USING COMPUTER VISION TO EXPLORE THE EFFECTS OF BAIT ADDITIVES ON INVASIVE ANT BEHAVIOUR

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Para a melhor mãe do mundo

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Summary

Invasive ants are among the most widespread and destructive invaders globally, causing severe ecological and economic damage. As a result, they are a common target of management efforts, which often fail. This thesis aims to lay the groundwork for new management strategies that leverage insect learning by manipulating individual and collective preferences and behaviours. Specifically, we aim to identify bait additives that enhance foraging behaviour, thereby increasing recruitment to and consumption of toxic baits. Additionally, we examine how ants, both individually and collectively, respond to spinosad, a promising slow-acting toxicant.

First, we investigated the effects of seven potential neuroactive chemicals on the cognitive abilities of Argentine ants, *Linepithema humile*, using bifurcation mazes. Despite demonstrating that these ants are fast associative learners, none of the seven chemicals tested significantly interfered with their ability to learn spatial or olfactory associations. To address the limitations of bifurcation mazes, we developed a more complex, field-realistic foraging experiment. We found that low to intermediate concentrations of caffeine enhanced ant foraging by improving path straightness rather than speed. This suggests that moderate doses of caffeine in baits could boost ants' ability to locate the bait, thereby potentially improving bait efficacy.

To optimise bait formulations, we developed a non-invasive method capable of quantifying both overall consumption and feeding rate of small invertebrates. This method provided valuable insights into individual feeding patterns and preferences, highlighting that caffeine solutions are palatable. It also demonstrated that individual ants exposed to spinosad reduced their food consumption in subsequent feedings, a consequence of earlier food abandonment.

We hypothesised that control attempts often fail due to conditioned taste aversion, where ants avoid toxic-tasting baits. However, our results showed that ants did not develop such an association for sublethal doses of spinosad. Despite the negative impacts of spinosad on individual feeding, pre-exposure to the toxicant resulted in faster collective recruitment towards all food sources. Moreover, we found that larger group sizes result in lower spinosad-induced mortality, suggesting that ants collectively evade the toxicant, which could explain the lack of conditioned taste aversion observed.

Finally, we investigated whether adding protein to baits would result in preferential feeding to the reproductive members of the colony. Our results showed that the presence of protein in food resulted in slower collective recruitment, predominantly in four-day starved colonies. However, it remains unclear to what extent protein affected food distribution and thus toxicant delivery.

Overall, our findings offer a foundation for the development of new bait formulations that leverage insect behaviour, potentially improving the efficacy of management strategies.

Resumo

As formigas invasoras estão entre as espécies mais disseminadas e destrutivas a nível global, causando danos ecológicos e económicos severos. Consequentemente, são uma prioridade nos esforços de gestão de pragas, embora frequentemente sem sucesso. Esta dissertação visa desenvolver novas estratégias de gestão, aproveitando a capacidade de aprendizagem destes insetos, manipulando as suas preferências e hábitos, tanto a nível individual como coletivo. Especificamente, pretende-se identificar aditivos para iscos que melhorem os comportamentos de forrageamento, aumentando o recrutamento e consumo de iscos tóxicos. Foi ainda estudada a resposta individual e coletiva das formigas ao spinosad, uma toxina de ação lenta promissora.

Primeiro, investigámos os efeitos de sete potenciais químicos neuroativos nas funções cognitivas das formigas argentinas, *Linepithema humile*, usando labirintos bifurcados. Demonstrámos que estas formigas têm uma rápida capacidade de aprendizagem associativa, mas nenhum dos compostos testados interferiu na aprendizagem de associações espaciais ou olfativas. Para superar as limitações dos labirintos bifurcados, desenvolvemos uma experiência de forrageamento mais complexa e realista. Descobrimos que concentrações baixas a intermédias de cafeína melhoraram o forrageamento das formigas, otimizando a rota em vez da velocidade. Isto sugere que doses moderadas de cafeína nos iscos podem melhorar a capacidade das formigas de os localizar, aumentando a eficácia dos iscos.

Para otimizar as formulações de iscos, desenvolvemos um método não invasivo capaz de quantificar o consumo e a taxa de alimentação de pequenos invertebrados. Este método permitiu obter informações pertinentes relativas aos padrões de consumo e preferências individuais, destacando a palatabilidade das soluções de cafeína. Verificámos também que as formigas expostas ao spinosad reduziram o consumo de todos os alimentos nas refeições seguintes, uma consequência do abandono antecipado da comida.

Partimos do pressuposto de que a aversão ao sabor condicionada permite que as formigas evitem iscos tóxicos, resultando no fracasso das tentativas de controlo. No entanto, os resultados mostraram que as formigas não desenvolveram tal associação após a ingestão de doses subletais de spinosad. Apesar dos efeitos negativos deste químico na alimentação individual, uma única exposição prévia resultou num recrutamento coletivo mais rápido para todas as fontes de alimento. Descobrimos também que grupos maiores têm menor mortalidade induzida pelo spinosad. Estes resultados sugerem que as formigas evitam coletivamente os efeitos negativos dos compostos tóxicos, potencialmente explicando a ausência de aversão ao sabor condicionada.

Finalmente, investigámos se a adição de proteínas aos iscos resultaria numa alimentação preferencial dos membros reprodutivos da colónia. Os resultados mostraram que a presença de proteínas nos iscos resultou num recrutamento coletivo mais lento. No entanto, ficou por esclarecer se a adição de proteína afeta a distribuição do alimento e, consequentemente, do composto tóxico.

Em suma, os resultados desta dissertação fornecem uma base para o desenvolvimento de novas formulações de iscos que aproveitem o comportamento das formigas invasoras para melhorar a eficácia das estratégias de gestão de pragas.

Work arising from this thesis

This thesis is composed of the following six manuscripts, two of which are published in peer-reviewed journals, two available as preprints and two in preparation for publication:

A. **Galante H**, Czaczkes TJ (2024) Invasive ant learning is not affected by seven potential neuroactive chemicals. *Current Zoology*. https://doi.org/10.1093/cz/zoad001.

B. **Galante H**, De Agrò M, Koch A, Kau S, Czaczkes TJ (2024) Acute exposure to caffeine improves foraging in an invasive ant. *iScience*. https://doi.org/10.1016/j.isci.2024.109935.

C. **Galante H**, Czaczkes TJ, De Agrò M (2024) Three-dimensional body reconstruction enables quantification of liquid consumption in small invertebrates. *bioRxiv*. https://doi.org/10.1101/2024.06.14.599002.

D. **Galante H**, Forster M, Werneke C, Czaczkes TJ (2024) Invasive ants fed spinosad collectively recruit to known food faster yet individually abandon food earlier. *bioRxiv*. https://doi.org/10.5281/zenodo.12073127

E. **Galante H**, Hugo H, LeBoeuf AC, Czaczkes TJ (In Prep.) Larger group sizes increase resistance to a slow-acting toxicant in invasive ants.

F. **Galante H**, Hugo H, Czaczkes TJ, LeBoeuf AC (In Prep.) Presence of protein in baits alters food consumption and dispersion in an invasive ant.

During the course of this thesis I also contributed to manuscripts and projects which are not part of this work:

G. Wagner T, **Galante H**, Josens R, Czaczkes TJ (2023) Systematic examination of learning in the invasive ant *Linepithema humile* reveals fast learning and long-lasting memory. *Animal Behaviour*. https://doi.org/10.1016/j.anbehav.2023.06.012.

H. Wagner T, **Galante H**, Czaczkes TJ (2024) A high-throughput and sensitive method for food preference assays in walking insects. *bioRxiv*. https://doi.org/10.1101/2024.04.10.588882.

H. Galante author contributions

Complete author contributions are available at the end of each chapter.

A. **H. Galante:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization.

B. **H. Galante:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing - original draft, Writing - review & editing, Visualization, Supervision.

C. **H. Galante:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing - original draft, Writing - review & editing, Visualization, Supervision.

D. **H. Galante:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing - original draft, Writing - review & editing, Visualization, Supervision.

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F. **H. Galante:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing - original draft, Writing - review & editing, Visualization.

G. **H. Galante:** Conceptualization, Methodology, Investigation, Writing - review & editing.

H. **H. Galante:** Software, Validation, Formal analysis, Data Curation, Writing - review & editing, Visualization.

To curiosity

During my BSc field course in South Africa, I fell in love with *Camponotus fulvopilosus*. This newfound passion for ants led me to pursue a MRes, where I planned to study energetic efficiency in ant colonies. Unfortunately, COVID-19 disrupted these plans, and I couldn't complete my thesis using ants. Instead, I studied energetic efficiency in bees through a meta-analysis of published data. After finishing my MRes, I began applying for PhD programs in the UK, focusing primarily on invertebrates, especially ants.

However, an exciting opportunity arose in Germany: the project offered good funding, intriguing applied research questions, and a somewhat British supervisor. I applied on January 8, 2021, was interviewed on January 22, and met with Tommy again on January 27. Despite concerns about my lack of laboratory experience and uncertainties about moving to Germany, I was offered the position and accepted it on January 29. By February 5, I was dealing with a huge amount of paperwork, and on March 29, I moved to Germany. My first day in the laboratory was April 6, 2021. This was also the first time I ever trained an ant, a poor *Lasius niger* with an excessive amount of acrylic paint on her back.

Moving to Germany amid the peak of COVID-19 was challenging. Our initial fieldwork plans fell through, and I waited six months for a computer, but we adapted. In the lab, I gained hands-on experience: cleaning, sifting ants from dirt, plastering, making nests, and 3D printing. Initially working with Y-mazes, I soon realized I wanted to innovate beyond that. I developed semi-automated Raspberry Pi systems, appreciating the data they generated. However, analysing such large amounts of data was at times daunting and pushed me to self-learn computer vision techniques.

The PhD journey had its highlights: conferences in San Diego, Groningen, Rovereto, Bielefeld, and two Bavarian monasteries. Most notably, my time in Fribourg was a fantastic experience. Reflecting on this journey, I see my PhD as a wise financial and intellectual decision. I am grateful for the opportunity to work with ants, meet incredible and inspiring individuals, and develop myself. I am proud to say that I included all my data in this thesis, even if some chapters are quite preliminary, and that I learned a lot of new computational skills that will be extremely useful in my new job as a data scientist in the UK.

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Most people who meet me quickly learn that I love to complain, and that although ants make me happy, most people do not have the same effect. However, this section is dedicated to the small army who made this thesis, and the last three years, possible.

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mustard cake queen, and Hannes, my favourite evening therapist. A huge thank you to Scheremy for all the deep conversations and banter, not many people get me to walk over two hours uphill and much less to watch an entire football match.

Thank you to Adria for welcoming me with open arms in her lab in Switzerland, for being extremely supportive, for all the fun lunches, inspiring talks and hiring a bunch of awesome people! In particular, my Wonder Women Duo Marie-Pierre and Sanja who are always there for me with a safe place to vent, laugh and most importantly gossip. Of course, also a huge thank you to Matteo (in case you haven't heard… Jack learned how to use tool!), Ayma and Hugo for all the amazing, very expensive, evenings we spent in Fribourg. To Jeanne, not many people could make me climb up a cathedral and even less up a mountain just to eat a true Fribourgeois cheese fondue. Thank you for all the fun evenings in Regensburg and for showing me around Fribourg!

My sanity was mostly keep in check by a great group of PhD's and postdocs. Thank you to Melissinha for changing her mind about me, taking me to toy stores and baking a very high quality vegan cake. To Thomas for all the snacks and red bull, to Atzimba por todo el limón y sal, to Piuli for multiple food comas, to Ameka for the good vibes, to Daniel and Luisa for being great drinking buddies, to Nils for all the tequila shots at Tiki and to Mathilde for including me in all the Fun House parties. A few special thanks go to Lina for being the best office mate I could ever have asked for. So many great memories with you which I will never forget. Thank you for that beautiful happy birthday lab glove and a night of breaking and entering into your own house. Also to Massimo for being a great friend, for all the boardgame nights, dinners, Casper walks and for bouncing off work ideas and code with me. You really kept me sane. And finally, a huge thank you to Laure-Anne for always being there, a million breaks a day, entertaining food and supplements, curcuma lattes and going out to the city past bedtime! Sorry for being so annoying in the last couple months (or always)!

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I shall forever be worried I forgot to thank someone very important! Rest assured if I forgot you its because writing this thesis in time was a challenge and not because I don't value you! Thank you to everyone who was part of my academic journey and life (somewhat unhealthily mixed) and for putting up with me (I know it is hard!).

Although I won't miss the nomadic nature of academia and all of its broken bits, I will miss the permanent availability of instant like-minded friends it often provided. I really hope some of you stay in my life for many years to come.

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Continental

Às vezes é no meio de tanta gente
Que descubro afinal p'ra onde vou
E esta pedra
E este grito
São a história d'aquilo que sou

Maria Guinot, 1984
Silêncio e tanta gente

A brief overview of ants and their functional roles

If one were to throw an imaginary spear into a world inhabited solely by animals, one would most likely strike an insect. Their global biomass is estimated at approximately 200 megatons of carbon (Eggleton, 2020), composed of an estimated 5.5 million species (Stork, 2018). Ants represent less than 2% of the more than 900 000 insect species currently described (Wilson & Hölldobler, 2005; Schultheiss et al., 2022). However, global ant abundance is conservatively estimated at 20 quadrillion individuals, corresponding to around 24 megatons of carbon, exceeding the combined biomass of wild birds and mammals with around 2.5 million ants for every human (Schultheiss et al., 2022). This disproportionate abundance of ants, compared to their relatively low species diversity, suggests the benefits of social living outweigh its costs.

Eusociality, defined by cooperative brood care, an overlap of multiple generations before maturity and a clear division of labour across castes, is a phylogenetic rarity, being almost exclusive to arthropods (Wilson & Hölldobler, 2005). Living in a synergistically interacting group, where individuals sacrifice their own reproductive potential in favour of that of their peers, usually the queen(s), leads to a reduction in the genetic diversity of the population as well as an increase of the rate of parasite and disease transmission (Brahma et al., 2022). Nevertheless, by living as a group, ants are more energetically efficient (Hou et al., 2010). In part, this could be due to lower cognitive demands stemming from highly specialised individuals, each solving a simpler task (Kamhi et al., 2016).

Collective decisions, such as which food to exploit or where to nest, arise from competition between individual choices, without direct comparison of all available options. In this way, rationality at the group level emerges even when individuals make suboptimal decisions (Sasaki & Pratt, 2011; Dussutour & Nicolis, 2013). Ultimately, this allows ants to maximise foraging time (Deneubourg et al., 1990; Dussutour et al., 2006) and adapt to unique and dynamic environments (Dussutour et al., 2009; Reid, Sumpter & Beekman, 2011). In fact, ants are so efficient, they have inspired multiple optimisation models, such as the Ant Colony Optimisation (ACO) algorithm which mimics ant behaviour, generating a range of solutions to a problem and using their value to influence future solutions (Dorigo & Stützle, 2004, 2019).

Ants fullfill multiple functional roles which are amplified by their sheer abundance in almost all terrestrial ecosystems (Hölldobler & Wilson, 1990; Folgarait, 1998; Schultheiss et al., 2022). They excavate and aerate soil (Sanford, Manley & Murphy, 2009), contributing to

waste removal, nutrient cycling, seed dispersal (Lengyel et al., 2009; Stuble et al., 2014) and the creation of habitats for other organisms (Farji-Brener & Werenkraut, 2017). Additionally, they serve as vital scavengers on a global scale, playing a crucial role in invertebrate predation (Del Toro, Silva & Ellison, 2015; Ewers et al., 2015) and, at times, acting as biological control agents (Drummond & Choate, 2011; Offenberg, 2015; Anjos et al., 2022). Furthermore, ants can function as pollinators and influence vegetation patterns (Sagers, Ginger & Evans, 2000; Farji-Brener & Werenkraut, 2017; Pinkalski et al., 2018), reducing plant pathogens (Thornham et al., 2012; Offenberg & Damgaard, 2019; Offenberg, Jensen & Hansen, 2022) and increasing crop yield (Way & Khoo, 1992; Evans et al., 2011).

Ecological and economic damages of invasive populations

While native ants play a crucial ecological role, the proliferation of invasive populations, heightened by globalisation, has become a serious issue (Perrings et al., 2010; Bertelsmeier et al., 2017). These invasions are often associated with measurable environmental and/or economic impacts (Keller et al., 2011; Pyšek et al., 2020) thereby making the term an effective way of mobilising both people and funds (Pereyra, 2016). Global economies have incurred significant costs in response to what is now recognised as a major threat to biodiversity (IPBES, 2023). For example, Germany had estimated costs associated with invasive species of more than 9 billion euros over a 60-year period (Haubrock et al., 2021a), the United Kingdom faced costs ranging from 5.4 to 13.7 billion pounds over 45 years (Cuthbert et al., 2021), and Australia lost nearly 300 billion dollars over 60 years (Bradshaw et al., 2021). Notably, these figures represent conservative estimates, and the actual costs, which are likely to be much higher, are increasing ten-fold each decade in Europe, reaching billions of euros annually (Haubrock et al., 2021b). Invasive populations compete with native wildlife contributing to 60% of the documented global animal and plant extinctions disrupting ecosystems at multiple trophic levels (Bellard, Cassey & Blackburn, 2016; Vilà & Hulme, 2017; IPBES, 2023). Additionally, they are a great threat to global food security largely contributing to crop loss (Paini et al., 2016).

The colonisation process differs from ecological succession, a natural evolutionary process by which native populations replace each other over time and undergo natural dispersal (Chang & Turner, 2019), in that it is much faster, allowing non-native populations to migrate beyond their native range as a result of direct or indirect human interference (Falk-Petersen, Bøhn & Sandlund, 2006; Cassini, 2020). In order to become invasive, a population must undergo a number of transitions. First, it is introduced to an area in which it has no evolutionary history, becoming a non-native population. The population is then considered established once it survives and reproduces over consecutive generations without human intervention. Finally, if the population begins to spread, either in abundance and/or range, resulting in introduction and establishment of new populations beyond the original

introduction point, then the population is said to be invasive (Keller et al., 2011; Soto et al., 2024). Invasions represent biogeographical phenomena rather than taxonomic ones. In this way, invasion stages should refer to individual populations and not entire species (Colautti & MacIsaac, 2004; Richardson & Pyšek, 2012). This is important as the same species can have invasive populations in one site but not another (Kueffer, Pyšek & Richardson, 2013). Furthermore, it has been argued that invasive populations cannot remain eternally novel, such that the coexistence of local enemies must mean that an invasive population eventually becomes native (Carthey & Banks, 2012). Nevertheless, a species is commonly said to be invasive if it exhibits traits often associated with invasive potential or is known for its invasive populations.

Invasive ants are among the most widespread and destructive invaders globally, with 23 species recorded to have invasive populations (Siddiqui et al., 2021; Angulo et al., 2022). These ants are responsible for the destruction of electrical equipment and infrastructures (Bradshaw et al., 2016), causing severe allergic reactions to humans, often, but not always, through stinging (Kim et al., 2005; Hoffmann, 2006; Knight & Bangs, 2007), and acting as pathogen vectors (Moreira et al., 2005; Fontana et al., 2010). Conservative estimates suggest that the costs associated with invasive ants over the past 90 years exceed 10 billion dollars, translating into an annual global cost ranging from 119 to 398 million dollars (Angulo et al., 2022). These costs primarily affect agriculture, and are often a result of mutualistic associations with sap-sucking homopterans, such as aphids, which secrete honeydew, a sugary liquid which serves as an energy source for ants. In return, ants protect these aphids, increasing their abundance, leading to detrimental effects on the plants they infest (Buckley, 1987; Nielsen, Agrawal & Hajek, 2010; Lach, Tillberg & Suarez, 2010).

Moreover, invasive ants are able to locate, recruit and monopolise food sources more efficiently than their native counterparts (Holway, 1999; Arnan et al., 2018). This often results in competitive interactions which culminate in a decrease of native ant diversity (Holway & Suarez, 2006; Sarty, Abbott & Lester, 2007). In part, this stems from the tendency of ant species which have invasive populations, to produce small, energetically cheap workers, favouring large highly polydomous colonies of thousands, or even millions of workers (McGlynn, 1999; Walters & Mackay, 2005; Cremer et al., 2008). Large colonies are highly beneficial when exploiting a novel environment, often wining wars of attrition with native species (Adams, 2003). However, the impact of invasive ants extends beyond ant communities, negatively affecting the survival of avian, reptilian, and mammalian species (Suarez & Case, 2002; Suarez, Yeh & Case, 2005; Diffie, Miller & Murray, 2010). The spread of invasive ants shows no signs of slowing down, with new populations continually being discovered (Menchetti et al., 2023) and ant species with invasive populations being favoured in the pet trade due to them commonly being generalists of large spatial distribution (Gippet & Bertelsmeier, 2021).

Argentine ants as a versatile model organism

The Argentine ant, *Linepithema humile* (Mayr, 1868), is native to northern Argentina, eastern Bolivia, southern Brazil, Paraguay and Uruguay. However, its presence has now been recorded in 59 countries, making it one of the most widespread, and the fourth most costly, invasive ant species (Angulo et al., 2022; Wong, Economo & Guénard, 2023; Angulo et al., 2024). *L. humile* workers take, on average, around four months to develop from egg to adult at their optimal survival temperature of 26°C (Abril, Oliveras & Gómez, 2010). They are small (2-3 mm in length), uniformly reddish-brown to dark brown, with long antennal scapes and large compound eyes (Wild, 2008). Queens are larger (4-6 mm in length) and do not engage in mating flights (Figure 1). Instead, they mate with related males in their nests of origin (Keller & Passera, 1990). Colonies reproduce via budding, where even small fragments of ten workers and one queen can give rise to new colonies (Hee et al., 2000). This not only decreases the mortality bottleneck often associated with mating flights and claustral founding queens, but also the genetic variability of the population. Having low agression among daughter colonies allows *L. humile* to form supercolonies: a large number of nests spread over vast areas, on the scale of landscapes or even continents, where individuals from one nest can be brought to any other nest and accepted as nestmates (Tsutsui & Case, 2001; Heller, Ingram & Gordon, 2008). This unicolonial social structure is a hallmark of invasive ant populations, and is likely maintained through restricted gene flow between supercolonies, sibling mating and in some cases the periodic execution of queens (Cremer et al., 2008; Inoue, Ito & Goka, 2015).

Figure 1 – Developmental stages of the highly invasive Argentine ant, *Linepithema humile*. From left to right, eggs, larva, pupa, worker and queen.

The traits that make Argentine ants successful invaders also make them an ideal model organism. They are trophic generalists, preying and scavenging on a wide variety of protein sources and sugary liquids, having a high affinity for honeydew (Hölldobler & Wilson, 1990). This dietary versatility, coupled with their tolerance for a wide variety of chemicals (Galante & Czaczkes, 2024 and H. Galante, personal observations), makes them ideal candidates for pharmacological assays. Their unicolonial social structure allows for the

mixing of different colonies which is extremely useful for experimental design, for example to artificially manipulate colony size. Furthermore, colonies are polygynous, harbouring a large number of fertile queens, ranging from a few dozen to thousands, which not only makes it easier to collect viable fragments in the field, but also to rescue dying colonies. *L. humile* nests tend to be superficial and highly mobile, often located in the top layer of soil and litter. As a result, they readily adapt to various artificial nest conditions and cope well with frequent nest disturbances (Wild, 2008 and H. Galante, personal observations)). Moreover, as this species does not sting or bite, it is a much safer organism to work with than some of the other highly invasive ants such as *Solenopsis invicta* (Buren, 1972) or *Wasmannia auropunctata* (Roger, 1863).

The main caveat of maintaining Argentine ants in a laboratory is their propensity for escaping which could lead to infested buildings or even new populations being introduced. These ants are extremely good escape artists being able to squeeze through the smallest of gaps (H. Galante, personal observations). This, paired with them being fast mass-recruiters, using pheromone trails to communicate with and recruit large amounts of ants to an area (Flanagan et al., 2013; Yates & Nonacs, 2016), and having multiple highly mobile queens, heightens the dangers of escape. However, for the past three years we have kept thousands of workers and queens in closed, non-airtight, PTFE-coated boxes resting on mineral oil baths with no notable escapes. This has proven to be an extremely effective, cheap and low maintenance method to mitigate the potential risks of maintaining large colonies of invasive ants in the laboratory (H. Galante, personal observations). Argentine ants are one of the most extensively studied ant species, with research covering a wide array of topics, including invasion biology, evolutionary ecology, and collective behaviors such as decision-making and self-organization (Angulo et al., 2024).

Current methods in invasive ant management

Introduced populations of *L. humile*, like most other invasive ants, quickly spread and dominate invaded areas, causing ecological and economic damage. For this reason, they are often the target of control programs. Eradicating invasive populations is often unrealistic (Britton, Gozlan & Copp, 2011), due to a need for public and economic support and a lack of effective treatment options, making it unsustainable in the long term, especially with the escalating proliferation of invasive populations (Essl et al., 2011; Seebens et al., 2017), including of *L. humile* (Mévergnies et al., 2024). Furthermore, eradication could have serious ecological consequences. Ants play key ecological roles, and with invasive ant populations assuming dominance in affected ecosystems, eradication could lead to potential ramifications, which remain, for the most part, unexplored (Schifani, Giannetti & Grasso, 2024). Currently, Integrated Pest Management (IPM) stands as the prevailing approach for managing invasive populations. It leverages various control methods, aiming to exploit the

target's ecological vulnerabilities (Silverman & Brightwell, 2008). While each method on its own may lack efficacy, their synergistic application can diminish the target population to tolerable, non-damaging levels (Dara, 2019). Management efforts applied to *L. humile* have been extensively reviewed (Silverman & Brightwell, 2008; Hoffmann, Abbott & Davis, 2009; Hoffmann et al., 2016; Angulo et al., 2024), and a brief overview is presented here. These broadly fall into four categories: physical, biological, chemical, and behavioural.

Physical control of invasive ants is mostly used in experimental research rather than management. Repellent or sticky barriers applied to tree canopies are a common method in ant-exclusion experiments, although their use is usually limited to suitable trees (Juan-Blasco et al., 2011; Mestre et al., 2016). Small exclusion plots have also proven effective in repelling mound-forming ants for over a decade (Wardle et al., 2011). However, applying and maintaining physical barriers in larger areas incurs significant costs whilst also excluding non-target organisms (Piñol, Espadaler & Cañellas, 2012). Furthermore, implementing physical control in large invaded areas can lead to the target being trapped inside the area, thus requiring additional treatments (Rust, Haagsma & Reierson, 1996; Rust, Reierson & Klotz, 2003).

Alternatively, biological control introduces the target's natural enemies, such as predatory arthropods and parasitic wasps, to the invaded area. This approach often works best when the biological control agents are trophic generalists and when multiple species are introduced simultaneously (Stiling & Cornelissen, 2005). However, it can be hard to fully predict their effects on the invaded area and whether they will themselves become established and invasive. Nevertheless, this risk can be outweighed by the scale of the ecological and economic damage caused by the invasive species (Thomas & Willis, 1998). The main obstacle to biological control is identifying natural predators, which has not yet been possible for *L. humile* and most other invasive ants (Vega & Rust, 2001; Morrison, 2012). Fungal pathogens are promising biological control agents for ants (Folgarait & Goffré, 2023), although often unsuccessful due to collective immunity (Cremer, Armitage & Schmid-Hempel, 2007).

Chemical control is the preferred method for managing invasive ants. Insecticide barrier sprays are often used around crops to prevent workers from crossing. Chlorpyrifos, an organophosphate insecticide, have also been extensively used on soil surfaces (Tollerup et al., 2004; Silverman & Brightwell, 2008). However, social insects present a unique challenge as the reproductive part of a colony is often the most physically and behaviourally protected (Howse, Haywood & Lester, 2023). If the toxicant does not reach a large proportion of the queens, invasive ant populations are likely to recover, thus rendering spraying ineffective. Furthermore, spraying lacks target specificity, and can lead to chemical runoff and water

pollution which negatively affects non-target species (Vega & Rust, 2001; Rust, Reierson & Klotz, 2003; Gentz, 2009).

Alternatively, baiting programmes, delivered in granular, gel, or liquid forms, leverage the social behaviour of ants for long-term suppression while minimising environmental harm (Cooper et al., 2008). Baits typically consist of a low-concentration delayed-effect toxicant combined with carbohydrates such as sucrose (McCalla et al., 2020). The delayed action of the toxicant provides enough time for mass recruiting ants to build foraging trails towards the bait and disseminate the toxicant throughout the colony before its effects become noticeable (Rust, Reierson & Klotz, 2004; Silverman & Brightwell, 2008). Liquid baits are preferred by ants over gel baits (Renyard et al., 2024), resulting in high mortality rates and population declines (Tollerup et al., 2004; Greenberg, Klotz & Rust, 2006; Nyamukondiwa & Addison, 2011, 2014; McCalla, Milosavljević & Hoddle, 2023). However, these are non-selective, require frequent reapplication, being both labour-intensive and costly (Daane et al., 2008).

Recently, polyacrylamide hydrogel beads were found to be a cheaper, easier to deploy, alternative to liquid baiting (Buczkowski et al., 2014; Buczkowski, Roper & Chin, 2014; Rust et al., 2015; Boser et al., 2017; Merrill et al., 2018). These porous beads absorb insecticide-laced sucrose solutions, acting as controlled-release bait dispensers (Tay et al., 2017). They have a relatively short activation window, mainly due to desiccation (Cabrera et al., 2021; Hoffmann et al., 2023) and are for the most part exclusively consumed by arthropods, most of which are invasive ants (Tay et al., 2020; Hoffmann, 2023). Biodegradable hydrogels, made from alginate, have now been developed and shown to be an effective control method for *L. humile* populations (Tay et al., 2017; McCalla et al., 2020). Moreover, adding species-specific attractants, such as synthetic trail pheromone (Choe et al., 2021), is a promising way to improve attractiveness to the baits, without attracting non-target organisms (Sunamura et al., 2024).

Despite the successful eradication of approximately 3000 discrete populations of *L. humile*, primarily through a single management program using chemical sprays (Hoffmann et al., 2016; Angulo et al., 2024), the efficiency of baiting programs remains significantly lower for *L. humile* than for other ant species (Buczkowski, Roper & Chin, 2014; Angulo et al., 2024). In fact, no eradication of an entire population of *L. humile* has been achieved using hydrogel beads despite consecutive attempts (Boser et al., 2017; Hoffmann et al., 2023; Angulo et al., 2024). Commonly used toxicants include imidacloprid, thiamethoxam, fipronil, boric acid and spinosad (Tollerup et al., 2004; Greenberg, Klotz & Rust, 2006; Cooper et al., 2008; Silverman & Brightwell, 2008; Greenberg, Tollerup & Rust, 2013). Mainly, fipronil, thiamethoxam and boric acid showed promising results in invasive ant control (Hooper-Bui & Rust, 2000; Cooper et al., 2008; Buczkowski et al., 2014; Buczkowski, Roper & Chin,

2014; Rust et al., 2015). However, most of these have now been banned, or are heavily regulated, in the European Union mainly due to their negative effects on pollinators and human health (European Chemicals Agency, 2010; Gan et al., 2012; European Food Safety Authority, 2013; Greenberg et al., 2014; Siviter & Muth, 2022; Milosavljević et al., 2024).

On the other hand, spinosad is considered to be an eco-friendly insecticide, mainly due to its low toxicity to mammals and fish (Bacci et al., 2016; Khan, 2018). It is a slow-acting neuroactive toxicant which acts by stimulating nicotinic acetylcholine and GABA receptors, leading to rapid nervous system excitation, and ultimately inducing a state of paralysis and eventually death (Salgado, 1998; Biondi et al., 2012). It is readily absorbed and metabolised (Santos & Pereira, 2020), showing similar toxicity to thiamethoxam and being registered for use in over 80 countries (Biondi et al., 2012; Milosavljević et al., 2024). For these reasons, spinosad is a promising toxicant for invasive ant management. Nevertheless, efforts should still be made to minimise its usage, as it can be harmful to non-target insects at low doses (Martelli et al., 2022). Future control is also likely to involve genetic pest management, which disrupts insect behaviour, introducing genetically engineered DNA sequences into wild conspecific populations. However, this is still in its infancy, having not yet been applied to invasive ant management, and has the potential for the engineered genes to spread uncontrollably (Harvey-Samuel, Ant & Alphey, 2017).

Using bait additives to manipulate ant behaviour

Chemical control attempts are costly and often result in ecosystem contamination (Sánchez-Bayo, van den Brink & Mann, 2011; Dhuldhaj, Singh & Singh, 2022). Nevertheless, these are necessary when preventive measures fail to halt the introduction of new populations. Invasive ant management often falls short, likely due to the high costs associated with large-scale deployment (Rust, Reierson & Klotz, 2003; Nelson & Daane, 2007), as well as a lack of sustained bait consumption, resulting from competition with natural food sources and the active abandonment of foraging trails (Silverman & Brightwell, 2008; Zanola, Czaczkes & Josens, 2024). Therefore, maximising bait consumption within the first few hours is crucial. Behavioural control aims to exploit the natural behaviours of the target species (Polajnar et al., 2015; Dara, 2019). For example, providing alternative sugary solutions, which compete with honeydew, can disrupt ant-hemipteran mutualisms. This maintains the positive effects of ants on plants, while reducing their protection of hemipterans, which negatively affect the plants they infest (Baker, Van Vorhis Key & Gaston, 1985; Correa et al., 2023; Jensen et al., 2023). Moreover, ants prefer complex nectars over simple sugars (Blüthgen & Fiedler, 2004), which presents an opportunity to further disrupt these mutualistic relationships by incorporating additives into baits. For example, queens and larvae are known to require more protein than workers (Feldhaar, 2014; Csata & Dussutour, 2019). Thus, the addition of protein to baits could lead to a higher amount of the toxicant reaching the reproductive part

of the colony (Markin, 1970; Baker, Van Vorhis Key & Gaston, 1985; Abril, Oliveras & Gómez, 2007).

Chemical additives can alter foraging behaviour by manipulating individual preferences. Bees have been shown to prefer sucrose solutions laced with neonicotinoid pesticides, even if these led them to eat less food overall (Kessler et al., 2015). In ants, synthetic trail pheromone was shown to enhance bait attractiveness, increasing consumption (Greenberg & Klotz, 2000; Choe et al., 2021), and caffeine to act as a repellent or an attractant depending on the extract and concentration used (Majid et al., 2018; Yeoh, Dieng & Majid, 2018; Madsen & Offenberg, 2019). Moreover, lacing sugary solutions with morphine resulted in ants developing a non-addictive preference for solutions containing it (Mogensen et al., 2024). Interestingly, whilst bees tend to forage on nuptial nectar, which is sucrose-dominated with high amounts of GABA and alkaloids, ants are predominantly found in extranuptial nectaries, which are hexose-rich having a richer yet less variable amino acid chemical profile, mainly composed of serine, alanine and tyramine (Balduino et al., 2023). Amino acids can influence nectar taste and/or odour, acting as attractants or repellents (Hendriksma, Oxman & Shafir, 2014; Pacelhe et al., 2019; Nicolson, 2022).

The ability to learn and remember allows animals to adapt to their environment during their lifetime, enhancing their chances of survival. Ants rely on multimodal cues, such as spatial and olfactory information, forming long-lasting associations rapidly (Dupuy et al., 2006; Huber & Knaden, 2018; Piqueret, Sandoz & d'Ettorre, 2019; Buehlmann, Aussel & Graham, 2020; Wenig, Bach & Czaczkes, 2021). However, like most animals, ants suffer from cognitive biases, where the perceived value of various options depends on previous experiences (d'Ettorre et al., 2017; Wendt et al., 2019; Oberhauser et al., 2020; De Agrò et al., 2021). These natural preferences and deviations from rationality can be exploited to influence foraging preferences. Leveraging natural behaviours could improve management attempts, increasing target specificity and reducing the use of pesticides (Farina et al., 2020; Estravis-Barcala, Palottini & Farina, 2021). Argentine ants, as well as most other invasive ants, are mass recruiters, known to strongly follow pheromone trails more so than to rely on visual memory (Von Thienen, Metzler & Witte, 2016). However, they do not solely rely on pheromone trails when navigating (Reid, Sumpter & Beekman, 2011), but also on path integration and learnt visual landmarks (Card, McDermott & Narendra, 2016; Freas & Spetch, 2024a, 2024b). In fact, *L. humile* are excellent learners, capable of quickly forming strong spatial and olfactory associations with sugary rewards (Rossi et al., 2020; Wagner et al., 2023; Galante & Czaczkes, 2024). Hence, targeting learning could be one way of increasing bait consumption. If a bait additive were to improve an individual's ability to locate food or return to its nest, reducing foraging time, it could result in the earlier formation of pheromone trails to the bait. These trails would likely outcompete those leading to natural food sources, being rapidly reinforced, enhancing recruitment, and

consequently, the consumption of the bait and its toxicant (Czaczkes, Grüter & Ratnieks, 2013).

Learning and short-term memory, which ranges from minutes to hours, are the main targets of management attempts, as these rely on immediate molecular actions which can be more easily manipulated (Kandel, Dudai & Mayford, 2014). Similarly, plants are known to lace nectar with bioactive secondary metabolites that have no nutritional value, but can interfere with pollinator cognition, altering their behaviour (Nepi, 2017; Stevenson, Nicolson & Wright, 2017; Bogo et al., 2019; Mustard, 2020; Estravis-Barcala, Palottini & Farina, 2021). Promising bait additives include neuroactives, such as alkaloids, biogenic amines and non-protein amino acids (see Table 1 in (Galante & Czaczkes, 2024)). For example, bumblebees fed nicotine showed memory improvements and a reduced ability to reverse learn, which could result in increased flower fidelity (Baracchi et al., 2017). In honeybees, dopamine increased the perceived value of sucrose solutions, improving olfactory learning and memory retrieval (Huang et al., 2022). Similarly, non-protein amino acids improved associative learning and memory retention (Carlesso et al., 2021).

Caffeine is a particularly interesting additive as it is cheap, widely available, and at low doses often beneficial. Throughout the day, as energy is required, neurons break down adenosine triphosphate (ATP), producing adenosine as a by-product. As more energy is used, more adenosine is produced and released extracellularly, eventually binding to adenosine receptors. In mammals, adenosine binding reduces neuronal activity by enhancing the release of GABA, the main inhibitory neurotransmitter. Moreover, adenosine binding inhibits the release of neurotransmitters such as dopamine, associated with reward and motivation, glutamate, responsible for promoting neuronal activity, and acetylcholine, closely involved in cognitive plasticity, memory, and attention. In doing so, adenosine promotes relaxation and drowsiness, allowing the brain to rest proportionally to how active it has been (Ribeiro, Sebastião & de Mendonça, 2003; Villanueva-García et al., 2020). Caffeine, having a similar molecular structure to adenosine, can bind to adenosine receptors without activating them. By blocking the adenosine receptors, caffeine prevents adenosine from binding to them, increasing alertness (Sugimachi et al., 2016; Reichmann, 2022). In honeybees, caffeine leads to longer lasting olfactory memory associations (Wright et al., 2013), increasing foraging frequency and quadrupling colony-level recruitment (Couvillon et al., 2015). Furthermore, it boosts learning, and when combined with arginine, it improves memory retention, resulting in increased foraging activity (Estravis-Barcala, Palottini & Farina, 2021; Marchi, Palottini & Farina, 2021). In bumblebees it has been linked to increased pollination (Thomson, Draguleasa & Tan, 2015) and improvements in odour associations (Arnold et al., 2021; De Ibarra & Rands, 2021). In ants, it was reported to improve conditioning and memory, albeit decreasing food consumption (Cammaerts, Rachidi & Gosset, 2014).

Automation and computer vision as research tools

Computer vision seeks to mimic human capabilities, applying algorithms to the analysis and interpretation of visual data obtained from images or videos. Here, I provide a brief overview of the benefits of using computational tools in scientific research (see Egnor & Branson, 2016 and Lürig et al., 2021 for reviews).

The use of visual representations to capture natural behaviours and patterns is crucial for research. Technological advancements have made it easier to acquire and analyse large volumes of images and videos, which could not otherwise be processed in real time (Hol, Lambrechts & Prakash, 2020; Baltiansky et al., 2021; Maya et al., 2023; Håkansson et al., 2024). Automated recording systems can significantly reduce human-induced biases in animal behaviour studies and allow for longer observation periods (Papadakis et al., 2012). However, extracting useful information from large visual datasets can be challenging. Traditional image analysis techniques are labor-intensive, expensive, and prone to human error, often resulting in low-resolution data. In contrast, computer algorithms offer an objective, quantitative, and high-resolution approach to analysis, addressing many challenges associated with manual methods (Høye et al., 2021). Implementing computer vision techniques not only saves time by automating repetitive tasks but also ensures high reproducibility and transparency, preserving original data and documenting each step of the analysis.

The versatility of computer vision is evident in its wide range of applications. In medical research, for example, computer vision aids in intraoperative video analysis (Mascagni et al., 2022). In archaeology, algorithms analyse artifacts and site layouts (Brutto & Meli, 2012). Applications also include body language recognition in linguistics (Neidle, Sclaroff & Athitsos, 2001), food quality assessment (Mogol & Gökmen, 2014), characterisation of molecular structures (Nussinov & Wolfson, 1991) and the analysis of plant characteristics (Spalding, 2009). Additionally, computer vision has been widely used in animal behaviour studies, extracting behavioural indicators from images on various animals such as fish (Papadakis et al., 2012), mammals (Barnard et al., 2016), invertebrates (Høye et al., 2021), and even free-roaming animals recorded with drones (Koger et al., 2023).

Similar to hypothesis testing and statistics, computer vision requires planning before data collection. Technically, it is important that the images have sufficient resolution and adequate characteristics, such as an even background or consistent lighting. However, such considerations vary with the algorithms being used. For example, methods relying on background subtraction, where the goal is detecting moving objects from the difference between the current image and a reference image, are more sensitive to light changes than those using pose estimation, where the goal is to detect the position and orientation of an

animal or object (Mathis et al., 2018; Nath et al., 2019; Pereira et al., 2022). Generally, if a human can reliably and consistently identify an object or behaviour of interest in an image, a computer algorithm can be trained to do so faster and more accurately. However, understanding how one intuitively identifies an object or pattern can be challenging in itself. Clear definitions are a vital part of research, even when not using computer vision. For example, ethograms, a list of behaviours or actions exhibited by an animal, must be carefully created such that different observers follow the same rules when classifying similar behaviours (Stanton, Sullivan & Fazio, 2015).

Despite its benefits, the adoption of computer vision faces resistance due to its perceived complexity, a steep learning curve, and concerns about job displacement (Spencer, 2018; Kozak et al., 2020). Nevertheless, the increasing accessibility of artificial intelligence and open-source software is largely lowering these barriers. For example, open-source, inexpensive scanners can now be used to generate computational models of animals (Plum & Labonte, 2021), which can later be used to generate training data for machine learning models, thus removing the time-consuming step of manually annotating data (Plum et al., 2023). Ultimately, researchers must weigh the initial learning costs of using computer vision algorithms against its long-term benefits. Embracing these technologies can free them from tedious manual tasks while dramatically increasing not only the volume but also the quality of experimental data.

Structure and outline of the thesis

Hydrogel baits are a promising tool in invasive ant management. Yet, to date, there have been no successful eradication attempts using them against *L. humile*. Typically, these baits consist of a toxicant-laced sucrose solution (Angulo et al., 2024), despite ants' preference for complex mixtures (Blüthgen & Fiedler, 2004). This thesis aims to identify bait additives which enhance foraging behavior and thus increase the consumption of toxic baits. Mainly, it explores the potential of neuroactives to influence ant behavior by interfering with learning and memory and how a neuroactive toxicant, spinosad, as well as protein interfere with collective food transfer.

Chapter 1 provides a brief overview of the effects of neuroactive chemicals on learning and memory in Hymenoptera, and highlights the lack of studies investigating these effects on ants. Additionally, it used a classical method, bifurcation mazes, to explore the effect of seven potential neuroactives on learning and memory in the highly invasive ant *Linepithema humile*. It demonstrated that Argentine ants are excellent and fast learners of both spatial and olfactory cues, albeit suggesting none of the seven chemicals tested interfered with the ant's cognitive abilities. Bifurcation mazes are fast and convenient, yet the binomial nature of the data they generate, paired with their simplicity, resulted in ceilling effects. Therefore,

while none of the chemicals tested impeded learning, they could have potentially enhanced it were the task more challenging. To tackle this, Chapter 2 used a more complex and field-realistic open landscape foraging experiment. This approach, combined with computer vision algorithms used to track the ants over time, revealed a dose-dependent effect of caffeine on ant foraging.

At the individual level, *L. humile* ingest extremely small amounts of food, typically in the nanoliter range, making it impossible to quantify consumption using traditional methods. Understanding and quantifying ant feeding behaviour is crucial to determine the amount of active ingredient being ingested, and thus adjust concentrations as needed, minimising the amount of chemicals used. Furthermore, feeding behaviour can reveal individual's food preference. In Chapter 3, using computer vision algorithms, ant bodies were three-dimensionally reconstructed over time while ants were exposed to a gradient of sucrose and caffeine solutions. Considering the body of Argentine ants expands during feeding, this new non-invasive method provided estimates of not only crop load, but also consumption rate.

Chapter 4 aimed to understand if ants, at both the individual and the collective level, perceived and avoided a sublethal dose of spinosad, a slow-acting toxicant. Mainly, it explored if ants were capable of conditioned taste aversion, where individuals form a delayed association between a solution and the negative symptoms induced by its ingestion. Following this, Chapter 5 established survival rates of different concentrations of spinosad across varying group sizes. Moreover, using fluorescence imaging, it aimed to elucidate some of the mechanisms by which ant collectively evade the effects of the toxicant, even upon ingesting and spreading it. Finally, Chapter 6 aimed to understand if the presence of protein in a solution results in it being differentially shared throughout the colony. Theoretically, the presence of protein in a bait should result in it reaching queen and brood faster and/or at higher amounts, whilst simultaneously reducing the amount of workers which come in contact with it.

Ultimately, the methods and results developed in this thesis contribute to our understanding of ant behaviour and cognition, and provide the foundation for the development of new behavioural control methodologies which effectively manipulate invasive ant behaviour.

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Chapter 1

Invasive ant learning is not affected by seven potential neuroactive chemicals

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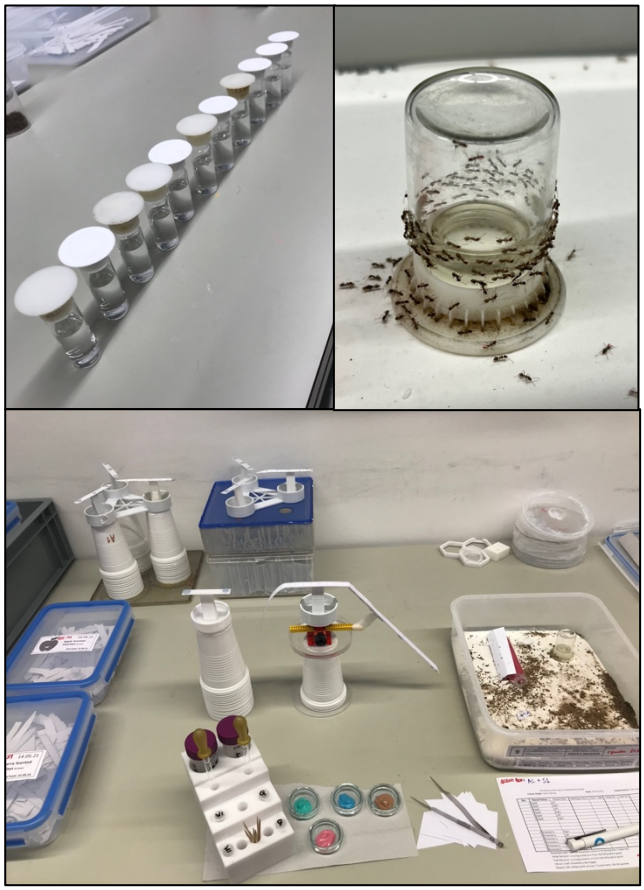
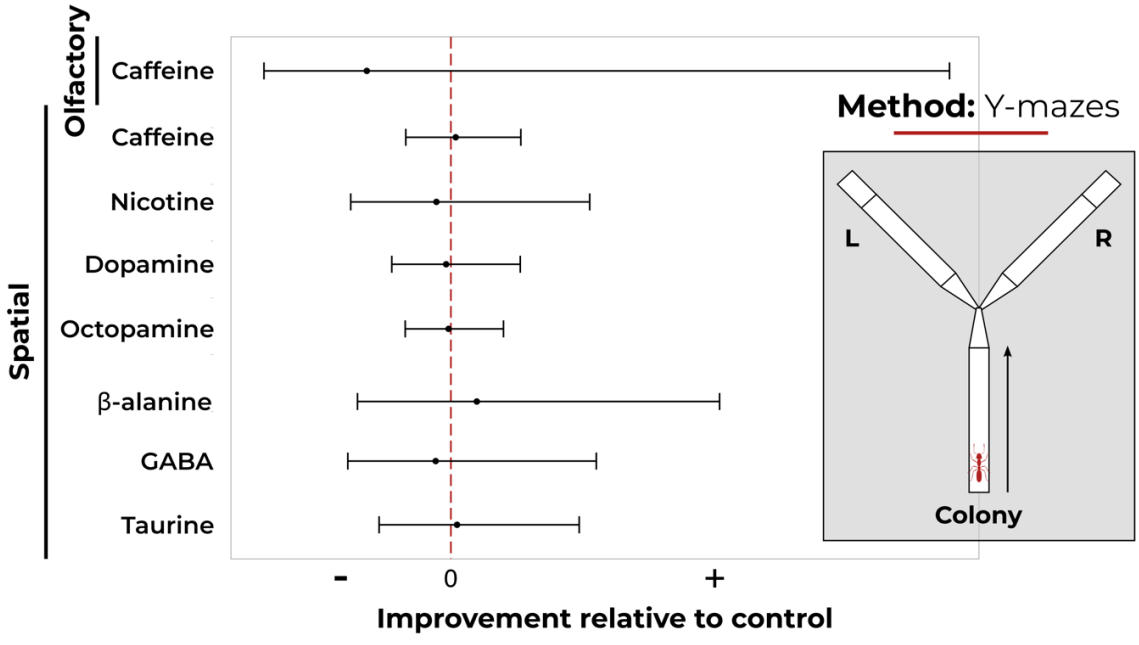
Abstract

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: β-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.

neuroactive chemicals • caffeine • associative learning • memory



Conclusion: None of the tested chemicals influenced learning!



Introduction

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. 2023). 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. 2023).

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 behaviour (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 β-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 β-alanine and GABA has been reported to improve associative learning and memory retention in bees. However, when ingested prior to conditioning, GABA, β-alanine and taurine hindered learning, but not memory retention, which was surprisingly improved by β-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).

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

	Chemical	Species	Effect
Alkaloids	**Caffeine** (see (Mustard, 2014) for a review)	*Apis mellifera*	Increased foraging frequency and waggle dancing, quadrupling colony-level recruitment (Ishay & Paniry, 1979; Couvillon et al., 2015). Elicited feeding preference (Singaravelan et al., 2005). Enhanced motivation and cognitive performance in complex learning tasks (Si, Zhang & Maleszka, 2005). Affects memory formation but not early long-term memory (Mustard et al., 2012). Longer lasting olfactory memory associations which can last several days (Wright et al., 2013). Increases learning performance. Memory retention increases when caffeine is mixed with arginine (Marchi, Palottini & Farina, 2021).
		Bombus impatiens	Increased pollination of flowers offering moderate concentrations of caffeine in nectar (Thomson, Draguleasa & Tan, 2015). Interaction of octopamine and tyramine with caffeine eliminated aversion to caffeine while enhancing visitation rate (Muth et al., 2022).
		Bombus terrestris	Lowered overall food consumption (Tiedeken et al., 2014). Short-lived decrease in handling times and improvement in odour associations (Arnold et al., 2021).
		Vespa orientalis	Enhanced motor activity, appetite for proteins and exaggerated response to optic and acoustic stimuli (Ishay & Paniry, 1979).
		Myrmica sabuleti	Increased linear speed, conditioning ability, and memory. Decreased food consumption (Cammaerts, Rachidi & Gosset, 2014a).
		Other ant species	Ineffective in killing household ants. Can act as a repellent or an attractant depending on the extract and concentration used (Yeoh, Dieng & Majid, 2018; Ab Majid et al., 2018; Madsen & Offenberg, 2019).
	Nicotine	*Apis mellifera*	Elicited feeding preference (Singaravelan et al., 2005). Increased sucrose sensitivity and improved olfactory learning retention (Thany & Gauthier, 2005). Partial repellent potentially enhancing cross-pollination (Köhler, Pirk & Nicolson, 2012).
		Bombus terrestris	Lowered overall food consumption (Tiedeken et al., 2014). Enhanced memory for floral traits and reduced ability to reverse learn (Baracchi et al., 2017).
		Myrmica sabuleti	Enhanced cognitive abilities and increased locomotion. Decreased food consumption (Cammaerts, Rachidi & Gosset, 2014).

(Continued on next page)

Table 1 - Continued

	Chemical	Species	Effect
Biogenic amines	**Dopamine** (see (Giurfa, 2006; Verlinden, 2018) for a review)	*Apis mellifera*	Decreased sucrose responsiveness (Scheiner et al., 2002). Blocking of dopaminergic receptors suppresses aversive learning (Vergoz et al., 2007). Regulates motor behaviour (Mustard, Pham & Smith, 2010). Reduced punishment perception (Agarwal et al., 2011). Impairs appetitive memory consolidation (Klappenbach, Kaczer & Locatelli, 2013). Increased likelihood of visiting training feeder (Linn et al., 2020). Increased perceived value of sucrose solution and improved olfactory learning and memory retrieval (Huang et al., 2022). Improved learning success and might regulate optimal motivational or attentional levels (Raza et al., 2022).
		Nasonia vitripennis	Interferes with appetitive learning (Lenschow et al., 2018).
		Lasius niger	Linked to long-term memory consolidation, independent of short-term memory formation (Wissink & Nehring, 2021).
	Octopamine (see (Giurfa, 2006; Farooqui, 2012) for a review)	*Apis mellifera*	Increased sucrose responsiveness (Scheiner et al., 2002). Reduced sucrose response thresholds (Pankiw & Page, 2003). 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, Galindo-Cardona & Oskay, 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).
		Nasonia vitripennis	Receptor antagonist disrupts appetitive learning (Lenschow et al., 2018).
		Lasius niger	Necessary for appetitive learning of olfactory cues (Wissink & Nehring, 2021).
Non-protein amino acids	**β-alanine** (see (Nepi, 2014) for a review)	*Apis mellifera*	Improved associative learning and memory retention. If ingested prior to conditioning, it hinders learning but improves memory retention (Carlesso et al., 2021).
		Bombus terrestris	Higher walking index and lower feeding, flying and stationary indices (Bogo et al., 2019).
		Vespa orientalis	Inhibited nest construction behaviour (Bouchebti et al., 2022).
	GABA (see (Nepi, 2014) for a review)	*Apis mellifera*	Regulated the specificity of associative olfactory memory (Hosier, Buxton & Smith, 2000). Exerted a modulatory role in memory formation depending on the training strength (Raccuglia & Mueller, 2013). Decreased activity levels (Mustard, Jones & Wright, 2020). Improve associative learning and memory retention, but hinders learning if ingested prior to conditioning (Carlesso et al., 2021).
		Bombus terrestris	Lower flying index (Bogo et al., 2019).
		Osmia bicornis	Higher motor activity (Felicioli et al., 2018).
		Vespa orientalis	Inhibited nest construction (Bouchebti et al., 2022).
		Oecophylla smaragdina	Elicited preference (Madsen & Offenberg, 2019).
		Lasius niger	Elicited preference (Madsen & Offenberg, 2019).
	Taurine (see (Nepi, 2014) for a review)	*Apis mellifera*	If ingested prior to conditioning, it hinders learning but improves memory retention (Carlesso et al., 2021).

See (Nepi, Grasso & Mancuso, 2018; Mustard, 2020) for reviews on the effects of nectar secondary metabolites on insect pollinators.

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: β-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. 2023). 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), β-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 Hymenopterans. 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 μmol 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 μmol mL^{-1} (Thany and Gauthier 2005; Cammaerts et al. 2014b; Baracchi et al. 2017). 10.55 μmol mL^{-1} of dopamine or octopamine were mixed with 9.94 μmol mL^{-1} of ascorbic acid to reduce oxidation of the biogenic amines (Scheiner et al. 2002; Linn et al. 2020). β-Alanine, GABA and taurine were used at 0.27 μmol mL^{-1}, 0.73 μmol mL^{-1}, and 0.32 μmol 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, 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 honeybees (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 flavouring (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 μmol 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, the Y-maze arm in which it was located, and the odour 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 (wi) 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).

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.

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 R2 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.

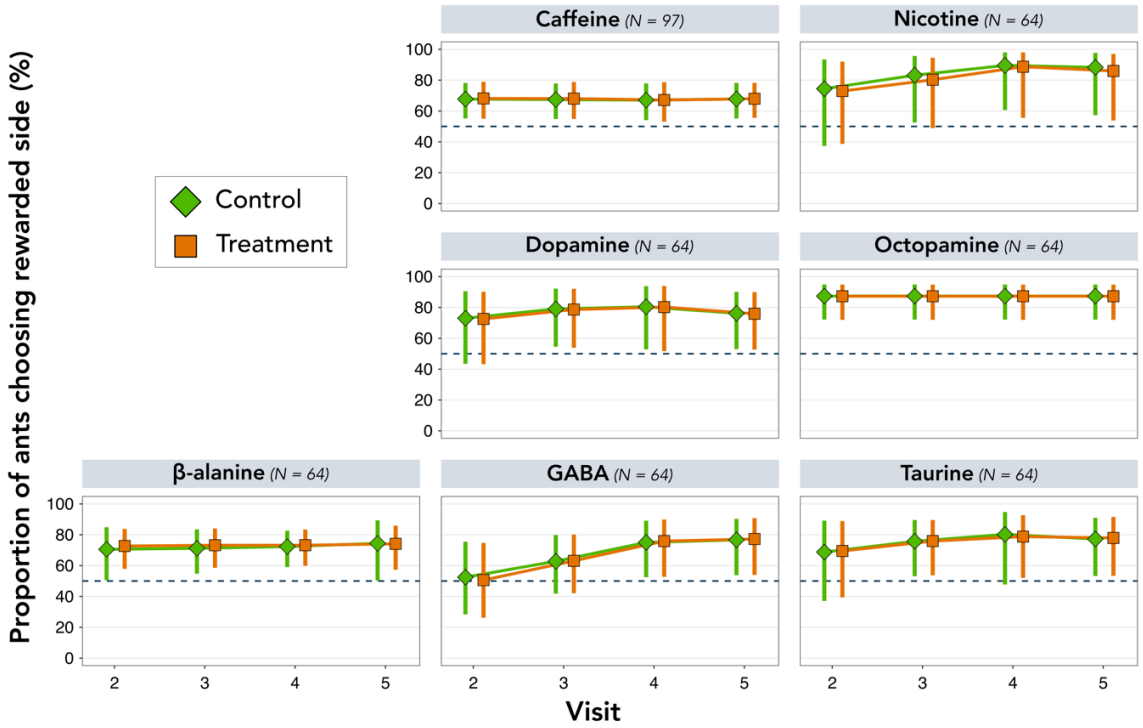


Figure 1 - Ants learn to associate a sucrose reward with an arm of a Y-maze over consecutive visits across experiments and treatments. Circles represent the proportion of ants choosing the rewarded side of the maze as their final choice and whiskers the respective 95% unconditional confidence intervals 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.

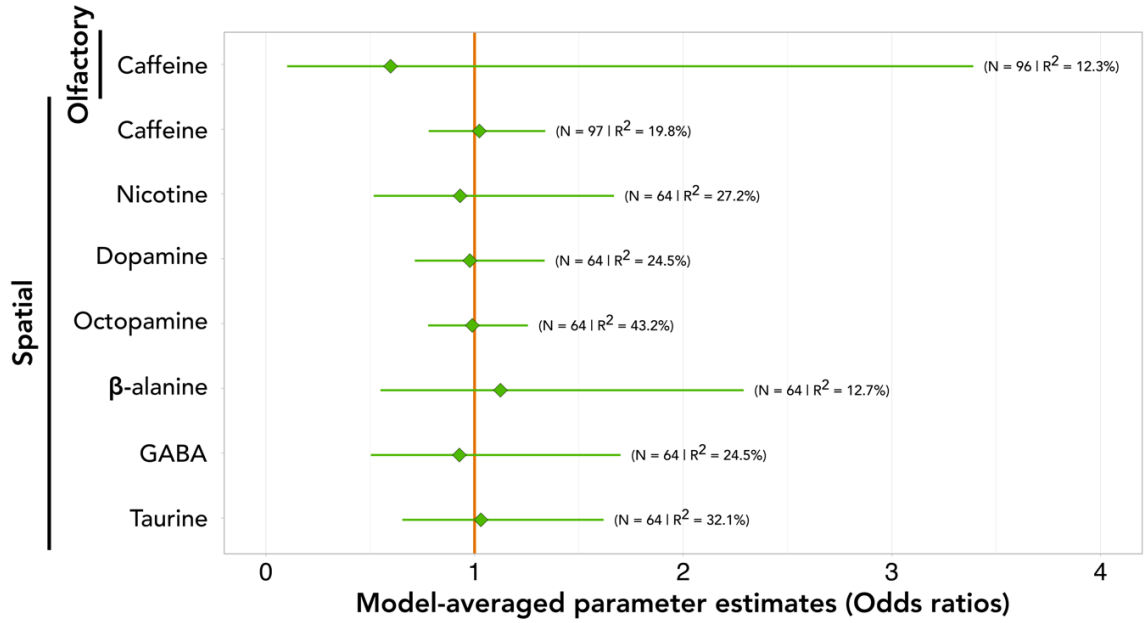


Figure 2 - Effect of seven potential neuroactive chemicals on the olfactory and spatial associative learning of *L. humile*. Circles represent the estimates obtained from model-averaging with shrinkage and whiskers the 95% unconditional confidence intervals. The odds ratio compares the odds (probability 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. R2 refers to the goodness of fit of the model which explains the most variance in the data for each set of candidate models.

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 β-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, β-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) 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. 2023). 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 Hymenopterans (Table 1). Honeybees prefer sucrose solutions laced with up to 0.52μmol 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, 5.15μmol 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 effects of the neuroactive chemicals used are time-dependent, and therefore this study

could have missed the chemical activation window. In fact, honeybees fed 1.04μmol 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 0.05μmol mL^{-1} and 0.51μmol 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 (>10.32μmol 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. 2023). 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.

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. 2024) 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, Palottini & Farina, 2021; Muth et al., 2022).
- β-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.

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 μmol mL^{-1} or 0.16 μmol 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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Declaration of interests

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

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

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Chapter 2

Acute exposure to caffeine improves foraging in an invasive ant

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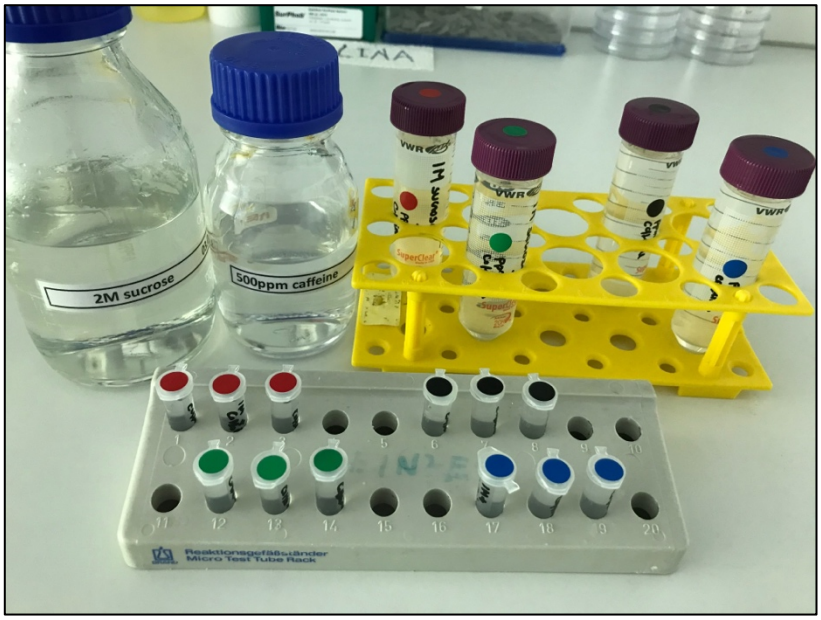
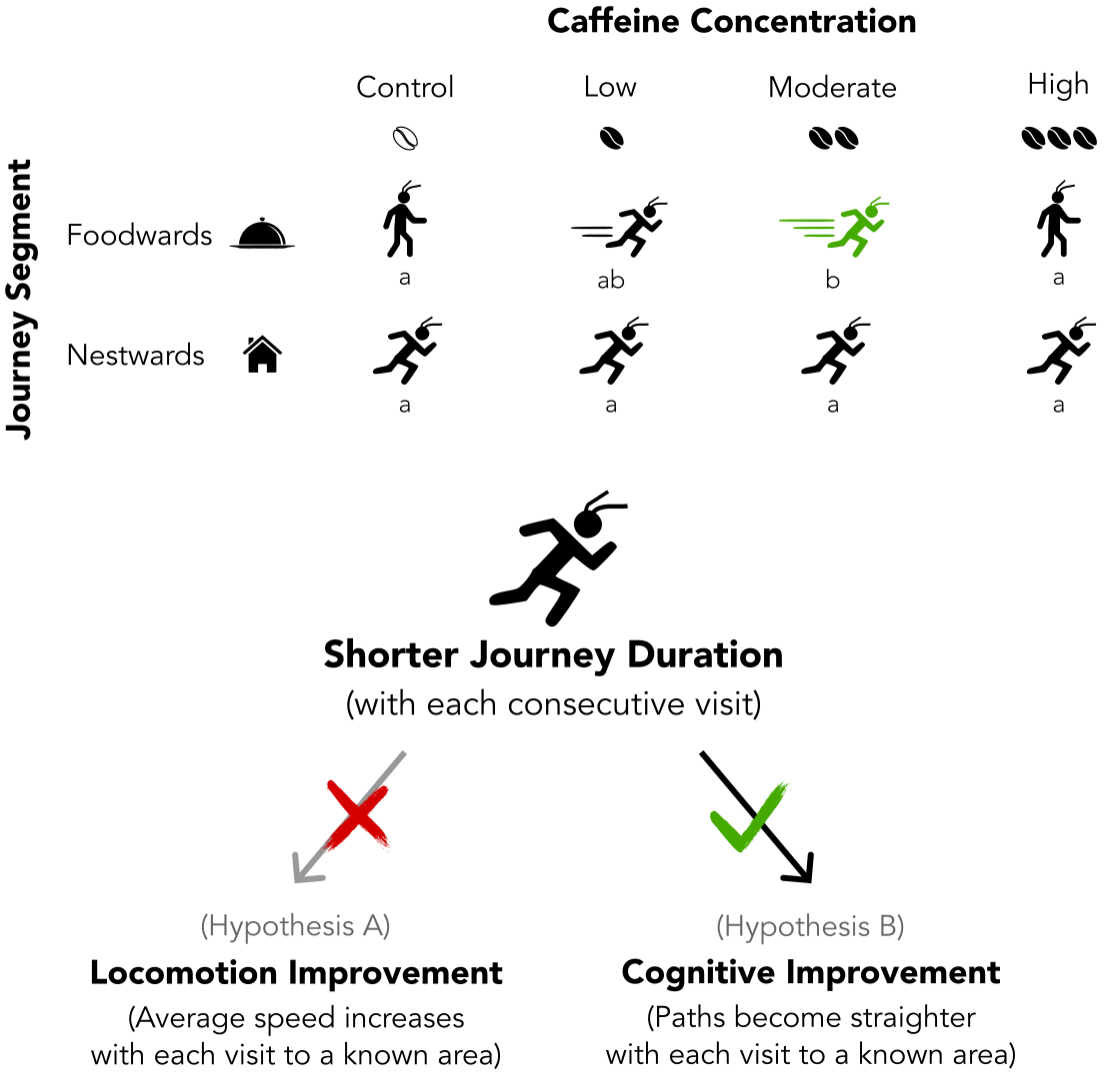
Abstract

Argentine ants, *Linepithema humile*, are a particularly concerning invasive species. Control efforts often fall short likely due to a lack of sustained bait consumption. Using neuroactives, such as caffeine, to improve ant learning and navigation could increase recruitment and consumption of toxic baits. Here, we exposed *L. humile* to a range of caffeine concentrations and a complex ecologically relevant task: an open landscape foraging experiment. Without caffeine, we found no effect of consecutive foraging visits on the time the ants take to reach a reward, suggesting a failure to learn the reward's location. However, under low to intermediate caffeine concentrations ants were 38% faster with each consecutive visit, implying that caffeine boosts learning. Interestingly, such improvements were lost at high doses. In contrast, caffeine had no impact on the ants' homing behaviour. Adding moderate levels of caffeine to baits could improve ant's ability to learn its location, improving bait efficacy.

invasion biology • ants • caffeine • foraging • navigation • learning • memory


2M sucrose
500ppm caffeine


Caffeine Concentration
Control
Low
Moderate
High
Journey Segment
Foodwards
Nestwards
a
ab
b
a
a
a
a
Shorter Journey Duration
(with each consecutive visit)
(Hypothesis A)
Locomotion Improvement
(Average speed increases
with each visit to a known area)
(Hypothesis B)
Cognitive Improvement
(Paths become straighter
with each visit to a known area)

Introduction

In Europe, the costs associated with invasive alien species are increasing ten-fold every decade, amounting to billions of euros (Haubrock et al., 2021). Among these invasive species, *Linepithema humile* (Mayr, 1868) is particularly damaging, considered one of the most ecologically harmful and costly invasive species worldwide (Lowe et al., 2000; Angulo et al., 2022): invasive ants can outcompete native ants, leading to declines in biodiversity and abundance, which in turn, can have a variety of direct and indirect effects on other non-ant species (Holway et al., 2002; Silverman & Brightwell, 2008; Alvarez-Blanco et al., 2021). Given the severity of their impact, invasive ants are frequently considered a top priority for conservation and pest management programs (Hoffmann et al., 2016; Merrill et al., 2018; Buczkowski & Wossler, 2019; Suiter et al., 2021).

Studying foraging and navigation in *L. humile* can offer both fundamental insights into insect navigation, whilst potentially offering new approaches for their control. As central place foragers, ants must actively seek out food sources and bring them back to the nest. Navigation relies predominantly on two mechanisms: path integration and the use of learnt information. Path integration combines compass information (Wehner & Müller, 2006; Müller & Wehner, 2007; Wajnberg et al., 2010; Wystrach et al., 2014) with an odometer (Wittlinger, Wehner & Wolf, 2006) to continuously track the ant's position relative to a reference point, usually either the nest or a frequently visited feeding site (Wehner, 2008). This global vector allows ants to return back to the reference point even when navigating through featureless and novel environments. Simultaneously, many insects also employ view-based navigation. They are able to take a panoramic snapshot of a goal, such as their nest, a food source, or a point along a path and later compare it with their current view (Collett & Cartwright, 1983; Cartwright & Collett, 1987; Wystrach & Beugnon, 2009; Wystrach, Beugnon & Cheng, 2011). Importantly, view-based navigation differs from path integration in that it does not exclusively rely on idiothetic cues. Instead, it is grounded in an individual's ability to perceive and learn environmental cues (Knaden & Graham, 2016). Nevertheless, both mechanisms greatly depend on an individual's memory retrieval capabilities (Wystrach et al., 2020). However, whether these navigational mechanisms rely on the same neurological foundations (Webb & Wystrach, 2016; Zeil, 2023), and the extent to which *L. humile* rely on these mechanisms remains unclear. Nonetheless, recent work highlights the use of path integration in other trail-laying species (Card, McDermott & Narendra, 2016; Freas & Spetch, 2024a, 2024b) and *L. humile* has been shown to be a fast learner of multimodal cues (Rossi et al., 2020; Wagner et al., 2023; Galante & Czaczkes, 2024).

Pharmacological interventions offer an excellent tool for dissecting the neural and cognitive basis of behaviours such as navigation (Scheiner et al., 2002; Buehlmann et al., 2020; Kamhi,

Barron & Narendra, 2020). Moreover, they provide the opportunity to manipulate, and potentially steer, the behaviour of animals. This seems to have already been adopted by various plants, which spike their nectar with secondary metabolites, some of which influence neural activity (Mustard, 2020; Muth et al., 2022; Nicolson, 2022). Such chemicals have the potential to artificially manipulate insect behaviour (Baracchi et al., 2017; Wink, 2018; Carlesso et al., 2021). In this way, deploying neuroactives in artificial baits may be a promising way of enhancing control efficacy. If a bait additive could enhance the individual's homing behaviour, pioneer ants ingesting it might return to their nest faster, likely leading to increased recruitment to the bait relative to scouts which collected food without such additives. This in turn may result in baits with such additives outcompeting other food sources. Additionally, were the bait additive to improve the acquisition and use of learnt information during navigation, this could result in faster round trips to the bait, with each additional visit further reinforcing the pheromone trail, increasing recruitment to and consumption of the bait (Czaczkes, Grüter & Ratnieks, 2013).

Caffeine is especially interesting in this regard, as it is naturally occurring, cheap, and well-studied. In honeybees, it has been shown to elicit feeding preference (Singaravelan et al., 2005) and to enhance motivation and cognitive performance during complex learning tasks (Si, Zhang & Maleszka, 2005). Additionally, it increased foraging frequency and waggle dancing, quadrupling colony-level recruitment (Couvillon et al., 2015). Interestingly, it seems to hinder learning but not memory formation (Mustard et al., 2012), although it is reported to improve long-term olfactory associations in honeybees (Wright et al., 2013). Similarly, caffeine improved bumblebee odour association learning (Arnold et al., 2021) and increased flower pollination (Thomson, Draguleasa & Tan, 2015). However, it lowered bumblebee food consumption (Tiedeken et al., 2014). In ants, it likely alters food value perception, acting as both an attractant and a repellent, depending on the plant extract and concentration used (Yeoh, Dieng & Majid, 2018; Majid et al., 2018; Madsen & Offenberg, 2019). Furthermore, caffeine has been reported to improve learning and memory in Myrmica sabuleti ants, albeit at the cost of decreased food consumption (Cammaerts, Rachidi & Gosset, 2014).

Contrastingly, recent work suggests a general lack of effect of an intermediate concentration of caffeine on both spatial and olfactory associative learning in *L. humile* (Galante & Czaczkes, 2024). However, that study focused on a single concentration of caffeine and overlooked its potential effects on foraging performance. Additionally, it tested learning in a binary choice Y-maze setup, which proved to be a very easy task for the ants, leading to a ceiling effect which further obscured the potential effects of caffeine. Here, we subject *L. humile* to a wider range of caffeine concentrations and a more complex and ecologically relevant task: an open landscape foraging experiment. Such a challenging task overcomes ceiling effect issues. By using an automated tracking system, we collected high resolution

data of different segments of the foraging journey, as opposed to simple associative learning binary choices, studying not only the effect of caffeine on learning and memory, but also on locomotion and navigation.

Materials and Methods

Colony maintenance

Linepithema humile (Mayr, 1868) were collected from Portugal (Proença-a-Nova) and Spain (Girona) between April 2021 and January 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 x 22.2 x 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 September 2021 and March 2022 using 14 colonies divided into donor/recipient pairs. Focal ants left their original colony (donor) but returned to a different colony (recipient) to unload the contents of their crop. This ensured donor colonies, and consequently focal ants, were never exposed to caffeine prior to the experiment.

Chemicals and solutions

Caffeine (CAS 58-08-2) was obtained from Sigma-Aldrich (Taufkirchen, Germany). 1M sucrose solutions (Südzucker AG, Mannheim, Germany) mixed with different caffeine concentrations were used as treatments. Identical 1M sucrose solutions were used as controls. Caffeine concentrations were chosen based on previous reports of their effects on Hymenopterans. Caffeine solutions ranged from a low, naturally occurring concentration (Singaravelan et al., 2005; Couvillon et al., 2015) of 25ppm (0.13μmol ml-1) to an intermediate concentration of 250ppm (1.29μmol ml-1), and a high concentration of 2000ppm (10.30μmol ml-1), previously reported as the LD50 of honeybees (Detzel & Wink, 1993). A double-blind procedure was applied to all solutions used in order to minimize experimenter bias.

Open landscape spatial learning

A challenging and ecologically relevant experimental paradigm was developed to study the effects of different concentrations of caffeine on spatial learning and memory. This setup consisted of an A4 (210 x 297 mm) disposable paper overlay platform, surrounded by a water moat, with a single 1cm entrance, attached to a fixed top-view Raspberry Pi HQ

camera setup. An *ad libitum* sucrose solution drop (positive stimulus), either pure or laced with 25ppm, 250ppm or 2000ppm of caffeine, was positioned at the entrance of the platform (see blue drop in Figure 1B). A single ant from a donor colony, placed on the table to the right of the setup, was allowed onto the platform via a mobile drawbridge, was marked with acrylic paint while drinking, and returned to its paired recipient colony. Meanwhile, the disposable overlay was replaced, ensuring the removal of any pheromone trails laid, and a new *ad libitum* drop of the solution used during the first visit placed on one of the sides of the platform (see green drops in Figure 1B). After unloading, the ant was placed back onto the platform's entrance where it was video recorded and the time it spent in the colony, the time it took to reach the reward, to drink and to return to the colony was noted for each visit. Ants were not prevented from using available room cues, as for example the ceiling light. Overall, each ant carried out five consecutive visits to the platform: a priming visit, with the reward placed at the entrance of the platform, followed by four visits with the reward either always on the left or the right side of the landscape. 142 individuals were tested, with a complete run taking between 35 to 155 minutes.

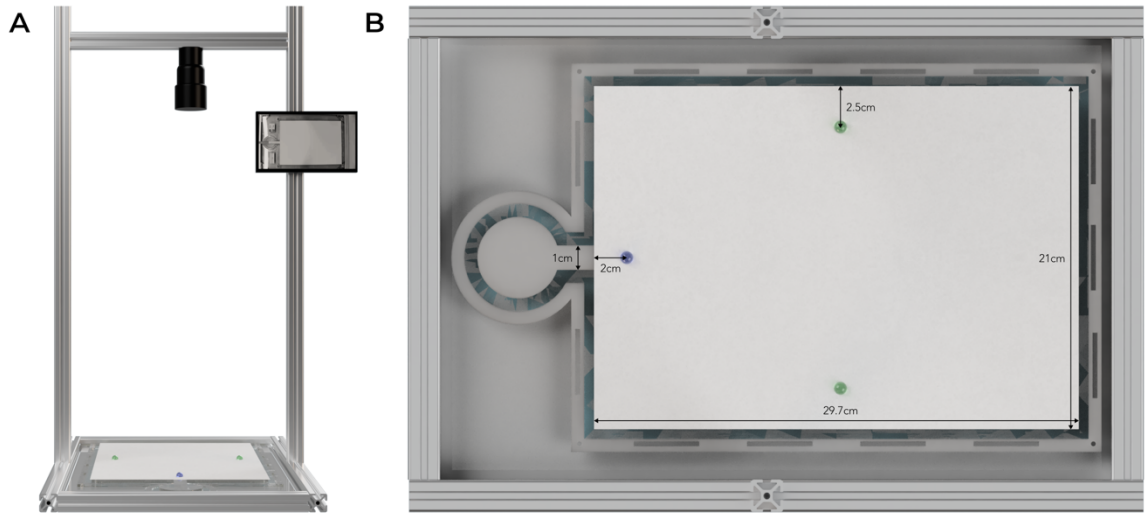


Figure 1 – Open landscape experimental setup. **(A)** Front view. A4 (210 x 297 mm) disposable paper overlay platform, surrounded by a water moat, with a single 1cm entrance, attached to a fixed top view Raspberry Pi HQ camera setup. **(B)** Top view. Blue drop represents the location of the sucrose solution drop (positive stimulus), either the control or the treatment on the ant's first visit (2cm from the middle of the platform's entrance). Green drops represent the possible location of the positive stimulus during the remaining visits (2.5cm from either the left or right side of the platform – each ant experienced the stimulus exclusively on one side).

Data extraction and processing

DeepLabCut version 2.2.2 (Mathis et al., 2018; Nath et al., 2019) was used to automate video analysis, allowing for the acquisition of coordinates for each ant's head and gaster, as well

as those of each corner of the A4 platform and the centre of the solution drop at every frame (videos recorded at 30fps). In total 142 ants were recorded, however five of them were excluded as they were unable to find the reward within 20 minutes in at least two of their five visits to the landscape. Furthermore, 11 ants failed to locate the reward in one of their five visits and one other was lost in the colony before it could do its final visit, therefore those visits were removed. 10 other visits were excluded due to tracking associated issues (specified in the detailed statistical analysis and code). Overall, 526 visits of 136 ants were analysed. Python version 3.7.13 (Rossum & Drake, 2009) was used to standardise the ants' coordinates by ensuring the same corner of the A4 platform was used as the origin of the cartesian referential of all videos. The known dimensions of the A4 were further used to convert coordinates from pixels to millimetres. To account for DeepLabCut tracking errors, any ant movement exceeding two millimetres per frame was considered implausible and subsequently removed. This is due to biological constraints that limit ants' movement to a maximum speed of around one millimetre per frame (Burford et al., 2018). In this process, an average of 0.1% [0%, 0.8%, N = 526] of frames were eliminated from each video. The times at which an ant reached and left the reward were automatically derived from the tracking data. These were used to define the ant's foodward and nestward journeys. The foodward journey encompassed the time from when the ant initially entered the landscape until it reached the reward for the first time. The nestward journey was taken as the last time the ant left the reward until it left the landscape. For each visit, the mean instantaneous speed and path tortuosity were then calculated separately for both the foodward and nestward journeys. Mean instantaneous speed represents the average distance travelled by the ant within a frame (1/30s), while path tortuosity quantifies the total distance covered by the ant relative to the minimum possible distance it could have travelled, providing a measure of path straightness. Importantly, exclusion criteria and extracted variables were defined a priori in a pre-registration with a few notable deviations. Mainly, the 25ppm and 250ppm caffeine treatments were added at a later stage and the second visit to the landscape was analysed together with all others in order to better understand the effect of consecutive visits on navigation.

Statistical analysis

The complete statistical analysis output, and the entire dataset on which this analysis is based, is available from Zenodo (https://doi.org/10.5281/zenodo.8413979).

All graphics and statistical analysis were generated using R version 4.2.1 (Wickham, 2016; Kassambara, Kosinski & Biecek, 2021; R Core Team, 2022; Wickham, 2022). Foodward and nestward journey duration were analysed using mixed effects cox proportional-hazards models (Therneau & Grambsch, 2000; Therneau, 2022). Note that for the survival analysis of foodward journey duration the 11 ants which failed to find the reward in one of their five

visits were included yet censored. Mean instantaneous speed and path tortuosity were analysed with linear mixed-effects models (Bates et al., 2015). DHARMa (Hartig, 2022) was used to assess linear model assumptions and MuMIn (Bartoń, 2022) to obtain a measure of goodness of fit. Analysis of variance tables were used to test the effects of the regressions coefficients (Fox & Weisberg, 2019). Estimated marginal means of linear trends and contrasts were obtained using the emmeans package (Lenth, 2022) with Bonferroni adjusted values accounting for multiple testing. Consecutive visit effects were assumed to be relatively linear as previously shown in Y-maze experiments performed on *L. humile* (Galante & Czaczkes, 2024; Wagner et al., 2023). We avoid the use of p-values, and their associated binary decision of significant/nonsignificant, instead reporting effect size estimates and their respective 95% confidence intervals shown throughout the results section as (estimate [lower limit, upper limit, N = sample size]). Nevertheless, should they be of interest, these are reported in the supplementary materials.

Results

There was no clear effect of caffeine concentration and/or the number of consecutive foraging visits to the landscape on the time ants spent drinking the reward (84s [min = 71s, max = 107s]).

Low to intermediate caffeine concentrations lead to foodward path optimisation

The time it took for an ant to reach the reward upon entering the landscape was influenced by both caffeine concentration and the number of consecutive visits to the landscape, as well as the interaction between the two (Figure 2 and Table S1). On average ($\bar{x}$), across treatments and visits, the foodward journey took 212s [$\bar{x}$ min = 63s, $\bar{x}$ max = 372s].

Under control conditions, ants showed little improvement in the time taken to reach the reward with every consecutive visit (5.6% [-12.6%, 23.8%, N = 537]). Meaning, if an ant initially took 300s to find the reward, after three consecutive visits to the open landscape, used henceforth as the example, it is likely to find the reward in 252s [133s, 428s]. However, when exposed to 25ppm of caffeine, each consecutive visit is likely to result in a 27.8% [5.7%, 50.0%, N = 537] faster foodward journey (113s [38s, 252s]). This effect is almost doubled in the 250ppm caffeine treatment, where ants are likely to find the reward 43.5% [19.5%, 67.6%, N = 537] faster with every consecutive visit (54s [10s, 156s]). However, the benefits observed at lower to intermediate caffeine concentrations are lost when ants are fed 2000ppm of caffeine (3.1% [-20.5%, 26.8%, N = 537] | 273s [118s, 525s]). Furthermore, the effect of consecutive visits is 38.0% [7.8%, 68.2%, N = 537] higher in ants under the 250ppm caffeine

treatment when compared to control-treated ants and 40.4% [6.6%, 74.2%, N = 537] higher when compared to the 2000ppm caffeine treatment (Figure 3 and Table S2).

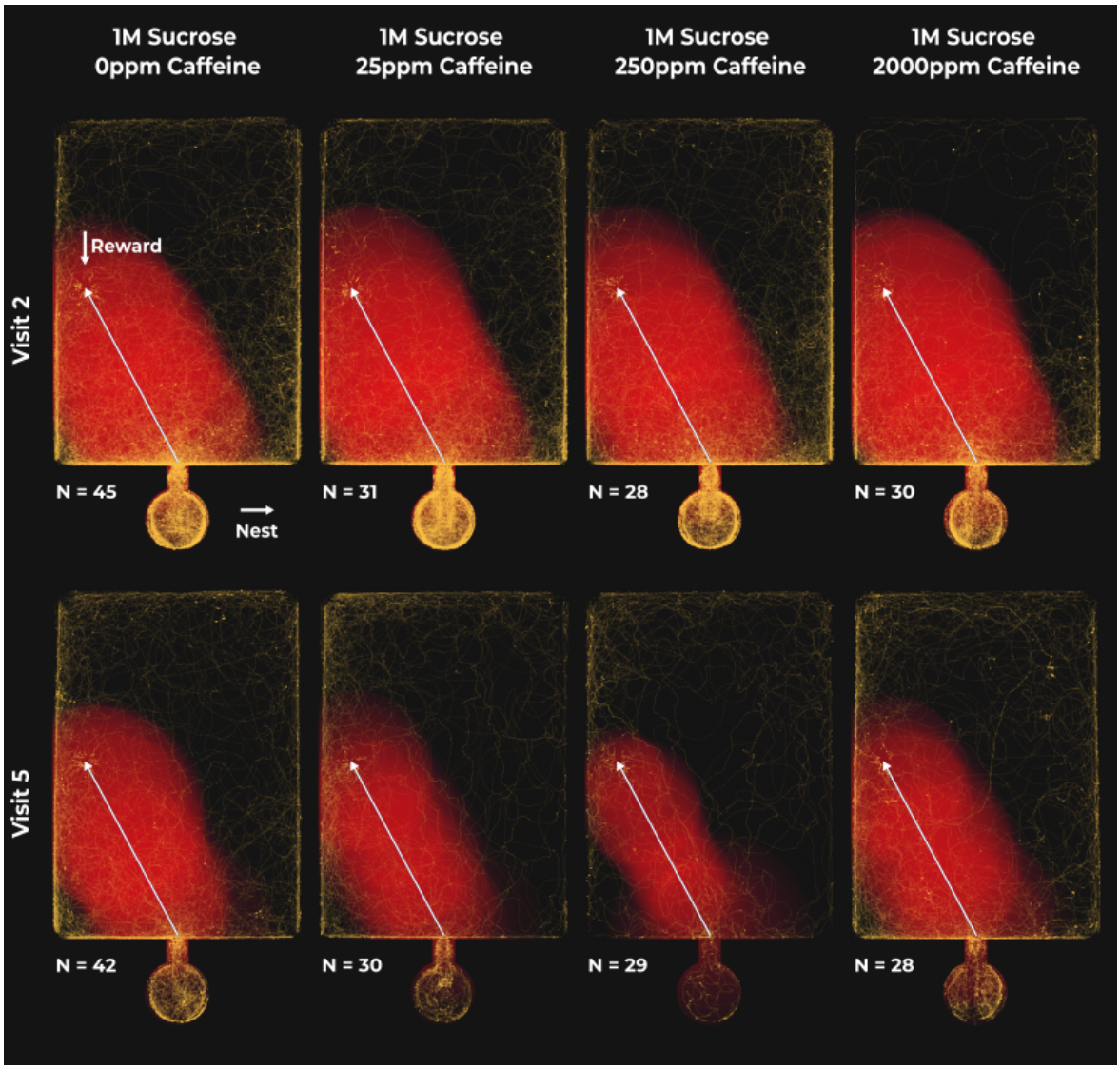


Figure 2 – Dose-dependent effect of caffeine on consecutive foodward visits to the open landscape. Red areas highlight the average deviation of all ant paths from the shortest route (white line) at each time point. Time normalisation sets the starting point of each path at 0% and the final point at 100%, with 1% increments translating to 12s increments at most (less for shorter journeys). Larger areas surrounding the shortest path indicate larger deviations from it, while brighter regions suggest greater overlap between deviations at different time points implying a lack of directional movement. Note that closer adherence to the straightest line results in smaller, dimmer red areas, which translates into shorter times spent in the landscape and therefore fewer yellow points on the graph. Yellow points highlight the paths taken by individual ants, from first entering the landscape until first reaching the reward, with brighter spots indicating overlapping trajectories. To aid visualization, the paths of ants reaching the reward on the right side of the landscape were mirrored.

Notably, caffeine did not affect mean instantaneous speed during the foodward journey (Table S5). However, from the first (17.5 mm/s [min = 9.0 mm/s, max = 25.5 mm/s, N = 134]) to the last (18.6 mm/s [min = 7.4 mm/s, max = 27.7 mm/s, N = 129]) visit to the landscape, mean instantaneous speed increased by 0.3 mm/s [0.1 mm/s, 0.6 mm/s, N = 526] per visit (Table S6). The logarithm of path tortuosity remained constant with each consecutive visit both under control conditions (0.4 [0.0, 0.8, N = 526]) and under the 2000ppm caffeine treatment (0.4 [-0.1, 0.9, N = 526]). However, 25ppm (0.9 [0.4, 1.4, N = 526]) and 250ppm caffeine treatments (0.8 [0.3, 1.3, N = 526]) exhibited an increase in path straightness with each consecutive visit (Table S10). Importantly, effects in the logarithmic scale are often exponential in the linear scale. Meaning, if an ant had a path tortuosity of 20 on its first visit to the landscape, after three consecutive visits, we would expect it to have a path tortuosity of 6.0 [1.8, 20.0] under the control treatment, and a path tortuosity of 1.8 [1.0, 8.1] under the 250ppm caffeine treatment.

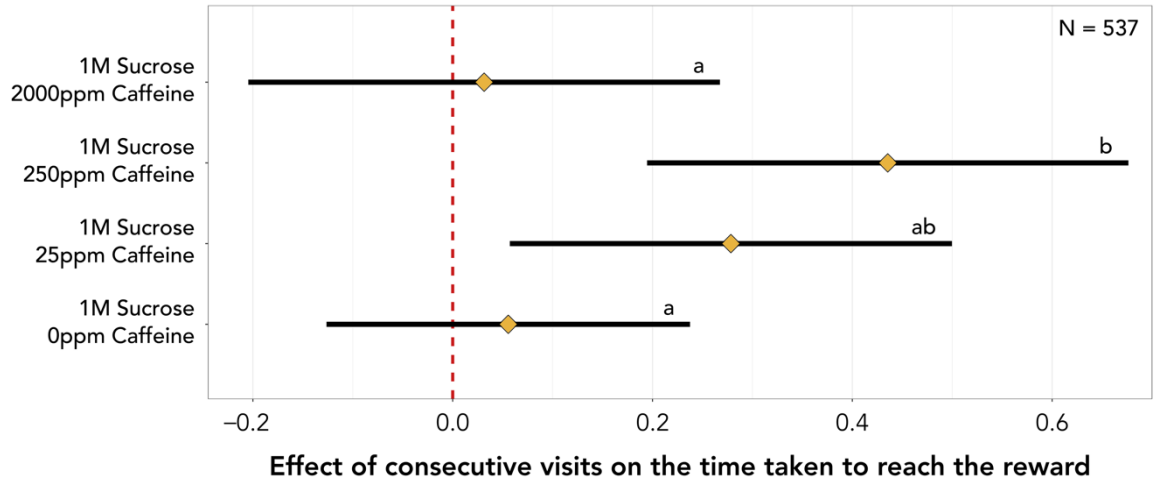


Figure 3 – Effect of consecutive visits on the time an ant takes to reach the reward since first entering the landscape for each caffeine treatment. Diamonds represent the estimated marginal means of linear trends obtained from the mixed effects cox proportional-hazards model and whiskers the respective 95% confidence intervals (see Table S2). Estimates of 0 (red dashed vertical line) indicate no effect of consecutive visits, whilst estimates > 0 or < 0 indicate that ants are more or less likely, respectively, to reach the reward faster with consecutive visits. If the 95% confidence intervals include an estimate of 0 it is likely that there is no effect of consecutive visits. Letters reflect statistical differences between treatments based on the estimated confidence intervals.

Caffeine had no effect on the nestward journey

The time it took for an ant to exit the landscape upon last touching the reward was influenced by the number of consecutive visits to the landscape, but not by the amount of ingested caffeine (Figure 4 and Table S3). On average ($\bar{x}$), across treatments and visits, the nestward journey took 44s [$\bar{x}$ min = 30s, $\bar{x}$ max = 57s].

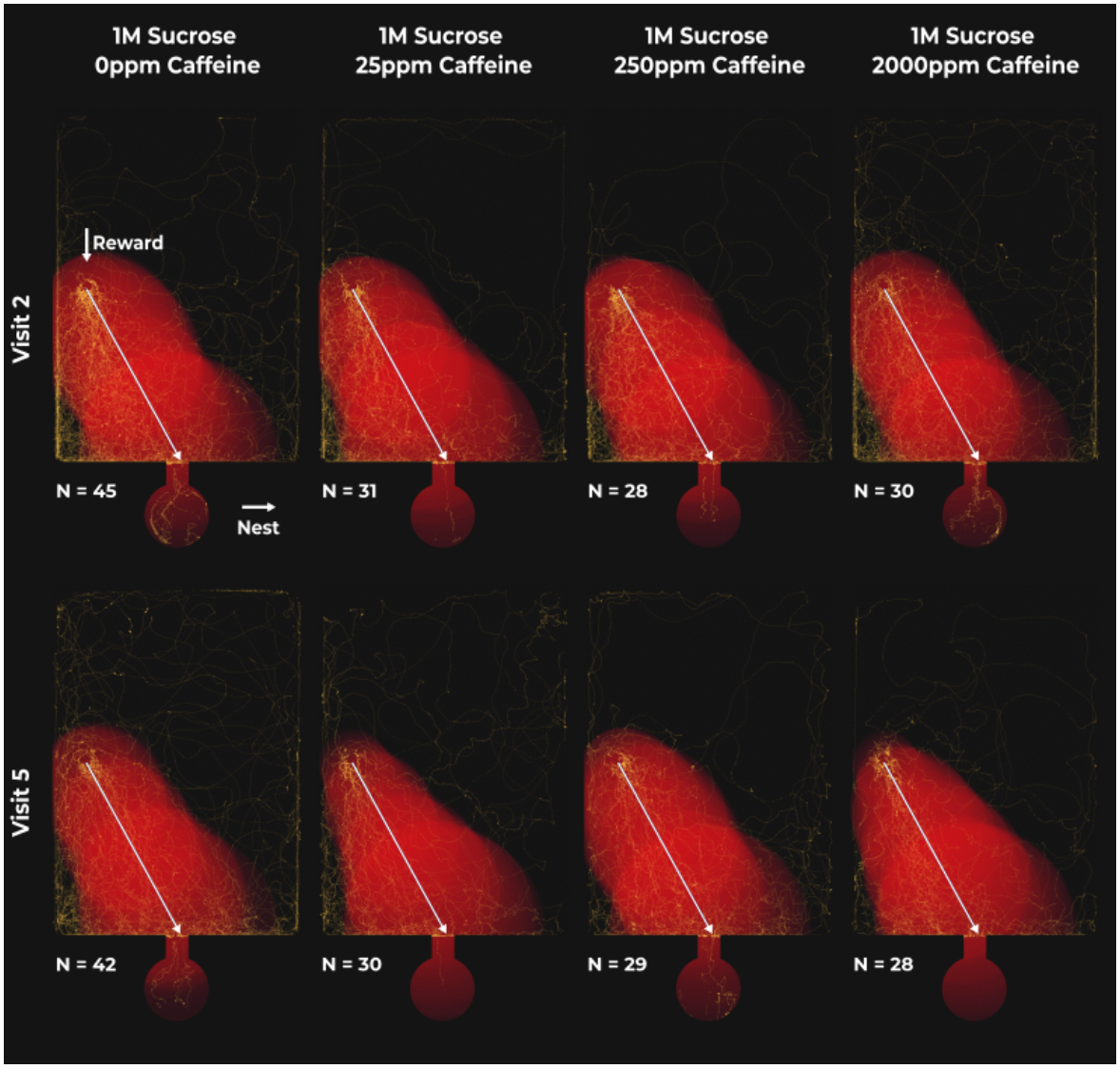


Figure 4 – No effect of caffeine on consecutive nestward journeys. Red areas highlight the average deviation of all ant paths from the shortest route (white line) at each time point. Time normalisation sets the starting point of each path at 0% and the final point at 100%, with 1% increments translating to 2s increments at most (less for shorter journeys). Larger areas surrounding the shortest path indicate larger deviations from it, while brighter regions suggest greater overlap between deviations at different time points implying a lack of directional movement. Note that closer adherence to the straightest line results in smaller, dimmer red areas, which translates into shorter times spent in the landscape and therefore fewer yellow points on the graph. Yellow points highlight the paths taken by individual ants, from last touching the reward until leaving the landscape, with brighter spots indicating overlapping trajectories. To aid visualization, the paths of ants presented a reward on the right side of the landscape were mirrored.

With each consecutive visit ants are likely to be 11.0% [2.8%, 19.2%, N = 526] faster at returning to the nest (Table S4). Meaning, if an ant initially took 30s to return to its nest, after three consecutive visits to the open landscape, it is likely to return in 21s [16s, 28s],

regardless of its consumption of caffeine (Figure 5). Contrastingly, mean instantaneous speed (14.5 mm/s [min = 4.9 mm/s, max = 26.9 mm/s, N = 526]) was constant throughout consecutive visits and treatments (Table S7). Furthermore, albeit unaffected by caffeine, the logarithm of path tortuosity decreased by 0.07 [0.02, 0.11, N = 526] per visit (Tables S11 and S12). Therefore, if an ant had a path tortuosity of 4 on its first visit to the landscape, after three consecutive visits, we would expect it to have a path tortuosity of 3.2 [2.9, 3.8].

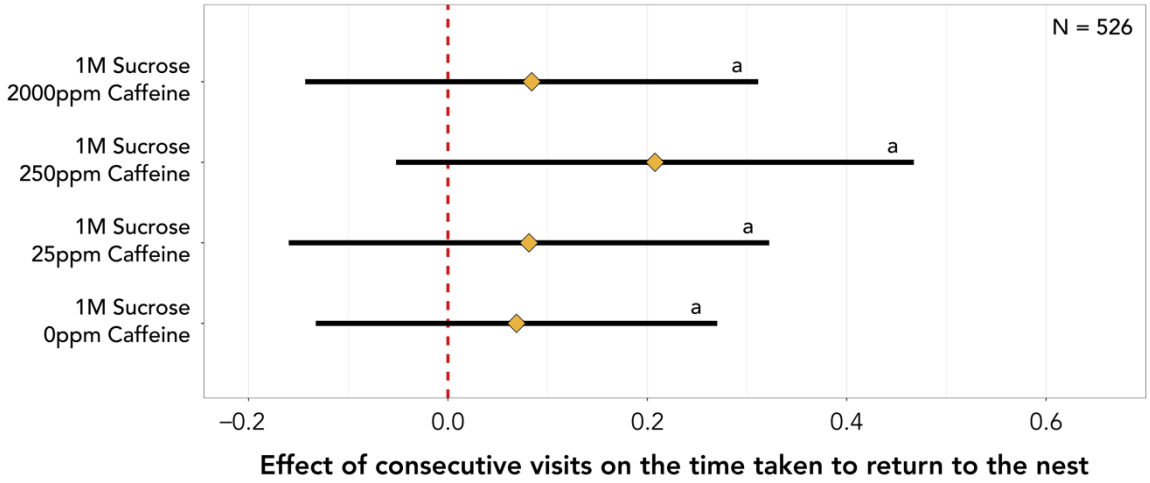


Figure 5 – Effect of consecutive visits on the time an ant took to return to the nest since leaving the reward for the last time for each caffeine treatment. Diamonds represent the estimated marginal means of linear trends obtained from the mixed effects cox proportional-hazards model and whiskers the respective 95% confidence intervals. Estimates of 0 (red dashed vertical line) indicate no effect of consecutive visits, whilst estimates > 0 or < 0 indicate that ants are more or less likely, respectively, to return to the nest faster with consecutive visits. If the 95% confidence intervals include an estimate of 0 it is likely that there is no effect of consecutive visits. Letters reflect statistical differences between treatments based on the estimated confidence intervals.

Discussion

Low (25ppm) and intermediate (250ppm) concentrations of caffeine shortened the foodward journey over consecutive visits (Figure 2 and 3). This was not due to an increase of mean instantaneous speed, but rather an increase in path straightness with each consecutive visit under these treatments. Thus, caffeine is likely dose-dependently boosting learning, as a straighter path suggests the ant knows the location of the reward (Figure 2). However, the improvements gained were lost at high doses of caffeine (2000ppm). This suggests a hormetic dose-response pattern, where caffeine is toxic at high doses, but when ingested in smaller amounts has the opposite effect, stimulating biological function. Similar results were previously found in bees for a variety of chemicals (Cutler & Rix, 2015), including caffeine (Wright et al., 2013). In honeybees, higher doses of caffeine have resulted

in a reduced likelihood of bees to respond to a conditioned odour (Mustard et al., 2012) and 2000ppm of caffeine has previously been found as the LD50 of honeybees (Detzel & Wink, 1993). In humans, low to moderate caffeine dosages often have stimulant and performance-enhancing effects. On the other hand, high doses may be associated with aversive somatic effects, including sleep disruption and increased anxiety and agitation, all of which can contribute to impaired fine motor control (Kaplan et al., 1997; Smith, 2002; Pallarés et al., 2013; Souza et al., 2022). In this way, it seems reasonable that the loss of effect at high doses of caffeine is likely due to its toxicity.

Interestingly, caffeine had no clear impact on nestward journey duration (Figures 4 and 5). Much as in the foodward journey, consecutive visits did not alter the speed at which the ants moved. However, ants moved slower, on average, during the nestward journey when compared to the foodward journey, possibly due to being heavier when their crop is full. Independently of caffeine, over consecutive visits nestwards paths became straighter, which translated into shorter nestward journey durations across treatments. In part, the lack of effect of caffeine observed could be due to the nestward journey durations being an order of magnitude shorter than foodward journey durations. The asymmetry between the two journey segments could be a result of the ants often returning to the entrance of the open landscape during their foodward journey but taking fewer detours during their nestward journey. Thus, any positive effects caused by caffeine would be harder to detect as there is less room for improvement.

Nevertheless, in invaded areas, ants need to navigate more complex environments, relying on multimodal cues. Under such conditions, where learning and memory play a key role, caffeine might have an impact on the nestward journey as well. In fact, we note a slight trend towards faster nestward journeys over consecutive visits in the 250ppm treatment (Figure 5). The range over which panoramic views offer navigational guidance is smaller in denser environments and larger in open landscapes (Zeil, Hofmann & Chahl, 2003). In this way, under the experimental conditions used, ants are likely to have heavily relied on view-based navigation during the foodward journey. However, in their invasive range, and considering the temporary nature of their nests, *L. humile* habitats are likely to be denser and richer in landmarks. Therefore, ants will not only rely on visual cues but also olfactory ones. Caffeine has been shown to improve olfactory associations in both honeybees and bumblebees (Wright et al., 2013; Arnold et al., 2021). Furthermore, ants have been shown to use olfactory landmarks to locate food and their nest (Helmy & Jander, 2003; Steck, Hansson & Knaden, 2009; Huber & Knaden, 2018) with a combination of visual and olfactory navigational tools leading to increased performance (Steck, Hansson & Knaden, 2011). Thus, the observed navigational improvements are likely to not only be maintained, but potentially increased under natural conditions.

Physiologically, the lack of effect of caffeine during the nestward journey, in contrast to the foodward journey, implies caffeine selectively targets distinct navigational mechanisms. Path integration and view-based navigation are thought to be encoded by different brain regions (Grob et al., 2024). The central complex is thought to directly modulate locomotion and be responsible for path integration (Webb & Wystrach, 2016; Stone et al., 2017). On the other hand, view-based navigation and learning are thought to be predominantly based on the mushroom bodies (Buehlmann et al., 2020; Kamhi, Barron & Narendra, 2020). Nevertheless, new models suggest a more complex interaction of both with other brain regions during navigation (Wystrach, 2023). Considering the lack of effect of caffeine on locomotion during the entire foraging journey, paired with an increase in path straightness during the foodward journey but not the nestward journey, it is likely that caffeine predominantly affected the mushroom bodies, and therefore view-based navigation, over the central complex.

In fact, caffeine is known to interact with invertebrate ryanodine receptors, prompting the release of intracellular calcium stores (Mustard, 2014). Pisokas et al. proposes a separation of the directional and learning components of path integration, suggesting that the foundation of path integration memory may involve molecular processes linked to CaMKII signalling pathways. These proteins are central to synaptic plasticity, learning and memory and an increase in calcium influx can rapidly enhance their activity. Moreover, studies have associated both CaMKII and caffeine with long-term memory formation in crickets (Mizunami et al., 2014; Sugimachi et al., 2016). Combined with our results, these findings suggest caffeine is likely interfering with ant learning and memory.

Alternatively, low to intermediate caffeine concentrations could be acting by enhancing alertness and motivation. However, caffeine did not impact crop load or consumption rate in *L. humile*, suggesting it was either not perceived by the individuals or did not influence reward value (Galante et al. In prep.). Nevertheless, across treatments, ants have a strong tendency to stay close to the edges of the open landscape (positive thigmotaxis). This behaviour appears to decrease under intermediate doses of caffeine (Figure 2, Visit 5) which could suggest caffeine is increasing foraging motivation. This could be due to the effects of caffeine as an adenosine receptor antagonist, potentially leading to increased levels of acetylcholine which might improve cognitive alertness (Gold, 2003). Moreover, a decrease of GABA, a neurotransmitter thought to impair olfactory memory in bees (Boitard et al., 2015), might reduce forgetting of the location of the caffeinated rewards. In turn, this could generate addiction-like symptoms potentially increasing the ant's motivation to forage on such rewards. However, a recent study exploring visual learning in bumblebees shows GABA promoting flower fidelity (Calderai et al., 2023). Caffeine has been shown to increase responsiveness and activity in jumping spiders (Humphrey, Helton & Nelson, 2019) as well as having an effect on locomotion in a variety of insects (Mustard, 2014). However, we show

no effect of caffeine on ant mean instantaneous speed throughout the foraging journey, further suggesting caffeine-mediated foraging improvements are not a consequence of motor efficiency but rather of cognitive enhancement.

Similarly to Si, Zhang & Maleszka (2005), we found that caffeine has no effect in a simple Y-maze spatial and olfactory association learning task (Galante & Czaczkes, 2024), but its effects become visible in a more complex and field-realistic experiment. Consecutive visits did not alter foodward journey duration in control treated ants (Figure 3). This implies that three visits to the reward were not sufficient for the ants to learn and/or memorise its location. However, previous work has demonstrated that *L. humile* are excellent spatial and olfactory learners often requiring as little as one visit to the reward to learn its characteristics and location (Rossi et al., 2020; Wagner et al., 2023; Galante & Czaczkes, 2024). We were thus successful in creating a more complex and challenging experiment, which arguably better depicts natural conditions. Furthermore, this enabled us to exclude ceiling effects potentially observed in previous experiments which might have masked any effects resulting from exposure to caffeine (Galante & Czaczkes, 2024). Ceiling effects are a major challenge of simpler learning setups, and open landscape tasks a suitable solution to this problem.

Intermediate concentrations of caffeine improved foraging in Argentine ants, demonstrating that adding neuroactive substances to toxic baits may hold potential as a novel approach to improve invasive insect control. Future work, ideally under natural conditions, should test whether caffeine interacts with the chosen toxicant, and if it also improves recruitment and visitation rates in ants, as previously shown in honeybees and bumblebees, respectively (Couvillon et al., 2015; Thomson, Draguleasa & Tan, 2015). As new invasive ant species start to become established worldwide (Menchetti et al., 2023), there is growing urgency in finding ways to improve control efforts. Adding low dosages of caffeine to toxic baits may be a cheap and easily deployable method of boosting learning of bait location, potentially leading to increased recruitment to and consumption of the toxicant, and ultimately improved control.

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Declaration of interests

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

H. Galante: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing - original draft, Writing - review & editing, Visualization, Supervision. **M. De Agrò:** Methodology, Validation, Writing - review & editing. **A. Koch:** Investigation, Writing - review & editing. **S. Kau:** Investigation, Writing - review & editing. **T. J. Czaczkes:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Chapter 3

Three-dimensional body reconstruction enables quantification of liquid consumption in small invertebrates

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Abstract

Quantifying feeding patterns provides valuable insights into animal behaviour. However, small invertebrates often consume incredibly small amounts of food. This renders traditional methods, such as weighing individuals before and after food acquisition, either inaccurate or prohibitively expensive. Here, we present a non-invasive method to quantify food consumption of small invertebrates whose body expands during feeding. Using the markerless pose estimation software DeepLabCut, we three-dimensionally track the body of Argentine ants, *Linepithema humile*. Using these extracted markers, we developed an algorithm which computationally reconstructs the ant's body, directly measuring volumetric change over time. Moreover, we provide measures of accuracy and quantify the ant's feeding response to a range of sucrose concentrations, as well as a gradient of caffeine-laced sucrose solutions. Small invertebrates are often prolific invasive species and disease vectors, causing significant ecological and economical damage. Understanding their feeding behaviour could be an important step towards effective control strategies.

consumption rate • crop load • caffeine • invasion biology • ants • pose estimation

Introduction

A fundamental aspect of science is measuring quantities. However, all devices are subject to error, making it impossible to obtain truly exact measurements. This becomes increasingly difficult as scale decreases (Jenkins & Cook, 2004; Deshpande et al., 2014). In biology, small animals such as mosquitoes, flies or ants often ingest volumes in the nanolitre range (Paul & Roces, 2003; Wong et al., 2008; Jové et al., 2020). This is problematic when studying animal behaviour as equipment capable of measuring such small volumes with both precision and accuracy is prohibitively expensive (Councill et al., 2021). Additionally, such devices often work exclusively under tailored conditions which are seldomly possible when studying natural behaviours (see Calisi & Bentley, 2009 for a review). Yet, feeding behaviour provides valuable insights into an animal's perception of the world, elucidating their preferences and cognitive capabilities (Kim & Smith, 2000; Oberhauser, Koch & Czaczkes, 2018). Additionally, feeding is often linked to nutritional status and can be a measure of animal welfare (Vaudo et al., 2016; Carvajal-Lago et al., 2021). Finally, feeding is also particularly important in pharmacological interventions (Devineni & Heberlein, 2009; Vinauger et al., 2018) and can play a crucial role in the development of effective invasive species control methodology (Nigg et al., 2004; Carrasco et al., 2019; Galante & Czaczkes, 2024; Galante et al., 2024). Thus, an affordable and reliable method for quantifying feeding behaviour in small invertebrates would be extremely valuable.

Currently, one of the most common methods of measuring feeding in small invertebrates is the capillary feeder assay. This involves quantifying the depletion of liquid food from a glass capillary, thereby providing a measure of the ingested volume (Ja et al., 2007). However, this approach requires the animal to drink upside-down from a vertical capillary, potentially restricting the method's applicability to animals who can do so. Moreover, the food used is often dyed to facilitate the measurement of depletion from the capillary, which can impact feeding behaviour by altering food consumption (Shell et al., 2021). Lastly, since the assay directly measures food depletion rather than the actual volume ingested by the organism, it is highly sensitive to evaporation and spillage (Diegelmann et al., 2017).

Gravimetric approaches are a widely used alternative, where an individual, or the food source, is weighed before and after feeding to quantify consumption (Rotheray, Osborne & Goulson, 2017; Straw & Brown, 2021). However, for small animals, this requires an extremely sensitive scale, which is both expensive and cumbersome to work with. Furthermore, measuring the weight of a dynamic object is complex, often requiring individuals to be anaesthetised before and after the feeding event, which may alter their behaviour and could influence food consumption (Mailleux, Deneubourg & Detrain, 2000; Poissonnier, Jackson & Tanner, 2015; Gooley & Gooley, 2023). More importantly, weighing does not provide information about consumption rate patterns during feeding and is

strongly influenced by external factors, such as liquids which were not ingested adhering to the individual's body.

Another popular alternative takes advantage of dyes, whether food-grade or fluorescent, or of other chemical tracers (Nigg et al., 2004; Řehoř et al., 2014; Wu et al., 2020; Sakuma & Kanuka, 2021). While these methods may offer easy quantification, they often come with steep equipment costs and provide indirect measures of food ingested through spectral analysis or similar processes. These chemicals can potentially alter feeding behaviour, and often interact with other food components, such as protein, which can interfere with quantification (Marmé et al., 2003; Baltiansky et al., 2021). Additionally, dyes can stain the individual's tissue and methodology often requires the sacrificing of the individual post-consumption.

Dedicated methods have previously been developed to address the complexity of quantifying feeding in small invertebrates. The flyPAD system, for example, can measure capacitance changes when an individual touches the food source (Itskov et al., 2014; Henriques-Santos, Xiong & Pietrantonio, 2023). This method has proven useful for characterising feeding behaviour, offering an indirect measure of consumed solution based on the time spent touching the reward, assuming linear consumption rates. However, this system is costly and requires rigorous maintenance and cleaning. Moreover, the non-open-source nature of the original hardware and software presents a limitation. Importantly, it also requires individuals to be anesthetised and placed in a small artificial chamber, which could alter their behaviour and food consumption (Mailleux, Deneubourg & Detrain, 2000; Poissonnier, Jackson & Tanner, 2015; Gooley & Gooley, 2023).

Here, we aim to address the limitations of currently available methods, developing a high-resolution, high-throughput and non-invasive system to quantify food consumption of small invertebrates. The body of many insects (Hymenoptera, Diptera, Hemiptera, Lepidoptera), some arachnids (Ixodida, Araneae, Opiliones) and worms (Annelida, Nematoda) visibly expands as they ingest food. Quantifying this expansion over time allows for measures of volumetric increase, and consequentially of liquid ingested. A similar approach has previously been developed by approximating volume consumption from two-dimensional tracking of body shape (Sola & Josens, 2016; Hol, Lambrechts & Prakash, 2020). We expand on this, using the well-documented open-source software DeepLabCut (Mathis et al., 2018; Nath et al., 2019), by tracking body shape in a three dimensional space. Using these extracted markers, we developed an algorithm which computationally reconstructs the animal's body over time, thus directly measuring volumetric changes. In this way, we eliminate the error associated with approximating volume from area, which occurs when using a two-dimensional system for volume estimation (Sola & Josens, 2016; Hol, Lambrechts & Prakash, 2020). This method allows

animals to move freely, without the need for anaesthesia or the use of dyes. Furthermore, it not only directly quantifies the volume of food ingested but also provides consumption rates over time. Understanding feeding patterns provides essential insights of animal behaviour and can be crucial for the study of disease vectors and transmission as well as the development of invasive pest control methodology.

Materials and Methods

The system we developed takes advantage of the fact that some animals' bodies expand during feeding to accommodate for the ingested food, enabling us to quantify feeding patterns. The process can be divided into five steps, detailed below. First, the user should build a setup, adapted to the animal being studied, which is capable of recording the feeding events with sufficient quality (Step 1). Next, using DeepLabCut three-dimensional pose estimation software (Mathis et al., 2018; Nath et al., 2019) key points of the individual's body are extracted from the videos at each time point (Step 2). Once these three-dimensional coordinates are obtained, volumetric changes of the tracked body over time can be quantified using one of the seven methods proposed (Step 3). Finally, our simple graphical user interface (GUI) allows the user to select the initial and final times of the feeding event (Step 4) and fit a model to the individual's volume over time (Step 5). This approach provides both the total amount of food ingested during the feeding event and the individual's consumption rate.

System specifications

Step 1 - Physical setup

In most cases, a basic recording configuration, consisting of two perpendicular cameras, capturing a top-view and side-view of the features of interest, should suffice. Nevertheless, additional cameras, placed at varying angles may be required, particularly if recording subjects mid-flight (Maya et al., 2023; Håkansson et al., 2024), for example. Regardless of the number of cameras used, it is imperative these are stereo-configured and time-synchronized to ensure overlapping views and simultaneous visibility of features of interest across multiple cameras, allowing for the feature to be reconstructed in a three-dimensional space. Once the system is built, it is important to take calibration images, for example by using a checkerboard. The calibration step will track known points across all cameras simultaneously, computing intrinsic (optical centre and focal length of the camera) and extrinsic (camera location in the three-dimensional space) parameters for each camera. This step is critical for successful triangulation, as it combines different views of the same point, in order to determine its location in the three-dimensional scene. For further details we recommend consulting the DeepLabCut user guide for 3D pose estimation (Mathis et al., 2018; Nath et al., 2019). The specific physical setup we developed to validate the volume estimation method with ants is described below.

Step 2 - Three-dimensional pose estimation

DeepLabCut version 2.3.8 (Mathis et al., 2018; Nath et al., 2019) was used to track points of interest in 3D. Two distinct DeepLabCut networks, one for each camera, were trained and later used during experimental validation and application. Each network was trained on 15 manually labelled frames from 28 videos (840 labelled frames and 14 280 points labelled in total). The two networks tracked 17 points each, 15 points following anatomical landmarks (Figure 1) in the animal's body (in this case, ants), and two reference points on the feeding platform (described below) allowing for the standardisation between pixels and millimetres (1 px = 1.38 ± 0.11 mm, N = 538). The network trained on recordings from camera A was trained to 100 000 iterations, with a train error of 3.59 pixels and a test error of 5.73 pixels. The network trained on recordings from camera B was trained to 110 000 iterations, with a train error of 3.75 pixels and a test error of 3.74 pixels. All videos were recorded at 30 frames per second and cameras were stereo calibrated using a 10x8 checkerboard. The output of each video triangulation consisted of a file containing the three-dimensional cartesian coordinates of each of the 17 points tracked at each frame of the video, alongside a likelihood value indicating the model's confidence in the point coordinate estimation. Points with a likelihood below 60% were discarded during triangulation.

Step 3 - Volume estimation methods

Python version 3.7.13 (Rossum & Drake, 2009) was used to process the triangulated points obtained from DeepLabCut. The algorithm developed loads the cartesian coordinates for each recording, uses the reference points to scale values from pixels to millimetres, and estimates the volume of the target feature using seven different approaches (Figure 1).

The first approach, which we named the Base method, quantified the volume of the convex hull: the simplest polygon encompassing a set of points, in this case formed by the 15 tracked points around the ant's gaster. While this method is simple and requires no assumptions of the shape of the animal's body, it creates straight lines between the points rather than curves.

Thus, in order for it to represent the true volume of the object, one would need to track an infinite number of points describing its shape. Yet, labelling points is time consuming and pose estimation is computationally costly. Thus, the Spline approach expands on this simple method, by interpolating 100 points for each of the seven splines (smooth, bendable lines used to connect points in a continuous, curvilinear manner) created by the object's main axes (highlighted in different colours in Figure 1), thus creating a more detailed skeleton, at the cost of assuming the object is curvilinear.

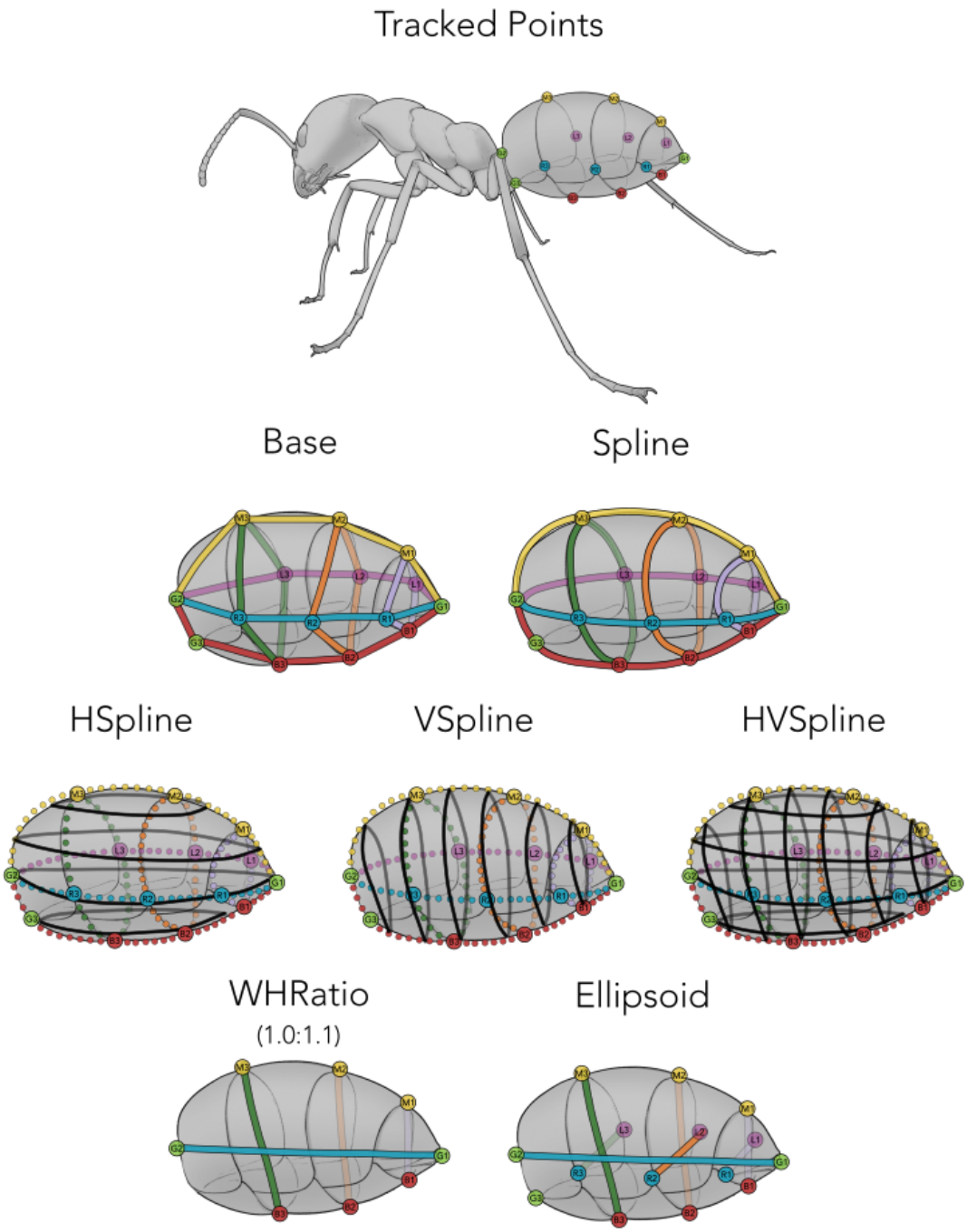

Figure 1 – Anatomical landmarks tracked using three-dimensional pose estimation and how these were used to estimate volume using seven approaches. The **Base** method simply connects the 15 tracked points using straight lines. **Spline** connects these points by fitting 100 interpolated points to curves. **HSpline** and **VSpline** further interpolate points along horizontal and vertical connections of the previously generated points, respectively. **HVSpline** combines both horizontal and vertical connections. **WHRatio** uses a species-specific width to height ratio to approximate the skeleton to an ellipsoid, and **Ellipsoid** does so but without using a predefined ratio.

Increasing the level of complexity, the HSpline and VSpline methods further interpolate points along horizontal and vertical connections of the previously generated points,

respectively, creating an even fuller skeleton without the need for tracking more points. The HVSpline method simply combines both of these methods by filling the skeleton both vertically and horizontally. For validation purposes, we include the WHRatio method, as described by Sola & Josens (2016). This method approximates the shape of an ant's gaster to an ellipsoid, using a two-dimensional image and a species-specific width-to-height ratio. Finally, the Ellipsoid approach approximates gaster shape to an ellipsoid, but without using a predefined ratio. Instead, it takes the maximum width and the maximum height values from the tracked points.

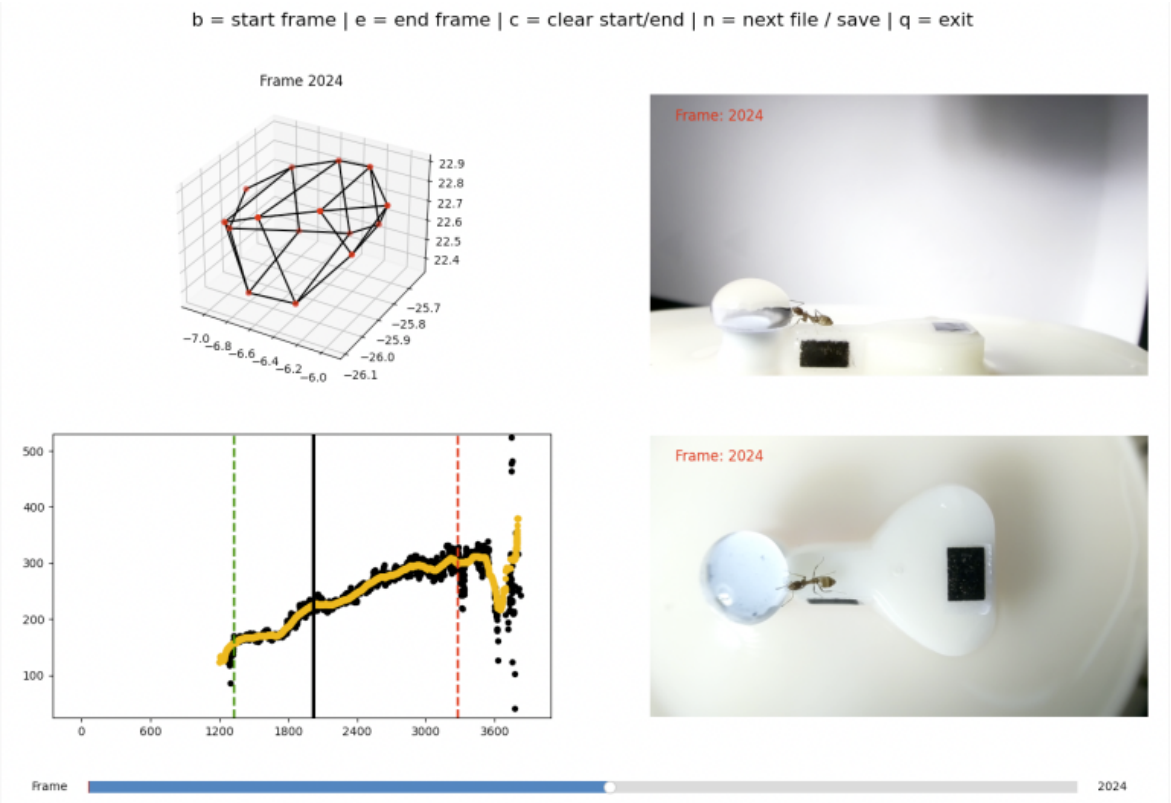

Figure 2 – Graphical user interface (GUI) designed to facilitate manual selection of the start (green dashed line) and end point (red dashed line) of the feeding event. The GUI displays the volume over time interactive plot, with points in black showing the raw volume estimates and those in orange a rolling average of these values. By moving the slider, the user can select a frame of interest, displaying it in each camera as well as a 3D plot of the tracked points. This allows the user to validate both the three-dimensionally tracked points and the volume calculations. The manually selected frames are automatically saved and the GUI allows the user to analyse recordings in multiple sessions, resuming from the last recording analysed.

Step 4 and 5 - Measuring ingested volume and consumption rate

To allow the user to verify if the tracking and volume estimation worked as expected, we designed a simple graphical user interface (GUI). When launched, this GUI plots a graph

of the estimated volume at each time point with a slider which can be adjusted to a frame of interest. By moving the slider, the user can select a frame of the video. This is then shown for both cameras and a 3D plot of the object's reconstruction (in our case, the ant gaster) is also displayed for that frame (Figure 2). Additionally, since the recordings do not necessarily begin and end with the feeding event, we manually select two time points which represent its beginning and end time. To facilitate this, the GUI allows the user to visualise the data being analysed and identify the time at which the individual started and stopped feeding. The indexes of these frames are then automatically saved for later use and the user can choose to inspect a new video or resume the process at a later time. The GUI automatically identifies which videos have not yet been inspected and appends the new data to the same file, allowing the user to seamlessly distribute the process across multiple sessions. In order to quantify feeding behaviour, we extract two main variables from each recording: crop load, a measure of how much food was ingested, and consumption rate, a measure of how fast this food was consumed over time. Using the manually selected start and end points of the feeding event and reducing noise by applying a rolling average filter for each five seconds, we fit a linear regression to the estimated volumes over time. The slope of the linear regression is taken as the consumption rate of that individual, and the regression equation used to calculate the initial and final volume from which crop load is then calculated.

Quantifying feeding patterns of Argentine ants

In order to validate and apply the developed algorithm, we conducted three experiments using Argentine ants, a globally invasive species. Firstly, we assessed the method's accuracy by measuring its agreement with gravimetric estimates. To do this, starved ants were initially weighed, then recorded whilst ingesting food, and once full, weighed again. Secondly, we fed ants a gradient of sucrose solutions. As sucrose concentration increases, so does the solution's viscosity. Ants are expected to prefer and consequently ingest more food when the sucrose concentration - and thus the energy provided by the solution - is higher. However, the high viscosity of these solutions is expected to decrease the rate at which the ants can ingest the food (Sola & Josens, 2016; Fujioka, Marchand & LeBoeuf, 2023). Lastly, we fed the ants a range of caffeine-laced sucrose solutions in order to assess their preference or aversion for them. Caffeine has been reported to decrease foraging times, likely due to its beneficial cognitive effects (Galante et al., 2024). Nevertheless, understanding if ants will be attracted or repelled to baits containing caffeine, consuming them at different rates and/or absolute amounts, is crucial if the addition of additives such as caffeine becomes common practice in invasive ant management.

Colony maintenance

Linepithema humile (Mayr, 1868) were collected from Portugal (Proença-a-Nova) 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 x 22.2 x 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 January and October 2022 using 571 ants from nine colonies.

Solutions

Sucrose solutions (Südzucker AG, Mannheim, Germany) following a geometric sequence ranging from 0.125M to 2M were used as treatments. Caffeine (CAS 58-08-2) was obtained from Sigma-Aldrich (Taufkirchen, Germany). 1M sucrose solutions mixed with different caffeine concentrations were used as treatments. Caffeine concentrations were chosen based on previous reports of their effects on Hymenopterans. Caffeine solutions ranging from naturally occurring concentrations (Singaravelan et al., 2005; Couvillon et al., 2015) to those reported as the LD50 of honeybees (Detzel & Wink, 1993) were used: 25ppm (0.13μmol ml-1), 250ppm (1.29μmol ml-1) and 2000ppm (10.30μmol ml-1), respectively.

Experimental validation – Accuracy measurements

Method accuracy was measured by both weighing ants before and after drinking (N = 104), and by feeding them a 138nL drop of 0.125M sucrose solution created by a nanolitre microinjection system (N = 10) whilst simultaneously recording them using our validation setup (described below). The difference between these and the volume estimation for each individual were then used as a measure of accuracy. All weight measurements were performed using an ultra-high-resolution scale (Sartorius Micro SC 2, Göttingen, Germany) with a precision of 0.0001mg. Calibration was carried out using a 2.001mg calibration weight ensuring the scale's accuracy. To establish a consistent starting temperature, two 0.2mL plastic tubes were cooled on ice for five minutes. Each tube was weighed three times. Following this, an ant was placed inside each tube, and the tubes were again cooled on ice for five minutes to minimise ant movement. The tubes were then weighted three times to determine ant weight. After this, the ants were allowed to recover for five minutes before being placed in the volume estimation setup. Ants were provided either 0.5M or 1M sucrose solution ad libitum. Post-recording, the ants were returned to the same 0.2mL tubes and put on ice for another five minutes before undergoing their final weighing. At the end of

each experimental day, all ants used were reintegrated into their respective colonies. In order to compare the weight of solution consumed by each individual with the estimated volume consumed, the density of the sucrose solutions used was determined. 1.5mL plastic tubes were placed on ice for five minutes, and later weighed three times. Using a precise repetitive pipette HandyStep touch (Brand GmbH, Wertheim, Germany), the tubes were then filled with 0.5mL of the solution, cooled on ice for another five minutes, and subsequently weighed three times. Using the average density of each solution, the weight of solution consumed by each individual was then converted to volume. The density of the 0.5M sucrose solution was measured as 1.06 ± 0.02 g/mL (N = 17) and that of the 1M solution as 1.11 ± 0.04 g/mL (N = 22).

Experimental application - Volume estimation setup

Our setup (Figure 3) consisted of a resin 3D-printed platform (Ø 45mm, height 5mm) filled with water to prevent the ants from escaping. Within the platform, there was an internal structure (height 7mm) with a maximum width of 11mm at its broadest point. This structure tapered down to a 3mm width channel that extended a length of 6mm, ultimately leading to a well (Ø 4.5mm) where the solution being tested was placed. This entire setup was attached to two fixed Raspberry Pi HQ camera systems equipped with 6mm wide-angle lenses (Raspberry Pi Foundation, Cambridge, United Kingdom). One camera captured a top-view of the ant whilst the other recorded a side-view. The cameras were synchronized by implementing a client-server architecture within the same network, ensuring simultaneous triggering. Focal ants were placed on the largest part of the internal structure and recorded for the entirety of the drinking event having access to ad libitum solution. Once full, the ant was removed from the platform and returned to its respective colony. Using this setup, we provided the ants with both a range of sucrose solutions, as well as a gradient of caffeine-laced sucrose solution, in this way quantifying the ant's feeding response to different food sources.

Data extraction and processing

In total 571 ants were recorded across all experiments. However, 146 recordings were discarded from the analysis (specified in the detailed statistical analysis and code), predominantly due to incorrect volume estimation resulting from the ant drinking at non-optimal positions. These ants were identified during the use of the GUI which allows the user to select initial and final time points for the feeding event. To exclude these, we simply did not select these time points, thus saving them automatically as empty values, making it easy to exclude them. As detailed above, all recordings were processed using DeepLabCut 3D pose estimation, and both crop load and consumption rate was obtained for each individual by fitting a linear regression to the estimated volumes over time. Overall, 114 ants were analysed for the accuracy measurements (experimental validation), and the seven

volume estimation approaches proposed used. For the method's experimental application, 311 ants were fed either pure sucrose or caffeine-laced sucrose dilutions, and their consumption was estimated by using the HVSpline method exclusively.

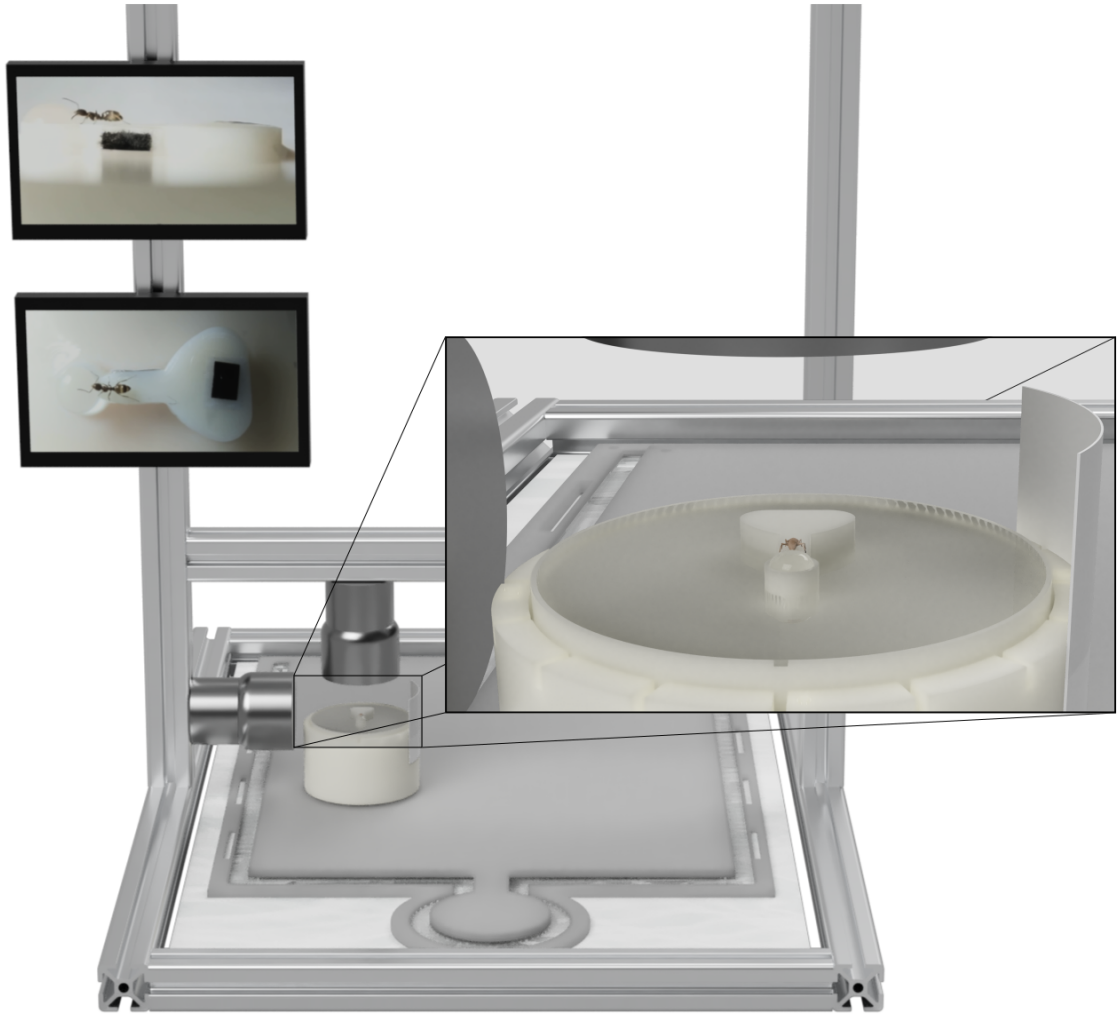

Figure 3 - Volume estimation experimental setup. Resin 3D-printed platform (Ø 45mm, height 5mm) filled with water. Within the platform, an internal structure (height 7mm) with a maximum width of 11mm at its broadest point. This structure tapers down to a 3mm width channel that extends a length of 6mm, ultimately leading to a well (Ø 4.5mm) where the solution being tested is placed. The entire setup is attached to a fixed dual-camera system equipped with 6mm wide-angle lenses recording simultaneously a top-view and a side-view.

Statistical analysis

The complete statistical analysis output, and the entire dataset on which this analysis is based, is available from Zenodo (https://doi.org/10.5281/zenodo.11655204).

All plots and statistical analysis were generated using R version 4.2.2 (Wickham, 2016, 2022; R Core Team, 2022). All measurements were analysed using linear mixed-effects models

(Bates et al., 2015). DHARMa (Hartig, 2022) was used to assess linear model assumptions and MuMIn (Bartoń, 2022) to obtain a measure of goodness of fit. Analysis of variance tables were used to test the effects of the regression's coefficients (Fox & Weisberg, 2019). Estimated marginal means and contrasts were obtained using the emmeans (Lenth, 2022) package with Bonferroni adjusted values accounting for multiple testing. We avoid the use of p-values, and their associated binary decision of significant/nonsignificant, instead reporting effect size estimates and their respective 95% confidence intervals shown throughout the results section as (estimate [lower limit, upper limit, N = sample size]).

Results and Discussion

Experimental validation – Accuracy measurements

As stated in the introduction, traditional methods of quantifying consumption are subject to considerable error at small volumetric scales. This makes it extremely difficult to establish a ground truth value and thus to estimate the accuracy of new methods. Nevertheless, here we attempt to do so by assessing the agreement between gravimetric measurements, and each of the seven proposed methods of estimating ingested volume (Figure 4). The Ellipsoid volume estimation method agreed most with weight, albeit producing consistently lower estimates, on average, by 62nL [45nL, 79nL, N = 728]. In part, this is due to the scale being particularly prone to error at small values, and due to the necessary density conversion. Moreover, weight measures the mass an individual gained or loss, regardless of if this was due to ingestion or simply due to a part of the solution being attached to the individual's body. At larger scales, this is often negligeable, but at the nanolitre scale a small drop of solution on the individual's body will lead to significant deviations from accurate measurements. This is highlighted by the strong positive correlation between the difference of weight and volume estimation and their mean. Such correlation is driven by weight values being, for the most part, higher than estimated volumes (Figure 4).

In addition, we compared the estimated volumes with a solution of known volume, 138nL, which was consumed in its entirety (see Statistical Analysis and Code). In this case, the Ellipsoid volume estimation method also agreed best with the known volume, albeit producing on average 53nL [24nL, 81nL, N = 70] lower estimates. Importantly, at the nanolitre scale, a liquid drop suffers from large evaporation rates. Thus, the discrepancy between the estimated volume and the available solution is likely due to evaporation. The exact amount of solution evaporated cannot be quantified as we did not control for the time it takes for ants to first contact the drop. However, we estimate that ants took between two and five minutes to reach the solution and therefore expect an absolute minimum evaporation loss of 25nL (see Statistical Analysis and Code).

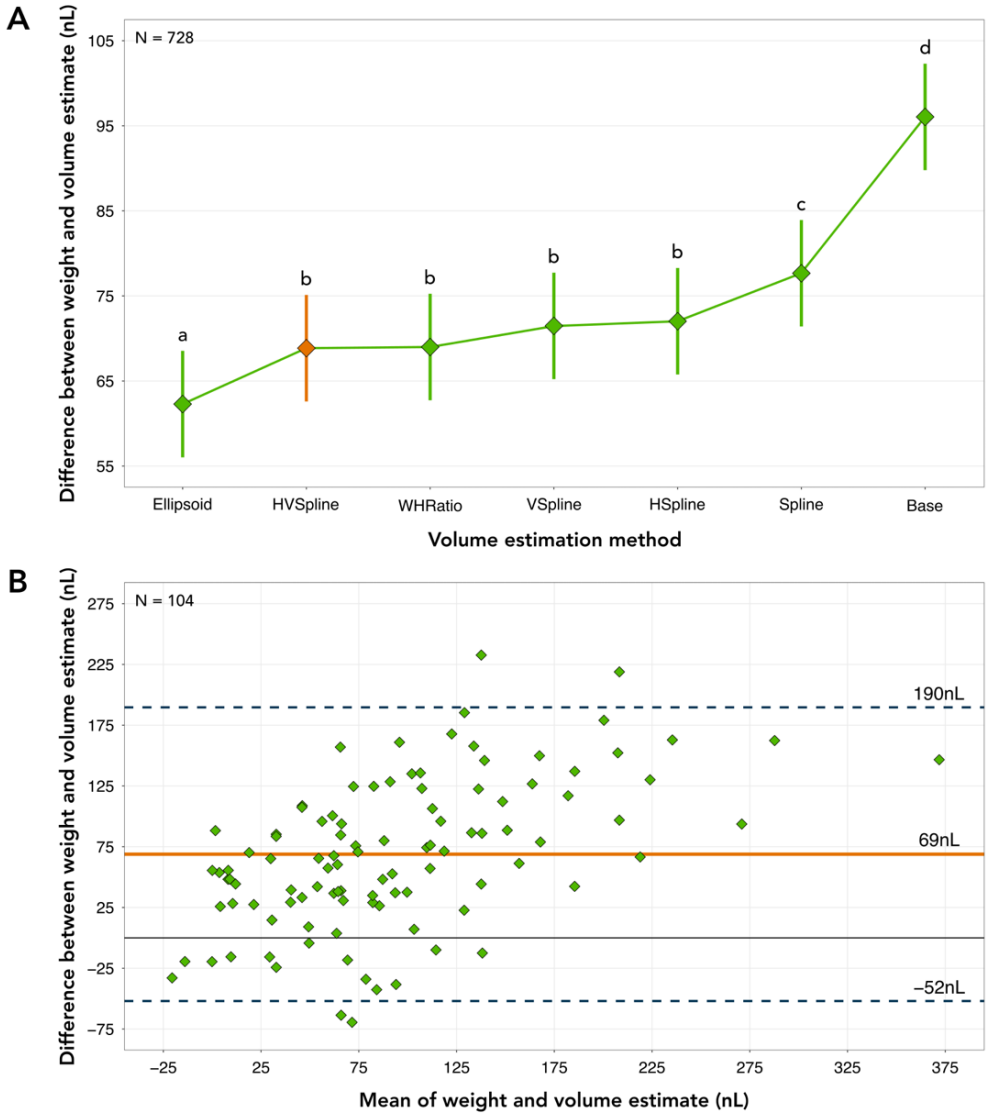

Figure 4 – Comparison between the volume estimates obtained from each of the methods proposed, against a gravimetric measure of consumption. **(A)** Accuracy of volume estimates compared to weight measurements. Diamonds represent the estimated marginal means obtained from the linear mixed-effects model and whiskers the respective standard errors. Letters reflect statistical differences between the agreement of each volume estimation method and the weight measurement based on the estimated confidence intervals. The Ellipsoid method agrees best with weight (62nL [45nL, 79nL], N = 728). However, we optted for the HVSpline method (69nL [52nL, 86nL], N = 728), as weight measures have significant associated error, and this volume estimation method can be used with a wider variety of shapes. **(B)** Bland–Altman plot for the agreement between weight measurements and the HVSpline volume estimates. Diamonds represent individual feeding events, and the orange line the average difference between the two methods, used as an estimate of accuracy. Dashed lines represent the 95% limits of agreement. Note that there is a strong positive correlation between the mean of the two measurements and their difference, which is predominantly driven by the weight measurements (see Statistical Analysis and Code). Moreover, in almost all cases, the weight measurement is higher than the estimated volume.

Even though the Ellipsoid method best agrees with both weight and a solution of known amount, we opted for the second highest agreeing method, the HVSpline. This is because, whilst an ant gaster, due to its natural shape, is correctly approximated to an ellipsoid, such approximation is not reasonable for most other organisms. Furthermore, since weight measurements are likely to be overestimating consumption, opting for the most agreeing volume estimation method might not be accurate, as in principle, such method would likely also overestimate consumption. The HVSpline method maintained a comparably high level of agreement to weight (69nL [52nL, 86nL, N = 728]) and known solution volume (55nL [27nL, 84nL, N = 70]) in comparison to the Ellipsoid method, whilst relying on fewer a priori assumptions of the organism's shape, being therefore more transferable to other organisms.

Experimental application – Higher sucrose concentrations increase crop load but decrease the consumption rate of an invasive ant

To assess if the method proposed is able to detect known behavioural patterns, we applied it to Argentine ants feeding on a wide range of sucrose solutions. As sucrose concentration increases, so does the energy provided by the solution. However, with an increase in sucrose, the viscosity of the solution also increases. Ants ingest food through a sucking-pump system which creates negative pressure through muscle contraction, driving fluid into the mouth (Paul, Roces & Hölldobler, 2002; Falibene, Rössler & Josens, 2012). In this way, ants are able to vary their consumption rate (Josens & Roces, 2000), for example by increasing pumping frequency, whilst maintaining a constant volume taken per pump contraction (Falibene & Josens, 2008; Falibene, Gontijo & Josens, 2009). Higher viscosity makes it harder for ants to ingest food, leading to longer feeding times to reach the same crop load. Interestingly, if viscosity is artificially manipulated, and a low sucrose concentration solution is made to be highly viscous, ants rapidly decrease their pump frequency, resulting in demotivation for the food source (Lois-Milevicich, Schilman & Josens, 2021). In some species, high viscosity solutions can even trigger a switch from trophallaxis – where ants ingest a liquid and later share it with their nestmates – to pseudotrophallaxis – where ants hold a liquid drop between their mandibles through surface tension and later share it with their nestmates without ingestion (Fujioka, Marchand & LeBoeuf, 2023).

As expected, our data suggests that an increase in sucrose concentration results in a relative increase of crop load, further reinforcing that individual ants are capable of regulating their food intake (Figure 5). Ants collecting diluted solutions reach lower crop loads (0.125M: 306nL [177nL, 435nL, N = 269]; 0.25M: 278nL [151nL, 405nL, N = 269]) than those provided higher molarity solutions (0.5M: 360nL [235nL, 486nL, N = 269]; 1M: 361nL [235nL, 487nL, N = 269]; 2M: 379nL [254nL, 504nL, N = 269]). A similar pattern has been previously observed for Argentine ants, where crop load increases with sucrose concentration, albeit

with such increases being lost at concentrations higher than 2M (Sola & Josens, 2016). Moreover, crop load volumes obtained in our experiment are generally higher than those previously reported, which reach a maximum around 200nL (Sola, Falibene & Josens, 2013; Sola & Josens, 2016). Such differences could be due to previous studies using Argentine ants from their native range, whilst we used ants from their invasive range, which could potentially influence feeding behaviour. Additionally, the ants used in our study were kept in considerably smaller colonies and starved for a shorter period of time when compared to previous work. Feeding patterns are highly variable and dependent on multiple factors. For example, in the ant Camponotus mus, increases in sucrose concentration were shown to increase crop load (Josens, Farina & Roces, 1998), with feeding varying with colony starvation (Josens & Roces, 2000).

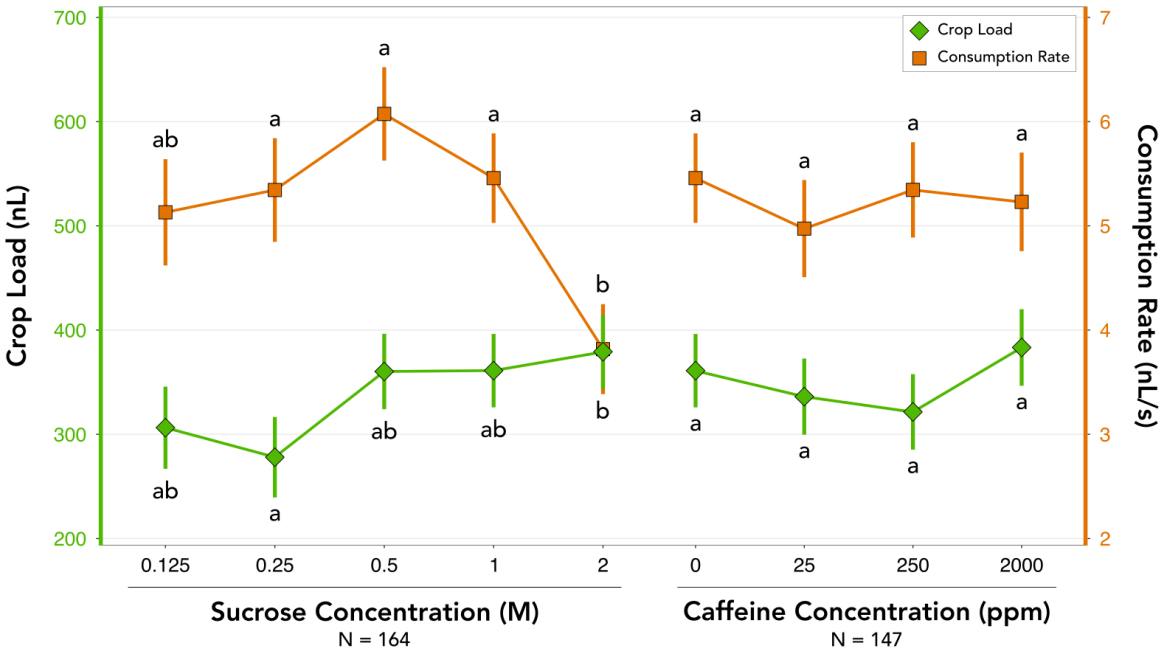

Figure 5 – Quantifying invasive ant feeding behaviour by three-dimensionally reconstructing their body during feeding. Diamonds and squares represent the estimated marginal means obtained from the linear mixed-effects models and whiskers the respective standard errors. Letters reflect statistical differences between treatments which are connected based on the estimated confidence intervals. Similarly to what can be found in literature, we find a trend towards increased crop load with increasing sucrose concentrations, with consumption rate decreasing as sucrose concentration increases. This reflects the trade-off between solutions which have high sucrose molarity having high energy output whilst being hard to drink from due to high viscosity. Overall, the presence of caffeine appears to have no effect on either crop load or consumption rate. This suggests ants are not averse to it or simply cannot detect its presence.

Consumption rate (Figure 5) was relatively constant at lower sucrose concentrations (0.125M: 5.1nL/s [3.6nL/s, 6.6nL/s, N = 269]; 0.25M: 5.3nL/s [3.9nL/s, 6.8nL/s, N = 269]), reaching its maximum at 0.5M (6.1nL/s [4.7nL/s, 7.4nL/s, N = 269]) and decreasing at high concentrations (1M: 5.5nL/s [4.1nL/s, 6.8nL/s, N = 269]; 2M: 3.8nL/s [2.5nL/s, 5.2nL/s, N = 269]). Importantly, the consumption rates obtained in our experiment are almost five-fold those reported in Sola and Josens (2016) for the same species, which reach a maximum around 1.6nL/s. However, in this previous work, feeding time was taken as the time during which the ant's mandibles touched the solution, an indirect measure of ingestion, and these were considerably longer than those we observed. Interestingly, between experiments ants were kept on 0.5M sucrose solutions which could explain the peak consumption rate at this concentration. This is expected, as ants are more likely to be demotivated by solutions which are more diluted than those they have previously experienced (Cassill & Tschinkel, 1999; Wendt et al., 2019), whilst being physically slowed down by the high viscosity of higher concentration solutions (Lois-Milevicich, Schilman & Josens, 2021).

Experimental application – Caffeine presence has no effect on invasive ant feeding behaviour

Additionally, we took the opportunity to feed the ants a range of caffeine-laced sucrose solutions. Caffeine has been reported to decrease Argentine ant foraging times, likely due to its beneficial cognitive effects. For this reason, caffeine was suggested as a suitable additive to slow-acting baits, as it might lead to increases in recruitment and consumption, thus potentially improving current invasive ant management (Galante et al., 2024). Understanding the reason for this improvement is important, as for example, caffeine could simply be improving food palatability. Thus, it is important to study if caffeine has an effect on the feeding behaviour, either the total volume ingested or the rate at which it is ingested, in order to assess if the reported neuroactive effects of caffeine are in part a result of increased preference and motivation for caffeine-laced solutions.

The presence of caffeine (Figure 5) had no clear effect on either crop load (0ppm: 361nL [235nL, 487nL, N = 269]; 25ppm: 336nL [208nL, 464nL, N = 269]; 250ppm: 321nL [195nL, 448nL, N = 269]; 2000ppm: 383nL [257nL, 510nL, N = 269]) or consumption rate (0ppm: 5.5nL/s [4.1nL/s, 6.8nL/s, N = 269]; 25ppm: 5.0nL/s [3.5nL/s, 6.4nL/s, N = 269]; 250ppm: 5.3nL/s [3.9nL/s, 6.8nL/s, N = 269]; 2000ppm: 5.2nL/s [3.8nL/s, 6.7nL/s, N = 269]). This suggests caffeine has no effect on reward value perception, either because ants can't detect its presence or are indifferent to it. Thus, the addition of caffeine to toxic baits is likely to result in foraging improvements driven by the alkaloid's effect on learning and memory rather than on motivation. On average, individual ants weighed 0.5mg ± 0.1mg (N = 145), meaning ants under the 25ppm of caffeine treatment ingested on average 16.8mg of caffeine per kilogramme of body mass [10.4mg/kg, 23.2mg/kg] and those under the 250ppm

treatment 160.5mg/kg [97.5mg/kg, 224mg/kg]. For reference, humans ingest under 10mg/kg per day at most (Verster & Koenig, 2018).

System strengths, limitations, and potential improvements

The method proposed allows researchers to quantify the feeding behaviour of small invertebrates, not only when it comes to the total amount of food ingested, but also the rate at which food is ingested. It takes advantage of the fact that the bodies of some animals expand while feeding, and three-dimensionally reconstructs their body over time, and thus reducing the number of approximations required to estimate ingested volume.

The method is high throughput, with recordings taking on average 3.51 ± 0.87 minutes (N = 571) per individual and ants drinking on average 1.5 ± 1.1 minutes (N = 466), which is similar to previously reported average drinking times of 1.4 minutes for the same species (Galante et al., 2024). The pose estimation process can be time consuming, depending on the available computational resources, but, for the most part, it does not require human intervention. We estimate that data processing, using the GUI, allowed us to look at over 40 videos per hour. Moreover, the system is affordable, as it can be assembled with different materials, such as cameras which are already available, or built with Raspberry Pi HQ camera systems which are extremely affordable and convenient.

One limitation of our specific system was that the accuracy of the tracked points, and thus of the three-dimensional reconstruction and volumetric measurements was compromised when the individual ant wasn't correctly positioned in reference to the cameras. This led us to exclude a large number of recordings for which quantifying feeding behaviour was not possible. In our case, this was mitigated by a high sample size. However, if possible, building a recording system with more cameras would mitigate the need for the animals to be in specific positions, enabling for example the tracking of animals mid-flight (Maya et al., 2023; Håkansson et al., 2024). This could overcome limitations such as occlusions, perspective distortions or ambiguity in depth estimation. Importantly, whilst the system is internally consistent, meaning the results of different treatments are always comparable to each other, external consistency, and thus comparison across different experiments, will rely entirely on the standardisation of the reference points and the accuracy of the camera calibration. To overcome this, we suggest, for example, using camera lenses with microscope calibration scales.

During our accuracy measurements for the experimental validation, we were able to directly compare our method with the traditional gravimetric approach. We found that weighing was not only more time consuming, especially as usually three replicates for each weighing are required, but also resulted in a substantial number of individuals being lost

due to excessive anaesthesia or during transfer to the scale. Interestingly, ants which were anesthetised and used in the gravimetric accuracy measurements, had a lower crop load (max = 299nL, N = 104) and consumption rate (max = 4.1nL/s, N = 104) than those estimated for similar sucrose solutions (Figure 5) which were not anesthetised and weighed (0.5M: 360nL at 6.1nL/s; 1M: 361nL at 5.5nL/s). This is likely due to the stress induced by cooling anaesthesia further reinforcing the benefits of a non-invasive method which does not require anaesthesia. In fact, ants which were anesthetised, either by cooling or carbon dioxide, were previously shown to be less willing to feed on sugary solutions (Mailleux, Deneubourg & Detrain, 2000).

The measuring device proposed here is extremely useful for the quantification of feeding behaviour in ants, especially invasive ones, which tend to be small yet expand during feeding. For Argentine ants, consumption rate was for the most part linear. However, the method is flexibly applicable to animals whose feeding dynamics are not constant over time, by simply fitting a different model to the data. We predict the system would also be useful for disease vector studies, for example, elucidating feeding patterns in mosquitoes, potentially leading to new control methodology. Importantly, the fact that the method is non-invasive, means it is also suitable for larger insects, which could be reliably weighed, but in this way would be less disturbed, thus making it easier to capture their natural behaviour, even in field settings if required. Finally, in principle, this system could also be applied to non-living objects, for example allowing the measure of volumetric changes in medical applications, where equipment usually has a high cost.

Overall, the proposed method of directly estimating volumetric changes over time is much faster and less invasive than most currently used methods. Considering it is currently almost impossible to establish a ground truth measurement of the feeding behaviour of small invertebrates, we believe we successfully demonstrated the method is both accurate and capable of detecting known behavioural patterns. Additionally, we demonstrate its potential to measure feeding preference and chemical perception. Ultimately, this could provide important insight on the preferences of disease vectors, such as mosquitoes feeding on different blood types, and have direct impacts on invasive ant management strategies.

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Declaration of interests

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

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

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Chapter 4

Invasive ants fed spinosad collectively recruit to known food faster yet individually abandon food earlier

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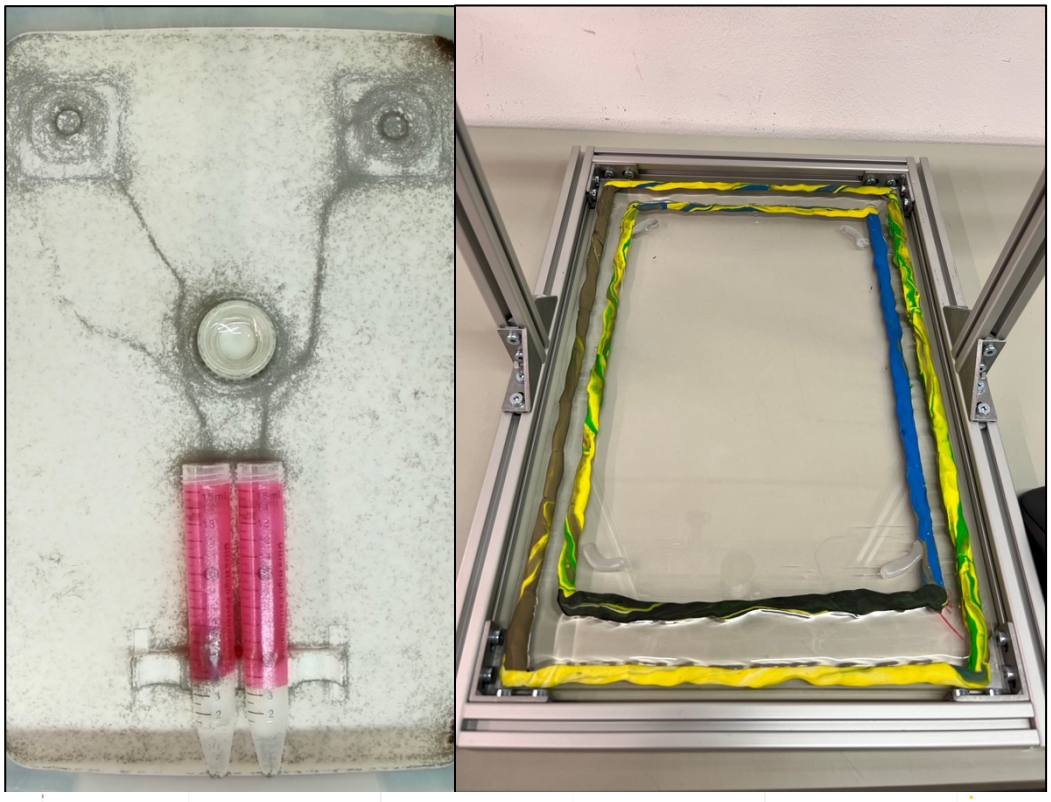
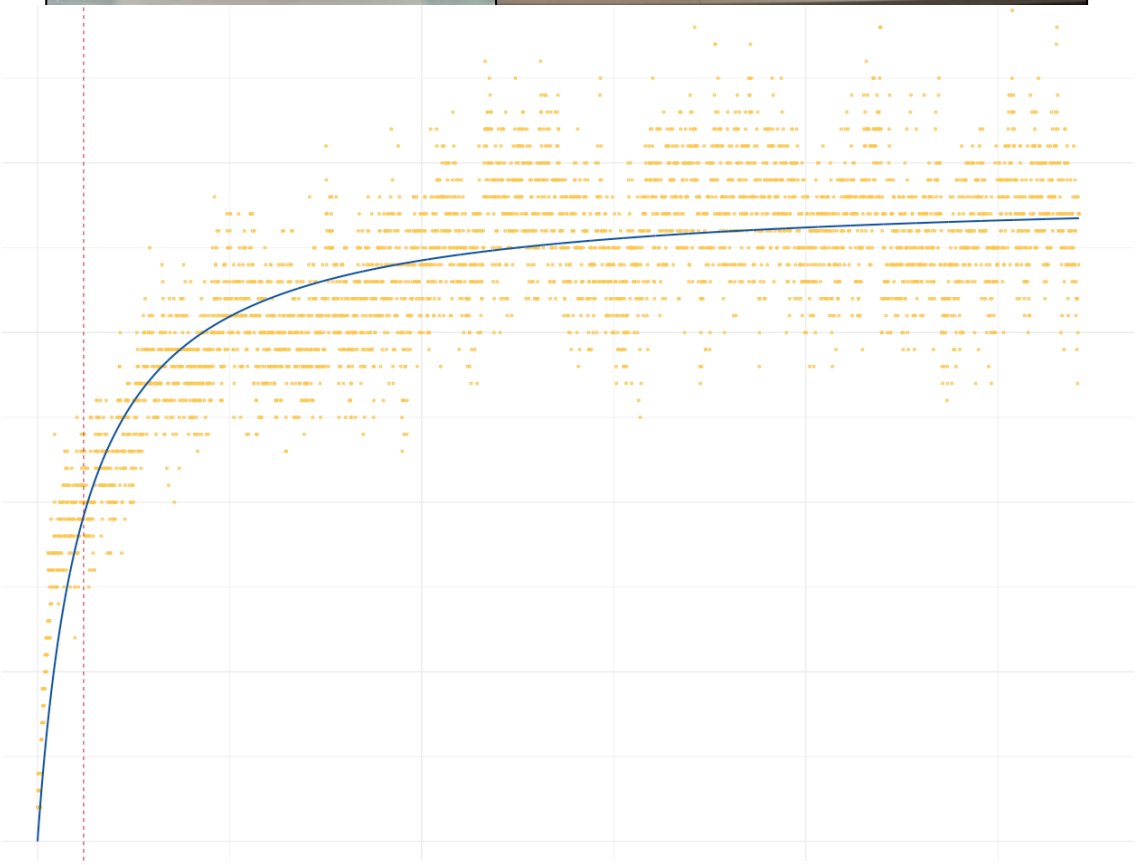
Abstract

Current management strategies applied to invasive ants rely on slow-acting insecticides which aim to delay the ant's ability to detect the poison until its effects are noticeable. Despite this, most control efforts are unsuccessful, likely due to bait abandonment and insufficient sustained consumption. Conditioned taste aversion, a learned avoidance of a particular taste, is a crucial survival mechanism which prevents animals from repeatedly ingesting toxic substances. However, whether ants are capable of this delayed association between food taste and subsequent illness remains largely unexplored. Here, we exposed colonies of the highly invasive Argentine ant, *Linepithema humile*, to a sublethal dose of the slow-acting insecticide spinosad. We combined measurements of individual-level feeding patterns with quantification of collective preferences and foraging dynamics to investigate the potential effects of the toxicant on behaviour. Collectively, ants preferred an odour associated with a previously experienced food, even if this contained spinosad, over a novel one. However, at the individual-level, previous exposure to spinosad resulted in reduced food consumption, as a consequence of earlier food abandonment. Moreover, while control-treated colonies recruited slower to a food source which tasted like a previously experienced one, spinosad-exposed colonies recruited equally fast to both novel and familiar foods. Although it appears that ants are unable to develop a conditioned taste aversion to sublethal doses of spinosad, ingestion of even small amounts of the toxicant strongly influences foraging behaviour. Understanding the subtle effects of slow-acting pesticides on ant cognition and behaviour can ultimately inspire the development of more efficient control methodologies.

delayed-action pesticide • conditioned taste aversion • foraging • recruitment • invasion biology





Introduction

Argentine ants are among the most widespread and destructive invaders globally, responsible for significant ecological and economic damage (Silverman & Brightwell, 2008; Angulo et al., 2022, 2024). Current pest control efforts often rely on chemical baiting. However, no successful eradication of an entire population of *L. humile* using slow-acting toxic hydrogel beads has been reported. This is thought to be due to low sustained bait uptake and the active abandonment of foraging trails to baits (Silverman & Brightwell, 2008; Zanola, Czaczkes & Josens, 2024). We hypothesise that this could, in part, be due to ants becoming averse to the taste of the toxicants in the baits. Spinosad, a slow-acting neuroactive toxicant, acts by stimulating nicotinic acetylcholine and GABA receptors, inducing rapid nervous system excitation, resulting in insect paralysis and death (Salgado, 1998; Biondi et al., 2012). Due to its low toxicity to mammals and fish, it is a promising eco-friendly insecticide for invasive ant management (Bacci et al., 2016; Khan, 2018). However, it is unclear if ants are able to detect its presence in the baits and if they become averse to it once its negative post-ingestion effects begin.

Learning allows animals to adapt to environmental changes throughout their lives. Associative learning, in particular, links an unconditional stimulus (a stimulus that causes a specific response without prior learning) with a conditional one (a stimulus that can be perceived but does not result in that specific response). Once these stimuli are associated, detecting the conditional stimulus triggers a response similar to that caused by the unconditional stimulus. This type of learning often requires multiple pairings of the unconditional and conditional stimuli, which must occur in close temporal proximity (Pavlov, 1927; Rescorla & Wagner, 1972; Dickinson, 2012). However, conditioned taste aversion (CTA), a form of classical conditioning, is an exception to these requirements.

CTA is a learned avoidance of a particular taste, developed when an initially neutral taste is associated with post-ingestion malaise, a general feeling of discomfort often linked to illness. This mechanism is crucial for survival, as it prevents animals from repeatedly ingesting toxic substances. In mammals, where CTA has been extensively studied, it is characterized by four main features: 1) a single conditioning trial is sufficient for forming long-lasting aversion (Steinert, Infurna & Spear, 1980; Rosas & Bouton, 1996); 2) conditioning can occur with a long delay of up to several hours between the conditional and unconditional stimuli (Nachman, 1970); 3) it is more easily achieved with a novel taste rather than familiar ones (Bernstein, 1999); and 4) illness is more easily associated with food taste than with other sensory cues, unless odour is compounded with taste (Garcia & Koelling, 1966; Palmerino, Rusiniak & Garcia, 1980).

Animals can learn to avoid odours associated with toxic food through both pre-ingestion and post-ingestion mechanisms. In honeybees, these mechanisms are mediated by different neurotransmitters: dopamine pathways primarily mediate direct aversion based on food unpalatability, while serotonin is involved in CTA signalling (Wright et al., 2010; Wright, 2011; Lai et al., 2020). Harnessed honeybees have been shown to develop aversions to bitter, toxic substances due to the physiological consequences of ingestion, with the malaise generated by these substances leading to a decreased response to odours which were previously appetitive (Ayestaran, Giurfa & Sanchez, 2010). On the other hand, honeybees did not develop a conditioned taste aversion to ethanol (Varnon et al., 2018). This suggests, that although honeybees are capable of CTA, not all compounds which likely cause post-ingestion malaise can be successfully associated with their negative effects.

In crickets, CTA is similar to that in mammals in that a single trial pairing is sufficient to achieve long-term memory retention. However, it differs in that the interval between food ingestion and toxin exposure has to be relatively short, under one hour (Lyu & Mizunami, 2022). Snails also exhibit CTA, which remains intact with repeated presentations of the conditioned stimulus and is selective for novel tastes (Nakai et al., 2020). Additionally, other insects such as moth larvae (Dethier, 1980), grasshoppers (Bernays & Lee, 1988; Simões, Ott & Niven, 2012), and fruit flies (Babin et al., 2014; Kobler et al., 2020) have been shown to form CTA.

Ants, known for their strong associative learning and reliance on multimodal cues, are incredibly fast learners (Knaden & Graham, 2016; Arenas & Roces, 2018; Oberhauser et al., 2019; Piqueret, Sandoz & d'Ettorre, 2019; Czaczkes & Kumar, 2020). Specifically, Argentine ants, *Linepithema humile*, form long-lasting olfactory associations often after a single experience (Rossi et al., 2020; Wagner et al., 2023; Galante & Czaczkes, 2024). Moreover, while most ant research focuses on appetitive learning, ants can also form associations through aversive learning, such as odour-heat associations (Desmedt et al., 2017) or the avoidance of quinine, a bitter substance (Wenig, Bach & Czaczkes, 2021). However, whether they are capable of conditioned taste aversion remains largely unexplored.

In this study, we provided *L. humile* colonies with spinosad-laced sucrose solutions to determine the extent to which they can develop a conditioned taste aversion for this toxicant. Using Y-mazes, we compared collective preferences for two odours before and after colonies were fed flavoured spinosad-laced sucrose solutions. Additionally, we quantified individual feeding patterns and collective foraging dynamics in order to understand if spinosad is detected and aversive to the individuals, but also how these effects translate to collective decision making and foraging. Ultimately, understanding how ants react to toxicant-laced baits and how their foraging behaviour changes in response to this is crucial for the design of effective control strategies. If ants become averse to slow-

acting toxic baits, this could drastically reduce the effectiveness of control attempts, and explain the general lack of success of current methodology.

Materials and Methods

To determine whether ants can form a conditioned taste aversion to sublethal doses of spinosad and to assess the influence of this slow-acting toxicant on collective foraging behaviour we combined three types of experiments. Firstly, we tested multiple individuals from each colony for their innate preference for two odours, apple and strawberry. We then fed 10% of each colony one of four treatments: apple-flavoured sucrose, strawberry-flavoured sucrose, apple-flavoured spinosad-laced sucrose, or strawberry-flavoured spinosad-laced sucrose. Approximately 24 hours later, we again tested multiple individuals from each colony for their post-ingestion preference for the same odours. We then quantified the amount and rate at which individuals which were not directly fed the treatment drank on either a food source with a novel taste-odour or one matching that of the treatment. Finally, we provided entire colonies two food sources - one with a novel taste-odour and one matching the previously experienced odour - and quantified their collective foraging dynamics.

Colony maintenance

Linepithema humile (Mayr, 1868) were collected from Portugal (Alcácer do Sal and Proença-a-Nova) and Spain (Girona) between May 2021 and November 2022. Ants were split into colony fragments, containing three or more queens and 200-1000 workers, kept in non-airtight plastic boxes (32.5 x 22.2 x 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. From these established colony fragments, standardised experimental colonies (henceforth colonies) composed of 500 workers (nurses and foragers, randomly chosen), two queens and as little brood as possible were created. Colonies were then allowed to acclimate for one week and provided with ad libitum 0.5M sucrose solution and water. After one week, colonies were deprived of carbohydrates for four days prior to testing, ensuring high foraging motivation (Figure 1A). Experiments were conducted between November and December 2022 using 19 colonies.

Chemicals and solutions

Spintor (44% w/w spinosad), a commercial insecticide, was obtained from Corteva Agriscience (Brussels, Belgium). 1M sucrose solutions (Südzucker AG, Mannheim, Germany) mixed with 0.25ppm of spinosad were used as treatments. This concentration

was chosen based on previous mortality reports suggesting it as a potential LD_{50} of *L. humile* kept in groups of 10 individuals (Galante et. al. In Prep.). Identical 1M sucrose solutions were used as controls. All solutions were mixed with 2µL/mL of either apple or strawberry food flavouring (Seeger, Springe, Germany). Scented paper overlays, used during the Y-maze preference test, were stored for at least one week prior to the experiments in airtight plastic boxes (19.4 × 13.8 x 6.6 cm) containing a glass petri dish with 0.5mL of either apple or strawberry food flavouring. Polyacrylamide hydrogel beads, used as food sources in the collective foraging test, were prepared one day prior to use. This was done by mixing 10mL of one of the four treatment solutions with approximately 80mg beads (around 8 beads) in a 15mL plastic tube. The beads were left to absorb the solution for about 24 hours at 6°C. For ease of use, the beads were cut in half before being placed on a platform, ensuring they lay flat.

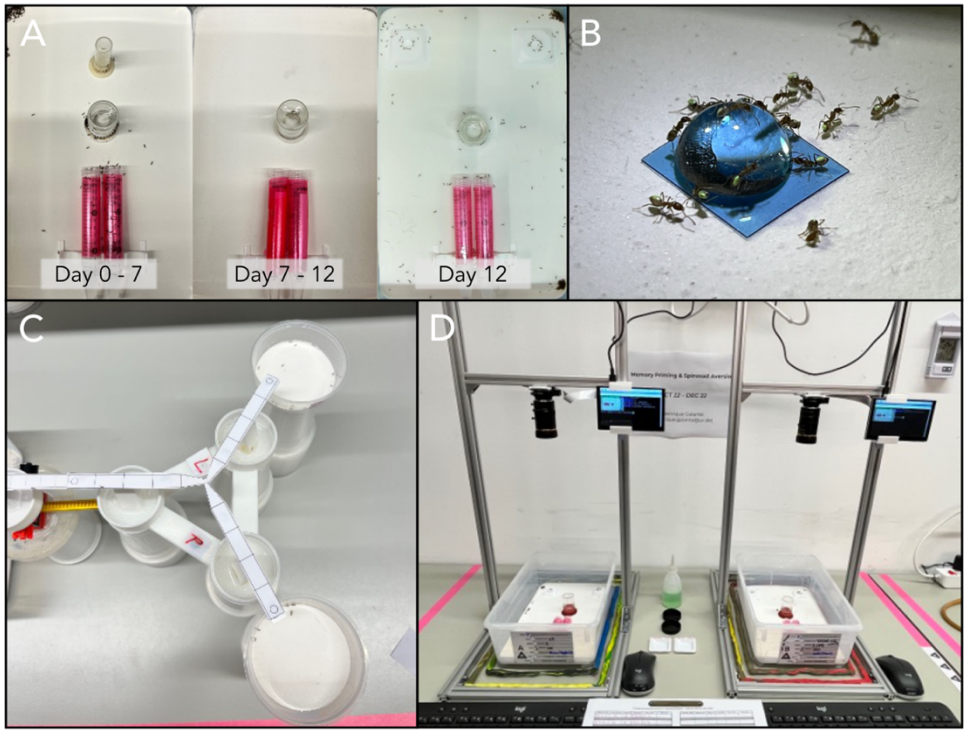


Figure 1 - Overview of experimental setups and timeline of data collection. **(A)** Standardised experimental colonies (500 workers, 2 queens) were maintained from day 0 to day 7 with *ad libitum* access to both 0.5M sucrose solution and water. On day 7, colonies were deprived of carbohydrates for four days. On day 11, colonies were tested for their innate preference for two odours and then fed one of four possible treatments. On day 12, colonies were assessed for their post-ingestion preference, the feeding patterns of approximately 30 workers were measured, and collective foraging dynamics were recorded while colonies foraged on two food sources. **(B)** Polyacrylamide hydrogel bead containing one of four possible treatments, fed to 50 individuals which were marked with acrylic paint during ingestion. **(C)** Y-maze preference test setup connected to a colony, allowing individuals to make a choice between two odours. **(D)** Raspberry Pi HQ camera systems used to record collective foraging dynamics.

Y-maze innate preference test

After depriving the colonies of carbohydrates for four days, approximately 50 individuals were given access to a drawbridge connected to a Y-maze (Figure 1B). The Y-maze consisted of three 10cm long, 1cm wide arms, tapering to 2mm at the bifurcation (Czaczkes, 2018). One arm of the Y-maze had a removable paper overlay scented with apple flavour, while the other arm had one scented with strawberry flavour, both novel stimuli for the ants. This setup allowed the ants to choose a side without any prior learning, thus measuring their innate preference for the two scents. To control for potential side biases due to brain lateralization, we tested the 50 individuals in two sequential trials with 25 ants each. In the second trial, the arm of the Y-maze in which the paper overlays were placed was swapped. Once an ant chose a side, it was removed from the maze and kept separately until all 50 ants had made their choices, after which they were returned to their original colony. In total 918 individuals from 19 colonies were used.

Treatment administration

Following the initial preference test, 50 workers from each colony were fed in small groups of three to five ants outside their nest with one of the four treatment solutions via polyacrylamide hydrogel beads (Figure 1C). These ants were marked with acrylic paint and kept in a separate box (19.4 × 13.8 x 6.6 cm) with ad libitum access to the treatment solution until all individuals were fed. This number, representing around 10% of the colony, corresponds to the expected proportion of foraging individuals in a colony, which usually falls within 10-20% of the workers in a colony (Lewis, Pollard & Dibley, 1974; Bruin, Röst & Draisma, 1977; Porter & Jorgensen, 1981; Hölldobler & Wilson, 2009). This method ensured a standardised amount of food was ingested by each colony regardless of treatment. Argentine ants visibly expand while feeding, which allowed us to confirm all treated individuals ingested the solutions provided. This approach also demonstrates that the solutions were palatable and thus it is unlikely there was any pre-ingestion aversion. Once fully fed, treated workers were returned to their respective colonies and allowed to share food and information through trophallaxis.

Y-maze post-ingestion preference test

Approximately 24 hours later, the preference test was repeated with another batch of 50 ants, again divided into two groups of 25. These ants had presumably been exposed to the treatnebt solutions through trophallaxis. Ants were allowed onto a Y-maze with scented paper overlays, and their preference for the odour of the flavour with which they were treated was measured and later compared to their innate preference for that scent. After testing, ants were returned to their colony. In total 841 individuals from 19 colonies were recorded.

Quantification of feeding patterns

Following the final Y-maze test, around 30 unmarked workers from each colony were removed and their feeding patterns, both total volume ingested and consumption rate, were quantified using 3D body reconstruction following Galante, Czaczkes & De Agrò (2024). Half of the individuals tested were fed a sucrose apple solution and the other half a sucrose strawberry solution. The setup consisted of a resin 3D-printed platform where the test solution was placed, attached to a Raspberry Pi HQ camera system and tracked using DeepLabCut version 2.3.8 (Mathis et al., 2018; Nath et al., 2019). The same DeepLabCut networks trained in Galante, Czaczkes & De Agrò (2024) were used, but the location of the cameras was adjusted and thus a new calibration performed (1px = 1.05 ± 0.20mm, N = 505). Each ant was recorded for its entire feeding event and removed from the platform once full, yet not returned to its colony of origin. In total 516 feeding events were recorded, 11 of which were excluded due to unsuccessful tracking and 102 manually removed due to the ants being in an unsuitable position for 3D reconstruction. Additionally, 49 recordings were removed due to a poor regression fit of volume over time ($R^2 < 80\%$) and four due to implausibly large crop load values. In total, 350 feeding events were analysed.

Collective foraging dynamics

Finally, each colony was given access to two food sources, a sucrose apple solution and a sucrose strawberry solution. These solutions were provided as polyacrylamide hydrogel beads and placed on plastic platforms. Empty platforms were added when colony starvation began, ensuring ants were familiar with them. These were positioned approximately 3cm from each side, 4cm from the back, 13cm apart from each other, and 15cm from the nest entrance. To account for discovery time, which could significantly impact recruitment to one food source over the other (Sumpter & Beekman, 2003), one ant was placed at each food source after both beads were added. Colonies were recorded for four hours at one frame per second while foraging (Figure 1D). In total, 19 colonies were recorded.

Automated quantification of ant count

ImageJ/Fiji (Schindelin et al., 2012; Schneider, Rasband & Eliceiri, 2012) was used to automatically count the number of ants at each feeder during the collective foraging experiment. An ImageJ macro was developed and applied to each frame (videos recorded at 1fps). First, a gaussian blur filter (radius = 0.4 pixels) was applied to the red colour channel of each frame. Following this, background subtraction was performed using a rolling ball method (radius = 2 pixels), the image's contrast was enhanced (saturated pixels = 10%) and the Yen's thresholding method was used (Yen, Chang & Chang, 1995). Watershed separation was applied to separate connected individuals. Pixels of intensity lower than 150 were removed, and colony-specific regions of interest (ROI) were set as

circles (Ø 80 pixels) around each of the two hydrogel bead feeders. Using particle analysis, all between 15 and 200 pixels in size and between 0.3 and 1.0 in circularity within each of the ROI's were counted. The output of this was a file containing the number of ants at each food source for every frame of each video. To validate the method, manual counts were performed blindly on ten images of each colony (N = 190). Selected images were evenly distributed across the duration of the videos. Manual ant count and ImageJ derived ant count had an almost perfect agreement of 98.6% (Spearman's rank correlation coefficient, N = 380). Thus, ImageJ was as reliable as the manual counts, and therefore considered to be accurate.

Quantification of collective foraging dynamics

Collective foraging followed a general pattern for all colonies and feeders. Initially, ant count rapidly increased (recruitment phase), reaching a maximum value which remained stable for a long period of time (plateau phase), and eventually began gradually decreasing (decline phase). For each of the two food sources in each colony, the end of the recruitment phase was taken as the point at which the maximum ant count was first reached. Similarly, the end of the plateau phase was taken as the point at which the maximum ant count was last reached. For all analysis, the decline phase was excluded, as it was most likely a result of the visible desiccation of the polyacrylamide beads (Cabrera et al., 2021), and thus food depletion. Moreover, the length and rate of both the plateau and the decline phase did not differ across treatments (see Statistical Analysis).

To quantify the rate at which colonies recruited to each food source, and the maximum ant count reached at each of them, a non-linear least-squares Michaelis-Menten model (Michaelis & Menten, 1913) was fit to the ant count over time data. Originally, the model describes biochemical kinetics and relates biochemical reaction rate to substrate concentration. However, the same formula can be applied to collective foraging dynamics, where the ant count at a feeder is a function of time and both the maximum ant count reached at a food source (Countmax) and the time at which the ant count at a food source reaches half the maximum count (KM).

$$Count = \frac{Count_{max} \times time}{K_M + time}$$

Statistical analysis

The complete statistical analysis output, and the entire dataset on which this analysis is based, is available from Zenodo (https://doi.org/10.5281/zenodo.12073127).

All graphics and statistical analysis were generated using R version 4.2.2 (R Core Team, 2022; Wickham, 2016, 2022). Y-maze preference test proportion data was analysed using a beta regression (Cribari-Neto & Zeileis, 2010) and model fit assessed using lmtest (Zeileis & Hothorn, 2002). Individual feeding patterns and collective foraging dynamics were analysed using linear mixed-effects models (Bates et al., 2015) with DHARMa (Hartig, 2022) used to assess linear model assumptions and MuMIn (Bartoń, 2022) to obtain a measure of goodness of fit. Analysis of variance tables were used to test the effects of the regression's coefficients (Fox & Weisberg, 2019). Estimated marginal means and contrasts were obtained using emmeans (Lenth, 2022) with Bonferroni adjusted values accounting for multiple testing. We avoid the use of p-values, and their associated binary decision of significant/nonsignificant, instead reporting effect size estimates and their respective 95% confidence intervals shown throughout the results section as (estimate [lower limit, upper limit, N = sample size]).

Results

Ants prefer the odour associated with a previously experienced food over a novel one

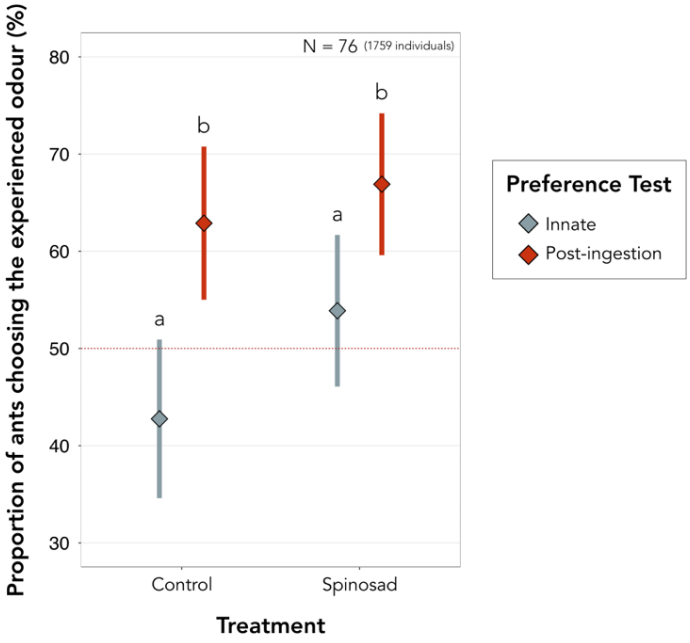


Figure 2 - Ants prefer the odour associated with a previously (approximately 24h) experienced food over a novel one, despite having no innate preference for it. Diamonds represent the estimated marginal means obtained from the beta regression model and whiskers the respective 95% confidence intervals. Estimates of 50% (red dashed horizontal line) suggest individuals have no preference for either odour. Letters reflect statistical differences between treatments based on the estimated confidence intervals. A total of 1759 individuals from 19 colonies were tested in 76 preference tests, with each test measuring the preference of around 25 ants for either odour.

Colonies had no innate preference for any odour, suggesting that both apple and strawberry odour were originally equally preferred (Control: 43% of the ants preferred the odour which would later be associated with the food [35%, 51%, N = 76]; Spinosad: 54% [46%, 62%, N = 76]). However, approximately 24 hours after each colony was fed one of the four treatments, its preference for the odour of the experienced food was higher than before its ingestion (Control: 63% [55%, 71%, N = 76]; Spinosad: 67% [60%, 74%, N = 76]). Previously experiencing a food taste increased the preference for its odour by 20% [9%, 32%, N = 76] when ants were fed sucrose, and by 13% [2%, 24%, N = 76] when ants were fed spinosad-laced sucrose (Figure 2).

Exposure to spinosad decreases the amount of food ingested through earlier food abandonment

Ants consumed food with a novel taste (1.9nL/s [1.6nL/s, 2.2nL/s, N = 350) faster than food with the same taste of a previously experienced food (1.7nL/s [1.4nL/s, 2.0nL/s, N = 350]), regardless of previous exposure to spinosad (Figure 3). However, exposure to spinosad led to a 24% [4%, 43% N = 350] decrease in overall food consumption relative to ants which were not exposed to the toxicant (Control: 136nL [117nL, 155nL, N = 350]; Spinosad: 112nL [95nL, 130nL, N = 350]).

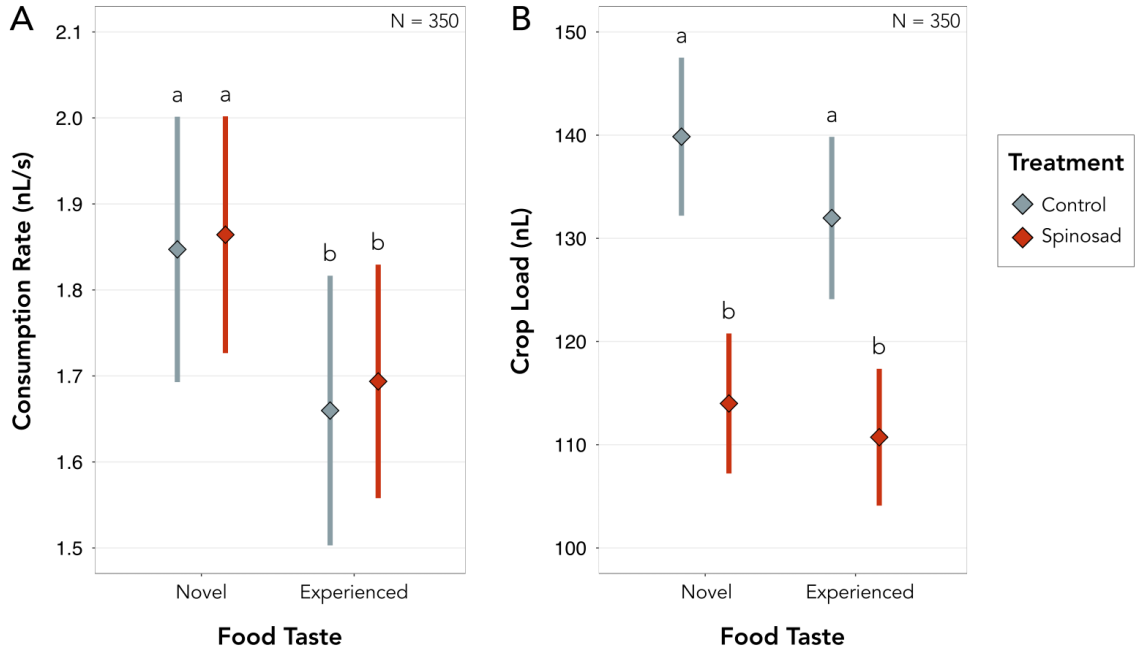


Figure 3 - Ants consume food with a novel taste faster than that which has the same taste of a previously experienced food. Ants which ingested spinosad, a slow-acting poison, generally consume less food overall compared to those fed on the sucrose controls. Notably, as consumption rates are not affected by the presence of spinosad, decreases in crop load must result from shorter drinking events, and thus the earlier abandonment of the current food source. Diamonds represent the estimated marginal means obtained from the linear mixed-effects models and whiskers the respective standard errors. Letters reflect statistical differences between treatments based on the estimated confidence intervals.

Ants recruit slower to a food source which tastes the same as a previously experienced food

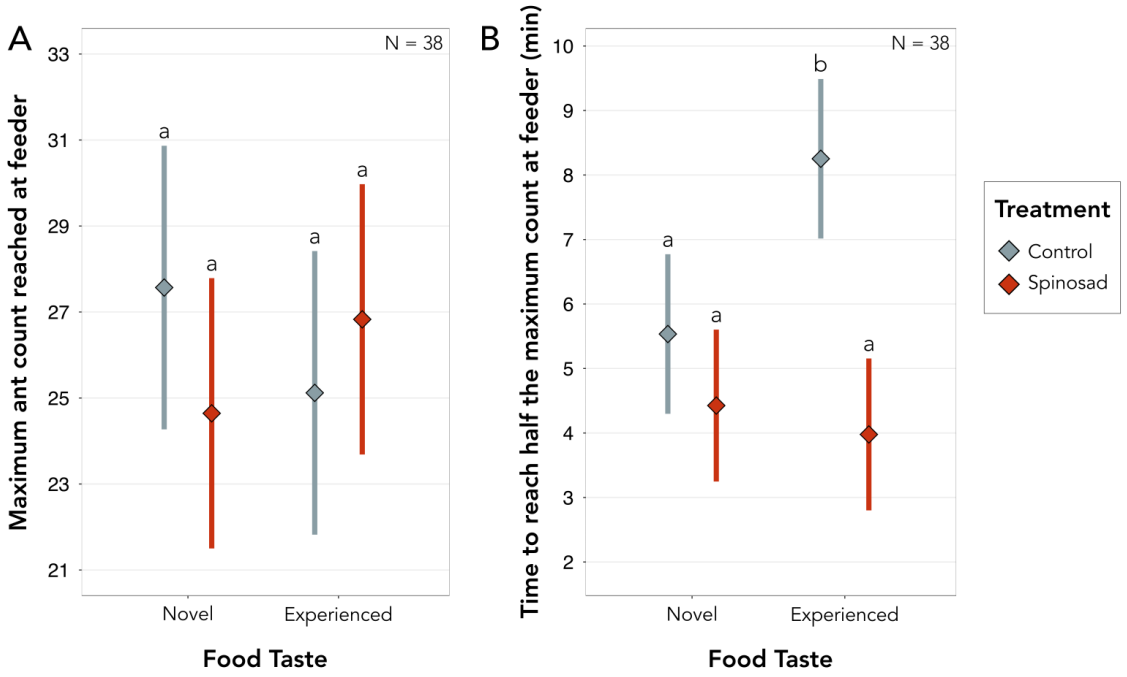


Figure 4 - Ant colonies recruit the same number of ants to both available food sources, regardless of which food they experienced previously. However, under control conditions, colonies take longer to recruit towards a food source which tastes the same as a previously experienced one, when compared to a food source with a novel taste. Notably, this effect is lost when the previously experienced food contained spinosad, a slow-acting poison. Importantly, ants treated with an apple-flavoured food show a tendency to recruit slower towards an apple tasting food, but only when this is placed on the left feeder. Moreover, the maximum number of ants recruited to a food source was affected by both odour and side, such that it reached higher values when either apple-flavoured food was placed on the right feeder or strawberry-flavoured food was placed on the left feeder (see Statistical Analysis). Diamonds represent the estimated marginal means obtained from the linear mixed-effects models and whiskers the respective standard errors. Letters reflect statistical differences between treatments based on the estimated confidence intervals.

Ant colonies recruited the same total number of ants to both available food sources, regardless of whether they were previously exposed to spinosad (Novel: 25 [16, 33, N = 38]; Experienced: 27 [18, 35, N = 38]) or simply to sucrose (Novel: 28 [19, 37, N = 38]; Experienced: 25 [16, 34, N = 38]). The recruitment dynamics towards each feeder differed with treatment (Figure 4). Under control conditions, colonies took longer to recruit towards the food which tasted the same as the previously experienced one (Novel: 6min [2min, 9min, N = 38]; Experienced: 8min [5min, 12min, N = 38]). Interestingly, this effect was lost in colonies which were previously exposed to spinosad (Novel: 4min [1min, 8min, N = 38];

Experienced: 4min [1min, 7min, N = 38]). Surprisingly, ants treated with an apple-flavoured food showed a tendency to recruit slower towards an apple tasting food, but only when it was placed on the left feeder. Moreover, the maximum number of ants recruited to a food source was affected by both odour and side, such that it reached higher values when either apple-flavoured food was placed on the right feeder or strawberry-flavoured food was placed on the left feeder. Taken together, it would appear that ants have an intrinsic preference for both strawberry-flavoured food and to turn right. Previous work has suggested *L. humile* workers have a slight preference of 58% for strawberry odour over 42% for apple (Wagner et al., 2023). However, previous work has also suggested these ants have a tendency to be left lateralised (Galante & Czaczkes, 2024, Poissonnier et. al. In Prep.). Given this, and since we believe such biases are satisfactorily accounted for by the full-factorial design of the experiment, we predominantly focused on the effects of pre-exposure to a food taste and to spinosad.

Discussion

Ants prefer the odour of a known food, over that of a novel food, regardless of spinosad exposure (Figure 2). Previous studies have also shown that ants strongly prefer the first odour associated with food which they experience after food deprivation (Oberhauser, Bogenberger & Czaczkes, 2022). Had ants been capable of forming a conditioned taste aversion (CTA) to spinosad, they would have been expected to actively prefer a novel food taste over a previously experienced one when this was paired with the toxicant. The lack of aversion observed could be due to the sublethal dose of spinosad used not causing post-ingestion malaise. In fact, during the experiment we observed a general lack of mortality, which we anecdotally quantified. In control-treated colonies, we observed a worker mortality of 5.4% ± 1.9% (N = 3), with a mortality of marked individuals which were directly fed the spinosad-laced solution of 7.3% ± 6.5% (N = 3). Similarly, spinosad-treated colonies had a mortality of 8.3% ± 2.3% (N = 5), with a mortality of marked individuals which were directly fed the treatment of 7.0% ± 2.5% (N = 5). This represents a spinosad associated mortality of around 3%, which was unexpected as previous work suggested Argentine ants, in groups of four individuals, fed a similar dose of spinosad had a mortality likelihood of 19% 24 hours post-ingestion. This is likely a consequence of group size, such that mortality is higher in small groups than in larger groups for the same toxicant dose (Galante et. al., In Prep.).

Spinosad-laced sucrose solutions were readily accepted by the ants, likely because they either could not detect the presence of the toxicant or did not recognise it as toxic on a first contact. However, even at the extremely small dose of spinosad used, which is around 600 times less than that typically used in field conditions (Milosavljević et al., 2024, Pedraza et. al., In prep.), spinosad had a clear post-ingestion effect: individuals exposed to the toxicant

showed a decrease in overall consumption of roughly 20% on all foods ingested afterwards, even though none of these foods contained spinosad. This suggests a single exposure to spinosad makes ants ingest less food for at least a day post-exposure. This may be driven by either physiological or cognitive processes. Moreover, while ants ingested food with a novel taste at a faster rate than food with a familiar taste, consumption rate was not affected by exposure to spinosad (Figure 3). Thus, the observed decrease in crop load must have been a result of shorter drinking events and the earlier abandonment of the food source. However, trail pheromones have been found to alter Argentine ant's subjective evaluation of food, increasing their acceptance (Rossi et al., 2020). Thus, during collective foraging, the decrease in food consumption found at the individual level might be mitigated by the presence of trail pheromone.

CTA is often characterised by a single conditioning trial with a long temporal separation between food ingestion and post-ingestion malaise (Nachman, 1970; Steinert, Infurna & Spear, 1980; Rosas & Bouton, 1996). However, crickets have been shown to successfully form associations only when the two stimuli were separated by less than one hour (Lyu & Mizunami, 2022). In our case, the sublethal dose of spinosad used might have taken too long to cause post-ingestion malaise, which could have prevented the formation of CTA. As a reference, a field-realistic concentration of 150ppm spinosad showed a survival likelihood of 72% after one hour whilst 0.25ppm resulted in a survival likelihood of 81% after 24 hours (Galante et. al., In Prep.). With that said, the level of spinosad used did cause a reduction in feeding of around 20%, which would be consistent with ongoing malaise.

Under the control conditions, colonies took longer to recruit to previously experienced food sources than to novel ones (Figure 4). This was likely due to ants which foraged on a novel food source ingesting it faster, and thus return to the nest sooner, than those feeding on a previously experienced food taste. Mass-recruiting ants, such as *L. humile*, rely on pheromone trails during foraging, with ants laying more pheromone towards higher quality food sources (Latty et al., 2017). Small initial differences between pheromone trails often result in large recruitment differences and in most cases in preference for one food source over the other, a phenomenon often called symmetry breaking (Beckers et al., 1990; Sumpter & Beekman, 2003; Detrain & Deneubourg, 2008; Grüter et al., 2012). Interestingly, despite initial differences in recruitment, control-treated colonies reach the same number of individuals at both food sources and no symmetry breaking was observed. This lack of preference for one food source over the other is thought to be one way by which small colonies exploit multiple food sources in order to avoid large-scale conflicts (Nicolis & Deneubourg, 1999) which would be too costly since the monopolisation of resources through cooperative defence cannot be efficiently implemented by small colonies (Franks & Partridge, 1993; Mailleux, Deneubourg & Detrain, 2003). Moreover, the lack of symmetry breaking observed is likely a consequence of both food sources being of equally high

energetic value (Price et al., 2016) and due to the negative feedback generated by overcrowding at a food source (Czaczkes, Grüter & Ratnieks, 2013; Wendt, Kleinhoelting & Czaczkes, 2020). The lack of brood in the colony is also known to decrease collective foraging asymmetry (Portha, 2002) and this was also likely potentiated by the two food sources being in close proximity to each other (13cm apart and 15cm from the nest entrances).

The initial recruitment differences observed in control-treated colonies were not present in spinosad-treated ones. Spinosad exposure resulted in equally fast recruitment for food sources of both novel and experienced taste, and even slightly faster recruitment than control colonies overall (Figure 4). However, individual level consumption rates did not differ with exposure to spinosad. Colonies feeding on a novel food source would still ingest it faster, and thus return to the nest sooner, than those feeding on a previously experienced food taste. A potential explanation for this would be that exposure to spinosad results in weaker pheromone trail deposition or affects the ant's ability to detect pheromone trails. Ants are capable of regulating pheromone deposition (Hölldobler, Stanton & Markl, 1978; Beckers, Deneubourg & Goss, 1992; Jackson & Châline, 2007), and exposure to spinosad resulted in reduced consumption of all food sources. When pheromone trail intensity is low, the decision of individual ants to deposit pheromone will have a disproportionate effect on the relative trail strength (Price et al., 2016). Moreover, weaker pheromone trails could lead ants to rely more on private information, such as learnt memories. Argentine ants have been shown to predominantly rely on social cues, such as pheromone trails, more heavily than on private information (Aron et al., 1993; Von Thienen et al., 2014; Von Thienen, Metzler & Witte, 2016). However, other mass-recruiting ants have been shown to combine both information sources for more efficient foraging or even to rely more heavily on route memories, which are often more accurate than pheromone trails, when these conflict with social cues (Evison et al., 2008; Grüter, Czaczkes & Ratnieks, 2011; Czaczkes et al., 2011). Similarly, Argentine ants have been shown to choose between trails randomly when pheromone concentration is low (Von Thienen et al., 2014).

Alternatively, spinosad could induce a state of hyperactivity, causing ants to move faster, be more motivated to find food or even forage more efficiently. In fact, Argentine ants fed moderate doses of caffeine, a neuroactive chemical, have been shown to have improved foraging (Galante et al., 2024). Spinosad, predominantly acting on nicotinic acetylcholine receptors, has also been identified as an antagonist of GABA receptors (see Millar & Denholm, 2007 and Kirst, 2010 for reviews). GABA, the main inhibitory neurotransmitter in invertebrates (Nepi, 2014) is often linked to an animal's ability to forget, and thus adapt to its environment (Boitard et al., 2015). By preventing the release of GABA, spinosad could lead ants to fixate on initially appetitive spinosad-laced sucrose solutions, potentially explaining the observed improvements in recruitment from spinosad exposed colonies.

Nevertheless, a recent study showed GABA promoting flower fidelity, suggesting its effects are complex (Calderai et al., 2023). Interestingly, sub-lethal doses of the neonicotinoid imidacloprid, also an antagonist of nicotinic acetylcholine receptors, were shown to shift colony-level preference in the invasive ant Lasius neglectus towards toxicant-laced solutions (Frizzi et al., 2022), even though imidacloprid has been shown to impair olfactory learning and memory in honeybees (Yang et al., 2012; Williamson & Wright, 2013). However, were this the case, control attempts using spinosad as the toxicant would likely have been more successful, unless such effects are lost at high doses, as they are for caffeine and imidacloprid (Frizzi et al., 2022; Galante et al., 2024).

The sublethal dose of spinosad used in our experiment did not lead the ants to form a conditioned taste aversion, potentially because of its low toxicity and the high energy content of the sucrose solutions provided. Nevertheless, conditioned taste aversions could still be present under field-conditions, where toxicant doses are higher. If this is the case, a potential way to address CTA in management efforts would be to leverage these aversive associations. For example, using toxic baits with one taste-odour initially, and once individuals formed a CTA to that taste-odour, cycle through different flavours such that individuals would always be driven towards novel foods, yet ingestion of the toxicant would remain. However, this approach would need to be formally tested as natural food sources would still be competing with the baits. Ultimately, identifying a toxicant which is not only palatable but also has no direct impact on food consumption is crucial for the development of effective control strategies.

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Declaration of interests

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

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

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Chapter 5

Larger group sizes increase resistance to a slow-acting toxicant in invasive ants

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In Prep.

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Abstract

Invasive ant management often fails. This is commonly thought to be a consequence of insufficient bait consumption and the active abandonment of foraging trails which lead to toxicants. On the other hand, ants are known to modify their network structures in response to pathogens, mitigating disease. However, whether similar adaptive behaviours occur in response to already ingested toxicants remains largely unexplored. Spinosad, is a promising slow-acting insecticide due to its low toxicity to mammals and fish and its widespread registration in over 80 countries. Here, we investigate the effects of spinosad, both in its pure form and as a commercial pesticide, on the survival of *L. humile*. Moreover, we explore how group living and conspecific interactions influence toxicant-induced mortality. We combine the results of three experiments which assessed ant mortality at various doses of spinosad in groups of four, ten, twenty-five, fifty, and one hundred ants, as well as in colonies composed of one hundred workers, one queen, and brood. Preliminary results indicate that larger group sizes, rather than the presence of a queen and brood, lead to reduced spinosad-associated mortality. Future work, using data from these experiments, will elucidate the mechanisms by which ants, at the collective level, evade spinosad even after ingesting it. Ultimately, understanding and potentially manipulating these collective responses to toxicants will be an important step towards more effective ant control strategies.

invasion biology • delayed-action pesticide • mortality • ants • group living


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Hypothesis

Ants are known to adjust their network structure in response to pathogens, thus mitigating the effects of disease (Stroeymeyt et al., 2018). However, it remains unclear whether similar adaptive behaviours occur in response to toxicants in food sources. Spinosad, a slow-acting insecticide with low toxicity to mammals and fish, is widely recognized for its eco-friendly profile and is registered for use in over 80 countries (Biondi et al., 2012; Bacci et al., 2016; Khan, 2018; Santos & Pereira, 2020). Spinosad acts as a neuroactive agent by interacting with nicotinic acetylcholine and GABA receptors, inducing rapid nervous system excitation, paralysis, and ultimately death (Salgado, 1998; Biondi et al., 2012).

Previous research suggests that sublethal doses of spinosad reduce overall food consumption, potentially compromising its effectiveness as a toxicant (Galante et al., 2024). In this study, we aim to establish the mortality rates associated with different doses of spinosad in the highly invasive Argentine ant, *Linepithema humile*. Additionally, we propose that social interactions within ant colonies significantly decrease toxicant-induced mortality. Future work, leveraging fluorescence imaging data from these experiments, will explore the mechanisms by which ants evade the effects of spinosad while ingesting and sharing it. Understanding what concentrations of spinosad are required for high mortality, while minimising its use, is crucial for sustainable control strategies. However, such concentrations might have to be extremely high to overcome social immunity behaviours, making the use of toxicants such as spinosad not only prohibitively expensive but also environmentally harmful. Ultimately, understanding how ants, as a large group, evade the negative effects of toxicants is crucial to later disrupt such defensive strategies, and improve the success rate of invasive ant management.

Materials and Methods

To assess spinosad-induced mortality at different concentrations of the toxicant, both in its pure form and as a commercially available pesticide (Spintor, 44% spinosad), we combined three experiments. Initially, we quantified mortality using a geometric sequence ranging from 0.0625ppm to 1ppm of spinosad in its commercial pesticide form, administered to groups of four workers. Next, we conducted collective experiments where colonies consisting of 100 workers, one queen, and brood were exposed to 150ppm of spinosad in its pure form. This was paired with administering the same exact solutions to groups of 10 workers. Building on these findings, we systematically investigated the effect of group size on spinosad-induced mortality by administering 150ppm of spinosad, under standardised conditions, to groups of 10, 25, 50, and 100 workers.

Spintor-induced mortality in groups of 4 workers

Colony maintenance

Linepithema humile (Mayr, 1868) were collected from Spain (Girona) in November 2021. 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 in May 2022 using 320 workers from a single colony.

Chemicals and solutions

Spintor (44% w/w spinosad), a commercial insecticide, was obtained from Corteva Agriscience (Brussels, Belgium). 1M sucrose solutions (Südzucker AG, Mannheim, Germany) laced with varying spinosad concentrations, following a geometric sequence ranging from 0.0625ppm to 1ppm, were used as treatments. An identical 1M sucrose solution was used as the control. All solutions contained 1.5% of blue food colouring (Rosenheimer Gourmet Manufaktur GmbH, Germany). Argentine ants expand during feeding (Galante, Czaczkes & De Agrò, 2024), which allowed us to verify food was palatable. Polyacrylamide hydrogel beads were used as the food sources and prepared one day prior to use. This was done by mixing 10mL of each solution with approximately 80mg of beads (around 8 beads) in a 15mL plastic tube. Beads were left to absorb the solution for approximately 24 hours at 6°C.

Artificial nests

Following the methods proposed by Pedraza *et al.* (2023), groups of four ants were kept in Petri dishes (Ø 5.5cm) with mesh lids. Each Petri dish contained a small portion of plaster of Paris with a sponge. The upper part of the sponge was embedded in the plaster while the lower part was immersed in water, ensuring the plaster remained permanently moist and could serve as a water source for the ants (Figure 1). Two setups were used simultaneously, totalling 160 workers tested in parallel. First, ants were given sucrose solutions containing 0ppm, 0.25ppm, 0.5ppm, and 1ppm of spinosad. Ant mortality was recorded 16 times over a 5-day period, approximately three times a day. Next, a different group of ants was exposed to sucrose solutions containing 0ppm, 0.0625ppm, 0.125ppm, and 0.25ppm of spinosad. Ant mortality was counted 21 times over a 9-day period, also approximately three times a day. The solutions were provided as hydrogel beads placed on

2mL plastic tube lids. Dead individuals were kept in their respective Petri dishes until the end of the experiments.

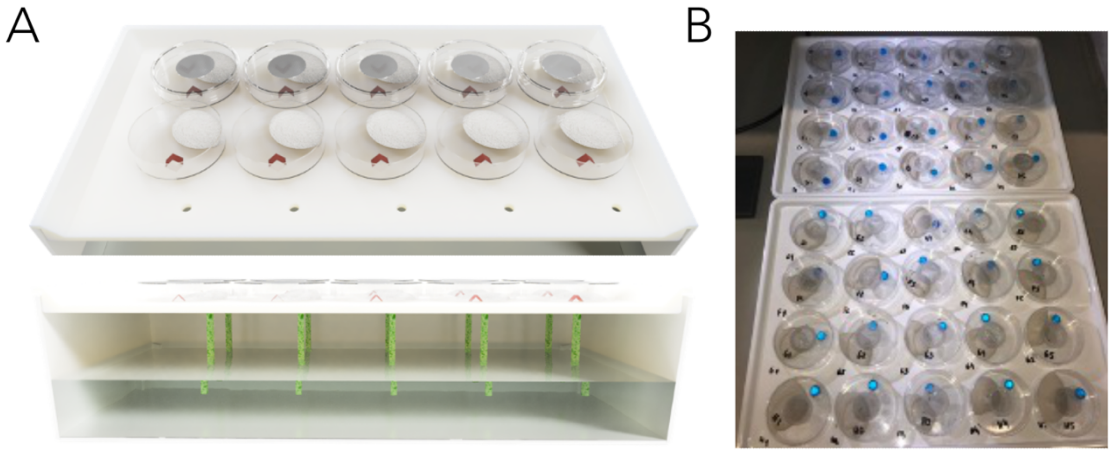

Figure 1 - Experimental setup used to assess spintor-induced mortality in groups of four workers following Pedraza et al. (2023). **(A)** Petri dishes with plaster of Paris, connected through a sponge to a water tank, ensuring permanent access to water. **(B)** Feeding of ants via blue dyed polyacrylamide hydrogel beads imbibed with either the control or the treatment solutions.

Spinosad-induced mortality in colonies of 100 workers versus groups of 10 workers

Colony maintenance

Linepithema humile (Mayr, 1868) were collected from Spain (Girona) in March 2023. Ants were split into colony fragments, containing over 30 queens and around 5000 workers, kept in non-airtight plastic boxes (26.8 x 19.6 x 11.0 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 in a constant climate chamber (HPP750, Memmert GmbH, Schwabach, Germany) at 26 °C ± 1°C and 45% ± 10% relative humidity with *ad libitum* access to water. Between experiments, ants were fed *ad libitum* 0.5M sucrose solution and *Drosophila melanogaster* twice a week. From these established colony fragments, standardised experimental colonies (henceforth colonies) composed of approximately 100 workers (nurses and foragers, randomly chosen), one queen and brood of all stages were created. Experiments were conducted between July and August 2023 using 2800 workers and 28 queens from four colonies. Additionally, 300 ants from established colony fragments were used to assess spinosad-induced mortality in groups of 10 workers.

Chemicals and solutions

Pure spinosad (CAS 168316-95-8) was obtained from Sigma-Aldrich (Taufkirchen, Germany). A 1M sucrose solution (Cristal, Zurich, Switzerland) laced with 150ppm of

spinosad was used as the treatment. An identical 1M sucrose solution was used as the control. Both solutions contained 50ppm of Atto 490LS, a fluorescent anionic dye obtained from Atto-Tec GmbH (Siegen, Germany). Moreover, both solutions contained 0.12% acetone (CAS 67-64-1), used as a solvent, ensuring spinosad miscibility. Throughout the experiments, solutions were stored at -20°C, thawed, and thoroughly mixed before each use. Colonies of 100 workers were provided with 200µL (effectively *ad libitum*) of either the control or treatment solution via a cotton plugged 0.2mL plastic tube. Groups of 10 workers were provided with 500µL (effectively *ad libitum*) of the exact same solutions as a drop placed on a piece of parafilm.

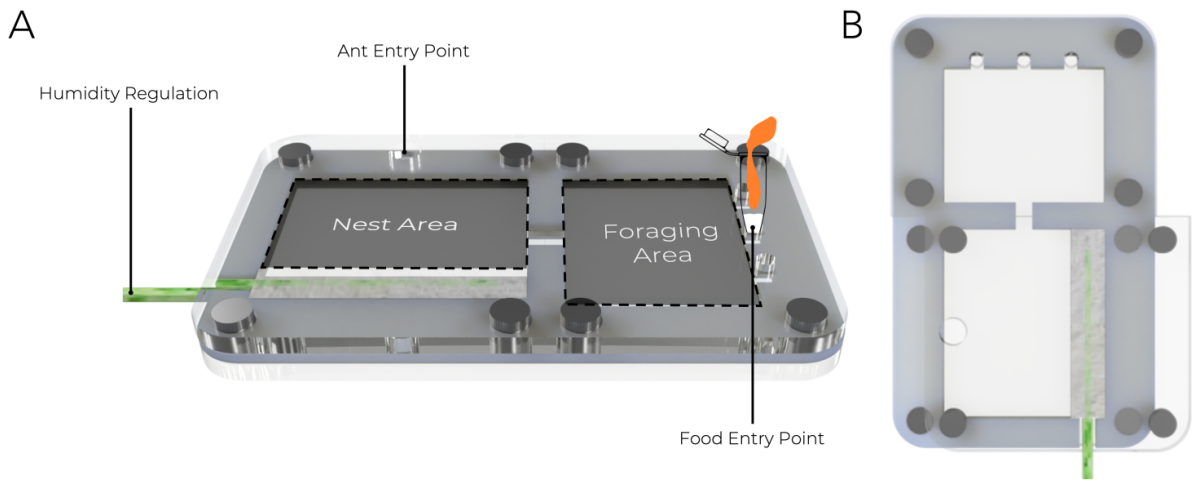

Figure 2 – Custom-made artificial nests used to assess spinosad-induced mortality in colonies of 100 workers. **(A)** Nests (128 x 78 x 10 mm) were designed with a foraging area (40 x 48 mm, 1920 mm²) and a nest area (56 x 48 mm) containing a 56 x 10 mm plaster of Paris section, adapted from Pedraza et al. (2023) for humidity regulation, providing a usable area of 2128 mm². The foraging and nest areas were connected by an 8 x 5 mm channel. **(B)** The top layer of the nest was divided into two pieces: one acting as a lid for the nest area and the other for the foraging area. The foraging area lid featured three equally spaced holes (Ø 4 mm), corresponding to the 4 x 4 mm feeding openings in the foraging area. The nest area lid had a single hole (Ø 8 mm) for ant insertion, which was done by using a PTFE-coated funnel.

Artificial nests

Colonies consisting of approximately 100 workers, one queen, and brood were housed in custom-made artificial nests (Figure 2). These nests measured 128 x 78 x 10 mm and were designed with a foraging area (40 x 48 mm, 1920 mm²) and a nest area (56 x 48 mm) containing a 56 x 10 mm plaster of Paris section, adapted from Pedraza et al. (2023), providing a usable area of 2128 mm². The foraging and nest areas were connected by an 8 x 5 mm channel. Additionally, custom vertical stands were developed which accommodate six artificial nests, facilitating their placement in an oil bath and continuous connection to

a water source, thereby optimising space use. The nests were composed of three layers: a bottom layer of 4 mm anti-reflective AMIRAN® glass, a middle layer of 2 mm 3D extruded polylactic acid (PLA), and a top layer of 4 mm polymethyl methacrylate (PMMA). The bottom and middle layers were permanently fixed using cyanoacrylate glue, while the top layer was non-permanently attached using 16 neodymium magnets (Ø 8 mm). These magnets were inserted into pre-cut holes in the layers and secured with cyanoacrylate glue. The top layer of the nest was divided into two pieces: one acting as a lid for the nest area and the other for the foraging area. The foraging area lid featured three equally spaced holes (Ø 4 mm), corresponding to the 4 x 4 mm feeding openings in the foraging area. The nest area lid had a single hole (Ø 8 mm) for ant insertion, which was done by using a PTFE-coated funnel. Once the ants were inside the nest area, the lid was closed, covering the insertion hole, and the channel to the foraging area was temporarily sealed. Ants were allowed to acclimate for approximately one hour, during which they typically nested in the designated nest area. Subsequently, the channel to the foraging area was opened, and its lid was secured, enabling ants to access the empty foraging area. Colonies were then deprived of food for 24 hours.

Nests were then transferred in pairs to the recording setup, where one was provided access to a sucrose solution and the other to a sucrose solution containing 150ppm of spinosad. The recording setup, for the most part following Baltiansky *et al.*, (2021), consisted of two cameras: a top one recording the colonies, and a bottom one capturing the fluorescent dye in the food source, which was placed in the middle feeding hole of the foraging area. Additionally, the system contained bright blue LEDs emitting light within the 400-500 nm wavelength range required to excite the fluorescent dye. Colonies were recorded continuously for four hours at approximately eight frames per second. Although exact mortality rates could not be quantified due to unreliable initial ant counts, occasional deaths during transfer or acclimation, and individuals escaping from the nests, mortality was remarkably low. In fact, no signs of spinosad-induced paralysis or twitching were observed and for the most part, ant survival was higher than 90%.

To ensure the efficacy of the treatment solution, groups of 10 workers were placed in Petri dishes in the laboratory and exposed to the exact solutions used in the collective experiments. Ant mortality was monitored over a three-hour period, approximately every half hour. Additionally, considering the possibility of photodegradation of spinosad due to the constant blue light used in the fluorescence recording system, we repeated the mortality assessment of ants in groups of 10 for two hours under this recording setup, under the same conditions used during the collective experiment.

Spinosad-induced mortality in groups of 10, 25, 50 and 100 workers

Colony maintenance

Linepithema humile (Mayr, 1868) were collected from Spain (Girona) in November 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 in November 2023 using 1098 workers from a single colony.

Chemicals and solutions

Pure spinosad (CAS 168316-95-8) was obtained from Sigma-Aldrich (Taufkirchen, Germany). A 1M sucrose solution (Südzucker AG, Mannheim, Germany) laced with 150ppm of spinosad was used as the treatment. An identical 1M sucrose solution was used as a control. All solutions contained 1.5% of blue food colouring (Rosenheimer Gourmet Manufaktur GmbH, Germany). Moreover, all solutions contained 0.12% acetone (CAS 67-64-1), used as a solvent, ensuring spinosad miscibility. Ants were provided with 200µL (effectively *ad libitum*) of either the control or treatment solution via a cotton plugged 0.2mL plastic tube.

Figure 3 - Experimental setup used to assess spinosad-induced mortality in groups of 10, 25, 50 and 100 workers. Ants were housed in plastic boxes measuring 75 x 75 mm, with a plaster of Paris floor and talc-ethanol coated walls. The plaster floor featured an indentation created with half a microscope slide measuring approximately 26 x 37.5 x 2 mm. Above this indentation, a 52 mm diameter Petri dish base was embedded into the plaster and covered with red transparent paper, providing ants with space to nest underneath it. Plastic tubes (2mL) filled with water and plugged with cotton were provided as *ad libitum* water sources.

Artificial nests

Ants were housed in plastic boxes measuring 75 x 75 mm with lids, with a plaster of Paris floor and talc-ethanol coated walls (Figure 3). The plaster floor featured an indentation created with half a microscope slide measuring approximately 26 x 37.5 x 2 mm. Above this indentation, a 52 mm diameter Petri dish base was embedded into the plaster and covered with red transparent paper, providing ants with space to nest underneath it. Plastic tubes (2mL) filled with water and plugged with cotton were provided as *ad libitum* water sources. Ants were allowed to acclimate and were deprived of food for approximately 19 hours. Following this, ants were allowed unrestricted access to either the control or the treatment solution for about five hours. Initial and final worker counts were recorded, and photographs of all individuals (both alive and deceased) were taken at the end of the experiment.

Statistical Analysis

All graphics and statistical analysis were generated using R version 4.2.2 (R Core Team, 2022; Wickham, 2016; Kassambara, Kosinski & Biecek, 2021; Wickham, 2022). Survival data for ants kept in groups of four and ten individuals was analysed using cox proportional-hazards models, and hazard ratios were compared against the respective control treatments (Therneau & Grambsch, 2000). The spinosad-induced mortality in groups of 10, 25, 50 or 100 workers proportion data was analysed using a beta regression (Cribari-Neto & Zeileis, 2010) and model fit assessed using lmtest (Zeileis & Hothorn, 2002). Analysis of variance tables were used to test the effects of the regression's coefficients (Fox & Weisberg, 2019). Estimated marginal means and contrasts were obtained using emmeans (Lenth, 2022) with Bonferroni adjusted values accounting for multiple testing. We avoid the use of p-values, and their associated binary decision of significant/nonsignificant, instead reporting effect size estimates and their respective 95% confidence intervals shown throughout the results section as (estimate [lower limit, upper limit, N = sample size]).

Preliminary Results

Spintor-induced mortality in groups of 4 workers

Control-treated ants kept in groups of four had a survival probability of 96% (N = 80) 24 hours after being fed a sucrose-only solution. Survival gradually decreased over time, reaching 82% after 120 hours. At this point, it dropped drastically to 41%, eventually hitting its lowest value of 38% at 216 hours when the experiment ended (Figure 4). This is in line with previous work suggesting that starvation-induced mortality for Argentine ants begins after approximately five days (120 hours) of food deprivation (Pedraza et al., In Prep.). Ants fed 0.0625ppm of spinosad had a hazard ratio of 0.91 [0.45, 1.8, N = 40]. The hazard ratio compares the mortality observed in a given treatment to that observed in a reference group,

in this case, the control-treated ants. A ratio of one suggests no difference between the hazard rates of the two groups, while a ratio greater than one indicates higher mortality in the treatment group, and a ratio less than one indicates lower mortality compared to the reference group. Similarly to ants fed 0.0625ppm spinosad, ants exposed to 0.125ppm had a comparably high survival probability to control-treated ants (0.62 [0.29, 1.3, N = 40]). However, ants fed 0.25ppm spinosad had a higher mortality risk (2.72 [1.65, 4.5, N = 80]), with a survival probability of 81% 24 hours after exposure, decreasing to 46% at 120 hours post-exposure (48h: 68%; 72h: 59%; 96h: 48%). Ants fed 0.5ppm and 1ppm of spinosad had significantly higher mortality compared to control-treated ants (0.5ppm: 14.71 [8.47, 25.5, N = 40]; 1ppm: 22.69 [12.93, 39.8, N = 40]). The survival probability for the 0.5ppm group was 35% after 24 hours and 10% for the 1ppm group after 24 hours. Both treatments reached their lowest survival probability of 2% at 72 hours.

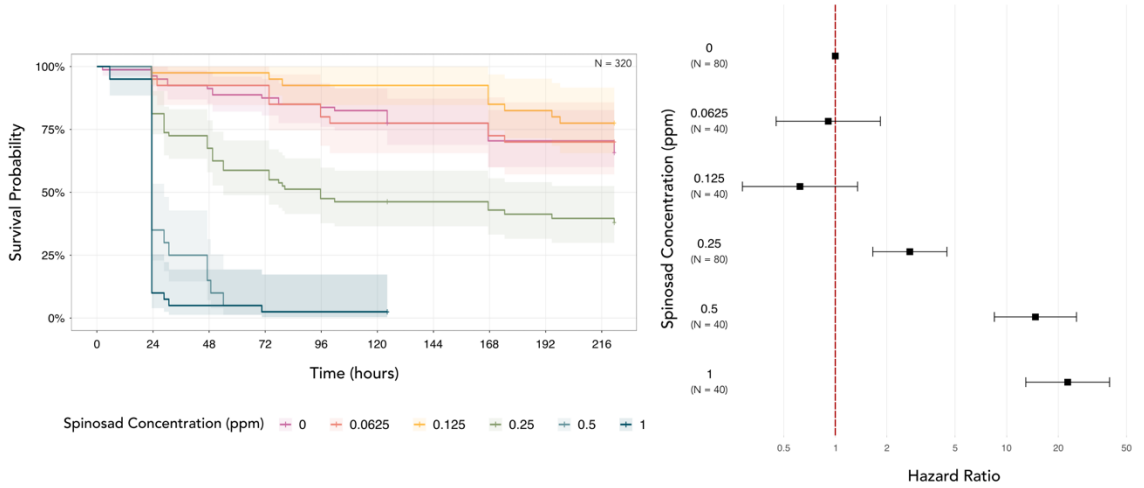

Figure 4 - Spintor-induced mortality in groups of four workers. Ants fed 0.0625ppm and 0.125ppm of spinosad exhibited similar survival probabilities to those of control-treated ants. In contrast, ants fed 0.25 ppm of spinosad experienced higher mortality rates, with survival probabilities stabilising around 50%, suggesting this concentration as the LD_{50} for Argentine ants kept in groups of four individuals. Ants fed 0.5 ppm and 1 ppm of spinosad suffered from extremely high mortality, with 98% of the ants dying 72 hours post-ingestion. Importantly, starvation-induced mortality for Argentine ants has been shown to begin after approximately five days (120 hours) of food deprivation (Pedraza et al., In Prep.).

Spinosad-induced mortality in colonies of 100 workers versus groups of 10 workers

The spinosad-induced mortality of colonies consisting of 100 workers, one queen, and brood could not be directly quantified. Nevertheless, Figure 5 shows that the proportion of live ants inside the nest at the end of the experiment was generally above 90%, and when it wasn't, this was not a result of spinosad exposure. In future, since all colonies were recorded during the experiment, obtaining effective ant counts should be possible. Regardless, we present this data as it shows a striking lack of mortality at 150ppm of spinosad after four

hours. To verify that this lack of mortality was not due to the toxicant solution being ineffective, we administered the exact same control and treatment solutions to ants kept in Petri dishes in groups of ten in the laboratory (Figure 6). After 3 hours, the survival probability of control-treated ants was 100% (N = 60), but for spinosad-treated ants, it was 15% (N = 60) after the same period (0.5h: 100%; 1h: 72%; 1.5h: 38%; 2h: 20%; 2.5h: 17%). We hypothesised that the constant blue light under the fluorescence recording setup could result in photodegradation of the poison, thus rendering it ineffective. For this reason, we repeated the experiment with groups of ten ants under the intense blue light of the setup. In this case, control-treated ants had a survival probability of 98% (N = 90), while spinosad-treated ants had a survival probability of 13% (N = 90) after 2 hours (0.5h: 100%; 1h: 71%; 1.5h: 13%). Considering the mortality of the control-treated ants under the blue light setup as the reference value, the hazard ratio of the control-treated ants in the laboratory was 0.42 [0.04, 4.80, N = 60]. However, the mortality of the spinosad-treated ants in both the blue light setup (104.15 [25.48, 425.80, N = 90]) and the laboratory (48.58 [11.80, 200.10, N = 60]) was considerably higher.

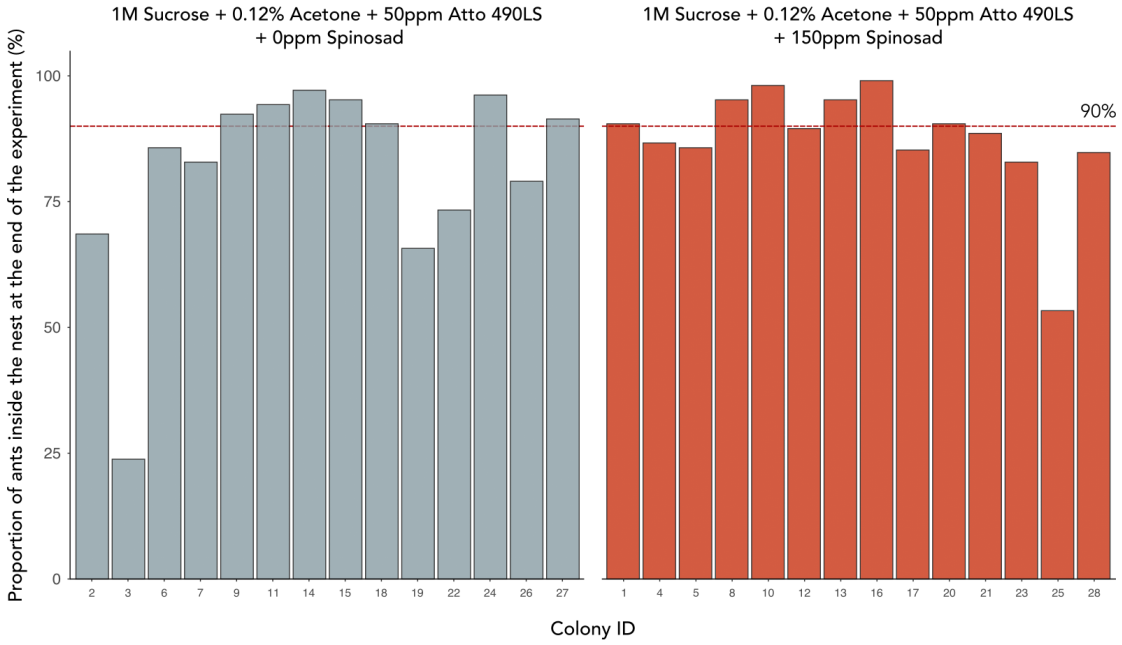

Figure 5 – Proportion of ants inside each nest at the end of the experiment. Exact mortality rates could not be quantified due to unreliable initial ant counts, occasional deaths during transfer or acclimation, and individuals escaping from the nests. Nevertheless, mortality was remarkably low, and no signs of spinosad-induced paralysis or twitching were observed. For the most part, ant survival was higher than 90% (horizontal red dashed line). It is worth noting that, for most cases where the proportion of ants inside the nest is low, for example for colony 3 and 25, these are due to a high number of ants escaping the artificial nests, which can be confirmed through the recordings.

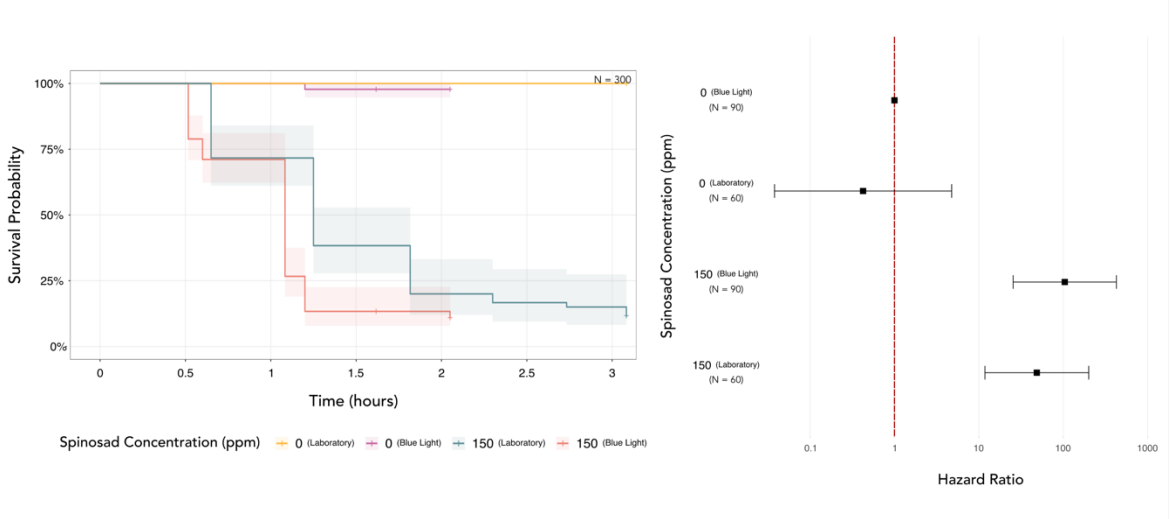

Figure 6 - Spinosad-induced mortality in groups of ten workers. Under both laboratory conditions and those of the fluorescence recording system, ants fed 150ppm of spinosad exhibited drastically lower survival probabilities when compared to control-treated ants. Considering mortality did not differ between experimental conditions, the solutions used were effective and spinosad was not photodegraded by the blue light produced by the recording system. Thus, the striking differences in mortality observed between colonies of 100 workers, one queen and brood, and groups of ten workers are likely attributable to differences in group size and/or the presence of a queen and brood.

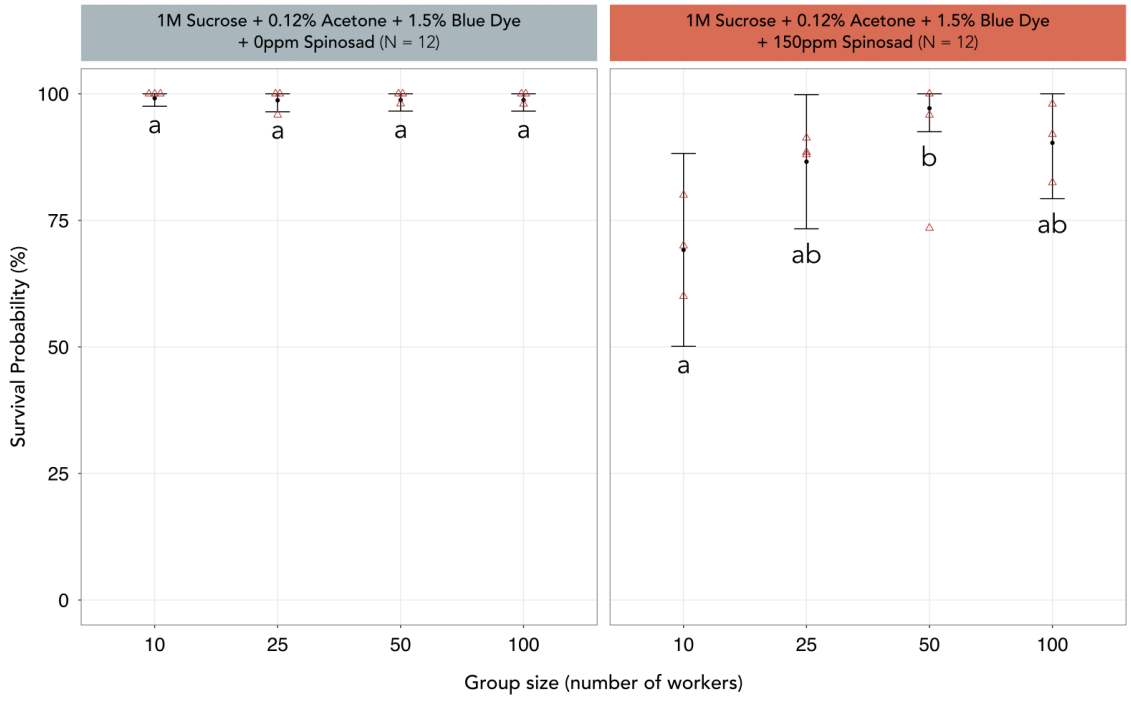

Figure 7 - Spinosad-induced mortality in groups of 10, 25, 50 and 100 workers. Larger group sizes lead to higher survival probabilities when ants are exposed to 150ppm of spinosad. Black circles represent the estimated marginal means obtained from the beta regression model and whiskers the respective 95% confidence intervals. Red triangles represent the survival probability of each replicate. Letters reflect statistical differences between group sizes based on the estimated confidence intervals.

Spinosad-induced mortality in groups of 10, 25, 50 and 100 workers

Control-treated ants kept in groups of 10 (99.1% [98%, 100%, N = 24]), 25 (98.7% [97.1%, 100%, N = 24]), 50 (98.8% [97.2%, 100%, N = 24]), or 100 individuals (98.8% [97.2%, 100%, N = 24]) had equally high survival rates. However, spinosad-induced mortality was affected by group size (Figure 7). Ants in groups of 10 fed 150 ppm of spinosad had a survival rate of 69.2% [55.5%, 82.8%, N = 24]. In contrast, ants exposed to spinosad in groups of 25 (86.6% [77.1%, 96.1%, N = 24]), 50 (97.1% [93.8%, 100%, N = 24]), and 100 individuals (90.3% [82.4%, 98.2%, N = 24]) showed increasing survival rates. Specifically, the survival rate of spinosad-fed ants in groups of 10 individuals was lower than in groups of 50 by -28% [-7.6%, -48.4%, N = 24], and by -21.1% [-44.0%, 1.7%, N = 24] when comparing to groups of 100.

Preliminary Conclusions

In small groups of four individuals, spinosad concentrations as low as 0.5ppm or 1ppm resulted in extremely high mortality 24 hours post-ingestion, with 65% and 90% mortality probability, respectively (Figure 4). Field-realistic doses of 150ppm of spinosad (Milosavljević et al., 2024, Pedraza et al., In Prep.) given to small groups of ten workers had an even faster effect, resulting in a large mortality probability between 80% and 87% after two hours (Figure 6). However, colonies of 100 workers, one queen, and brood exposed to the same 150ppm of spinosad solution showed a striking lack of mortality after four hours (Figure 5). This was despite the colonies ingesting the toxicant early on, sharing it across the colony, and reaching similar overall fluorescence intensities, and thus poison quantities, as control-treated colonies. Similar results were previously observed, where colonies of 500 workers and one queen exposed to 0.25ppm of spinosad had extremely low mortality despite an expected mortality of 19% 24 hours post-ingestion (Galante et al., 2024).

While the shift in spinosad-induced mortality could be influenced by group size, it could also by a result of the presence of a queen and brood. To explore this, we fed groups of 10, 25, 50, and 100 workers a sucrose solution containing 150ppm of spinosad for approximately five hours, keeping all groups under standardised conditions. Our findings were consistent with the previous results, showing higher mortality rates when ants are kept in smaller groups of ten individuals (Figure 7). However, mortality was lower (20-40% five hours post-ingestion) than what would have been expected from the previous experiment (80-87% two hours post-ingestion). We hypothesise this is because ants were kept in plastic boxes with a plaster floor and a nest, which they acclimated to for approximately one day. Contrastingly, when mortality was higher, ants were almost immediately exposed to the toxicant and kept in empty Petri dishes. Thus, it could be that in a more natural setting, where a nest was provided, not all individuals directly fed on the food source and more food was transferred between individuals. Nevertheless, our results

opens up intriguing research questions and emphasise that collective behaviour cannot always be directly extrapolated from individual behaviour (Sasaki & Pratt, 2011; Dussutour & Nicolis, 2013).

Group size has long been thought to have strong effects on collective organisation (see Dornhaus, Powell & Bengston, 2012 for a review). Moreover, previous studies have shown that ants have a lower lifespan when kept alone compared to when kept in groups of ten (Koto et al., 2015; Wang et al., 2016). At the collective level, ants are known to resist pathogen-associated mortality primarily by engaging in sanitary actions and reducing social contact. These measures are particularly effective against pathogens such as fungi, which infect an individual's body surface and can be mechanically removed and disinfected (Hughes, Eilenberg & Boomsma, 2002; Stroeymeyt et al., 2018; Stockmaier et al., 2023). However, the presence of social immunity behaviours in response to an ingested toxicant shared throughout the colony remains largely unexplored.

Potential behavioural mechanisms could include the avoidance of the toxicant-laced food or a reduction in its consumption. In fact, pre-exposure to sublethal doses of spinosad has been shown to lower consumption of all food sources (Galante et al., 2024). Moreover, ants could regurgitate food when the negative effects of the slow-acting toxicant start to become apparent. However, the fluorescent data obtained from colonies of 100 workers fed 150 ppm of spinosad suggests that neither regurgitation nor noticeable alterations in feeding are occurring. Ants appear to ingest similar amounts of food compared to the control-treated colonies, and, for the most part, there was no fluorescence detected outside the ants' bodies.

Alternatively, ants which directly consume the toxicant-laced food could concentrate the toxicant. Previous studies have demonstrated that despite frequent trophallactic interactions, the sharing of food between individuals, food within the colony does not become evenly mixed (Greenwald, Eckmann & Feinerman, 2019). However, if this were the case, we would expect to observe high mortality among donor ants and a general avoidance of the food by recipient ants. This does not appear to be happening, as most ants show fluorescent traces of food by the end of the experiment, and no signs of paralysis or twitching associated with spinosad poisoning have been observed. Instead of concentrating the toxicant, ants might be diluting it through a high trophallaxis rate. Similar dilution processes have been shown to reduce mortality caused by fungal infections (Novak & Cremer, 2015). Additionally, ants are capable of filtering particles as small as 0.88μm (Glancey et al., 1981) and can modify food, adding compounds through trophallaxis (Koto et al., 2015; Meurville & LeBoeuf, 2021). Moreover, ants have been observed to self-medicate using their highly acidic venom to reduce infection from contaminated food (Tragust et al., 2020). However, it remains unclear how the addition of compounds via trophallaxis might interfere with the neuroactive effects of spinosad.

While the mechanisms by which ants avoid the negative effects of ingested toxicants are unknown, the fluorescence data obtained in this study holds promise to shed light on these mechanisms once technical challenges are resolved. One potential approach to overcome social immunity could involve increasing the dosage of spinosad. However, it is uncertain whether ants would develop an aversion to higher doses, or if such concentrations would remain economically and environmentally feasible (Martelli et al., 2022). Ultimately, understanding how ants collectively respond to toxicants in baits, and whether these behaviours can be manipulated, will be crucial for developing more effective ant control strategies.

Supplementary Material

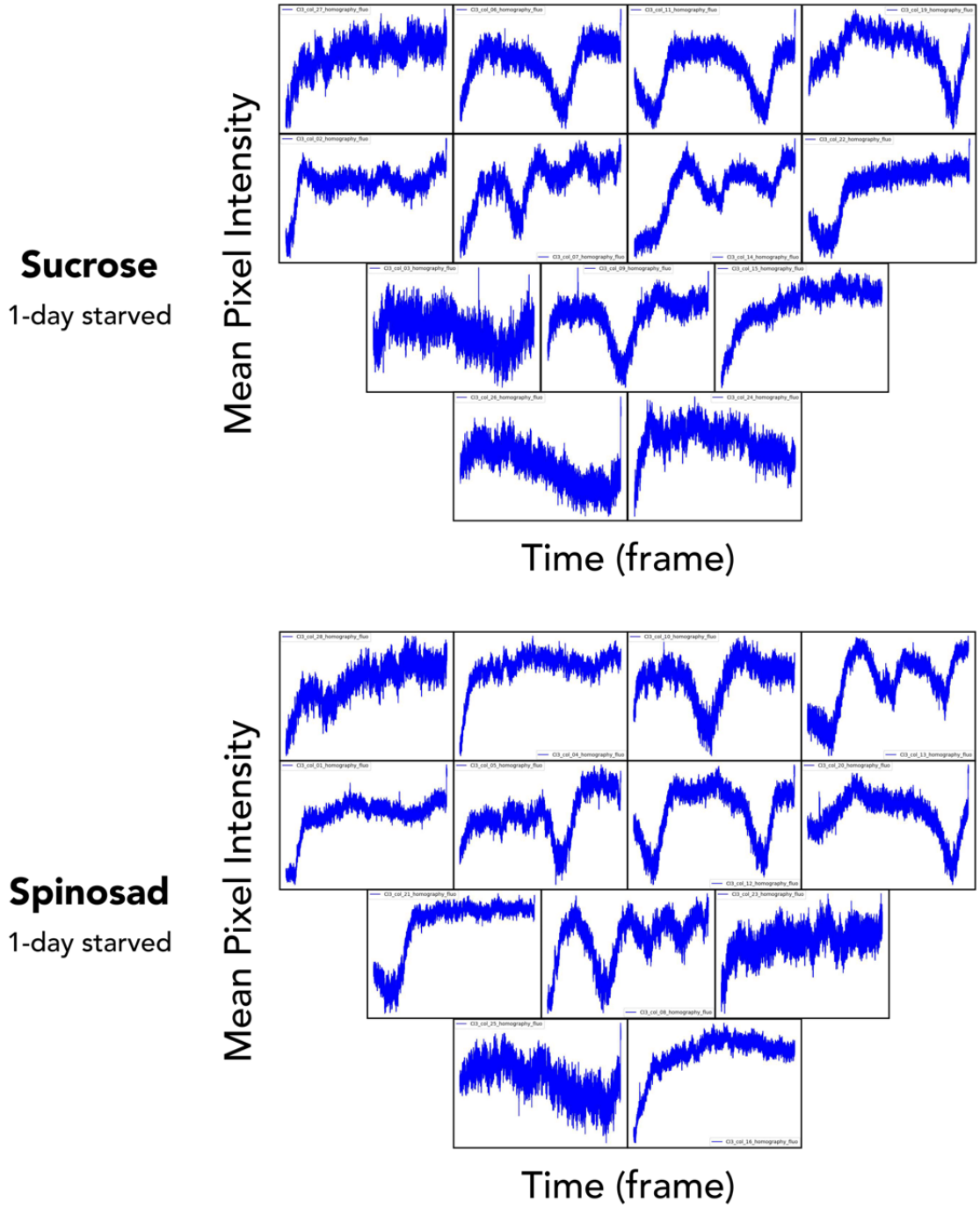

Figure S1 – Mean pixel intensity over time for each experimental colony represents the amount of fluorescent dye present in both the foraging and nest areas, indicating how much food was ingested by the ants over the four-hour experimental period. While this is preliminary data, there appears to be no obvious difference between treatments. Moreover, this data highlights that the spinosad-laced sucrose solution was palatable and readily ingested. Importantly, considering no fluorescent food was found on the nest floor, this suggests ants retain the toxicant inside their bodies during its active period. Nevertheless, no mortality was observed. It remains unclear whether, once food consumption plateaus, the food is redistributed among individuals. Once technical difficulties are overcome, this data will allow us to quantify not only food transfer but also how much food, and thus spinosad, effectively reaches the queen and brood.

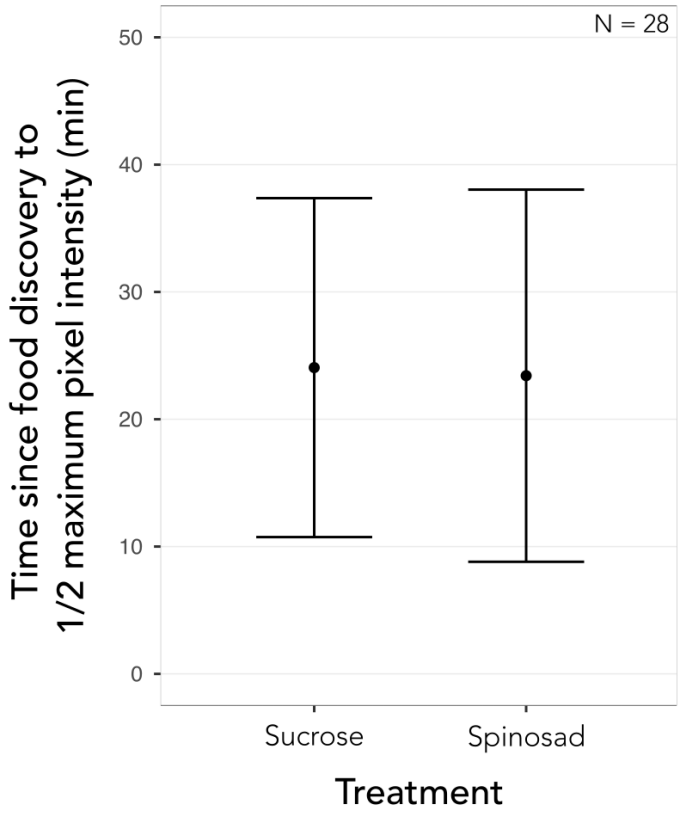

Figure S2 – Note that while most colonies immediately found the food source and began ingesting it, this was not the case for all. Therefore, we accounted for colony-specific food discovery times, removing any data before this point. Pixel intensity represents the amount of fluorescent dye present in both the foraging and nest areas of each colony, serving as a measure of how much food was ingested by the ants over the four-hour experimental period. While this is preliminary data, we observe no difference between treatments. This demonstrates that the spinosad-laced sucrose solution was recruited to and ingested at similar rates as sucrose solutions without the toxicant. Importantly, we note that preliminary data suggests colonies reach similar maximum pixel intensity values, regardless of treatment.

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Declaration of interests

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

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

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Chapter 6

Presence of protein in baits alters food consumption and dispersion in an invasive ant

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In Prep.

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Abstract

Invasive ants are widespread and cause extensive ecological and economic damage. Current management strategies often fail, likely due to low sustained bait consumption. Recently, the addition of neuroactive chemicals to toxicant-laced baits has been proposed as an improvement to management programs. However, deploying chemicals at large scales poses high environmental risks. Here, we explore the potential of protein as a safer bait additive. Ant queens and larvae require higher protein levels, while workers primarily rely on carbohydrates. Therefore, adding protein to baits could result in a more targeted delivery of the toxicant to the reproductive part of the colony. Our preliminary results suggest that colonies starved for four days have a higher foraging motivation and faster recruitment to food compared to colonies starved for one day. Furthermore, ants fed baits containing egg protein isolate took longer to reach half the maximum amount of food compared to control-treated ants. This effect, notably stronger in four-day starved colonies, suggests that protein may reduce food uptake. However, quantifying collective food transfer will be crucial to understand if, despite overall reduced food ingestion, more toxicant reaches the queen and brood due to changes in the trophallactic network. Ultimately, we hypothesise that a combination of sucrose and protein-enriched sucrose baits, deployed in tandem, could improve management efforts. This would allow ants to regulate nutrient intake while continuously ingesting toxicants, potentially improving bait uptake and control efficacy.

invasion biology • egg protein • ants • foraging • trophallaxis

Hypothesis

The addition of protein to baits is a promising strategy for more efficient invasive ant management, particularly since workers, queens, and brood have different nutritional requirements (Markin, 1970). Specifically, queens and larvae require higher protein levels, while workers primarily rely on carbohydrates (Feldhaar, 2014; Csata & Dussutour, 2019). This approach leverages natural collective behaviours, using ants' ability to regulate nutrient intake to deliver the toxicant to the reproductive component of the colony, the main target of management efforts, in a more direct manner.

Ants prefer complex nectars over simple sugars (Blüthgen & Fiedler, 2004). Chemical additives have been shown to alter foraging behaviour by manipulating preference. For example, bees have been shown to prefer sucrose solutions laced with neonicotinoid pesticides, even if these led them to eat less food overall (Kessler et al., 2015). In ants, synthetic trail pheromone has been shown to enhance bait attractiveness and increase consumption (Greenberg & Klotz, 2000; Choe et al., 2021), while morphine has resulted in ants developing a non-addictive preference for solutions containing it (Mogensen et al., 2024). However, protein has much lower risks associated with large-scale deployment than neuroactive chemicals, which are often toxic at high doses and whose effects on behaviour are harder to assess (Cutler & Rix, 2015; Baracchi et al., 2017; Frizzi et al., 2022; Galante et al., 2024).

Importantly, protein ingestion is highly regulated by the colony, as high protein diets can be toxic (Arganda et al., 2017). The presence of brood, its developmental stages, and previous access to protein are all important factors when a colony decides to forage on proteinaceous sources (see Csata & Dussutour, 2019 for a review on nutrient regulation in ants). In fact, ants have been shown to prefer sucrose solutions all year-round, while protein demand is greatest in winter and spring (Rust et al., 2000; Mathieson, 2011). Therefore, a combination of carbohydrate baits in autumn and spring and of protein-enriched baits in winter and spring could result in more efficient invasive ant management. To further assess the effects of starvation on foraging dynamics we conducted the same experiment with one day and four days starved colonies. Understanding if protein is more readily shared with the reproductive component of a colony, even when not artificially starved, will be crucial if its addition is ever to be implemented in the field.

Materials and Methods

Colony maintenance

Linepithema humile (Mayr, 1868) were collected from Spain (Girona) in March 2023. Ants were split into colony fragments, containing over 30 queens and around 5000 workers, kept

in non-airtight plastic boxes (26.8 x 19.6 x 11.0 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 in a constant climate chamber (HPP750, Memmert GmbH, Schwabach, Germany) at 26 °C ± 1°C and 45% ± 10% relative humidity with ad libitum access to water. Between experiments, ants were fed ad libitum 0.5M sucrose solution and Drosophila melanogaster twice a week. From these established colony fragments, standardised experimental colonies (henceforth colonies) composed of approximately 100 workers (nurses and foragers, randomly chosen), one queen and brood of all stages, roughly standardised in number across colonies, were created. Experiments were conducted between April and July 2023 using 5700 workers and 57 queens from four colonies.

Chemicals and solutions

Egg protein isolate (see Supplementary Material for details) was obtained from Lee-Sport (Rothenburg, Switzerland). 1M sucrose solutions (Cristal, Zurich, Switzerland) enriched with 34.23mg/mL of egg protein, and thus with 1g of protein per 10g of sucrose, were used as treatments. Identical 1M sucrose solutions were used as controls. Both solutions contained 50ppm of Atto 490LS, a fluorescent anionic dye obtained from Atto-Tec GmbH (Siegen, Germany). Throughout the experiments, solutions were stored at -20°C, thawed, and thoroughly mixed before each use. Colonies were provided with 200µL (effectively ad libitum) of either the control or treatment solution via a cotton plugged 0.2mL plastic tube.

Artificial nests

Colonies consisting of approximately 100 workers, one queen, and brood were housed in custom-made artificial nests (Figure 1). These nests measured 128 x 78 x 10 mm and were designed with a foraging area (40 x 48 mm, 1920 mm²) and a nest area (56 x 48 mm) containing a 56 x 10 mm plaster of Paris section, adapted from Pedraza et al. (2023), providing a usable area of 2128 mm². The foraging and nest areas were connected by an 8 x 5 mm channel. Additionally, custom vertical stands were developed which accommodate six artificial nests, facilitating their placement in an oil bath and continuous connection to a water source, thereby optimising space use. The nests were composed of three layers: a bottom layer of 4 mm anti-reflective AMIRAN® glass, a middle layer of 2 mm 3D extruded polylactic acid (PLA), and a top layer of 4 mm polymethyl methacrylate (PMMA). The bottom and middle layers were permanently fixed using cyanoacrylate glue, while the top layer was non-permanently attached using 16 neodymium magnets (Ø 8 mm). These magnets were inserted into pre-cut holes in the layers and secured with cyanoacrylate glue. The top layer of the nest was divided into two pieces: one acting as a lid for the nest area and the other for the foraging area. The foraging area lid featured three equally spaced holes (Ø 4 mm), corresponding to the 4 x 4 mm feeding openings in the foraging area. The

nest area lid had a single hole (Ø 8 mm) for ant insertion, which was done by using a PTFE-coated funnel. Once the ants were inside the nest area, the lid was closed, covering the insertion hole, and the channel to the foraging area was temporarily sealed. Ants were allowed to acclimate for approximately one hour, during which they typically nested in the designated nest area. Subsequently, the channel to the foraging area was opened, and its lid was secured, enabling ants to access the empty foraging area. Colonies were then deprived of food for either one or four days.

Nests were then transferred in pairs to the recording setup, where one was provided access to a sucrose solution and the other to a sucrose solution containing egg protein. The recording setup, for the most part following Baltiansky et al., (2021), consisted of two cameras: a top one recording the colonies, and a bottom one capturing the fluorescent dye in the food source, which was placed in the middle feeding hole of the foraging area. Additionally, the system contained bright blue LEDs emitting light within the 400-500 nm wavelength range required to excite the fluorescent dye. Colonies were recorded continuously for four hours at approximately eight frames per second.

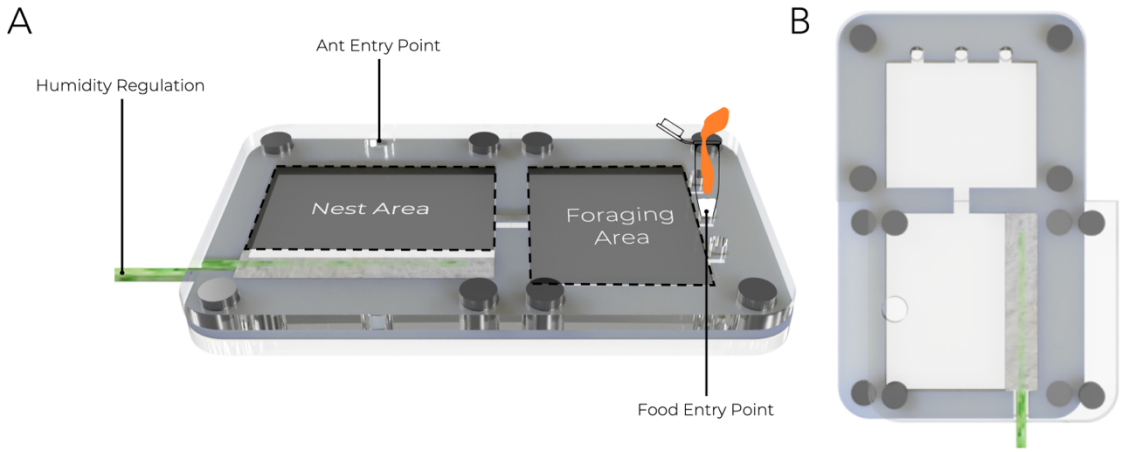

Figure 1 – Custom-made artificial nests used to assess spinosad-induced mortality in colonies of 100 workers. **(A)** Nests (128 x 78 x 10 mm) were designed with a foraging area (40 x 48 mm, 1920 mm²) and a nest area (56 x 48 mm) containing a 56 x 10 mm plaster of Paris section, adapted from Pedraza et al. (2023) for humidity regulation, providing a usable area of 2128 mm². The foraging and nest areas were connected by an 8 x 5 mm channel. **(B)** The top layer of the nest was divided into two pieces: one acting as a lid for the nest area and the other for the foraging area. The foraging area lid featured three equally spaced holes (Ø 4 mm), corresponding to the 4 x 4 mm feeding openings in the foraging area. The nest area lid had a single hole (Ø 8 mm) for ant insertion, which was done by using a PTFE-coated funnel.

Preliminary Results and Conclusions

By leveraging ants' natural dietary preferences and nutritional requirements, protein-enriched baits have the potential to not only increase the uptake of toxicants within colonies but also de-liver the toxicant in a more targeted manner (Markin, 1970; Baker, Van Vorhis Key & Gaston, 1985; Abril, Oliveras & Gómez, 2007). Mainly, through the addition of protein to baits, we aim to increase the amount of target food reaching the queen and brood or to decrease the time it takes for it to reach them.

Starvation often enhances the acquisition and retrieval of learned behaviours, likely due to changes in motivational states (Van Damme et al., 2021). Increases in motivation are particularly useful for experimental work. However, if the addition of protein is to be used in the field, understanding its effects on colonies that are not artificially starved is crucial. Moreover, ants tend to be more aggressive when fed protein and starved for longer periods (Poissonnier, Simpson & Dussutour, 2014). To assess the potential impacts of this on collective foraging behaviour and food transfer, we fed colonies either sucrose or protein-enriched sucrose solutions and tested colonies that were starved for either one day or four days.

Unsurprisingly, our preliminary results suggest that colonies starved for four days ingested more food and recruited more efficiently to it than those starved for one day (Figure 2 and 3). Furthermore, recruitment to food, standardised for discovery time, was approximately 20 minutes slower in one-day starved colonies than in four-day starved ones, regardless of the presence of protein in the food (Figure 4). This was expected, as higher levels of food deprivation and the depletion of reserves are likely to result in increased foraging motivation.

Ants fed baits containing egg protein isolate took longer to reach half the maximum amount of fluorescence, and thus of food, than control treated ants (Figure 4). This effect, which was considerably stronger in four-day starved colonies, could result from slower recruitment to the food source, supported by a general dislike for protein-based food sources (Wagner et. al., In. Prep.). However, this does not mean that the lower amount of toxicant ingested was not primarily given to the queen and brood. For example, it has been hypothesised that a forager may allocate less search effort in response to insect prey because its caloric content is greater than that of a single crop load of sucrose. Consequently, fewer foraging trips would be required to meet the nutritional demands of a colony when retrieving insect prey compared to carbohydrates (Fourcassié & Traniello, 1993).

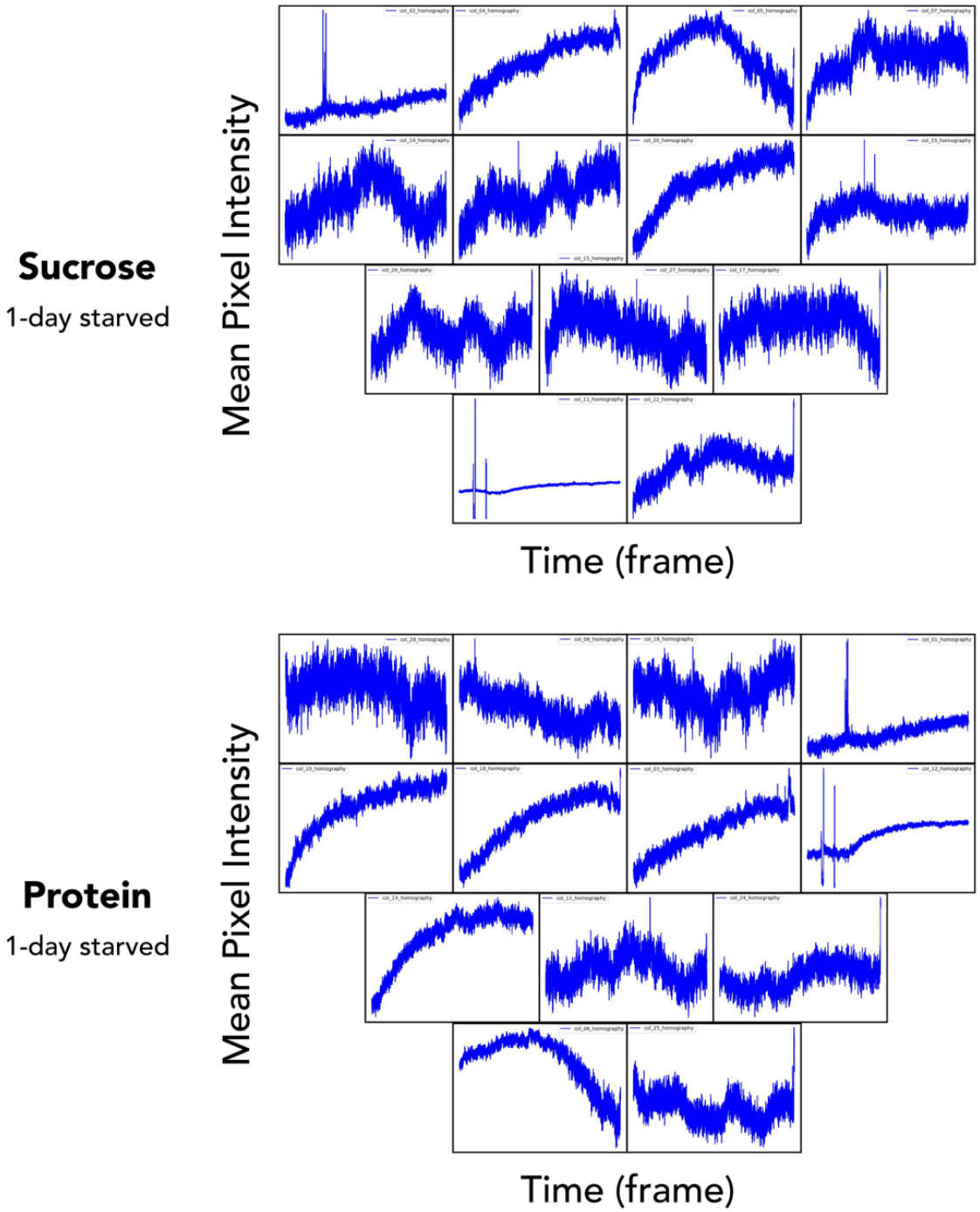

Figure 2 – Mean pixel intensity over time for each experimental colony represents the amount of fluorescent dye present in both the foraging and nest areas, indicating how much food was ingested by the ants over the four-hour experimental period. This data highlights that protein-enriched sucrose solutions were palatable and readily ingested. However, it suggests that one-day starvation might not be sufficient to ensure ants are motivated to forage. Once technical difficulties are overcome, this data will allow us to quantify not only food transfer but also how much food effectively reaches the queen and brood and how fast it does so.

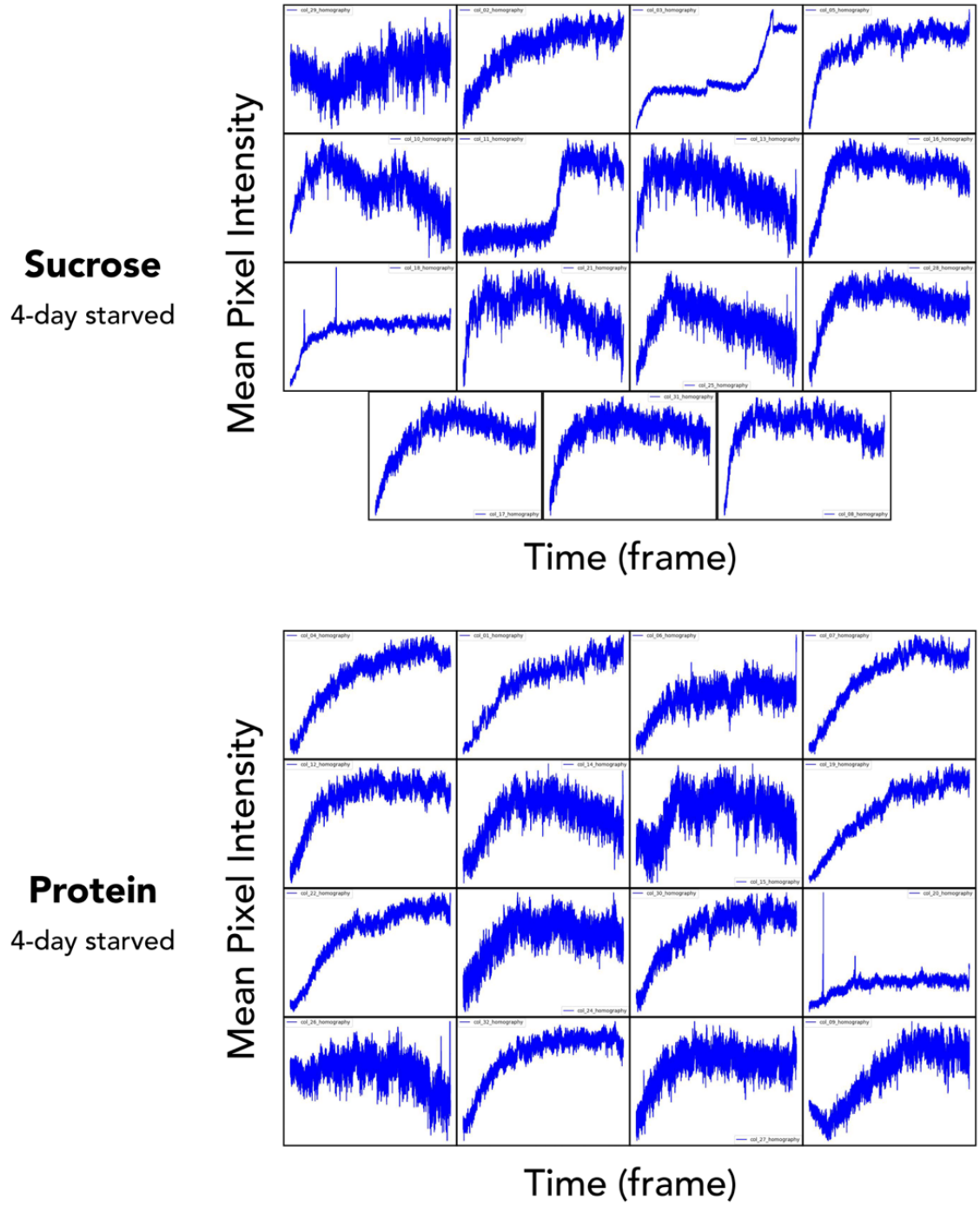

Figure 3 – Mean pixel intensity over time for each experimental colony represents the amount of fluorescent dye present in both the foraging and nest areas, indicating how much food was ingested by the ants over the four-hour experimental period. This data highlights that protein-enriched sucrose solutions were palatable and readily ingested. Once technical difficulties are overcome, this data will allow us to quantify not only food transfer but also how much food effectively reaches the queen and brood and how fast it does so.

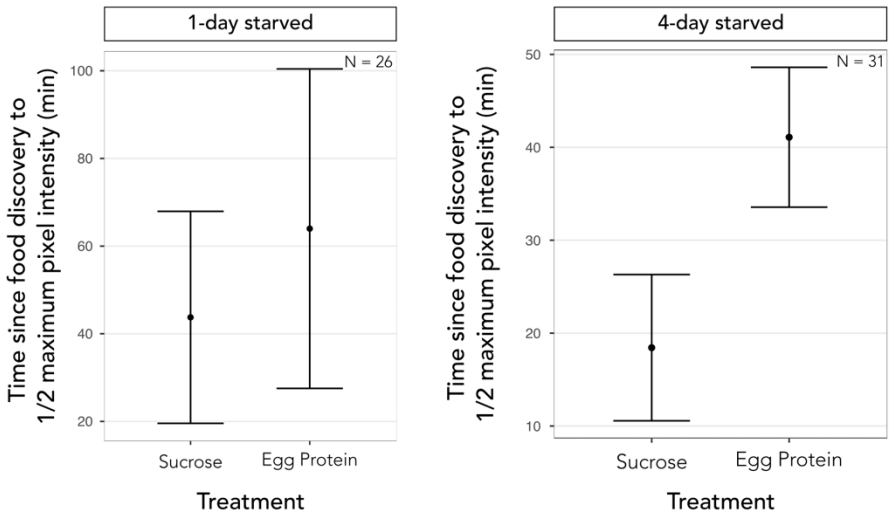

Figure 4 – Note that while most colonies immediately found the food source and began ingesting it, this was not the case for all. Therefore, we accounted for colony-specific food discovery times, removing any data before this point. Pixel intensity represents the amount of fluorescent dye present in both the foraging and nest areas of each colony, serving as a measure of how much food was ingested by the ants over the four-hour experimental period. While this is preliminary data, we observe that ants fed protein-enriched sucrose take longer to recruit to it than ants fed sucrose only, particularly in 4-day starved colonies where foraging motivation is higher.

Unfortunately, due to technical constraints, we have not yet been able to quantify collective food transfer through trophallaxis (Greenwald, Segre & Feinerman, 2015; Baltiansky et al., 2021). Previous work has suggested that *Monomorium orientale* ant queens, with or without brood, are not fed protein even six days after workers forage on it, and that protein uptake is delayed compared to food without it (Loke & Lee, 2006). However, based on our fluorescent recordings, it appears that brood is receiving food, and queens have been observed engaging in trophallaxis with full workers.

Ants have been shown to increase protein intake in winter and spring (Rust et al., 2000; Mathieson, 2011). However, the presence of protein baits, in addition to the natural protein sources available in the environment, could lead to over-ingestion of protein, eventually resulting in its avoidance. Thus, we hypothesise that the use of protein-enriched baits could steer ants towards protein-free food sources. Sucrose-rich baits often have a higher energetic value than natural sources of carbohydrates and can even disrupt ant-hemipteran mutualisms. (Correa et al., 2023; Jensen et al., 2023). Thus, using both types of baits in tandem could be beneficial. To facilitate ant choice in the field, each bait could be flavoured and coloured so that ants easily distinguish between them through associative learning. By providing a diverse range of food sources, all laced with a slow-acting toxicant, ants may preferentially ingest baits over natural food sources, thereby improving overall toxicant consumption and control efforts.

Supplementary Material

Composition of the natural egg protein isolate

Energy: 1600kJ / 381kcal
Fat: 0.0g, of which saturated fatty acids: 0.0g
Carbohydrates: 0.0g, of which sugars: 0.0g
Protein: 84.0g
Salt: 1.3g

Amino acid profile of the natural egg protein isolate

Leucine: 8.4g	Threonine: 4.5g	Glutamine: 12.9g
Isoleucine: 5.3g	Tryptophan: 1.6g	Glycine: 3.4g
Valine: 6.8g	Alanine: 6.1g	Histidine: 2.3g
Lysine: 6.3g	Arginine: 5.7g	Proline: 3.8g
Methionine: 3.7g	Asparagine: 10.1g	Serine: 6.8g
Phenylalanine: 5.8g	Cysteine: 2.7g	Tyrosine: 3.9g

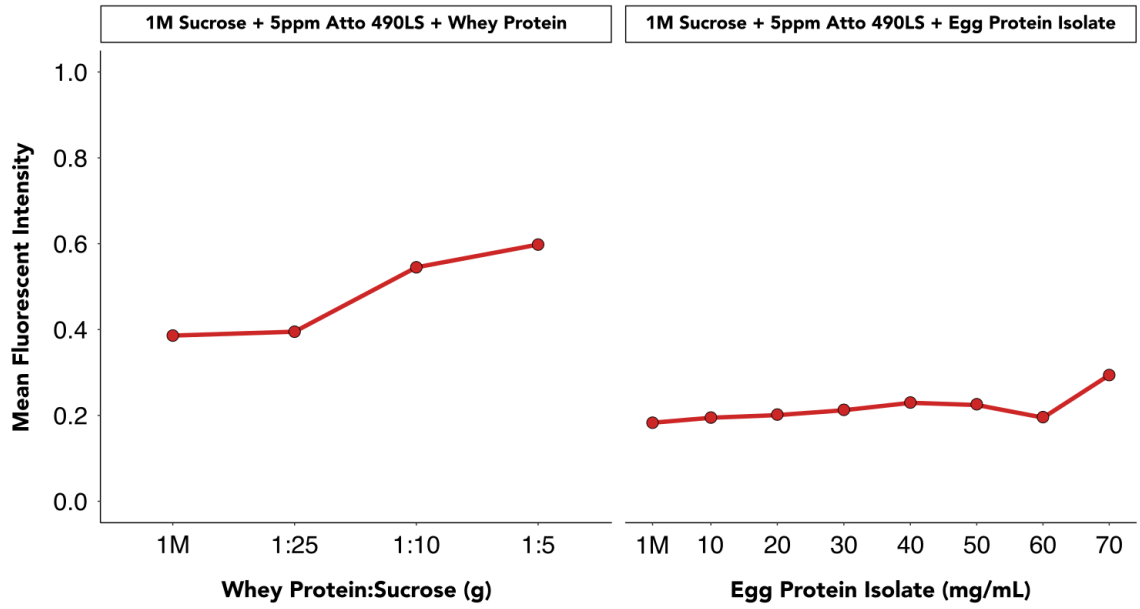

Figure S1 – Relationship between the mean pixel intensity of fluorescent food and protein *in vitro*. Protein has previously been shown to affect the intensity of fluorophores. Therefore, considering the ants' likely capability to concentrate and dilute solutions, it is essential to ensure that the presence of protein does not influence pixel intensity and thereby affect the quantification of food amount. We show that adding whey protein to the solution increases overall fluorophore intensity. However, even when using double the concentration of egg protein isolate used in our experiment, this does not affect pixel intensity. It is important to note that while the volume of solution and fluorophore used was kept constant to ensure comparable results, slightly higher volumes were used in the whey protein tests. This accounts for the higher overall pixel intensity values observed in the whey protein tests compared to those with egg protein isolate.

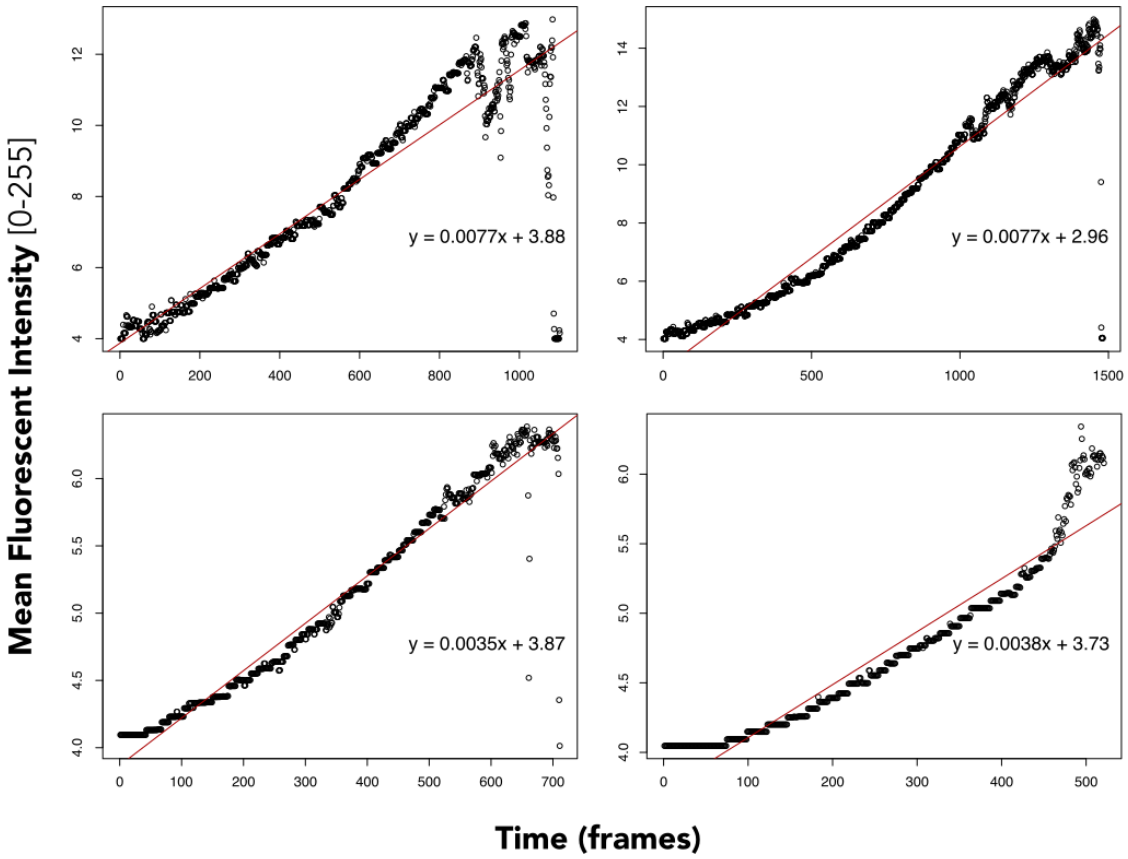

Figure S2 – Relationship between the mean pixel intensity of fluorescent food and its consumption over time *in vivo.* To quantify food consumption through fluorescence, we assume that an increase in the amount of dye ingested leads to a linear increase in mean pixel intensity. Argentine ants have been shown to have linear consumption rates (Galante, Czaczkes & De Agrò 2024). Therefore, individual ants were continuously recorded while ingesting fluorescently labelled food without interruptions. Our findings confirm that as food consumption increases linearly, there is a corresponding linear increase in mean pixel intensity. This relationship supports the validity of using fluorescence as a method to accurately measure food intake in Argentine ants.

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Declaration of interests

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

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

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General Discussion

Argentine ants spread rapidly in invaded areas, causing significant ecological and economic damages, making them frequent targets for control programs (Angulo et al., 2024; Mévergnies et al., 2024). Chemical control, the commonly preferred method, often struggles to reach the reproductive part of the colony, the primary target of invasive ant management efforts, as this is well protected (Howse, Haywood & Lester, 2023). Baiting programs, recently using hydrogel beads, leverage ant social behavior for long-term suppression while minimising environmental harm (Cooper et al., 2008). However, despite the eradication of approximately 3000 *L. humile* populations through chemical sprays, which are now rarely used due to a lack of target specificity (Hoffmann et al., 2016; Angulo et al., 2024), baiting remains less efficient for this species compared to others (Buczkowski, Roper & Chin, 2014; Angulo et al., 2024). Invasive ant management often falls short, likely due to the high costs associated with large-scale deployment (Rust, Reierson & Klotz, 2003; Nelson & Daane, 2007), as well as a lack of sustained bait consumption resulting from competition with natural food sources and the active abandonment of foraging trails (Silverman & Brightwell, 2008; Zanola, Czaczkes & Josens, 2024). Therefore, maximising bait consumption within the first few hours is crucial. The addition of chemical additives to baits could revolutionise control methods by leveraging ant cognition and behaviour. Ants rely on multimodal cues for navigation and can form strong spatial and olfactory associations with sugary rewards (Dupuy et al., 2006; Huber & Knaden, 2018; Piqueret, Sandoz & d'Ettorre, 2019; Buehlmann, Aussel & Graham, 2020; Rossi et al., 2020; Wenig, Bach & Czaczkes, 2021). Enhancing ants' ability to locate food and return to the nest more efficiently through bait additives could lead to earlier pheromone trail formation, increased recruitment and ultimately increased bait consumption.

Typically, baits consist of a toxicant-laced sucrose solution (Angulo et al., 2024), despite ants' preference for complex mixtures (Blüthgen & Fiedler, 2004). Moreover, many effective toxicants are now banned or heavily regulated in the European Union due to their negative effects on pollinators and human health (European Chemicals Agency, 2010; Gan et al., 2012; European Food Safety Authority, 2013; Greenberg et al., 2014; Siviter & Muth, 2022; Milosavljević et al., 2024). Thus, there is an urgent need for new methodologies for the effective control of invasive ants, particularly Argentine ants. This thesis aims to identify bait additives which enhance foraging behaviour and thus increase the consumption of toxic baits. Specifically, it explores the potential of neuroactives to influence ant behaviour by interfering with learning and memory and examines how a neuroactive toxicant, spinosad, as well as protein, interfere with collective foraging and food transfer.

Using neuroactive chemicals to manipulate ant behaviour

Promising bait additives include neuroactives such as alkaloids, biogenic amines, and non-protein amino acids, which have been shown to improve associative learning and memory retention in insects (Baracchi et al., 2017; Mustard, 2020; Carlesso et al., 2021; Huang et al., 2022). Chapter 1 highlights that *L. humile* are effective associative learners, requiring two visits to develop strong spatial associations and a single visit for olfactory associations. However, despite extensive literature on the effects of these chemicals on Hymenopterans (Table 1 in Galante & Czaczkes (2024)), our results showed no effect of the seven potential neuroactive chemicals tested. While this suggests none of the chemicals had a negative effect on learning, we cannot exclude the possibility of ceiling effects masking potential learning improvements, which could have been observed had a more challenging task been used. Alternatively, using poorer rewards could reduce motivation, potentially decreasing learning speed or quality and thus mitigating potential ceilling effects (Van Damme et al., 2021).

Among the neuroactives tested, caffeine was particularly interesting because it is cheap and widely available. Additionally, caffeine's effects on cognition and behaviour have been extensively studied (see Mustard, 2014 for a review). Caffeine is thought to act on mushroom body neurons, which are involved in learning and memory (Buehlmann et al., 2020), by acting as an adenosine receptor antagonist. Behaviorally, in honeybees, caffeine was shown to increase foraging frequency, quadrupling colony-level recruitment (Couvillon et al., 2015), to increase learning performance (Marchi, Palottini & Farina, 2021) and result in longer lasting olfactory memories (Wright et al., 2013). Similarly, in bumblebees, its presence led to increases in pollination (Thomson, Draguleasa & Tan, 2015) and improved odour associations (Arnold et al., 2021), albeit lowering food consumption (Tiedeken et al., 2014). In ants, it was suggested to increase speed, learning and memory at the cost of lower food consumption (Cammaerts, Rachidi & Gosset, 2014).

Given these reports, the lack of effect found in our experiment was surprising. We believe this discrepancy primarily stemmed from differences in methodology. In fact, there is a heavy bias in the literature towards the use of bees and the proboscis extension response (PER) paradigm, along with olfactory associative learning. It is likely that a simple task, such as a Y-maze, which Argentine ants easily solve (Wagner et al., 2023), either did not provide enough time for caffeine to act, or the caffeine-borne increases in alertness and cognitive arousal did not enhance an already maximised learning capacity (Si, Zhang & Maleszka, 2005).

To address this, in Chapter 2 we use a more complex, field-realistic experiment to assess the effects of caffeine on foraging. We show that low to moderate doses of caffeine shorten the

foodward foraging journey over consecutive visits due to an increase in path straightness rather than speed. However, high doses have no such effects. Secondary metabolites generally exhibit dose-dependent effects (Wright et al., 2013; Baracchi et al., 2017; Bogo et al., 2019; Carlesso et al., 2021). Caffeine specifically appears to follow a hormetic dose-response pattern, where it is toxic at high doses but stimulates biological function when ingested in smaller amounts. Interestingly, caffeine had no clear impact on nestward journey duration, likely because it predominantly affected the mushroom bodies, and therefore view-based navigation, over the central complex, and thus path integration (Webb & Wystrach, 2016; Stone et al., 2017; Kamhi, Barron & Narendra, 2020; Grob et al., 2024). However, in invaded areas where ants navigate more complex environments relying on multimodal cues, caffeine might impact the nestward journey as well. This would mean the observed effects of caffeine would not only be maintained but potentially even increased under natural conditions. Importantly, the foraging distances traveled by the ants in our setup were orders of magnitude shorter than those they would travel in a natural environment (Vega & Rust, 2003; Hogg et al., 2018). Thus, it is unclear if at longer foraging distances, where caffeine would remain in the ant's system for longer, there would be a difference in its effects. Moreover, it is unclear wether the reported effects would be diminished or enhanced at the collective level where ants share food across the colony and make collective foraging decisions (Meurville & LeBoeuf, 2021).

Feeding behaviour provides valuable insights into an animal's perception of the world, elucidating their preferences and cognitive capabilities (Kim & Smith, 2000; Oberhauser, Koch & Czaczkes, 2018). For example, how hungry an insect is can influence its sensitivity to toxins and impact motivation (Wu, Zhao & Shen, 2005). Quantifying feeding behaviour is particularly important in pharmacological studies (Devineni & Heberlein, 2009; Vinauger et al., 2018) and can play a crucial role in developing effective invasive control methods (Nigg et al., 2004; Carrasco et al., 2019; Galante & Czaczkes, 2024; Galante et al., 2024). In Chapter 3, we developed a non-invasive method to quantify food consumption in small invertebrates by measuring body expansion during feeding. This method allowed us to quantify not only overall food consumption, but also consumption rate. We demonstrated that an increase in sucrose concentration results in a relative increase in crop load. Consumption rate however, increases at low sucrose concentrations, yet rapidly decreases at higher molarities, due to the viscosity of the solution (Lois-Milevicich, Schilman & Josens, 2021). This reinforces that individual ants can regulate their food intake.

The caffeine associated improvements on ant foraging we found (Chapter 2) could be a result of increased motivation rather than enhanced cognition. However, using our volume estimation method to quantify food consumption (Chapter 3), we showed that the presence of caffeine, at the same doses used during the foraging experiment, had no effect on either crop load or consumption rate. Importantly, understanding individuals' feeding patterns

allowed us to quantify individual level caffeine intake. On average, ants fed low doses of caffeine ingested 16.8mg of caffeine per kilogram of body mass and those fed moderate doses 160.5mg of caffeine per kilogram of body mass. For reference, humans ingest under 10mg/kg per day at most (Verster & Koenig, 2018). Quantifying the effective amount of caffeine exposure needed for the desired behavioural effects can lead to optimised solutions and reduce chemical usage. Moreover, in the future, our system could be adapted for field settings, where ants are recorded during baiting programs in order to quantify their feeding behaviours in a natural environment. This would allow us to understand how their feeding behaviour changes during control attempts and precisely quantify bait abandonment, which could manifest not only as physical avoidance but also as decreased feeding.

Effects of spinosad on ant behaviour

Whilst neuroactive chemicals such as caffeine are promising for invasive ant control, resulting in faster foraging trips, it remains unclear whether these will lead to higher recruitment and ultimately increase toxicant consumption. Moreover, commonly used toxicants are often neuroactive compounds themselves (Millar & Denholm, 2007). Thus, and considering their desired slow-acting nature, it is important to understand their effects beyond mortality. Argentine ants have been shown to actively avoid trails leading to slow-acting poisonous baits (Zanola, Czaczkes & Josens, 2024). In Chapter 4, we explored one potential reason for this behaviour: conditioned taste aversion, the learned avoidance of a particular taste when an initially neutral taste is associated with post-ingestion malaise, a general feeling of discomfort often linked to illness.

We show that ants prefer the odour of a known food over a novel one, regardless of the presence of spinosad in the previously experienced food. This is in agreement with previous work suggesting that ants strongly prefer the first odour associated with food they experience after food deprivation (Oberhauser, Bogenberger & Czaczkes, 2022). Had ants developed a taste aversion towards sublethal doses of spinosad, they would have been expected to prefer a novel food taste over a previously experienced one had it been successfully paired with the toxicant. Nonetheless, even at an extremely low dose of spinosad, around 600 times less than typically used in field conditions (Milosavljević et al., 2024, Pedraza et al., In Prep.), spinosad had a clear post-ingestion effect. Individuals exposed to the toxicant showed a decrease in overall consumption of roughly 20% on all foods ingested afterwards, even though none contained spinosad. This suggests that a single exposure to spinosad causes ants to ingest less food for at least a day post-exposure. While ants ingested food with a novel taste faster than familiar-tasting food, their consumption rate was not affected by spinosad exposure. Thus, the decrease in crop load must have resulted from shorter drinking events and earlier abandonment of the food source, further highlighting the importance of quantifying feeding. Similar unintended

effects have been observed in ants exposed to commonly used pesticides, including decreased food intake, although this is not the case for all insecticides (Schläppi, Stroeymeyt & Neumann, 2021; Schläppi et al., 2023).

Under control conditions, colonies took longer to recruit to previously experienced food sources than to novel ones. This is likely because ants feeding on familiar-tasting food ingested it more slowly yet reached the same crop load. In other words, ants foraging on novel food drink faster and thus return to the nest sooner. Mass-recruiting ants, such as *L. humile*, rely on pheromone trails during foraging, with more pheromone being laid towards higher quality food sources (Latty et al., 2017). Small initial differences between pheromone trails often result in significant recruitment differences and a preference for one food source over another, a phenomenon known as symmetry breaking (Beckers et al., 1990; Sumpter & Beekman, 2003; Detrain & Deneubourg, 2008; Grüter et al., 2012). Interestingly, despite initial differences in recruitment, control-treated colonies reached the same number of individuals at both food sources, and no symmetry breaking was observed during the four-hour experimental period.

Contrastingly, the initial recruitment differences observed in control-treated colonies were not present in spinosad-treated ones. Spinosad exposure resulted in equally fast recruitment to both novel and familiar-tasting food sources and even slightly faster recruitment overall compared to control colonies. However, individual level consumption rates did not differ with exposure to spinosad. In this way, colonies feeding on a novel food source still ingested it faster, and thus returned to the nest sooner, than those feeding on a previously experienced food taste. Given that exposure to spinosad reduced consumption of all food sources, we hypothesise that it might also have resulted in weaker pheromone deposition or altered ant's perception of pheromone trails. Whilst quantifying pheromone deposition in *L. humile* is a challenge, future work should investigate whether Argentine ants react differently to artificial pheromone trails of equal intensity before and after ingesting spinosad.

Alternatively, spinosad could induce a state of hyperactivity, causing ants to move faster, be more motivated to find food, or even forage more efficiently. Spinosad primarily acts on nicotinic acetylcholine receptors and has also been identified as an antagonist of GABA receptors (see Millar & Denholm, 2007 and Kirst, 2010 for reviews). GABA, the main inhibitory neurotransmitter in invertebrates, is often linked to an animal's ability to forget and adapt to its environment (Boitard et al., 2015; Nepi, 2014). By preventing the release of GABA, spinosad could cause ants to fixate on initially appetitive spinosad-laced sucrose solutions, potentially explaining the observed improvements in recruitment from spinosad-exposed colonies. However, a recent study showed GABA promotes flower fidelity, suggesting its effects are complex (Calderai et al., 2023). Nevertheless, sub-lethal doses of

the neonicotinoid imidacloprid, also an antagonist of nicotinic acetylcholine receptors, were shown to shift colony-level preference in the invasive ant Lasius neglectus towards toxicant-laced solutions (Frizzi et al., 2022), even though imidacloprid has been shown to impair olfactory learning and memory in honeybees (Yang et al., 2012; Williamson & Wright, 2013). Interestingly, by blocking adenosine receptors, caffeine is likely to also decrease the release of GABA (Sugimachi et al., 2016; Reichmann, 2022). Thus, adding small doses of caffeine to baits could produce effects similar to those of sub-lethal spinosad, potentially increasing recruitment speed. Yet, similarly to spinosad, caffeine could also result in lower bait consumption. In Chapter 3, we show that caffeine has no impact on overall consumption or its rate. Yet, in this experiment, ants were not previously exposed to caffeine. Therefore, although our results suggest caffeine is initially palatable to ants, it remains uncertain if, once metabolised, ants become averse to it or ingest less of solutions containing it.

Fast-acting insecticides negatively affect bait consumption and trophallaxis (Ripa et al., 1999). This is because whilst higher doses typically cause higher mortality in individuals that feed directly on the baits, these have reduced potential for secondary mortality, as the donors die before they can share the insecticide. Lower doses typically have more potential for transfer yet may result in lower overall mortality if the insecticide becomes too dilute as it is shared via trophallaxis (Buczkowski, Roper & Chin, 2014). During the experiments in Chapter 4, we observed a general lack of mortality, which contrasted with the high mortality seen in Chapter 5 for Argentine ants kept in groups of four individuals. Initially, we assumed this was because we had purposefully chosen a sublethal dose as we wanted to test individuals post exposure. However, in Chapter 5, we show that, by living as a large group, ants are likely to better resist the toxicant's effects.

Ants are known to modify their network structure in response to pathogens (Stroeymeyt et al., 2018). Nevertheless, whether they also do so in response to toxic food is unknown. Our results suggest that when ants are fed toxicants in larger groups, their mortality is drastically decreased. The exact mechanisms driving this remain unclear. In part, it is likely an effect of food, and thus toxicant, dilution during trophallaxis. Donor ants fed thiamethoxam through hydrogel baits effectively transfer it to untreated recipient ants, albeit the level of recipient ant mortality is entirely dependent on the proportion of donor ants (Buczkowski, Roper & Chin, 2014). Luckily, due to the experimental protocol used, we know exactly how many workers ingested the poisonous solution, how much of it and how it was dispersed through the colony over time. Preliminary results suggest a fast spread of sucrose, and therefore of spinosad, likely driven by the small colony size, proximity to the bait and a lack of alternative foods. We hope, that once completely analysed, we will be able to determine what mechanism(s) allow ants, as a collective, but not as individuals, to avoid toxicant induced mortality. An anecdotal observation is that individuals displaying negative effects from spinosad appeared to be more cared for by healthy workers (H.

Galante, personal observations). As a neurotoxic compound, spinosad affects nicotinic acetylcholine receptors (Biondi et al., 2012). This results in rapid nervous system excitation, leading to paralysis and eventually death (Salgado, 1998). Therefore, by being fed and cared for during paralysis, affected ants may potentially avoid death. Understanding how long spinosad remains active inside the ant's system, and whether conspecific interactions such as trophallaxis can hasten its degradation, could provide valuable insights into the mechanisms behind ants' evasion of ingested toxicants.

Using protein for targeted delivery of a toxicant

Invasive ants often form supercolonies with highly connected nests spanning large geographical areas. However, members of individual nests primarily use local food sources, with minimal exchange of individuals or food among neighbouring nests (Buczkowski & Bennett, 2006). While this may be beneficial for field experiments, allowing for parallel treatments, it could pose a challenge for control attempts. Interestingly, colonies fed sucrose tend to focus on a single food patch, whereas foragers are more uniformly distributed across available patches when feeding on protein sources (Portha, 2002). Thus, the addition of protein to baits could help disrupt this tendency of ants focusing on local food sources. Moreover, it has long been hypothesised that adding protein to baits would increase the amount of toxicant reaching the reproductive part of a colony (Markin, 1970; Angulo et al., 2024). Queens and larvae require more protein than workers (Feldhaar, 2014; Csata & Dussutour, 2019). Thus, protein baits containing slow-acting toxicants could enhance either the quantity or speed at which the toxicant reaches the reproductive arm of the colony, assuming that foragers prioritise sharing proteinaceous food with colony members who need it most (Markin, 1970; Baker, Van Vorhis Key & Gaston, 1985; Abril, Oliveras & Gómez, 2007).

In Chapter 6, we aimed to explore how the presence of protein in baits altered collective dynamics and whether it translated into more food reaching the queen and brood. By adding protein to baits, we sought to either increase the overall amount of toxicant reaching the queen and brood or decrease the time it takes to reach them. In an effort to prioritise protein reaching the reproductive part of the colony, ants might reduce the number of trophallactic events the food goes through. This would be advantageous as it would minimise exposure to the toxicant, potentially maintaining bait consumption for longer. Interestingly, a reduction in the number of trophallactic interactions as a result of protein presence could be a natural way to disrupt the potential social immunity of ants to toxicants (Chapter 5).

Our preliminary results suggest that ants fed baits containing egg protein isolate took longer to reach half the maximum amount of fluorescence. This could result from slower

recruitment to the food source, supported by a general dislike for protein-based food sources (Wagner et. al., In. Prep.). However, this does not mean that the lower amount of toxicant ingested was not in fact given primarily to queen and brood. For example, it has been hypothesised that a forager may allocate less search effort in response to insect prey because its caloric content is greater than that of a single crop load of sucrose. Fewer foraging trips would thus be required to meet the nutritional demands of a colony when retrieving insect prey compared to collecting carbohydrates (Fourcassié & Traniello, 1993).

Importantly, contrary to sugary solutions, protein ingestion is highly regulated by the colony as high protein diets can be toxic (Arganda et al., 2017). Moreover, the presence of brood, its developmental stages and previous access to protein are all important when a colony decides to forage on proteinaceous sources or not (see Csata & Dussutour, 2019 for a review on nutrient regulation in ants). In fact, it has previously been shown that sucrose solutions are preferred year-round, while protein demand is greatest in winter and spring (Rust et al., 2000; Mathieson, 2011). Another consideration is that starvation often enhances the acquisition and retrieval of learned behaviors, likely due to changes in motivational states (Van Damme et al., 2021). Furthermore, ants tend to be more aggressive when fed protein and starved for longer periods (Poissonnier, Simpson & Dussutour, 2014). To further assess the effects of starvation on foraging dynamics we conducted the experiments in Chapter 6 with 4-day starved and 1-day starved colonies. Understanding if protein is more readily shared with the reproductive component of a colony, even when not artificially starved, will be crucial if its addition is ever to be implemented in the field.

Outlook and conclusion

This thesis lays the groundwork for the development of control strategies which leverage insect cognition, addressing a crucial gap in the control of invasive ant species. It studies potential bait additives and provides semi-automated methods to test their effects on learning bait location, food preference, and feeding behaviour. Mainly, it proposes caffeine, a cheap and widely available neuroactive chemical, as a bait additive that enhances ant learning and shortens foraging bouts. However, it remains to be explored whether adding caffeine to baits will improve recruitment and visitation rates in ants, as seen in honeybees and bumblebees (Couvillon et al., 2015; Thomson, Draguleasa & Tan, 2015). Moreover, it is vital to determine if repeated exposure to caffeine does in fact increase bait consumption or on the other hand results in ants developing an aversion to it. Importantly, combining neuroactive chemicals can alter their behavioural effects (Marchi, Palottini & Farina, 2021; Muth et al., 2022). Since many toxicants, particularly spinosad, are neuroactive themselves, understanding their interactions with caffeine at both the individual and collective level is crucial.

Given the lack of effect of caffeine observed in the Y-maze associative learning experiment (Chapter 1) and the effects found in the open landscape foraging experiment (Chapter 2), the lack of effect of the other six potential neuroactive chemicals tested should be re-evaluated. β-alanine, in particular, showed promise in improving foraging time in the Y-maze test. However, considering the infinite number of possible neuroactive additives, toxicants, and their concentrations, research should focus on finding one combination of these which improves bait consumption, results in high mortality, and uses low chemical doses, over attempting to study all potential neuroactive chemicals.

While integrating behavioural and chemical control holds promise for improving invasive ant management, reducing overall chemical use, and aiming for a more targeted delivery of the toxicant, using large quantities of chemicals in the field will always be damaging to an extent. Large-scale control efforts should be restricted to highly invaded areas, where native ants are unlikely to access the baits due to the fast monopolisation of resources by invasive ants (Holway, 1999; Arnan et al., 2018). Hydrogel beads are advantageous for their ease of dissemination, low cost, and rapid desiccation, which reduces environmental exposure to chemicals (Cabrera et al., 2021). Nevertheless, assessing the effects of the chemicals used on soil and plants is vital when formally testing these for generalised use. For example, caffeine has been linked to retardation in seedling growth in the laboratory (Mohanpuria & Yadav, 2009), yet is quickly biodegraded in agricultural soil (Topp et al., 2006).

Hydrogel beads are mainly consumed by arthropods, most of which are invasive ants (Tay et al., 2020; Hoffmann, 2023). Nevertheless, in an effort to further mitigate non-target exposure to the baits, recent work suggests that Argentine ants are more tolerant to bitter substances than other ant species or animals (Wagner et al., In Prep). The addition of a powerful bitter agent to the baits could be key to further prevent non-target exposure. However, understanding how such chemicals interfere with ant foraging when natural food sources are available will be crucial before deployment.

We demonstrate the nuanced effects of spinosad, which at sublethal doses leads to faster collective recruitment but reduces individual food consumption. Furthermore, we show that larger group sizes significantly decrease the mortality associated with toxicant ingestion. Future work should focus on understanding if the decreases in food consumption persist at high toxicant doses. Moreover, although we found that ants did not develop a conditioned taste aversion to sublethal doses of spinosad, it remains unclear if this neuroactive toxicant was not perceived by the ants, and thus not successfully associated with food taste, or if it directly interfered with learning and memory. Understanding if ants can develop conditioned taste aversions to higher doses of spinosad is also important. If they can, testing whether these aversions can be disrupted by cycling through different food

flavours, so that ants are always driven towards novel foods while still ingesting the toxicant, should be a priority.

This thesis produced a substantial amount of data that remains unexplored. In particular, the fluorescence tracking data from Chapters 5 and 6 could provide insights into how ants collectively avoid toxicant-induced mortality and whether protein is a suitable addition to baits. Understanding how trophallactic networks impact toxicant dissemination and whether these can be manipulated is crucial for disrupting social immunity. In fact, this is the first attempt to extend the concept of social immunity beyond pathogens. Quantifying the amount of toxicant reaching the queen and brood and assessing if this achieves a satisfactory level of mortality is key for successful invasive ant control.

Ultimately, this thesis has already laid the foundation for field trials conducted in Spain and opened new avenues for exploring group immunity in ants. Once effective strategies are identified, it is imperative to understand their potential for use with other invasive ant populations beyond Argentine ants, as these continue to expand (Menchetti et al., 2023). Understanding how invasive ants avoid control attempts is the first step in developing new methodologies and revolutionising ant management through cognitive control.

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