A high-throughput and sensitive method for food preference assays in walking insects

Thomas Wagner[1,*], Henrique Galante[1] and Tomer J. Czaczkes[1]

[1] Animal Comparative Economics Laboratory, Faculty of Biology and Preclinical Medicine, University of Regensburg, 93053 Regensburg, Germany

* Corresponding Author: t.wagner.science@gmail.com

Abstract

Insects pose significant challenges in both pest management and ecological conservation. Often, the most effective strategy is employing toxicant-laced baits, which must also be designed to specifically attract and be preferred by the targeted species for optimal species-specific effectiveness. However, traditional methods for measuring bait preference are either non-comparative, meaning that most animals only ever taste one bait, or suffer from methodological or conceptual limitations. Here we demonstrate the value of direct comparison food preference assays using the invasive and pest ant *Linepithema humile* as a model. We compare the food preference sensitivity of non-comparative (one visit to a food source) and sequential comparative (visiting one type of food then another) assays at detecting low levels of aversive quinine in sucrose solution. We then introduce and test a novel dual-choice feeder method for simultaneous comparative evaluation of bait preferences, testing its effectiveness in discerning between foods with varying quinine or sucrose levels. While the non-sequential assay could not detect aversion to 1.25mM quinine in 1M sucrose, the sequential comparative approach detected aversion to quinine levels as low as 0.94mM. The novel dual feeder method approach could detect aversion to quinine levels as low as 0.31mM, and also preference for 1M sucrose over 0.75M sucrose. The dual-feeder method, combines the sensitivity of comparative evaluation with high throughput, ease of use, and avoidance of interpretational issues. This innovative approach offers a promising tool for rapid and effective testing of bait solutions, contributing to the development of targeted control strategies. Moreover, the method can be easily modified for application to a wide range of walking insects, such as cockroaches, crickets, and beetles.

Keywords

bait evaluation – pest control – *Linepithema humile* – bitterness perception – bait aversion – bait attractiveness – invasive ants

Introduction

Ants are a major control concern: Pest ants pose significant economic threats due to their ability to infest agricultural crops, damage property, contaminate healthcare facilities, and contaminate stored food (Pereira and Stimac 1997; Josens et al. 2014). Invasive ants threaten biodiversity, disrupting ecosystem processes, and can become significant ecological problems themselves (Wetterer et al. 2001; Angulo et al. 2022).

One of the best current approaches for eradication or control of ants is to use baits with a slow-acting toxicant. This method often surpasses traditional spraying because it targets the entire colony, including the queen and brood, by allowing ant workers to carry the toxicant into the nest (Klotz et al. 2007; Hoffmann et al. 2016). Baits have been shown to be effective both in the field and in buildings (Hays and Arant 1960; Osae et al. 2011; Josens et al. 2017; Choe et al. 2021) .

However, controlling ants poses considerable challenges: two-thirds of eradication efforts targeting invasive ants have proven unsuccessful (Hoffmann et al. 2016). Alongside bait active bait abandonment or aversion (Wagner and Czaczkes 2024; Zanola et al. 2024) a significant challenge lies in the availability of high-quality natural food sources, which ants often prefer, resulting in inconsistent bait consumption (Rust et al. 2003; Silverman and Brightwell 2008). To effectively address this challenge, it is crucial to understanding the feeding preferences of ants. This information is vital for designing baits that effectively attract, and are consumed by, ants - particularly when they contain various ingredients such as toxicants, bittering agents, co-formulants, and attractors. If the bait, its additives, or its co-formulants do not align with the ants' preferences, it may be rejected, or dispreferred compared to other available food sources. Testing the acceptance of baits and the various co-formulants they contain is thus crucial.

Commonly used methods for determining food preference include presenting foods and baits simultaneously, known as cafeteria tests, or sequentially offering solutions, often associated with odour stimuli. Preference testing is then conducted by measuring the number of ants attracted to various food sources or their associated odours, or by quantifying the amount of food consumed (Hoey-Chamberlain and Rust 2014).

Cafeteria-style experiments serve as valuable tools for understanding preferences in various species. For example, in *Apis mellifera*, such experiments have revealed a preference

for a sucrose-ethanol solution over a pure sucrose solution in dual-choice feeding situations (Mustard et al. 2019). Similarly, ants have been observed favouring basic sugars combined with attractants in such experiments (Madsen et al. 2017), along with showing a preference for collecting food they lack more (Kaspari and Yanoviak 2001). Cafeteria-style experiments also shed light on how these preferences can be influenced by seasonal variations (Rust et al. 2000; Nondillo et al. 2014). In more applied settings, this approach is used to explore preferences for liquid or gel baits, as well as for wet or dry bait attractants (Baker et al. 1985; Nyamukondiwa and Addison 2014), or investigate bait particle size preference (Hooper-bùi et al. 2002).

The other commonly used method for testing ant preference is the sequential-choice test, in which individual ants are presented with a series of choices sequentially to determine their preferences, either directly or by associating each option with an odour. For instance, sequential-choice tests have demonstrated that ants exhibit a preference for the first odour encountered (Oberhauser et al. 2022), food sources without other nestmates around them (Wendt et al. 2020), or closer food sources (De Agrò et al. 2022). Preference can be influenced by various factors such as personal expectation, experience, social learning, and the timing of stimulus exposure (Rust et al. 2000; Grüter et al. 2011; Sasaki and Pratt 2013; Wendt et al. 2019; Oberhauser et al. 2022). Importantly, evaluation by comparison, where an individual's evaluation of a food source depends on how it compares to other options, interacts with the baseline evaluation of the food to shape feeding decisions.

To investigate comparative valuation, animals are usually trained to expect a certain quality or quantity of reward, which is then abruptly increased (positive incentive contrast) or decreased (negative incentive contrast) (Bitterman 1976; Couvillon and Bitterman 1984; Waldron et al. 2005; Kessler et al. 2015; Wendt et al. 2019; Daniel 2020). For instance, in a sequential preference test assay, *Lasius niger* ants were observed to value a sucrose solution relative to a previously experienced sucrose solution (Wendt et al. 2019): During training, ants encountered sucrose solution droplets of either low or high concentration. Then, during testing, all ants encountered a sucrose solution of medium concentration. Ants expecting low-quality food exhibited greater acceptance of medium-quality food, while those expecting high-quality food showed the opposite pattern. Further experiments suggested that these contrast effects stem from cognitive rather than mere sensory factors. Food

received within the nest from foraging ants influenced the perceived value of food of the ants receiving the food when they subsequently went out to forage (Wendt et al. 2019). These findings underscore the importance of comparative evaluation in decision-making processes. When it comes to preference testing and the development of novel baiting strategies, it is essential to address the limitations of traditional methods. For instance, the common practice of marking individuals (Couvillon and Bitterman 1984; Andrew et al. 2014; Wendt et al. 2019), required in order to test the trained individual ant in a sequential setup, can unintentionally induce stress and subsequently alter their preference. Preference can also be altered depending on which choice was presented first, or if the presented choice had an unexpected flavour (Oberhauser and Czaczkes 2018; Oberhauser et al. 2022). Presenting different choices simultaneously, but in different locations (as in cafeteria tests), can be vulnerable to side and location biases. Most critically, however, the cafeteria test approach prevents animals from making direct comparisons in real-time, thus potentially reducing their sensitivity. In response to these challenges, we have developed a new non-disruptive solution: the dual-choice feeder.

Here, we first demonstrate that comparative evaluation tests are much more sensitive than non-comparative tests for detection of a distasteful additive (quinine) to food. We then undertake a comparison between conventional sequential preference tests and our new dual-feeder method, and test the ability of the dual-feeder method to differentiate foods with added distasteful substances (quinine), and foods with lower levels of attractive substances (sucrose).

Materials & Methods

Colony Fragments Maintenance

Linepithema humile (Mayr, 1868) were collected from Girona, Spain in April 2021. Ants were split into colonies, each consisting of one or more queens and 300 to 1000 workers. The colonies were housed in plastic foraging boxes (32.5 cm x 22.2 cm x 11.4 cm), with plaster of Paris on the bottom and PTFE-coated walls to prevent escape. Each box contained several 15 ml plastic tubes covered with red transparent plastic slide, partially filled with water, and plugged with cotton, acting as nests.

Colonies were maintained under a 12:12 light:dark cycle at room temperature (21-25°C). Ants had continuous access to water via both the plugged tubes and a water feeder. Colonies were offered *ad libitum* 0.5M sucrose solution and freeze-killed *Drosophila melanogaster*, but deprived of food for 4 days prior to testing.

Experiment 1 – Quantifying food acceptance using the established sequential testing method

Ants and bees reduce their acceptance of a normally acceptable food source if they have previous recent experience with a better food source (Wendt et al. 2019; Daniel 2020). Thus, ants with a set expectation of highly acceptable food may be more sensitive in their response to less-acceptable food. The aim of this experiment was to test if ants with an expectation for pure sucrose solution show a reduced acceptance of solutions with small amounts of quinine. This is important, as before the dual-feeder can be tested and evaluated, an established method for testing comparative food acceptance must be demonstrated. This approach relies on comparing two groups of ants: naïve ants, and ants with recent previous experience of a comparator food source.

To quantify food acceptance by naive ants, a single ant was allowed access to a paper-covered 1x10m linear runway via a drawbridge, following Wendt et al. (2019). The ant encounters a drop of test solution at the end of the runway, and its behaviour was quantified. Acceptance was defined as sustained contact between the ant's mandibles and the sugar droplet within ten seconds. Furthermore, we noted whether the ants' abdomen was visibly expanded after 90 seconds, an indication of food ingestion. This specific time was chosen based on previous data, which indicated that the average drinking time for *L. humile*

ants of 1M sucrose is around 90 seconds (Wagner et al., unpublished data). Once an ant had been tested, it was removed from the platform and separated from the colony. Subsequently, both the paper cover and the sample solution were replaced. Various solutions with different sucrose-to-quinine concentration ratios were tested, including a control solution with 1M sucrose, and solutions with decreasing quinine concentrations (1.25mM quinine + 1M sucrose, 0.94mM quinine + 1M sucrose, 0.625mM quinine + 1M sucrose, and 0.3125mM quinine + 1M sucrose). These solutions were dispensed as a single droplet onto the end of the straight runway. In total, 143 ants from seven different colonies were tested over the five test treatments.

To qualify the acceptance of experienced ants, individual ants stemming from a 'donor' colony (home colony) were given access to a pure 1M sucrose droplet at the end of the straight runway. While drinking, the ant was marked with a dot of paint on the abdomen and was allowed to return to a 'recipient' colony nest (related colony). This was done to ensure that food was not introduced to the 'donor' colony, thus potentially changing the expectation of the ants which would be tested next. While the ant was in the recipient nest, the pure sucrose droplet was replaced with one of the sucrose/quinine solutions. The ant subsequently re-entered the straight runway via the drawbridge and was allowed to taste and feed on the test solution. In total, 199 ants from five different colonies were tested.

Experiment 2 – Quantifying ant acceptance using the dual-feeder method

The goal of this experiment was to validate the findings of the initial experiment while demonstrating the benefits of this novel dual-feeder method.

We developed a new feeder design, which was 3D-printed using a stereolithography resin printer (Prusa SL1, Prague). The feeder consists of two triangular wells which narrow into two parallel 0.35mm-wide channels, directing liquids to their tips through capillary action. The channels are separated by a narrow (0.30mm) gap (see Figure 1). This ensures that, regardless of which solution is contacted first, the ant will almost immediately come into contact with the alternative solution by touching it with its antennae, enabling an informed choice between the two solutions. Access to the feeder area is via a narrow bridge, ensuring that the ant is funnelled to the centre of the dual-feeder. Access to the dual-feeder is via a larger 'landing platform' – a raised platform surrounded by a water moat, and

covered by a linear paper runway 1 x 10cm, tapering to 2mm at the tip. Ants are allowed to walk onto a piece of paper in the nest, and then allowed to walk off the paper onto the landing platform paper overlay. From there, the ant walks without interference until it reaches the dual-feeder.

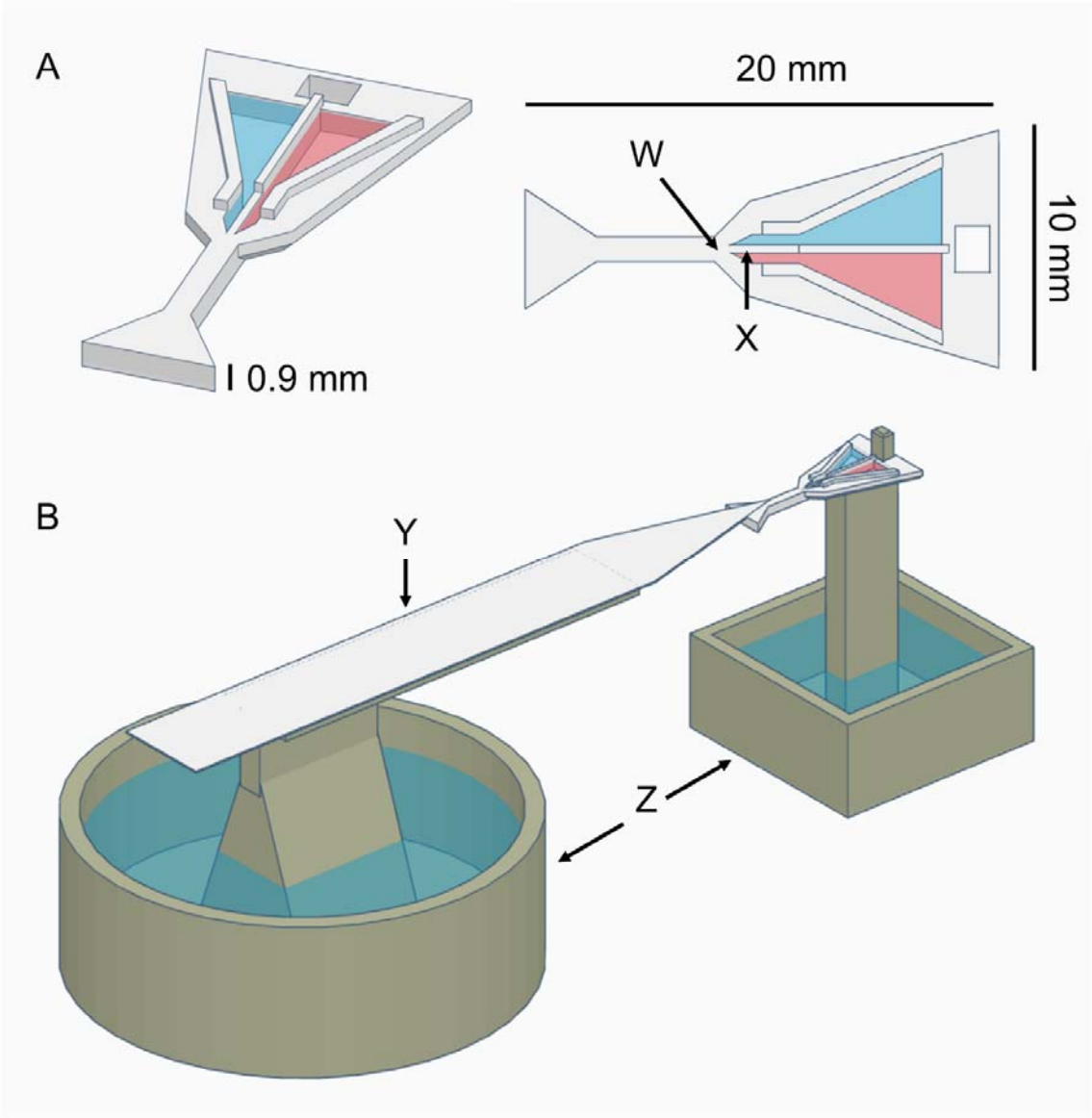

Figure 1: A: 3D model of the Dual-Feeder, W: Optimal drinking position, X: 0.30mm gap separation between the two wells containing solution 1 and 2 (blue and red coloured), D: Whole experimental setup display showing A connected with the landing platform Y and attached to a pillar Z, surrounded by water in a sink to prevent ants from escaping. 3D-model files for all printed parts are provided from https://doi.org/10.5281/zenodo.10953784.

Avoidance of quinine-containing solutions

Each well in the dual-feeder contained a choice between a comparator (here 1M sucrose solution) and a test sucrose-quinine solution (or a control), simultaneously presented, with variations in quinine concentration: 0.63mM quinine in 1M sucrose, 0.31mM quinine in 1M sucrose and 0.16mM quinine in 1M sucrose. The side containing the test solution was systematically varied between ants. Ants encounter one of the drinking channels first either with their mandibles or antennae and begin drinking. The initially encountered side and drinking time for each solution were recorded. In total, 179 ants from eight different colonies were tested.

Preference for higher molarity sucrose solution

Here, test solutions were of equal or lower sucrose concentration to the comparator, and contained no quinine. The following comparisons were tested: 1M vs 0.5M, 0.75M vs 0.5M, and 1M vs 1M. In total, 422 ants from nine different colony fragments were tested.

Statistical analysis

The complete statistical analysis output, and the entire dataset on which this analysis is based, is available from https://doi.org/10.5281/zenodo.10953784.

All graphics and statistical analysis were generated using R version 4.2.2 (R Core Team 2022) and ggplot2 (Wickham 2016).

Sequential test data (Experiment 1) was analysed using binomial generalized linear mixed-effect models (GLMM) (Bates et al. 2018). DHARMa (Hartig 2021) was used to assess linear model assumptions and MuMIn (Bartoń 2023) to obtain a measure of goodness of fit. Dual-feeder proportion data (Experiment 2) was analysed using beta regressions (Cribari-Neto and Zeileis 2010). Analysis of variance tables were used to test the effects of regression coefficients (Fox and Weisberg). Estimated marginal means and contrasts were obtained using the emmeans package (Lenth et al. 2024) with Bonferroni adjusted values accounting for multiple testing.

Results

Experiment 1 – Quantifying food acceptance using the established sequential testing method

Bitterness acceptance comparison between naive ants and experienced ants

We compared the acceptance rates of naive ants, which were exclusively exposed to the sucrose-quinine mix, with experienced ants, which first encountered a pure sucrose solution before being presented with the sucrose-quinine mix. In terms of initial (first 10 second) acceptance scores, experienced ants showed significantly lower food acceptance than naive ants, showing a 70% lower acceptance for 1.25mM quinine (GLMM: $z = 4.93$, $p < 0.0001$) and a 54% lower acceptance for 0.94mM quinine (GLMM: $z = 3.66$, $p = 0.0012$) (see Figure 2). However, no difference in acceptance between naive and experienced ants was observed in response to 0.63mM or 0.31mM quinine (17% and 10%. GLMM: $z = 1.78$, $p = 0.3734$ and $z = 0.10$, $p = 1$, respectively). Likewise, there was no difference in a 10 second acceptance response to pure 1M sucrose between naive and experienced ants (12%. GLMM: z-ratio = 0.27, $p = 1$). In contrast to the 10 second acceptance results, no difference in the 90 seconds acceptance score (consumption) was found between the groups for any of the quinine concentrations (22.5%, 30%, 9% and 7%. GLMM: 1M sucrose + 1.25mM quinine, $z = 2.11$, $p = 0.1755$; 1M sucrose + 0.94mM/0.63mM/0.31mM quinine, $z = 0.09$, $z = 1.23$, z-ratio = 0.08, $p = 1$ for all and pure 1M, 2%, $z = 0.58$, $p = 1$). On average, 5 ants were tested by one experimenter per hour.

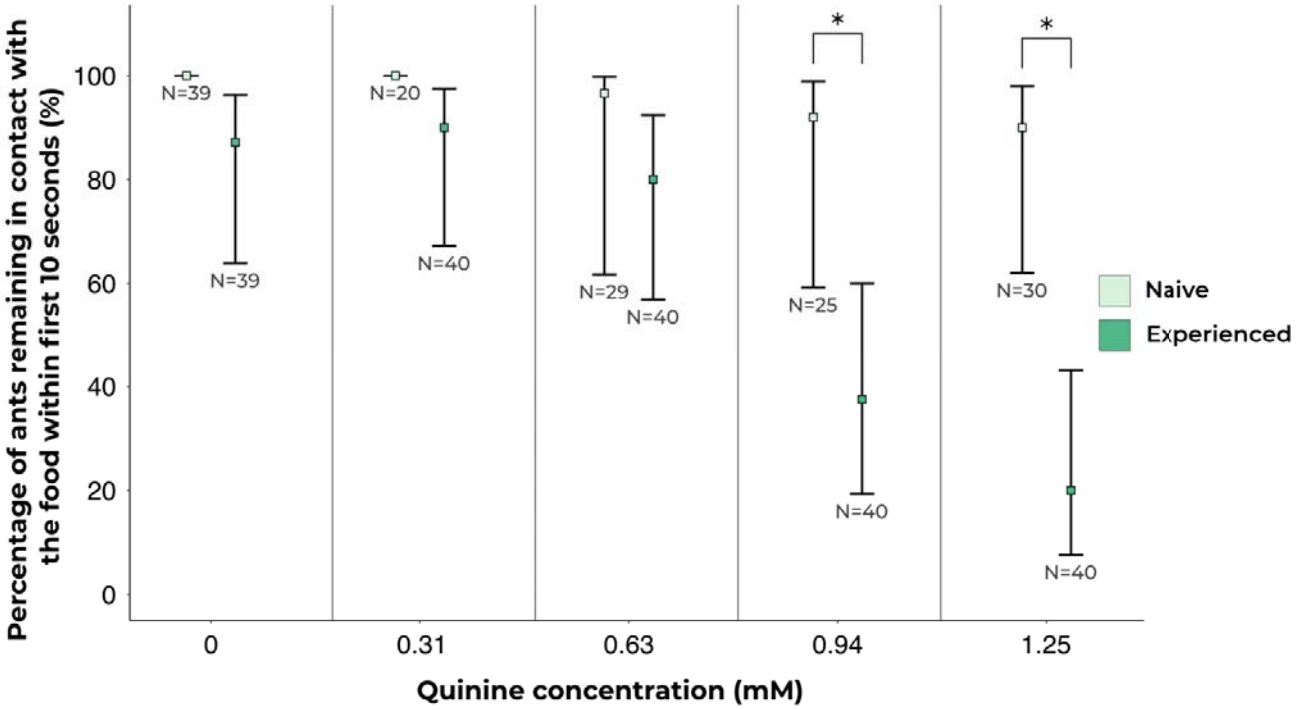

Figure 2: Comparison of acceptance between naive and experienced ants: Naive ants consistently accepted the presented food solutions without showing aversion, while experienced ants, tested on a comparative setup, exhibited aversion to sucrose solutions containing 0.94mM or 1.25mM quinine. Squares represent estimated marginal means obtained from beta regression models, whilst error bars represent the 95% confidence intervals associated with each estimate. Asterisks (*) denote pairs with statistical differences in preference.

Experiment 2 – Quantifying ant acceptance using the dual-feeder method

Avoidance of quinine-containing solutions

In this experiment, we evaluated the efficacy of our dual-feeder method by investigating the responses of ants to sucrose solutions infused with varying levels of quinine to assess bitterness rejection. The key measure explored was the proportion of time spent drinking from the first choice feeder solution, because ants which fully accept a liquid food source tend to remain feeding at it until satiated.

Ants significantly preferred solutions with no quinine, spending a greater proportion of time on the first choice feeder when presented with 1M sucrose solution compared to 1M sucrose solution with 0.63mM quinine (20.3%. Betareg: Chisq = 12.79, p = 0.0003). Additionally, ants showed a significant preference for 1M sucrose

solution compared to 1M sucrose solution with 0.31mM quinine (16.8%. Betareg: Chisq = 15.25, p = 0.0001). However, the comparison between 1M sucrose solution and 1M sucrose solution with 0.156mM quinine did not show a significant difference (5%. Betareg: Chisq = 0.89, p = 0.344). For a visual representation of the ants' preference for bitterness levels, see Figure 3. On average, 12 ants were tested by one experimenter per hour.

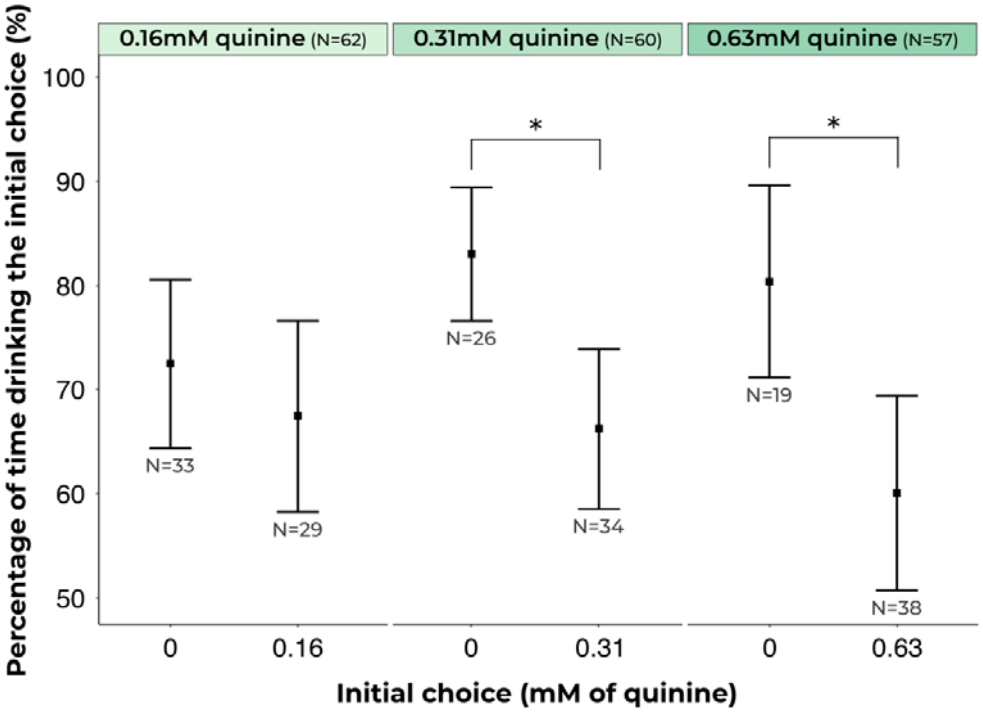

Figure 3: Comparison of drinking behaviour across different quinine concentrations: In our comparative simultaneous setup, where two solutions are live presented simultaneously in the dual feeder, ants exhibited a strong preference for pure sucrose solutions over sucrose solutions containing quinine, even when the concentration of quinine was low. This preference underscores the sensitivity of the dual feeder method compared to the method tested in the initial experiments, where ants were indifferent to sucrose solutions with low concentrations of quinine. Squares represent estimated marginal means obtained from beta regression models, whilst error bars represent the 95% confidence intervals associated with each estimate. Asterisks (*) denote pairs with statistical differences in drinking behaviour.

Preference for higher molarity sucrose solutions

Ants significantly preferred higher concentration sucrose solutions, spending a greater proportion of time on the first choice feeder when it was 1M sucrose solution compared to 0.5M sucrose solution (15.8%. Betareg: Chisq = 18.37, $p < 0.0001$) and also when compared to 0.75M sucrose solution (19.4%. Betareg: Chisq = 12.60, $p = 0.0004$, see Figure 4). The comparison of 1M sucrose solution to another 1M sucrose solution, serving as a negative control, did not yield differences in preference (1.2%. Betareg: Chisq = 0.24, $p = 0.6274$). On average, 12 ants were tested by one experimenter per hour.

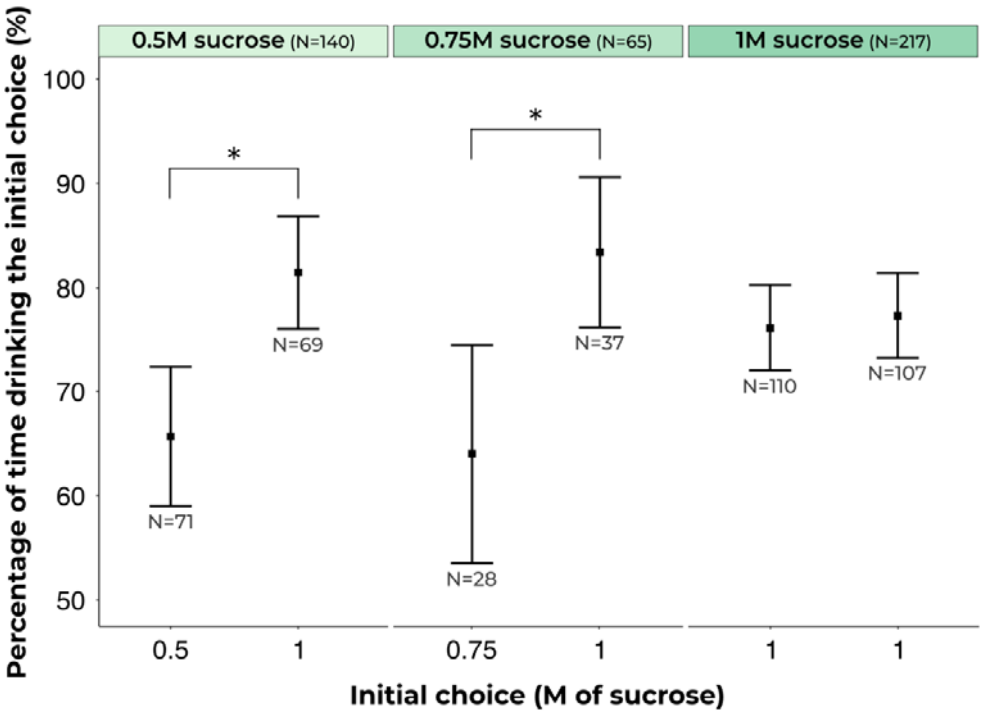

Figure 4: Comparison of drinking behaviour across different sucrose solution concentrations: Ants prefer higher sucrose molarity over smaller concentrations, and a comparison between two identical 1M sucrose solutions showed no preference, validating the sensitivity of the dual feeder method. The squares represent the estimated marginal means obtained from beta regression analysis, while the error bars indicate the confidence intervals derived from the same regression. Asterisks (*) denote pairs with statistical differences in drinking behaviour.

Discussion

Our results show that comparative evaluation is a more sensitive approach to quantifying ant feeding preference than absolute food acceptance. Our newly developed simultaneously comparative dual-feeder method is faster and easier to use than the alternative sequential comparative preference method. It also avoids triggering neophobia or sequential exposure effects, which have all been reported in ants (Oberhauser and Czaczkes 2018; Oberhauser et al. 2022). Perhaps most importantly, it seems to be more sensitive as well, able to detect preference at a lower level of quinine than the sequential method. Using the dual-feeder method, we demonstrated that ants are capable of differentiating between various levels of sweetness in sucrose solutions and bitterness in sucrose-quinine solutions. The dual-feeder method seems to be especially sensitive to differentiating food sources containing small amounts of disliked compounds.

The marked contrast between the response of naive ants to the moderately bitter solutions with those of ants expecting non-bitter solutions demonstrates that comparative preference evaluation is much more sensitive than absolute acceptance evaluation. However, at moderately low levels of bitterness (0.31mM quinine) no difference between naïve and experienced ants was observed, although data from the dual-feeder method demonstrates that they can, indeed, perceive this level of quinine. One possible reason why we see this difference between the preference testing approaches is that ants weigh their opportunity costs of investing time and energy searching for the previous food source against the known benefits of the present one. Furthermore, ants in the sequential comparative approach might receive feedback from their colony through trophallaxis as indicated in Wagner et al. (2023). Another possibility is that direct evaluation allows the ants to detect smaller differences than evaluations which require a quality stored in the ant´s memory to be compared to those currently being directly sensed.

Interestingly, the same, relatively high, proportion of ants drank to satiation (visible distention of the abdomen after 90 seconds of drinking) in all bitterness levels in the non-comparative and sequential tests. This may be a result of the constrained nature of the experimental design: having potentially rejected the second food source within the first 10 seconds, ants may have spent some time attempting to relocate the initial food source, only to discover that it was no longer available. They may have then decided to accept the only

available food source. It is quite possible that, had the ant encountered the less preferred food in an unrestricted area, it would have wandered off without consuming the food.

While clearly more sensitive than the simple acceptance test, the sequential preference testing approach suffers from a number of potential confounds. Firstly, ants have been shown to undervalue unexpected food flavours, even if those flavours are not in themselves distasteful (Oberhauser and Czaczkes 2018). Ants also prefer the option they are trained on first (Oberhauser et al. 2022). It is thus impossible to be certain if a reduction in acceptance towards the test food is due to a true dislike of the test food, an expectation disconfirmation effect, or simply because the first experienced food is always preferred. Furthermore, the tested ant must first return to the nest to unload from the first visit. This not only makes the procedure take longer, it also introduces food to the other nestmates, which may change their behaviour if they are subsequently tested (Provecho and Josens 2009; Sola and Josens 2016; Josens et al. 2016; Czaczkes et al. 2019; Reina and Marshall 2020; Wagner et al. 2023). To ensure the testing of truly naive ants, the colonies must be split into donor and recipient fragments, with tested ants only coming from donor fragments and only returning to recipient fragments. This doubles the number of colony boxes needed. Moreover, ants returning to a nest which is not their home nest tend to take longer to unload their crop, and are less likely to return to foraging (TW, TJC, Pers. Obs.). A final issue with the sequential testing approach is that ants may detect a difference between the training (first) and test (second) food, but choose not to reject it, because they consider the cost of rejecting it and going onwards to search for the better test food too high. Finally, this approach requires marking of ants, which may stress the ants, and may be difficult for 'nervous' species, such as the invasive Longhorn and Yellow Crazy ants (*Paratrechina longicornis*, *Anoplolepis gracilipes*). Given the lack of difference in response between the 1st and 2nd presented food in the control test, marking seemed to not influence acceptance in the current study.

The comparative sequential preference approach and our simultaneous comparative dual-feeder support each other in their results. Nevertheless, we think that the simultaneous comparative approach is superior. The dual-feeder has proven to have higher sensitivity than the sequential approach. This may be because the simultaneous evaluation reduces the impact of other factors, as mentioned above. Our behavioural tests thus demonstrate that *L. humile* can perceive quinine in sucrose solutions at concentrations at least as low as

0.31mM, and prefer pure sucrose solution. Only at very low quinine concentrations (0.16mM) could we detect no preference for the pure sucrose solution. This may be because the ants could not perceive the quinine – either because it is absolutely below their perception threshold, or because at these low levels perception of the quinine is masked by the sweetness of the solution. Alternatively, the ants may have perceived the quinine but not have found it aversive at such low levels.

The dual-feeder approach can also be used to differentiate preferences between attractive solutions with no aversive components. Testing with the dual-feeder method is rapid, enabling the examination of approximately 12 ants per hour for a minimally-trained experimenter, compared to sequential testing which permits only 5 ants per hour. Moreover, no marking or returning to the nest is needed, thereby avoiding many of the confounding issues mentioned above. In cafeteria tests, ants are also presented with multiple choices of different food sources simultaneously. However, contrary to our dual feeder, most individual ants in a cafeteria test may not encounter more than one food source, thus hindering their ability to perform a comparative evaluation.

Our dual-feeder system has demonstrated the enhanced sensibility and high throughput of simultaneous comparative preference tests. However, it's important to acknowledge certain limitations. The potential for lateralization in antennal function could influence the ants' perception of the solutions presented. Lateralization has been demonstrated in *Apis mellifera*, where bees respond more sensitively to odours when trained through their right antenna (Letzkus et al. 2006; Haase et al. 2011; Baracchi et al. 2018). Furthermore *Bombus terrestris* exhibits population-level olfactory lateralization, with the right antenna showing enhanced performance in odour learning (Anfora et al. 2011). Similarly, a right antenna lateralization has been observed in ants in the context of trophallaxis (Frasnelli et al. 2012). It may be that placing the test solution in the right well of the dual feeder may result in the assay higher sensitivity. Nonetheless, the design of our experiment, which involved alternating the placement of different solutions (sucrose and sucrose-quinine mixtures) on the left and right sides of the dual-feeder, minimizes the likelihood that any innate lateralization in antennal use would significantly impact the outcomes. Another limitation of the dual feeder method is that while positive results are indicative of detection as well as preference, negative results indicate either no preference or no antennal detection. Finally, in natural foraging scenarios, ants are likely to encounter conditions more akin to a cafeteria

test or sequential comparisons, where choices are influenced by prior experiences and interactions with nestmates, rather than the simultaneous presentation of options as in our dual-feeder setup. While our method demonstrates heightened sensitivity, it's important to note that the detection and disfavouring of a substance under these controlled conditions may not mirror absolute rejection in the wild. On the other hand, in natural settings, ants might exhibit reduced recruitment to less preferred sources, an aspect that our study did not directly investigate. We note that acceptance scores and recruitment intensity tend to correlate (Wendt et al. 2019).

Creating effective and species-specific baits is challenging due to the diversity of ant preferences and the complexity of integrating repellent substances without reducing bait attractiveness (Knight and Rust 1991; Nyamukondiwa and Addison 2014). Nyamukwondiwa and Addision (2011) note that developing attractive baits while including deterrents is a major bottleneck in the development of effective ant control strategies. Our dual-feeder method offers itself as a powerful approach for testing potential bait solutions quickly and effectively. As the global community faces growing concerns over the spread of invasive ant species, innovative and sustainable approaches in bait formulation have become a top priority (Bertelsmeier et al. 2015; Tay et al. 2017). We anticipate that the dual-feeder method we report here can contribute to these ongoing efforts. Moreover, by simply scaling the size of the apparatus, this approach could easily be deployed for testing a range of walking insects, such as cockroaches or beetles. With more extensive modifications, flying insects with antennae or palps which read beyond the mouthparts could also be tested.

ACKNOWLEDGEMENTS

We would like to thank Silvia Abril for providing ant colonies. Thomas Wagner and Henrique Galante were supported by an ERC starter grant (Cognitive control: 948181) to Tomer J. Czaczkes. Tomer J. Czaczkes was supported by a Heisenberg fellowship from the Deutsche Forschungsgemeinschaft (CZ 237/4-1). Many thanks to Cosmina Werneke, Laura Wögler, Leonie Dechant and Nick Wolter for conducting part of the experiments.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available online from https://doi.org/10.5281/zenodo.10953784.

AUTHOR CONTRIBUTIONS

T. Wagner: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Supervision. **H. Galante:** Software, Validation, Formal Analysis, Data Curation, Writing - Review & Editing, Visualization. **T. J. Czaczkes:** Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Literature

Andrew SC, Perry CJ, Barron AB, et al (2014) Peak shift in honey bee olfactory learning. Anim Cogn 17:1177–1186. https://doi.org/10.1007/s10071-014-0750-3

Anfora G, Rigosi E, Frasnelli E, et al (2011) Lateralization in the Invertebrate Brain: Left-Right Asymmetry of Olfaction in Bumble Bee, Bombus terrestris. PLoS ONE 6:e18903. https://doi.org/10.1371/journal.pone.0018903

Angulo E, Hoffmann BD, Ballesteros-Mejia L, et al (2022) Economic costs of invasive alien ants worldwide. Biol Invasions. https://doi.org/10.1007/s10530-022-02791-w

Baker TC, Van Vorhis Key SE, Gaston LK (1985) Bait-preference Tests for the Argentine Ant (Hymenoptera: Formicidae). J Econ Entomol 78:1083–1088. https://doi.org/10.1093/jee/78.5.1083

Baracchi D, Rigosi E, De Brito Sanchez G, Giurfa M (2018) Lateralization of Sucrose Responsiveness and Non-associative Learning in Honeybees. Front Psychol 9:425. https://doi.org/10.3389/fpsyg.2018.00425

Bartoń K (2023) MuMIn: Multi-Model Inference

Bates D, Kliegl R, Vasishth S, Baayen H (2018) Parsimonious Mixed Models

Bertelsmeier C, Luque GM, Hoffmann BD, Courchamp F (2015) Worldwide ant invasions under climate change. Biodivers Conserv 24:117–128. https://doi.org/10.1007/s10531-014-0794-3

Bitterman ME (1976) Incentive Contrast in Honey Bees. Science 192:380–382. https://doi.org/DOI: 10.1126/science.1257773

Choe D-H, Tay J-W, Campbell K, et al (2021) Development and Demonstration of Low-Impact IPM Strategy to Control Argentine Ants (Hymenoptera: Formicidae) in Urban Residential Settings. J Econ Entomol 114:1752–1757. https://doi.org/10.1093/jee/toab079

Couvillon PA, Bitterman ME (1984) The overlearning-extinction effect and successive negative contrast in honeybees (Apis mellifera). J Comp Psychol 98:100–109. https://doi.org/10.1037/0735-7036.98.1.100

Cribari-Neto F, Zeileis A (2010) Beta Regression in *R*. J Stat Softw 34:. https://doi.org/10.18637/jss.v034.i02

Czaczkes TJ, Beckwith JJ, Horsch A-L, Hartig F (2019) The multi-dimensional nature of information drives prioritization of private over social information in ants. Proc R Soc B Biol Sci 286:20191136. https://doi.org/10.1098/rspb.2019.1136

Daniel AM (2020) Scaling relative incentive value in honey bees, Apis mellifera. Learn Motiv 69:101614. https://doi.org/10.1016/j.lmot.2020.101614

De Agrò M, Matschunas C, Czaczkes TJ (2022) Bundling and segregation affect pheromone deposition, but not choice, in an ant. eLife 11:e79314. https://doi.org/10.7554/eLife.79314

Fox J, Weisberg S Nonlinear Regression, Nonlinear Least Squares, and Nonlinear Mixed Models in R

Frasnelli E, Iakovlev I, Reznikova Z (2012) Asymmetry in antennal contacts during trophallaxis in ants. Behav Brain Res 232:7–12. https://doi.org/10.1016/j.bbr.2012.03.014

Grüter C, Czaczkes TJ, Ratnieks FLW (2011) Decision making in ant foragers (Lasius niger) facing conflicting private and social information. Behav Ecol Sociobiol 65:141–148. https://doi.org/10.1007/s00265-010-1020-2

Haase A, Rigosi E, Frasnelli E, et al (2011) A multimodal approach for tracing lateralisation along the olfactory pathway in the honeybee through electrophysiological recordings, morpho-functional imaging, and behavioural studies. Eur Biophys J 40:1247–1258. https://doi.org/10.1007/s00249-011-0748-6

Hartig F (2021) Residual Diagnostics for Hierarchial (Multi-Level/Mixed) Regression Models [Computer software manual]. http://florianhartig.github.io/DHARMa/

Hays SB, Arant FS (1960) Insecticidal Baits for Control of the Imported Fire Ant, Solenopsis saevissima richteri1. J Econ Entomol 53:188–191. https://doi.org/10.1093/jee/53.2.188

Hoey-Chamberlain R, Rust MK (2014) Food and Bait Preferences of Liometopum occidentale (Hymenoptera: Formicidae). J Entomol Sci 49:30–43. https://doi.org/10.18474/0749-8004-49.1.30

Hoffmann BD, Luque GM, Bellard C, et al (2016) Improving invasive ant eradication as a conservation tool: A review. Biol Conserv 198:37–49. https://doi.org/10.1016/j.biocon.2016.03.036

Hooper-bùi LM, Appel AG, Rust MK (2002) Preference of Food Particle Size Among Several Urban Ant Species. J Econ Entomol 95:1222–1228. https://doi.org/10.1603/0022-0493-95.6.1222

Josens R, Mattiacci A, Lois-Milevicich J, Giacometti A (2016) Food information acquired socially overrides individual food assessment in ants. Behav Ecol Sociobiol 70:2127–2138. https://doi.org/10.1007/s00265-016-2216-x

Josens R, Sola F, Lois-Milevicich J, Mackay W (2017) Urban ants of the city of Buenos Aires, Argentina: species survey and practical control. Int J Pest Manag 63:213–223. https://doi.org/10.1080/09670874.2016.1239035

Josens R, Sola FJ, Marchisio N, et al (2014) Knowing the enemy: ant behavior and control in a pediatric hospital of Buenos Aires. SpringerPlus 3:229. https://doi.org/10.1186/2193-1801-3-229

Kaspari M, Yanoviak SP (2001) Bait Use in Tropical Litter and Canopy Ants—Evidence of Differences in Nutrient Limitation1. BIOTROPICA 33:207. https://doi.org/10.1646/0006-3606(2001)033[0207:BUITLA]2.0.CO;2

Kessler SC, Tiedeken EJ, Simcock KL, et al (2015) Bees prefer foods containing neonicotinoid pesticides. Nature 521:74–76. https://doi.org/10.1038/nature14414

Klotz JH, Rust MK, Greenberg L (2007) An Evaluation of Several Urban Pest Management Strategies to Control Argentine Ants (Hymenoptera: Formicidae). 50:8

Knight RL, Rust MK (1991) Efficacy of Formulated Baits for Control of Argentine Ant (Hymenoptera: Formicidae). J Econ Entomol 84:510–514. https://doi.org/10.1093/jee/84.2.510

Lenth R, Bolker BM, Buerkner P, et al (2024) emmeans: Estimated Marginal Means, aka Least-Squares Means

Letzkus P, Ribi WA, Wood JT, et al (2006) Lateralization of Olfaction in the Honeybee Apis mellifera. Curr Biol 16:1471–1476. https://doi.org/10.1016/j.cub.2006.05.060

Madsen NEL, Sørensen PB, Offenberg J (2017) Sugar and amino acid preference in the black garden ant Lasius niger (L.). J Insect Physiol 100:140–145. https://doi.org/10.1016/j.jinsphys.2017.05.011

Mustard JA, Oquita R, Garza P, Stoker A (2019) Honey Bees Apis mellifera Show a Preference for the Consumption of Ethanol. Alcohol Clin Exp Res 43:26–35. https://doi.org/10.1111/acer.13908

Nondillo A, Ferrari L, Lerin S, et al (2014) Foraging Activity and Seasonal Food Preference of Linepithema micans (Hymenoptera: Formicidae), a Species Associated With the Spread of Eurhizococcus brasiliensis (Hemiptera: Margarodidae). J Econ Entomol 107:1385–1391. https://doi.org/10.1603/EC13392

Nyamukondiwa C, Addison P (2014) Food preference and foraging activity of ants: Recommendations for field applications of low-toxicity baits. J Insect Sci 14:

Nyamukondiwa C, Addison P (2011) Preference of foraging ants (Hymenoptera: Formicidae) for bait toxicants in South African vineyards. Crop Prot 30:1034–1038. https://doi.org/10.1016/j.cropro.2011.03.014

Oberhauser FB, Bogenberger K, Czaczkes TJ (2022a) Ants prefer the option they are trained to first. J Exp Biol 225:jeb243984. https://doi.org/10.1242/jeb.243984

Oberhauser FB, Bogenberger K, Czaczkes TJ (2022b) Ants prefer the option they are trained to first. J Exp Biol 225:jeb243984. https://doi.org/10.1242/jeb.243984

Oberhauser FB, Czaczkes TJ (2018) Tasting the unexpected: disconfirmation of expectations leads to lower perceived food value in an invertebrate. Biol Lett 14:20180440. https://doi.org/10.1098/rsbl.2018.0440

Osae M, Cobblah M, Djankpa F, et al (2011) Development of a Bait System for the Pharaoh's Ant, *Monomorium Pharaonis* L. (Hymenoptera: Formicidae). West Afr J Appl Ecol 18:29–38. https://doi.org/10.4314/wajae.v18i1.70316

Pereira RM, Stimac JL (1997) Biocontrol options for urban pest ants. J Agric Entomol 14:231–248

Provecho Y, Josens R (2009) Olfactory memory established during trophallaxis affects food search behaviour in ants. J Exp Biol 212:3221–3227. https://doi.org/10.1242/jeb.033506

R Core Team (2022) R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Reina A, Marshall JAR (2020) Negative feedback may suppress variation to improve collective foraging performance. Ecology

Rust MK, Reierson DA, Klotz JH (2003) Pest Management of Argentine Ants (Hymenoptera: Formicidae)1. J Entomol Sci 38:159–169. https://doi.org/10.18474/0749-8004-38.2.159

Rust MK, Reierson DA, Paine E, Blum LJ (2000) Seasonal Activity and Bait Preferences of the Argentine Ant (Hymenoptera: Formicidae). 17:

Sasaki T, Pratt SC (2013) Ants learn to rely on more informative attributes during decision-making. Biol Lett 9:20130667. https://doi.org/10.1098/rsbl.2013.0667

Silverman J, Brightwell RJ (2008) The Argentine Ant: Challenges in Managing an Invasive Unicolonial Pest. Annu Rev Entomol 53:231–252. https://doi.org/10.1146/annurev.ento.53.103106.093450

Sola FJ, Josens R (2016) Feeding behavior and social interactions of the Argentine ant *Linepithema humile* change with sucrose concentration. Bull Entomol Res 106:522–529. https://doi.org/10.1017/S0007485316000201

Tay J, Hoddle MS, Mulchandani A, Choe D (2017) Development of an alginate hydrogel to deliver aqueous bait for pest ant management. Pest Manag Sci 73:2028–2038. https://doi.org/10.1002/ps.4616

Wagner T, Czaczkes TJ (2024) Corpse-associated odours elicit avoidance in invasive ants. Pest Manag Sci 80:1859–1867. https://doi.org/10.1002/ps.7916

Wagner T, Galante H, Tomer J. C (2023) Systematic examination of learning in the invasive ant Linepithema humile reveals fast learning and long-lasting memory. Anim Behav 203:41–52. https://doi.org/10.1016/j.anbehav.2023.06.012

Waldron FA, Wiegmann DD, Wiegmann DA (2005) Negative Incentive Contrast Induces Economic Choice Behavior by Bumble Bees. Int J Comp Psychol 18:. https://doi.org/10.46867/IJCP.2005.18.04.02

Wendt S, Kleinhoelting N, Czaczkes TJ (2020) Negative feedback: ants choose unoccupied over occupied food sources and lay more pheromone to them. J R Soc Interface 17:20190661. https://doi.org/10.1098/rsif.2019.0661

Wendt S, Strunk KS, Heinze J, et al (2019) Positive and negative incentive contrasts lead to relative value perception in ants. eLife 8:e45450. https://doi.org/10.7554/eLife.45450

Wetterer JK, Wetterer AL, Hebard E (2001) Impact of the Argentine Ant, *Linepithema humile* on the Native Ants of Santa Cruz Island, California. 38:13

Wickham H (2016). Package ggplot2: create elegant data Visualisations using the grammar of graphics http://ggplot2.org, https://github.com/hadley/ggplot2

Zanola D, Czaczkes TJ, Josens R (2024) Ants evade harmful food by active abandonment. Commun Biol 7:84. https://doi.org/10.1038/s42003-023-05729-7