).
	Dopamine (see <a href="#">Giurfa 2006</a> ; <a href="#">Verlinden 2018</a> for a review)	<i>Nasonia vitripennis</i>	Interferes with appetitive learning ( <a href="#">Lenschow et al. 2018</a> ).		
		<i>Lasius niger</i>	Linked to long-term memory consolidation, independent of short-term memory formation ( <a href="#">Wissink and Nehring 2021</a> ).		
	Octopamine (see <a href="#">Giurfa 2006</a> ; <a href="#">Farooqui 2012</a> for a review)	<i>Apis mellifera</i>	Increased sucrose responsiveness ( <a href="#">Scheiner et al. 2002</a> ). Reduced sucrose response thresholds ( <a href="#">Pankiw and Page 2003</a> ).		

Table 1. Continued

Chemical	Species	Effect	
Non-protein amino acids		Modulates the representation of floral rewards in dances by changing the processing of reward (Barron et al. 2007).	
		Shifted foragers to different resources, likely through altered reward representation (Giray et al. 2007).	
		Increased punishment perception (Agarwal et al. 2011).	
		Increased likelihood of scouting (Liang et al. 2012).	
		Increased use of private information (Linn et al. 2020).	
		Receptor antagonist disrupts appetitive learning (Lenschow et al. 2018).	
		Necessary for appetitive learning of olfactory cues (Wissink and Nehring 2021).	
	$\beta$ -Alanine (see Nepi 2014 for a review)	<i>Apis mellifera</i>	Improved associative learning and memory retention. If ingested prior to conditioning, it hinders learning but improves memory retention (Carlesso et al. 2021).
		<i>Bombus terrestris</i>	Higher walking index and lower feeding, flying and stationary indices (Bogo et al. 2019).
		<i>Vespa orientalis</i>	Inhibited nest construction behaviour (Bouchebti et al. 2022).
GABA (see Nepi 2014 for a review)	<i>Apis mellifera</i>	Regulated the specificity of associative olfactory memory (Hosier et al. 2000).	
		Exerted a modulatory role in memory formation depending on the training strength (Raccuglia and Mueller 2013).	
		Decreased activity levels (Mustard et al. 2020).	
		Improve associative learning and memory retention, but hinders learning if ingested prior to conditioning (Carlesso et al. 2021).	
		Lower flying index (Bogo et al. 2019).	
		<i>Bombus terrestris</i>	
		<i>Osmia bicornis</i>	Higher motor activity (Felicoli et al. 2018).
		<i>Vespa orientalis</i>	Inhibited nest construction (Bouchebti et al. 2022).
		<i>Oecophylla smaragdina</i>	Elicited preference (Madsen and Offenberg 2019).
		<i>Lasius niger</i>	Elicited preference (Madsen and Offenberg 2019)
Taurine (see Nepi 2014 for a review)	<i>Apis mellifera</i>	If ingested prior to conditioning hinders learning but improves memory retention (Carlesso et al. 2021).	

See Nepi et al. 2018; Mustard 2020 for reviews on the effects of nectar secondary metabolites on insect pollinators.

within 0-30 minutes since the end of the first visit, one of the two marked ants was allowed back onto the Y-maze. Its initial decision was recorded as the first maze arm in which it crossed a 2 cm reference line, and its final decision as the maze arm containing the drop it first touched. To account for a potential time-dependent effect of the neuroactive chemicals tested, the second marked ant was only allowed back onto the Y-maze 31–60 minutes after the end of the first visit. For the caffeine experiment, instead of two, five ants were initially marked. In this case, each ant's second visit occurred in increasing 30-minute intervals going up to over 120 minutes since the end of its first visit. This followed previous literature reporting delayed caffeine effects ranging between 30 and 120 minutes in honey bees (Mustard et al. 2012; Gong et al. 2021). From the second visit onwards, ants were allowed back onto the Y-maze as soon as possible. In total, each ant carried out five consecutive visits to the Y-maze: an initial one where it was marked and no data was collected, and four others where their choice was recorded. The treatment used, the Y-maze arm in which it was located and the elapsed time since the end of the first visit were randomly assigned to

each individual following a full factorial design. A total of 481 individuals were tested across seven experiments.

### Y-maze experimental setup: Olfactory learning

Y-mazes were also used to study the effects of caffeine on olfactory memory and learning. Scented paper overlays, used during testing, were stored in airtight plastic boxes (19.4 × 13.8 × 6.6 cm) containing an open glass petri-dish with 0.5 mL of either strawberry or apple food flavoring (Seeger, Springe, Germany) for at least a week prior to use. An individual ant from a donor colony was allowed onto a 10 cm linear runway covered by a scented paper overlay offering a sucrose solution drop (positive stimulus), either pure or laced with 1.29  $\mu\text{mol mL}^{-1}$  caffeine, at the end. The ant was marked while drinking and, upon satiation, was allowed to return to the paired recipient colony to offload its crop content. After unloading, the marked ant was allowed onto a Y-maze offering on one arm a paper overlay scented to match the odour experienced during training, and on the other arm the opposing odour (novel stimulus). The ants' initial and final choice was recorded as the first maze arm in which it crossed a 2 cm and an 8 cm reference line, respectively. The treatment used,

**Table 2.** Candidate model set and corresponding *a priori* hypothesis used for multimodel inference. All models used the proportion of ants choosing the rewarded side of the Y-maze as their final decision as the response variable and included data collection date, colony identity and ant identity as random effects. Additionally, spatial learning models included experimenter and colony starvation period as random effects.

	Model	Biological Hypothesis
Spatial	Null	Ants randomly choose a Y-maze arm.
	Visit	Learning improves over consecutive visits.
	Treatment	The neuroactive chemical interferes with learning.
	Reward Side	Ants have an intrinsic predisposition towards turning left or right.
	Elapsed Time	Recall strength, and therefore learning, is affected by the time memories had to consolidate.
	Treatment * Elapsed Time	The effects of the neuroactive chemical on learning are time dependent.
	Treatment * Visit	The neuroactive chemical interference varies with learning strength.
	Maximal	All the variables of interest contribute towards learning.
Olfactory	Null	Ants randomly choose a Y-maze arm.
	Treatment	The neuroactive chemical interferes with learning.
	Reward Side	Ants have an intrinsic predisposition towards turning left or right.
	Odour	Ants have an innate preference towards specific odours.
	Treatment * Odour	The neuroactive chemical might affect the ant's perception of the odour.
	Maximal	All the variables of interest contribute towards learning.

the Y-maze arm in which it was located, and the odor associated with the reward were randomly assigned to each individual following a full factorial design, testing 96 individuals.

### Statistical analysis

The complete statistical analysis output for all experiments, and the entire dataset on which this analysis is based, is available from Zenodo (<https://doi.org/10.5281/zenodo.7268444>).

All graphics and statistical analysis were generated using R version 4.2.1 (R Core Team 2022). Data wrangling used the `reshape2` (Wickham 2007) package and graphics were created using the `ggplot2` (Wickham 2016) package. Analysis was conducted by multi-model inference following an information theory approach (Anderson 2008). Generalised linear mixed models were fit using the `lme4` (Bates et al. 2015) package with binomial error distributions and estimated marginal means and contrasts were obtained using the `emmeans` package (Lenth 2022) with Bonferroni adjusted values accounting for multiple testing. An *a priori* set of hypotheses, and matching candidate models, was developed for each experiment (Table 2). The DHARMA (Hartig 2022) package was used to inspect the global model in each set, from which all other models can be derived, assessing model fit and ensuring model assumptions were met (Burnham and Anderson 2002). Conditional coefficients of determination, a measure of goodness of fit, were calculated for each model using the `MuMIn` (Bartoń 2022) package. The `AICcmodavg` (Mazerolle 2020) package was used to calculate Akaike's information criterion, adjusted for small sample sizes (AICc), and Akaike weights ( $w_i$ ) for each model. Model-averaged parameter estimates, standard errors and confidence intervals were then computed as a weighted mean of the set of candidate models. We avoid the use of p-values, instead reporting effect size estimates

and their respective 95% confidence intervals (Greenland et al. 2016) shown throughout the results section as (estimate [lower limit, upper limit],  $N$  = sample size).

### Results

Binomial generalised linear models were used to check for differences between the proportion of ants choosing the rewarded side of the Y-maze as their initial versus their final decision for each experiment (conditional  $R^2$  range of 20–69%,  $N = 8$ ). Post-hoc estimated marginal means, with Bonferroni-adjusted significance levels, based on each spatial learning model revealed small differences between initial and final decision (0.3–7.1%,  $N = 7$ ). However, the same method applied to the olfactory learning experiment revealed a relatively large difference between initial (76.5% [47.2%, 92.2%],  $N = 96$ ) and final decision (93.1% [69.7%, 98.8%],  $N = 96$ ). Across experiments, the proportion of ants choosing the rewarded side of the maze as their final decision was always higher than that of ants doing so as their initial decision. This suggests that ants often realised that they had entered the unrewarded maze arm and corrected their decision. Such corrections imply ants recall the location of the reward and are likely learning. As our aim was to explore the effects of different neuroactive chemicals on learning all statistical analysis used final decision as the response variable.

#### Ants learn to associate a reward with a scent and with a side of a Y-maze

All candidate models (Table 2) were fit using generalised linear models with binomial error distributions for each experiment. The conditional  $R^2$ , a measure of goodness of fit, for the model which explains the most variance in the data for

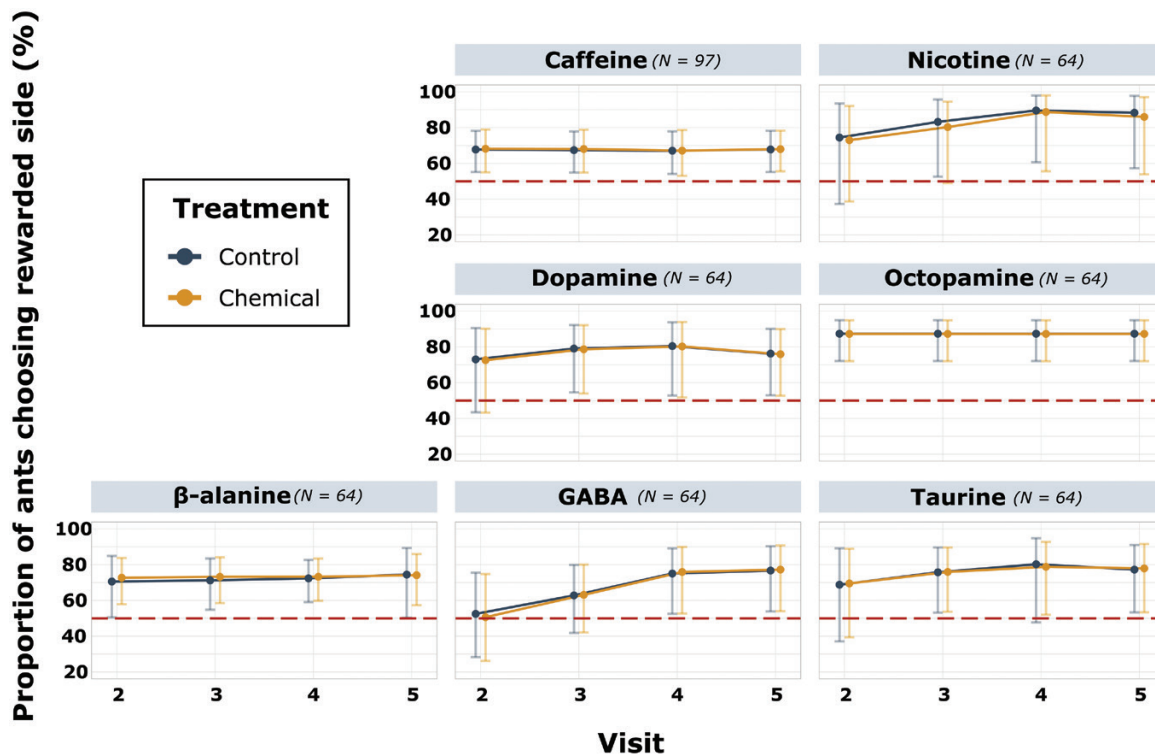
each set of candidate models is reported in Figure 2 (conditional  $R^2$  range of 12–43%,  $N = 8$ ). Estimated marginal means, with Bonferroni-adjusted significance levels, averaged over the treatments used and the side of the maze in which the reward was located, show that ants can associate both the apple (80.0% [63.0%, 90.4%],  $N = 48$ ) and strawberry (78.4% [63.0%, 88.5%],  $N = 48$ ) scents with the presence of a sucrose reward after a single training visit. Similarly, Figure 1 shows that ants can associate the presence of a reward with a side of a Y-maze and that learning tends to increase over consecutive visits. It is worth noting that for the octopamine experiment, ants had a significant innate side bias towards turning left. This same trend was seen across all experiments, although for all others it was not statistically significant (see ESM1).

### None of the chemicals tested influenced learning

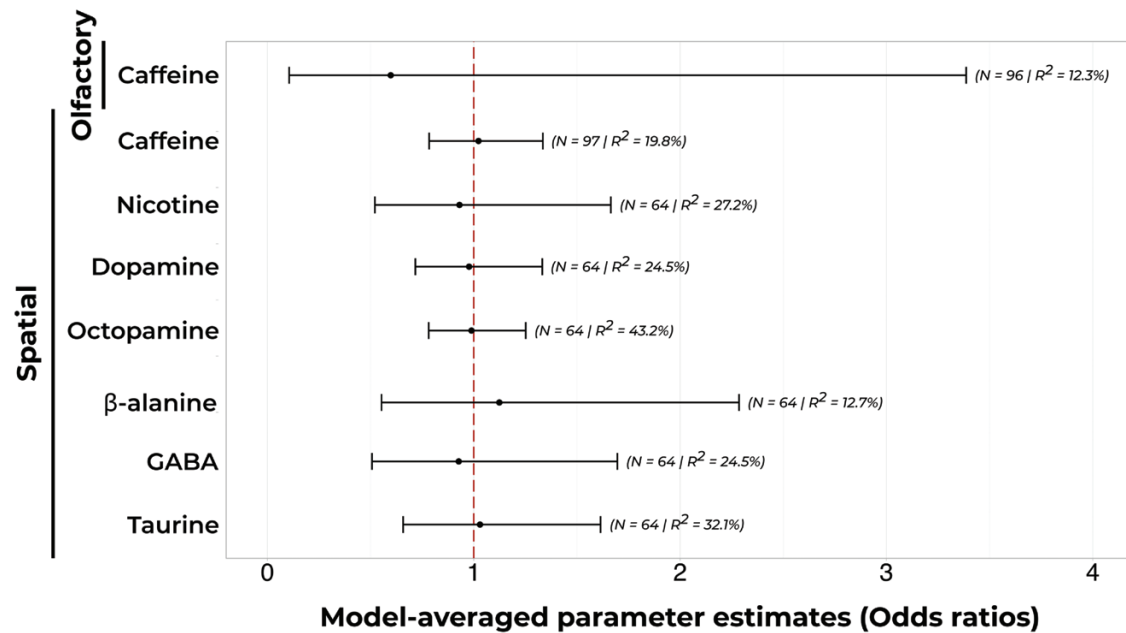
Parameter estimates for each experiment were obtained from model-averaging with shrinkage as odds ratios. Odds are the probability of an event occurring divided by the probability of the event not occurring. Odds ratios compare two odds, testing how the relationship between these two odds change given different conditions. Figure 2 shows the estimated odds ratios for each experiment comparing the odds of an ant under the influence of each chemical choosing the rewarded side of the Y-maze against the odds of an ant

under the influence of the respective control treatment doing so, if all other variables are kept constant. Odds ratios of 1 indicate no difference between the treatment and its control, whilst odds ratios  $> 1$  or  $< 1$  indicate that ants are more or less likely, respectively, to choose the rewarded side of the Y-maze under the influence of the neuroactive chemical when compared to the control. Our results suggest that none of the chemicals used interferes with *L. humile* associative learning.

Additionally, we collected data regarding the time each ant took from entering the Y-maze until it reached the reward (“In Duration”) and the elapsed time since each ant finished drinking the reward until it reached the entrance of the maze (“Out Duration”). Since the Y-maze represents a relatively short and straightforward distance, it is hard to detect small variations between treatments. Nevertheless, we performed a simple survival analysis, computing the probability of each ant reaching the reward or the nest, at specific points in time using Cox proportional-hazards models (see ESM1 for detailed analysis and figures). Ants treated with  $\beta$ -alanine (15.9s [6s, 465s],  $N = 128$ ) returned to the nest around 10 seconds faster (−42.9% [−11.1%, −83.9%],  $N = 256$ ) than control treated ants (24.5s [5s, 375s],  $N = 128$ ). Interestingly,  $\beta$ -alanine treated ants were on average 22.2s faster than control ones when returning to the nest after their fourth visit to the Y-maze but only 2.2–6.4s faster on other visits. Furthermore, albeit the confidence intervals cross 0%, dopamine (21.4% [−1.0%, 38.9%],  $N = 256$ )



**Figure 1.** Ants learn to associate a sucrose reward with an arm of a Y-maze over consecutive visits across experiments and treatments. Circles represent the proportion of ants choosing the rewarded side of the maze as their final choice and whiskers the respective 95% unconditional confidence intervals for each treatment. Estimates for each experiment were obtained from model-averaging with shrinkage and estimated marginal means were averaged over the side of the maze in which the reward was located and the elapsed time since the end of the first visit. This was done as the confidence intervals for the model averaged odds ratios for both reward side and elapsed time crossed 1, suggesting small differences between the categorical levels of these variables. The exception to this, with an odds ratio of 0.2 [0.07, 0.89], being the octopamine experiment which showed a relatively large side bias towards the left ( $L = 93\%$ ,  $R = 77\%$ ). However, since even ants with the reward on the right were able to learn the association, we average both sides. If the confidence intervals of each estimate include 50% (red dashed horizontal line), ants are considered to choose an arm of the Y-maze at random and therefore likely did not learn. Significance levels were adjusted using Bonferroni correction for multiple testing.



**Figure 2.** Effect of seven potential neuroactive chemicals on the olfactory and spatial associative learning of *L. humile*. Circles represent the estimates obtained from model-averaging with shrinkage and whiskers the 95% unconditional confidence intervals. The odds ratio compares the odds (probability of an event occurring divided by the probability of the event not occurring) of the ants choosing the rewarded side of the Y-maze under the influence of each neuroactive chemical against those of the corresponding control treatment. Odds ratios of 1 (red dashed vertical line) indicate no difference between the treatment and its control, whilst odds ratios  $> 1$  or  $< 1$  indicate that ants are more or less likely, respectively, to choose the rewarded side of the Y-maze under the influence of the neuroactive chemical when compared to the control. If the 95% confidence intervals include an odds ratio of 1 there is no significant difference between treatment and control.  $R^2$  refers to the goodness of fit of the model which explains the most variance in the data for each set of candidate models.

and octopamine (18.8% [−4.0%, 36.7%],  $N = 256$ ) seem to increase the time ants take to reach the reward. Control ants take around 20s to reach the reward (dopamine: 22.3s [7s, 132s],  $N = 127$ ; octopamine: 20.2s [7s, 187s],  $N = 128$ ) whilst treated ants take around 30s to reach the same destination (dopamine: 32.4s [7s, 307s],  $N = 127$ ; octopamine: 28.6s [6s, 431s],  $N = 128$ ). In this case, the fifth visit to the Y-maze seems to be the main driver of the effect with dopamine treated ants taking 34.5s longer to reach the reward and octopamine treated ones taking 20.1s longer, when compared to their respective controls. Throughout other visits, the effect is considerably smaller (dopamine: 1.6–4.8s; octopamine: 0.7–7.1s).

## Discussion

*Linepithema humile* are incredibly effective associative learners (Rossi et al. 2020; Wagner et al. 2022). Here, we show that a single training visit to a Y-maze is often enough for ants to develop a spatial association between the presence of a reward and an arm of the maze. Ants often correct their initial decision, further suggesting they are in fact learning. Over consecutive visits, the proportion of ants choosing the rewarded side of the maze increases until it plateaus, with three and four training visits showing similarly strong learning. Furthermore, after a single training visit, *L. humile* show an extremely strong preference for the scent they were trained with over a novel one. These results support previous work suggesting ants require as little as one experience to form a memory retaining it for up to three days (Dupuy et al. 2006; Josens et al. 2009; Huber and Knaden 2018; Oberhauser et al. 2019; Piqueret et al. 2019; Czaczkes and Kumar 2020).

Furthermore, across all experiments, ants seem to have an innate preference towards turning left, even if in most cases this does not hinder learning. Such preference is likely linked to brain lateralisation with a preference towards the left being shown in ants previously (Hunt et al. 2014).

None of the seven potential neuroactive chemicals tested showed a significant effect on spatial learning, with caffeine also not influencing olfactory associative learning. This is in contrast to the extensive literature on the effects of these chemicals on Hymenoptera (Table 1). Honeybees prefer sucrose solutions laced with up to  $0.52\mu\text{mol mL}^{-1}$  caffeine (Singaravelan et al. 2005) with topically delivered caffeine improving both motivation and cognitive performance of complex learning tasks at vastly greater concentrations (Si et al. 2005). Similarly,  $5.15\mu\text{mol mL}^{-1}$  caffeine was reported to increase conditioning ability and memory in ants (Cammaerts et al. 2014a). However, due to a positive publication bias (Nissen et al. 2016; Mlinarić et al. 2017), it is extremely hard to find null results to contextualise our findings. As an example, two unpublished Master's theses have studied the chronic effects of caffeine on honeybee learning, and both suggest a general lack of effect on learning performance (Malechuk 2009; Yusaf 2012).

The lack of effect we found in this study does not rule out these chemicals as influencing spatial learning in ants (see Box 1). Although the chemical concentrations used were chosen based on previous literature showing their effects on Hymenoptera, it could be that we missed the concentration at which they influence learning and memory. For instance, unnaturally high concentrations of nicotine deterred bumblebees, but lower nectar-relevant concentrations lead to attraction (Baracchi et al. 2017). Furthermore, it is likely that the

### Box 1. Future directions

Neuroactive chemicals are likely to influence learning and memory in ants. However, our work suggests that such effects might not manifest over short time periods. Thus, steering ant preference with neuroactive chemicals might not be ideally suited to application in pest control. Nevertheless, understanding how these chemicals influence learning and memory still offers significant mechanistic insights into the insect brain. Here, we propose some potential avenues of exploration which we think would be of particular interest:

- Focusing on olfactory learning, which is thought to take place in the acetylcholine receptor-rich antennal lobes and mushroom bodies.
- Using lower sucrose concentrations would reduce motivation, in theory decreasing learning speed or quality, which could help studying subtle effects induced by the chemicals – especially in the face of ceiling effects caused by excellent olfactory learning.
- Using different, more complex tasks, such as reversal learning or navigation in an open field (Galante et al. In prep.) would require more neural pathways to be activated and therefore could help expose effects induced by the chemicals.
- Testing learning, but also its extinction, could provide insights into how these chemicals impact long-term memory formation, consolidation, and retention.
- Using different concentrations and combinations of various nectar secondary metabolites seems to be promising – for example combining caffeine with arginine or octopamine and tyramine (Marchi et al. 2021; Muth et al. 2022).
- $\beta$ -alanine is a promising chemical for further testing, as it caused a small but significant (around 10 seconds) reduction in return time to the nest.
- Neuroactive chemicals could have an effect on other aspects of foraging, such as recruitment, by for example, affecting how individuals perceive pheromones.

effects of the neuroactive chemicals used are time-dependent, and therefore this study could have missed the chemical activation window. In fact, honeybees fed  $1.04 \mu\text{mol mL}^{-1}$  caffeine were more likely to remember a conditioned scent than the respective control at both 24 and 72 hours after conditioning, but not 10 minutes after conditioning (Wright et al. 2013). Nevertheless, honeybees fed  $0.05 \mu\text{mol mL}^{-1}$  and  $0.51 \mu\text{mol mL}^{-1}$  caffeine showed stronger memory retention at two and 24 hours post-treatment, with more recent treatment resulting in stronger recall (Gong et al. 2021). Similarly, high concentrations of caffeine ( $>10.32 \mu\text{mol mL}^{-1}$ ) lead to a significant decrease in memory retention five minutes post-treatment (Mustard et al. 2012). Here, we specifically focussed on short time frames, as we were exploring the potential for neuroactive chemicals to improve bait consumption in the field. Baiting is, however, both costly and time sensitive, with modern hydrogel bead delivery systems desiccating quickly, lasting up to two hours (Cabrera et al. 2021). For this reason, we focused on short-term effects with an activation window of up to two hours.

There is a significant literature bias toward bees as model organisms, often using the proboscis extension response (PER) paradigm and focusing on olfactory associative

learning. It could thus be that the contrast between our results and much of the published literature stems from species specific differences and/or methodological ones. It is possible that the chemicals studied target specific neurological pathways that are activated during PER experiments, but not during the ones we conducted. A wide range of acute doses of caffeine has been shown to affect learning but not memory in honeybees (Mustard et al. 2012). In addition, caffeine improved long-term memory, but does not seem to affect short-term memory (Wright et al. 2013). This suggests neuroactive chemicals have high specificity and therefore it is likely that different tasks are disrupted differently. In fact, caffeine and nicotine target acetylcholine receptors (AChR) which are abundant in the antennal lobes and mushroom bodies, the same areas thought to be responsible for appetitive olfactory learning in bees (MaBouDi et al. 2017; Mustard 2020). Contrastingly, spatial learning is thought to mainly occur at the level of the central complex (Ofstad et al. 2011), which might have less AChR expressed and might therefore remain unaffected by chemicals that target it. Nevertheless, work on fruit flies and grasshoppers suggests an overlapping presence of acetylcholine and GABA in these regions (Pfeiffer and Homberg 2014). Acetylcholine is an important neurotransmitter, likely linked to learning and memory in invertebrates. Since most of the chemicals tested interact with cholinergic neurons, it could be that, in ants, expression of AChR in the central complex is not as strong as in other invertebrates, or alternatively that acetylcholine is not the main driver of learning and memory in this group. The lack of an effect on olfactory learning in the current study could also be due to a ceiling effect: we replicate the previous finding that there is little to no room for improvement when it comes to olfactory associative learning in *L. humile* ants (Wagner et al. 2022). Thus, even if caffeine does improve olfactory learning in these ants, it would be hard for such an effect to be visible due to their already excellent natural learning.

Even though none of the chemicals tested showed an effect on learning and memory, we cannot exclude the possibility that these might interfere with foraging motivation through preference manipulation. Recently, sub-lethal doses of the neonicotinoid imidacloprid were shown to shift colony-level preference in the invasive ant *Lasius neglectus* (Frizzi et al. 2022). At the individual-level, orally administered serotonin decreases the amount of food ingested by treated ants (Falibene et al. 2012) whilst its antagonist, ketanserin, increases consumption (Josens et al. 2021). Furthermore, low doses of topically applied cocaine have been suggested to cause foraging bees to overestimate the value of floral resources, increasing sucrose responsiveness (Barron et al. 2009). However, even if seemingly promising, chemicals such as neonicotinoids and cocaine are unsuitable for pest control. Such chemicals are expensive, hard to procure and, in the case of pesticides, ecologically damaging. In fact, this is one of the main reasons we focused our efforts on affordable, naturally occurring chemicals.

Finally, recent studies suggest that, like in plants, a combination of different neuroactive chemicals might be key toward manipulating behavior. Honeybees fed  $0.05 \mu\text{mol mL}^{-1}$  or  $0.16 \mu\text{mol mL}^{-1}$  of caffeine showed improved learning performance, but no change in memory retention unless caffeine was mixed with arginine (Marchi et al. 2021). Moreover, octopamine and tyramine mixed with caffeine



altered bumblebee behavior, but not when present individually (Muth et al. 2022). However, considering the infinite possible combinations of chemicals at different concentrations, it seems that using neuroactive chemicals to artificially manipulate ant behavior might not be straightforward. Nevertheless, many promising avenues of research remain unexplored (see Box 1). Understanding how neuroactive chemicals influence learning and memory still offers significant mechanistic insights which could be leveraged towards improving invasive ant control.

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## Conflict of interest

The authors declare no conflict of interest related to this work.

## Ethical statement

We have conducted all experiments in accordance with the guidelines that are applicable to working with the model organism in the European Union. Colonies were kept in closed boxes under oil baths in order to prevent any escape.

## Author contributions

Conceptualization: H. G., T. J. C. Methodology: H. G., T. J. C. Software: H. G. Validation: H. G., T. J. C. Formal analysis: H. G. Investigation: H. G. Resources: T. J. C. Writing - original draft: H. G. Writing - review & editing: H. G., T. J. C. Visualization: H. G. Supervision: T. J. C. Project administration: T. J. C. Funding acquisition: T. J. C.

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