Chemicals on the cuticle of ants: their role in hygiene, navigation and kairomone signalling to termites

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Declaration

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree.

Signature:……

University of Sussex

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Summary

This thesis describes investigations of how chemicals present on the cuticle of ants impact three important features of social living in insects: hygiene and disease resistance; navigation; and interspecies chemical signalling.

Eusociality brings many benefits, but also has the potential to make insect colonies vulnerable to disease. In Chapter 2 of this thesis I investigate the role of the antimicrobial agent micromolide, in the Yellow meadow ant, *Lasius flavus.* Micromolide is found to be present on the cuticle of *L. flavus* workers, and is also found to be deposited onto a substrate by walking ants, revealing a possible mechanism for maintenance of sanitary nest conditions. Chapter 3 of this thesis focuses on navigation in *L. flavus*, specifically route-memory formation and the possibility of home-range markings providing a chemical cue via which ants can navigate from a food source to the nest. It was found that allowing ants to follow a pheromone trail to food increased the number of navigational errors made by returning ants, and that home-range markings did not provide effective guidance to ants returning to the nest. In Chapter 4, I report on a project undertaken during field work in Brazil into how cuticular hydrocarbons (CHCs) of the ant *Camponotus arborious* can act as kairomones when detected by *Nasutitermes corniger*, a common termite species. Experiments showed that *N. corniger* is less likely to repair experimentally opened tunnels in the presence of *C. arborious* CHCs, with 4 of 7 colonies tested blocking up tunnels, rather than rebuilding over CHC marked areas. Finally, Chapter 5 of this thesis discusses potential future projects, following on from the work presented in Chapters 2, 3 and 4.

Acknowledgements

Firstly, I would like to say a huge thank you to my main supervisor Jonathan Bacon. It is safe to say that without his guidance, and considerable patience, this thesis would never have been completed. I know that he has put considerable time and effort into helping me through this MPhil, and for that I will be eternally grateful. My second supervisor, Liz Hill, was also hugely important, allowing me lab space and access to equipment that made this degree possible.

I was very fortunate to work alongside a number of fellow social insect enthusiasts, whose advice was invaluable, foremost amongst whom is Tom Butterfield, another of Jonathan's students. Working with Tom was a pleasure, and his advice on experimental design, and especially on using GC-MS, helped me get to grips with the techniques needed to complete this degree. Kyle Shackleton helped immensely with his advice on statistics, and all the members of LASI contributed by making my working environment such a fun and inspiring place to be.

Last but not least, thank you to my parents, Paul and Julie Gallagher, who have supported me throughout my time studying. They are the ones who first impressed upon me the importance of education, and without them I would never have been able to undertake this MPhil, let alone complete it.

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Contents

1. Introduction

As I was just starting my MPhil, I had decided to undertake what I thought would be a relatively simple task. My initial aim had been to investigate the cuticular hydrocarbon (CHC) profile of *Lasius flavus,* giving me a chance to familiarise myself with the techniques required for GC-MS analysis and acquire a basic knowledge of the literature, to underpin my future work. It was during this time that I found something that changed the direction of my investigation. A colleague and I visited Keele University to use some equipment unavailable to us at Sussex. My colleague, PhD student Thomas Butterfield, was attempting to identify the trail pheromone of *L. flavus*, and was having some difficulties due to the minute volumes he was having to work with, and what seemed to be the high volatility of the compounds of interest. I was also having some difficulty (perhaps for no other reason than my own incompetence) in my aim of analysing the CHC profile of *L. flavus*. For these reasons we decided try something different.

Keele University is the academic home of Professor E. David Morgan and Dr Falco Drijfhout, two researchers who had invented and developed a method known as solid-sample injection in their GC-MS analysis of insect chemical secretions. This method involves sealing samples in small glass capillaries which are then heated to the required temperature, before the capillary is crushed and the sample injected in to the machine. This method does not require use of solvents, decreasing the risk of contamination and dilution of samples, and allows individual body parts or glands to be analysed (Morgan 1990, Bagneres & Morgan 1990). In an attempt to analyse the CHC profile of *L. flavus* without contaminating my sample by accidental inclusion of any of the various glands or their secretions present in ants, I used solid-sample injection to analyse the legs of *L. flavus* workers. The results of this rough experiment did not help me in my original aim, but it did show that there were what seemed to be large quantities of the compound micromolide on the legs that I had fired into the

machine, but not on other parts of cuticle, such as the head or thorax, that I had examined. Micromolide is a γ-Lactone first identified in the gaster of *L. flavus*, and described by Bergström & Lofqvist (1970) as 4-hydroxyoctadec-9-enolide. Subsequent studies have found micromolide to be a female produced sex pheromone in the stem girdler *Janus integer* (Cossé et al 2001), and have also determined the chemical structure of micromolide to be (4*R*, 9*Z*)-Octadec-9-en-4-olide (James et al 2003, Mori 2005). Micromolide has also been identified in tree bark (Ma et al 2005), where it was found to be effective against tuberculosis, and in the secretions of a parasitiod wasp larvae, where it is thought to act as an antimicrobial agent (Herzner et al 2013). I was also somewhat familiar with micromolide, as this compound had been found by PhD student Sam Jones, who I had worked with previously during my final-year undergraduate project, while he was performing an analysis of glands in the gaster of *L. flavus*. This raised an interesting question; why is this compound found on the legs of *L. flavus,* in what appears to be much higher concentrations than on the rest of the cuticle?

This is the question that I chose to investigate in Chapter 2 of this thesis. I assumed that micromolide played some role in maintaining hygienic conditions, either on an individual or colony-wide basis. As the use of micromolide as an antimicrobial agent in ants had not been previously considered, I decided to confirm or refute my initial observation regarding the distribution of micromolide over the cuticle of *L. flavus* workers. I also thought that the presence of micromolide on the legs of *L. flavus* may give some clue as to how micromolide may be used to maintain hygienic nest conditions. CHCs on the legs of ants are laid down as home-range markings, chemical signals that influence ant behaviour in a number of ways (Devigne & de Biseau 2012). In *L. flavus,* the presence of micromolide on the legs of workers would suggest that it is deposited on the ground just as the CHCs are, and that this may lead to micromolide being spread onto the nest substrate, which could in turn lead to more hygienic nest conditions. This was the second aim of Chapter 2, to confirm that micromolide is laid down by walking ants as a possible mechanism for spreading antimicrobials.

After this diversion from my initial plans, I attempted to return to my original area of interest, cuticular hydrocarbons in *L. flavus*, and their role as home-range markings and how that influences behaviour. As already mentioned, I had previously worked during my undergraduate degree with a PhD student, now PhD recipient, Sam Jones, on a study into navigational strategies of *Lasius niger* in low light and total darkness conditions. I knew from my final year project that *L. flavus*, like many other ant species, is capable of using visual cues to navigate. I also observed that *L. flavus* did not lay pheromone when generally foraging, only doing so once a food source had been located. I thought this must be a problem for ants that live almost entirely underground, as without visual cues or pheromone to guide them, it would become difficult for individual ants to navigate around the nest. I then considered that perhaps home-range markings could be used in navigation underground. Initially I attempted to get ants foraging in the dark; this proved difficult as the ants did not seem particularly keen to go exploring when the lights were turned off. I tried to combat this reticence by providing a pheromone trail to the food in an attempt to encourage the ants to start foraging. This worked in getting the ants to the food, but when I replaced the pheromone trail with CHC marked paper for the return journey the ants just seemed confused, even more so than I would have expected. I repeated the experiment with the lights on to try and see what was really going on (because I can't see in the dark either). To my surprise the ants remained just as confused, even in the presence of lights and visual cues. Again my initial plans had been somewhat upset, but once more interesting questions had been raised. How does following a pheromone trail influence memory formation in ants, and can ants switch between chemical cues? This led me to design the experiment presented here in Chapter 3 of this thesis. It seemed to me that olfaction must play a role in guiding these ants underground, and CHCs in the form of home-range markings offered a possible answer to this conundrum. Home-range markings make a likely candidate due to the way they often work in synergy with trail pheromones, for example, to produce colony specific foraging trails (Akino & Yamaoka 2005), or to increase recruitment to a food source

and stimulate pheromone deposition (Devigne et al 2004, Devigne & Detrain 2006). By examining this possibility, my aim was to expand the body of knowledge relating to navigation techniques in social insects.

I was very fortunate during my MPhil to get the opportunity to go on a field trip to Brazil. My supervisor Professor Jonathan Bacon and my colleague Thomas Butterfield had visited the University of São Paulo at Ribeirão Preto previously, and had both undertaken projects on termite behaviour. The species of interest was *Nasutitermes corniger*, a species that has been well studied and is common in Central and South America (Levings & Adams 1984). This species is arboreal, and builds conspicuous nests in trees, with covered foraging tunnels built on the surface of the tree, often reaching down to ground level. Tom was performing a study which involved opening these foraging tunnels, and finding how the presence of a predatory ant (held next to the tunnel break with forceps) affected the rebuild of the tunnel. This guided the design of my project, and again I decided to focus on the effect of ant CHCs on behaviour, but this time the behaviour in question was that of the termites. Ants are major predators of termites, so it seemed possible to me that just as ant behaviour can be influenced by ant CHC presence, the behaviour of a prey species could also be affected. In this way it is possible for ant CHCs to act as a kairomone, and my investigation into this possibility is presented as Chapter 4 of this thesis. Termites, like ants and other social insect groups, use CHCs in nestmate recognition (Howard & Blomquist 2005), and predator released hydrocarbons have previously been shown to act as kairomones (Silberbush et al 2010). It is for this reason that I considered the possibility of ant CHCs playing a role in mediating the relationship between these two ecologically important insect groups.

2. Micromolide on the cuticle of *Lasius flavus* and passive deposition of micromolide onto a substrate

Abstract

Eusociality has many benefits for insects, allowing greater exploitation of resources, more effective predator defence and increased reproductive fitness. But social living also leaves insect colonies vulnerable to attack from pathogens. To counteract this risk, social insects such as ants have developed a range of behavioural and chemical defences against disease. This study examines the use of the antimicrobial agent micromolide, in the ant *Lasius flavus,* using GC-MS to analyse extracts from the cuticle of different body parts of *L. flavus* workers, and from a paper substrate conditioned by allowing ants to walk over the substrate.

Micromolide was found to be present on the cuticle of *L. flavus* workers, and also on the paper substrate that had been walked on by *L. flavus.* Blocking of the acidopore significantly reduced the quantity of micromolide found on both the cuticle and the substrate, providing evidence that the Dufour gland is the only source of micromolide in *L. flavus.* This chapter then goes on to discuss a possible role for micromolide as a prophylactic defence against pathogens, as part of the social immunity of the ant colony.

Micromolide on the cuticle of *Lasius flavus* and passive deposition of micromolide onto a substrate

2.1 Background

The evolution of eusociality in insects has allowed bees, wasps, ants and termites to become dominant insect fauna, making up 75% of insect biomass in the Amazon rainforest (Holldobler & Wilson 1990). Ants in particular have a huge influence on the environment; in most terrestrial habitats they are a major predator of other insects and small invertebrates, and in Central and South America, leafcutter ants are the principle herbivores and a major pest species. Forming social groups allows greater resource exploitation, predator defence and reproductive fitness but it also leaves social insects vulnerable to attack from pathogens due to a high frequency of contact between individuals which can lead to an increased risk of disease transmission (Fefferman et al 2007). It has also been suggested that low levels of genetic variability leaves social insect colonies at greater risk of infection from pathogens, but studies into this have produced conflicting results. There is evidence that in *Bombus terrestris* genetic variability reduces transfer of pathogens between individuals (Shykoff & Schmid-Hempel 1991) and reduces parasite load (Bear & Schmid-Hempel 1999). There is also some evidence that greater inter-colony genetic variation leads to less variation in disease resistance between honey bee colonies (Tarpy 2003). However other studies have found that greater genetic variation between nestmates does not lead to improved disease resistance or a general increase in fitness (Fuchs et al 1996, Sundstrom & Ratnieks 1998, Neumann & Moritz 200, Costa & Ross 2003) and that the picture can be further complicated by factors such as the dose of the parasite and whether the parasite is generalist or specialist (Hughes & Boomsma 2004).

To cope with the risk associated with social living, ants have evolved various strategies to defend themselves and their colony from pathogens which can be split into two main categories: behavioural and chemical. The behaviours include self-grooming and

nestmate grooming (allo-grooming), and also the expulsion of infected ants from the nest.

2.1.1 Behavioural Defences

A number of studies have investigated the role of grooming in protection from various pathogens, and I will start by discussing these results. Reber et al (2011) examined the expression and impact of anti-fungal self- and allo-grooming in the ant *Formica selysi*. This study monitored how infecting individuals with the generalist entomopathogen fungus, *Metarhizium anisopliae,* affected self- and allo-grooming behaviour. This was done by dividing colonies into experimental groups 24 hours before testing, removing individuals from those groups, infecting them with various concentrations of spores and then reintroducing the individuals to the experimental group from which they were taken. They found that exposing these removed individuals to *M. anisopliae* spores produced an increase in self-grooming behaviour, suggesting that individuals could detect their infection with spores.

However, infection with fungal spores did not affect allo-grooming behaviour by the experimental group mates of the infected and reintroduced ants. There was no effect in terms of the duration of grooming of the re-introduced individual by nest mates, or the number of groomers, but an interesting result was that groups of ants that had previously had an infected individual reintroduced subsequently spent longer allo-grooming reintroduced individuals, regardless of whether they had been infected or not. This shows that the ants modulate their behaviour to protect themselves against the perceived level of threat. The study concluded that allo-grooming was a systematic constitutive prophylactic behaviour rather than behaviour elicited by the presence of pathogens. In contrast to these findings, Okuno et al (2012) found that in *Lasius japonicas* the reaction was completely the opposite. Exposure of individuals to a fungal parasite resulted in an increase in allo-grooming but not in self-grooming. This shows that strategy to combat infection differs greatly between ant species.

The result showing an increase in allo-grooming by ants previously exposed to fungal parasites is supported by another study by Ugelvig & Cremer (2007). This paper found that ants that had not previously been exposed to a fungal parasite were more susceptible to infection, suggesting that rather than increasing the risk of infection, social contact enhanced defence against pathogens. The same study also found that infection with a fungal parasite changed an individual's behaviour. Infected ants spent less time in the brood chamber, therefore limiting brood exposure to dangerous fungal parasites. This study also supports the idea that ants can sense if they become infected. Ants exposed to fungal spores which had been previously treated with UV light, making the spores non-infectious, did not change their behaviour regarding time spent in close proximity to brood (Ugelvig & Cremer 2007). This shows that ants can detect whether spores of fungal parasites are viable or not.

2.1.2 Chemical Defences

Grooming can also be used in conjunction with chemical defences. The metapleural gland is a common exocrine gland in ants typically located on the lower plate of the metapleuron (Yek & Mueller 2011) which has long been associated with production of antimicrobial compounds. Fernandez-Marin et al (2006) found that a number of ant species groom themselves after contacting the metapleural gland with their forelegs, and that this involves body movements that are quite different from normal grooming. This suggests that this type of grooming is undertaken specifically to spread antimicrobial secretions over the body. In *Atta columbica,* it was observed by Fernandez-Marin et al (2006) that after contacting spores of a fungal parasite this metapleural gland grooming increased, which, as found in other studies I have mentioned, suggests some ant species can change their behaviour, presumably to reduce the chances of becoming infected. It is not just behaviour that can change when ants are exposed to pathogens; Yek et al (2012) also found that in *Acromyrmex octospinosus* the volume and efficacy of secretions from the metapleural gland could

be affected by exposure to different fungal pathogens, with more virulent pathogens resulting in a higher volume of metapleural gland secretions, and greater inhibition of fungal-spore germination per unit of metaplueral gland secretion.

Fernandez-Marin et al (2006) also found that as well as spreading antimicrobial secretions on their own bodies, five species of *Atta* and *Acromyrmex* also applied these secretions to the nest, specifically the fungus gardens, the garden substrata as well as brood. They hypothesized that secretions are transferred from the metapleural gland to the legs, then from the legs to the mouth where they are applied to the surrounding environment. This is similar behaviour to that found in *Lasius neglectus* by Tragust et al (2013). This study found that *L. neglectus* workers treated infected brood with secretions from the poison gland. Workers first transferred the poison to their mouth parts through oral uptake and then applied it to infected brood. This behaviour may also have the benefit of protecting the brood-tending workers by making sure that any fungal spores that workers comes into contact with are already disinfected and so can be more safely collected in the infrabuccal pockets during brood care.

Applying antimicrobial agents from the exocrine glands of workers to brood and the nest environment has also been described by Tranter et al (2014). The leafcutter ant, *Acromyrmex subterraneus,* and the weaver ant, *Polyrhachis dives,* both have large poison glands, and the leafcutter also has a metapleural gland which produces antimicrobial secretions. The study found that when these glands were made non-functional by blocking up their ducts, brood developed infections whether they had been treated with a fungus or not, and nest material was also more likely to harbour fungal infection. This shows that these behaviours have a significant effect on microbial activity, and the ability to disinfect the whole nest is vital for survival in these species.

These previous studies have concentrated on active behaviours by ants to protect against infection by pathogens. The study described in this chapter will examine the effect of passive behaviour by *Lasius flavus* workers, and how this may lead to more hygienic nest conditions. Micromolide is a known antimicrobial agent previously identified in chemical extracts of *L. flavus* gasters (Bergström & Löfqvist 1970), and recent work in our Sussex laboratory has used GC-MS to locate micromolide to the Dufour gland (Jones et al in preparation). This compound occurs elsewhere in nature, for instance it has been found in the stem bark of *Micromelum hirsutum* (Ma et al 2005) and also in secretions of the larvae of the parasitiod wasp *Ampulex compressa*, where it is thought to have a role in sanitising the cockroach host body (Herzner et al 2013). However the use of micromolide by ants has not been described before, and this chapter investigates how it is used in disease control. To do this I extracted micromolide from the cuticle of *L. flavus* workers, as well as from a substrate trampled by walking ants.

2.2 Materials & Methods

2.2.1 Housing and maintenance of ants

All colonies were collected from meadowland near the University of Sussex campus. Colonies were stored in 30 cm by 30 cm plastic boxes with some soil from the colonies' original nest. Nest boxes were lined with Fluon to prevent escape. All colonies were queenless with ≈1000 workers and small amounts of brood. Colonies were fed twice weekly with honey water and protein jelly.

2.2.2 Method for extraction of micromolide from paper substrate

Two sets of substrate extractions were performed during this experiment: an initial exploratory extraction and a second set of substrate extractions performed at the same time as the extractions from the cuticle of *L. flavus* workers. Six colonies were used in the initial set of extractions and six different colonies were used for further paper and cuticle extractions. In the initial extraction, 5 cm diameter discs were cut from filter paper and washed in hexane for 10 minutes, and then washed in

dichloromethane for 10 minutes. The paper discs were then left in a fume cupboard until all solvents had evaporated, and were then used to line 5 cm diameter petri dishes which had been coated with Fluon. 40 ants were then removed from the sample colony and placed in a fridge at 5 ˚C for 1 minute to reduce locomotion and alarm response activity before their transfer to the paper lined petri dish. Once the ants had been transferred to the petri dishes they were provided with damp cotton wool and left to trample the paper for 24 hours. Whilst the paper was being conditioned, the ants were observed regularly to ensure high levels of survival, and to ensure that the ants were not depositing chemical secretions directly from the gaster onto the paper substrate. Once the paper had been conditioned, the ants were removed and returned to their colony. The conditioned paper discs were then cut into small pieces and placed in a large vial containing 3 mL of hexane with 0.15 µg/mL of tetradecalactone as an internal standard (IS). After 5 minutes the pieces of paper were removed and the sample was transferred in stages into an insert-containing GC-MS vial, while being blown down under nitrogen. Once the entire sample had been transferred, the sample was blown down to dryness and was re-suspended in 30 µL of hexane. The sample was then analysed using GC-MS (Perkin Elmer AutoSystem XL gas chromatograph, Perkin Elmer TurboMass mass spectrometer using electron impact ionisation). Following a splitless injection of 0.3 µL of sample, the oven was maintained at 80 ˚C for 2 minutes, then increased to 270 ˚C at 20 ˚C/minute, then to 285 ˚C at 3 ˚C/minute, then increased to 310 ˚C at 20 ˚C/minute and held there for 7 minutes. The method for the second set of substrate extractions was the same, but the concentration of the internal standard was reduced to 0.1 µg per 3 mL of hexane.

2.2.3 Method for extraction of micromolide from the cuticle

80 ants were taken from the foraging area of each of the six colonies. 40 ants from each colony were anaesthetised using CO_2 and had their acidopores blocked using nail varnish, while the other 40 ants from each colony were left with unblocked acidopores. The two groups from each colony were then transferred into 5 cm petri dishes lined with paper (prepared in the same way previously described in these

Materials & Methods), with access to water from damp cotton wool, and left for 24 hours. After 24 hours the ants were freeze killed and dissected under a light microscope to separate the legs from the head and thorax. The gasters were also removed and disposed of to avoid contamination of the samples from glands contained within the gaster. An extraction was then performed for each pair of legs (front, middle, back) and from the combined head and thorax. Body parts were submerged for 5 minutes in 200 µL of hexane containing 0.1 µg of tetradecalactone as an internal standard. The sample was then blown down to dryness and re-suspended in 30 µl of hexane. Samples were then analysed by GC-MS following the same protocol as outlined previously in these Materials & Methods.

2.2.4 Statistical analysis

All data were analysed in SPSS version 24. Comparisons between treatments across the whole data set were analysed using a paired t-test. Comparisons between samples (e.g. foreleg vs midleg) were performed using a one-way MANOVA.

2.3 Results

2.3.1 Identification of compounds of interest

Identification of compounds of interest (analyte micromolide and internal standard tetradecalactone) was performed by comparison of peak ion spectra (Figures 2.1 & 2.3) with previously published ion spectra for these compounds (Figures 2.2 & 2.4).

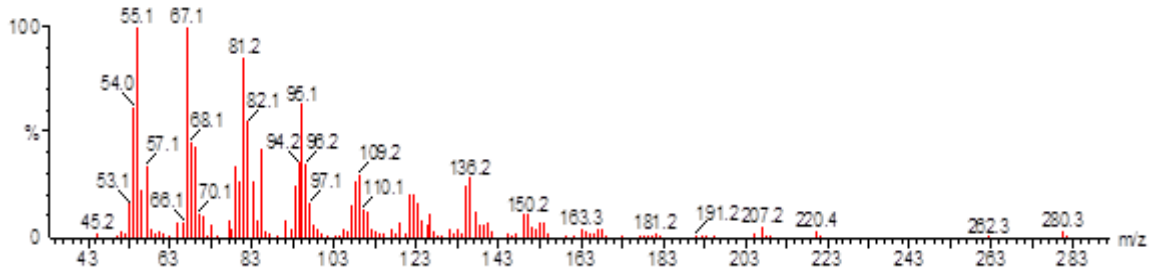

Figure 2.1: mass spectrum of micromolide peak from a paper extract of colony 4, the characteristic ions at *m/z* 280, 262 and 220 were detected.

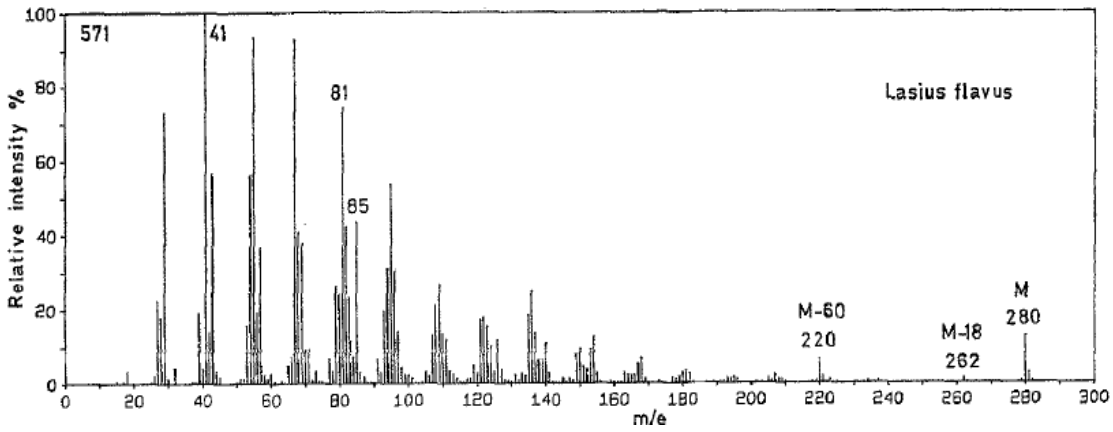

Figure 2.2: Published mass spectrum of 4-hydroxyoctadec-9-enolide (micromolide) from Bergstrom & Lovqvist (1970). The molecular ion (M) can be seen at *m/z* 280. Ions at *m/z* 262 and 220 are characteristic of micromolide corresponding precisely to those seen in Figure 2.1.

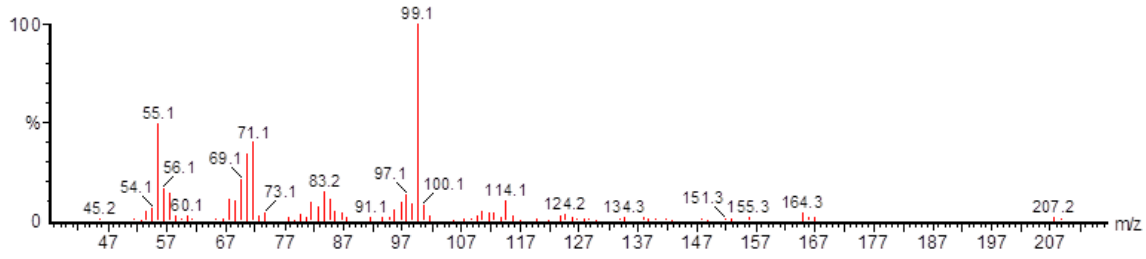

Figure 2.3: Ion spectrum taken for calibration data, the characteristic peak at *m/z* 99 reveals this compound to be my internal standard tetradecalactone.

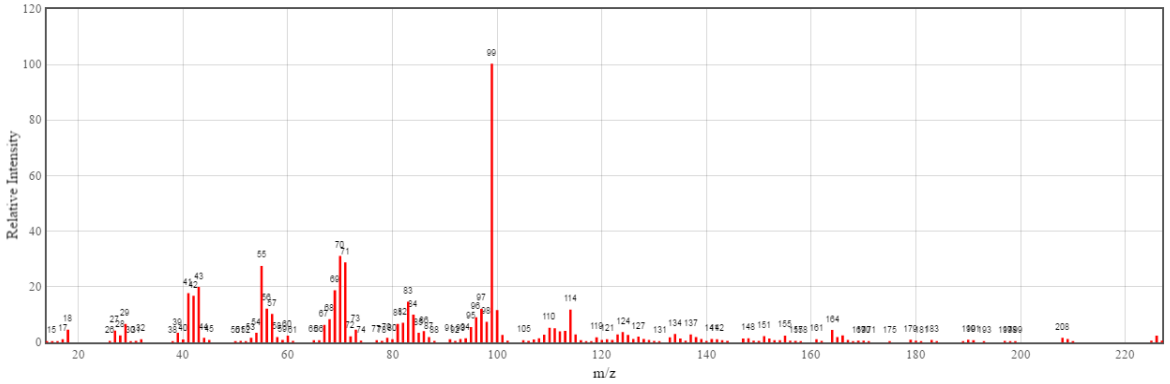

Figure 2.4: Ion spectrum for tetradecalactone, published by the National Institute of Standards and Technology (NIST), US Department of Commerce. The identifying ion in this ion spectrum is found at *m/z* 99, precisely in the position of my control standard above.

2.3.2 Passive deposition of micromolide by *L. flavus* workers on paper substrate

Having unequivocally demonstrated that *L. flavus* passively lays down micromolide on paper (Figures 2.1 & 2.2) I am now in a position to examine this more quantitatively (Figures 2.5 & Table 2.1). My initial set of extractions from a paper substrate showed clearly that micromolide was deposited onto a paper substrate by walking ants, and that this could be detected using GC-MS.

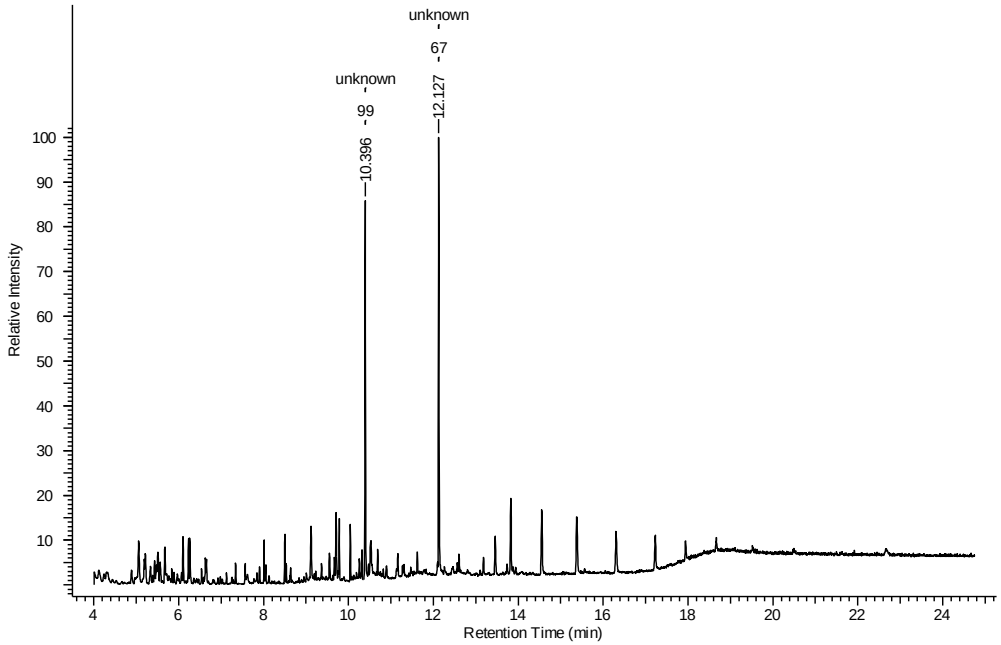

Figure 2.5: The chromatogram of the initial paper substrate extraction for colony C, with both my internal standard and micromolide clearly visible. The first large peak at a retention time of 10.4 minutes is the internal standard and the second large peak at a retention time of 12.1 minutes is micromolide identified as described previously.

As can be seen Figure 2.5, micromolide is easily detectable in samples extracted from the paper substrate with this chromatogram showing a large, clear peak representing micromolide. All samples from different colonies showed that micromolide had been laid down. The results by colony are shown in Table 2.1.

Colony	IS Peak Area (AU)	Micromolide peak Area (AU)	Ratio/ weight (ng) IS equivalent
B	1257842	1010145	0.803/3.6 ng
C	1427194	2075063	1.454/6.5 ng
D	1214403	1077286	0.887/3.9 ng
G	1397504	1618282	1.158/5.2 ng
I	1363921	2854993	2.093/9.4 ng
J	1343808	536600	0.399/1.8 ng

Table 2.1: The results of my initial extraction show that micromolide can be detected on the paper substrate trampled by *L. flavus*. In this extraction, 4.5 ng of internal standard was injected onto the column so by finding the ratio between the area, measured in arbitrary units (AU), of the internal standard (IS) peak and the micromolide peak, the quantity of micromolide extracted could be quantified.

2.3.3 Substrate and cuticle extraction: ants with blocked or unblocked acidopores

The results of the second set of extractions confirm that micromolide can be found on a substrate that has been conditioned by allowing *L. flavus* workers to trample the substrate, and that micromolide is also found on the cuticle of ants (Table 2.2). My results also show that blocking the acidopore of ants reduces the amount of micromolide deposited (Figure 2.6). The quantity of micromolide extracted from unblocked acidopore samples (mean=5.58 ng IS equivalent, SD = 7.64) was significantly greater than the quantity of micromolide extracted from the blocked acidopore sample (mean=0.34 ng IS equivalent, SD=0.74) using a paired t-test ($t(27)=3.83$, $p=0.001$). This strongly supports the hypothesis that the glandular source of micromolide in *L. flavus* is one or more of the glands (Dufour, poison or rectal) contained in the gaster and connected to the acidopore.

I also compared the quantity of micromolide extracted from the different body parts and substrate using a MANOVA, but I found no significant difference between these extracts ($F(4, 5)=1.35$, $p=0.39$, Wilk's $\Lambda=0.37$, partial $\eta^2=0.63$).

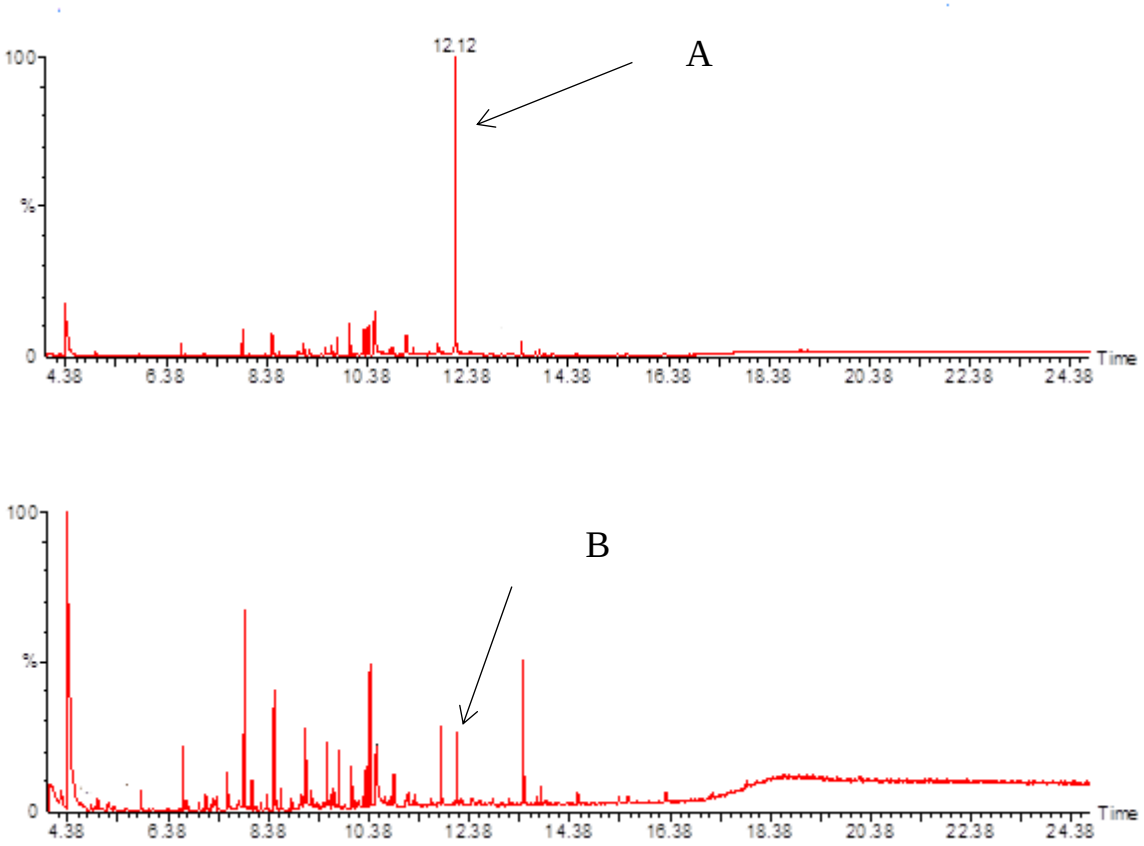

Figure 2.6: Blocking the acidopore reduces the amount of micromolide deposited on paper. Chromatogram A shows the extraction from the paper substrate trampled by ants (colony 3) with unblocked acidopores, the peak indicated by the arrow is micromolide. Chromatogram B shows an extraction from paper trampled by ants (colony 3) with blocked acidopores, again the peak for micromolide is shown with an arrow. The peak for micromolide in chromatogram B is smaller than for chromatogram A, which gives the impression that the other equivalent peaks are larger in B than in A. This is not the case because these chromatograms show the relative abundance of each compound.

	Paper extract		Foreleg extract		Midleg extract		Hind leg extract		Head & thorax extract	
Colony/ treatment	IS/ micromolide peak area	**Ratio/IS Equivalent on Column (ng)**	IS/ micromolide peak area	**Ratio/IS Equivalent on Column (ng)**	IS/ micromolide peak area	**Ratio/IS Equivalent on Column (ng)**	IS/ micromolide peak area	**Ratio/IS Equivalent on Column (ng)**	IS/ micromolide peak area	**Ratio/IS Equivalent on Column (ng)**
1/unblocked acidopore	1925295/ 809968	**0.421**	116068/ 1133785	**9.768**	MI	**NA**	MI	**NA**	116848/ 39865	**0.341**
1/blocked acidopore	819139/ 787529	**0.961**	6741/ NM	**NA**	19583/ NM	**NA**	MI	**NA**	11579/ NM	**NA**
2/unblocked acidopore	357984/ 5000501	**13.969**	32323/ 1013584	**31.358**	235485/ 3413228	**14.494**	230569/ 2186805	**9.484**	280380/ 123896	**0.442**
2/blocked acidopore	375845/ 617803	**1.644**	85882/ 272769	**3.176**	109921/ NM	**NA**	85900/ NM	**NA**	130505/ NM	**NA**
3/unblocked acidopore	506851/ 5957022	**11.753**	222376/ 646224	**2.906**	204990/ 80200	**0.391**	192861/ 202225	**1.049**	195913/ 132938	**0.679**
3/blocked acidopore	499897/ 329615	**0.659**	151099/ NM	**NA**	209535/ NM	**NA**	228763/ NM	**NA**	216065/ NM	**NA**
4/unblocked acidopore	542236/ 4315828	**7.959**	196432/ 257542	**1.311**	229462/ 123165	**0.537**	214785/ 234247	**1.091**	293716/ 45537	**0.155**
4/blocked acidopore	483461/ 643622	**1.331**	225395/ NM	**NA**	185898/ NM	**NA**	190321/ NM	**NA**	288888/ 5817	**0.020**
5/unblocked acidopore	2055329/ 804712	**0.392**	151692/ 3051115	**20.114**	159327/ 1408146	**8.838**	123796/ 1605917	**12.972**	127048/ 223862	**1.762**
5/blocked acidopore	1969110/ 153698	**0.078**	123289/ NM	**NA**	113691/ NM	**NA**	175941/ NM	**NA**	146005/ 5645	**0.039**
6/unblocked acidopore	4234651/ 741470	**0.175**	204955/ 445063	**2.172**	196969/ 142906	**0.726**	193181/ 43166	**0.223**	210473/ 171731	**0.816**
6/blocked acidopore	5798771/ 238817	**0.041**	307632/ 43423	**0.141**	252195/ NM	**NA**	204281/ 35065	**0.172**	193235/ 297674	**1.540**

Table 2.2: Results of extractions from paper and cuticle, blue rows show results for the unblocked acidopore treatment, yellow rows show the blocked acidopore treatment from six numbered colonies. MI denotes a miss injection into the GC-MS; NM denotes samples where no micromolide was found; NA (not applicable) denotes a ratio could not be calculated.

2.4 Discussion

When discussing my results, the first thing to note is that micromolide was consistently found on the cuticle of *L. flavus* workers, and that it was also found on a paper substrate that had been walked on by those same ants. It seems that that this micromolide deposition on the substrate is the result of walking on the paper rather than any specific behaviours, because none were observed during the experiment.

The second finding of this experiment is that the amount of micromolide extracted from both the cuticle of the ants and from the paper substrate is significantly reduced by the blocking of the acidopore of the ants. This suggests that the only source of micromolide in *L. flavus* workers is one or more of the glands found in the gaster, and that other glands such as the metapleural glands do not produce this compound. This agrees with previous work by Bergstrom & Lovqvist (1970) that was the first to identify micromolide (as 4-hydroxyoctadec-9-enolide) in the gasters of *L. flavus*. That study concluded that the specific gland responsible for the production of micromolide was likely to be the Dufour gland (located in the gaster), and this was confirmed by Jones et al (in preparation). The Dufour gland is closely associated with the poison gland and together they form a major component of the stinging apparatus found in extant ants, bees and wasps. These glands are thought to have originally been used in reproduction, the Dufour gland producing oily compounds to provide lubrication during egg laying (Attygale & Morgan 1984). The Dufour gland in extant ants is thought to have evolved to produce a variety of compounds with a variety of functions. In *Myrmica rubra* and *M. scabrinodis,* Dufour gland secretions are used to elicit foraging behaviour and as species-specific foraging-area marking (Cammaerts et al 1978). Dufour gland secretions are also used in foraging-area marking in two species of *Tetramorium* ant, *T. caespitum* and *T. impurum* (Cammaerts & Cammaerts 2000). The Dufour gland has also been shown to produce antibiotic compounds. Dufour gland secretions in the species *Cramatogaster pygmaear* has been found to be an

effective antibiotic against gram positive and gram negative bacteria (Quinet et al 2012) as well as their main function as a contact defensive secretion against arthropod antagonists. This close association between the Dufour and the poison gland may give some insight into the role of micromolide in *L. flavus* ants and how this evolved. As members of the subfamily Formicinae, *L. flavus* have a well-developed poison gland and reservoir, which produces and stores formic acid (Holldobler & Wilson 1990). Formic acid is mainly used as a defensive compound (Cavill & Robertson 1965), but also has been shown to have antibiotic properties (Graystock & Hughes 2011; Moreau 2013), and is used by ants to disinfect brood and nest material (Tragust et al 2013). It has been suggested that many of the compounds produced in the Dufour gland function as a spreading agent that increases the effectiveness of formic acid (Attygalle & Morgan 1984). It is possible that micromolide in *L. flavus* has evolved from simpler compounds produced in the Dufour gland which were originally used as spreading agents for formic acid, and as such micromolide is a component of a suite of chemicals used in defence against pathogens.

Another element of this study was investigating the distribution of micromolide over the cuticle of *L. flavus* workers. I did not find a statistically significant difference in micromolide distribution over the cuticle of the ants, but in my view this was primarily due to my sample size being too small for any differences to be properly elucidated. However if more tests were to be done, I predict that there would be a significant difference in the amount of micromolide found on various body parts. From my results it appears that more micromolide would be found on the legs of workers than on the head and thorax. I also predict that with more repeats of this experiment, more micromolide would be extracted from the paper substrate than from the head and thorax of ants. If this were to be the case, then it would raise an interesting question into the specific role micromolide plays in ants. The production of compounds such as micromolide has a cost for the individuals that produce it (Poulsen et al 2002).

Therefore, it would seem to be wasteful and inefficient for such compounds to be more abundant on a substrate than on the ant themselves. For this reason, I propose that micromolide may be used primarily as part of the social immunity of ant colonies rather than at the individual level. I also propose that this forms a prophylactic defence, as opposed to social defences which occur on demand such as social fever in honeybees, a defence whereby many bees within a colony raise their body temperature to heat-kill bacteria (Starks et al 2000).

Social immunity is of particular importance for ants such as *L. flavus*. This is because *L. flavus* forms colonies which live underground in soil, a nest material that has the potential to host high numbers of pathogens (Boomsma et al 2005). In addition to this *L. flavus* nests can be maintained for decades (King 1977), providing a spatially and temporally stable environment for pathogens. In such circumstances it would make sense for ants to invest significant resources into maintaining hygienic nest conditions. There are in fact a number of examples of behaviours that have a primary role in keeping nest material free from pathogens. The wood ant *Formica paralugubris* incorporates solidified resin from conifer trees into nest material which can inhibit the growth of pathogens (Christe et al 2003). Secretions from the metapleural gland of leafcutter ants and weaver ants play an important role in maintaining sanitary nest conditions, and are also important in the protection of brood against fungal pathogens (Tranter et al 2013). Indeed defences that form part of the colony's social immunity are of particular importance in protecting brood as they are unable to groom themselves, lack the glands that produce antimicrobial compounds (Holldobler & Wilson 1990), and are without a fully developed immune system (Tranter et al 2013).

My results suggest a possible mechanism through which micromolide could be distributed throughout the nest. How micromolide is concentrated on the legs of the ants is unclear, but this results in large quantities being deposited onto the substrate

through the simple process of ants walking in the nest. This mechanism would allow micromolide levels to build up over time. It would also result in higher concentrations of micromolide on areas of the nest that are most heavily populated by ants. Ants provide high levels of care for colony brood (Holldobler & Wilson 1990), and as a result of this one would expect high concentrations of workers in brood chambers of *L. flavus*. This would in turn produce high levels of micromolide on the nest material around brood, providing protection from pathogenic fungi to which brood are particularly vulnerable, allowing ant colonies to sustain themselves.

3. Getting Home: How food is found has effects on *Lasius flavus* nest bound navigation

Abstract

The ability of social insects to gather information from the environment and share that information with nestmates is one reason for their extraordinary success. When foraging, ants often use visual cues to help form a route memory which allows successful navigation from a food source, often found via a circuitous route, back to the nest. By laying trail pheromone from the food source to the nest, returning ants are able to share the location, and guide naïve nestmates to food. This study examines how the method of food discovery affects the formation of a route memory in *L. flavus* foragers, and if the guidance of a pheromone trail can be replaced with another chemical cue, home-range markings. Ants were allowed to forage on an H-shaped maze, under 4 different treatment conditions, and their return journey was observed, recording the errors made at each of the two trail bifurcations. In the first treatment ants were led to the food with a pheromone trail which was removed for the return journey. In the second treatment ants were allowed to explore the maze independently, and were provided with no pheromone for their return. In treatment 3 ants were provided with trail pheromone to and from the food, and in treatment 4 ants were led to the food with a pheromone trail, which was replaced with home-range markings for the return.

It was found that ants led to the feeder made more errors on a return than those that had explored independently, and that the provision of home-range markings did not decrease the number of errors made suggesting that *L. flavus* are unable to replace one chemical cue with another. These results show that method of food discovery affects route-memory formation.

Getting Home: How food is found has effects on *Lasius flavus* nest bound navigation

3.1 Background

Social insects are some of the most successful organisms on the planet, and one of the major reasons for this is the ability of individuals to privately gather and store information and then to share that information with nestmates. The ability to share information has been shown in many organisms, such as fish, birds and bats, to improve group foragers' ability to exploit resources (Templeton & Giraldeau 1996, Valone & Templton 2002). In vertebrates this can be visual information; for example, starlings (*Sturnus vulgaris*) can use visual information in deciding when to scrounge from conspecifics (Templeton & Giraldeau 1996).

In social insects such as ants this communication is often chemical, and performs a vital role in recruitment of nestmates to food sources, nestmate recognition, territorial and alarm behaviours (Jackson & Morgan 1993). Like all insects, ants have a waxy cuticle comprising of a mix of long chain hydrocarbons which reduce the evaporation of water across the cuticle and so protect themselves from desiccation (Gibbs 1998). However in ants other information is encoded in the profile of cuticular hydrocarbons (CHCs) and this allows for even greater communication (Martin & Drijfout 2009). CHCs play a vital role in nestmate recognition (Sturgis & Gordon 2012, Sturgis et al 2011) and are also used by ants to determine what role they should undertake at any one time. The composition of certain parts of the CHC profile can be influenced by exposure to environmental conditions, and this can be used by individual ants to inform what task (e.g. midden work or foraging) should be undertaken (Green & Gordon 2003).

Foragers for example are exposed to warmer drier conditions than other nest workers such as nurses, leading to a change in their hydrocarbon profile (Wagner et al 2001). This allows other workers to identify them as foragers and so change their own task when repeatedly encountering foragers trying to recruit them to exploit a new food source. CHCs are also laid down by walking ants to form home-range markings (Lenoir et al 2009). These home-range markings perform a number of functions. In *Lasius japonicas,* home-range markings combine with non-colony specific pheromone to produce colony specific trail-following behaviour (Akino & Yamaoka 2005). It has also been shown that ants act more aggressively to non-nestmates on territory that is marked with their own colony home-range markings, and that ants trespassing on an area marked by another colony tend to avoid aggressive encounters with the home colony (Akino et al 2005).

As home-range markings are laid passively by walking ants, their concentration on the substrate is determined by ant density. Therefore, intensity of home-range markings decreases with distance from the nest. This information can be used by foragers as a cue for distance from the nest, and so modulates recruitment behaviour (Devigne et al 2004, Devigne & Detrain 2006). Ants will lay more pheromone on paths marked with home-range markings, and an increase in home-range markings as a forager approaches the nest can stimulate the resumption of pheromone deposition in foragers that had stopped laying on their return journey from a food source (Devigne & Detrain 2006). The gradient in home-range marking is also important in the desert ant *Pogonomyrmex barbatus*. This species does not have a trail pheromone and uses the gradient of home-range markings on the midden surrounding the nest entrance to guide foragers back to the nest (Sturgis et al 2011).

Ants show the ability to use chemicals and olfaction in a number of tasks. However, during foraging, scout ants on unexplored territory are unlikely to have these cues available to them. This is where an ant's ability to interact with the environment allows it to gather information privately, and navigate effectively. For example it has been shown that orientation and navigation in ants is influenced by the earth's magnetic field (Anderson & Vander Meer 1993, Banks & Srygley 2003). This is particularly useful for ants that live in dark environments such as the rainforest, or that forage at night when visual cues may not be readily available. When visual cues are available, landmark recognition combined with path integration provides the basis for navigation (Collett & Collett 2002). Most insects, ants included, are able to recognise many visual features including colour, size, edge orientation, symmetry and motion. These features of landmarks are recorded as 'snapshots' that the ant can remember and use to navigate from one location to another (Judd & Collett 1998). Features such as landmarks and polarised light are often used to indicate direction, and distance is normally recorded by monitoring locomotive activity e.g. leg movement (Wittlinger 2006, Collett et al 2006). By combining these inputs, ants are able to use path integration to navigate back to the nest site, and the return route is often the shortest route available, in contrast to a circuitous outbound route. This shows that these methods of navigation often led to the most efficient outcome.

Lasius flavus is a common UK species, and workers spend almost all of their lives underground. In this subterranean environment, there is a complete lack of visual cues and so it seems reasonable to assume that olfactory cues are vital for all tasks. *L. flavus* does not lay pheromone whilst exploring the environment, only doing so once a food source has been discovered (personal observation). It is also the case that pheromone trails mainly serve to guide naïve workers to the newly discovered resource. Pheromone trails are generally not used to guide experienced ants, and it has been shown that experienced ants will choose their own private memory of a

route over the socially available information provided by pheromone trails (Grüter et al 2011). This was shown in an experiment with the related species *Lasius niger* where workers were trained to a feeder on a T-maze, and then presented with a pheromone trail that conflicted with the forager's memory of the food location. In this scenario foragers overwhelmingly chose to ignore the pheromone trail and instead followed their own memory (Grüter et al 2011). This experiment was repeated with *L. flavus* and produced the same result (Jones et al, in preparation).

So while it is clear that *L. flavus* has the ability to use visual cues to form a private route memory, it is not clear how *L. flavus* workers navigate underground in the absence of these cues. It was for this reason that I chose to investigate the possible role of home-range markings in underground navigation.

L. flavus can form colonies of ≈10,000 individuals and build large conspicuous mounds in grassland. The main food source of *L. flavus* is a variety of root aphid species which the ants farm for honeydew and on which they also predate in the summer (Pontin 1978). These aphids are often found in chambers that are dug out and maintained by the ants, and these chambers are likely to be reasonably permanent and so in principle attract enough traffic for an accumulation of home-range markings to occur. As there are no visual cues underground and workers do not lay pheromone until they have found a food source, it seemed possible that home-range markings may play a role in navigation within the large mounds built by colonies of *L. flavus*.

To investigate this, an experiment was designed to test the ability of workers to navigate from food source to nest in the absence of visual cues but while providing home-range markings. This experiment involved leading foragers to food so that they would be motivated to return to the nest. However, I found that when foragers were

led to food with pheromone they struggled to return to the nest, even when I provided visual cues. This was surprising as it has been shown that *L. flavus* workers are capable of quickly forming accurate route memories when allowed to explore a maze (Jones et al, in preparation). So I changed the design and focus of my experiment to investigate the difference between ants that had been recruited and led to a food source using pheromone trails with ants that had been allowed to explore a maze independently while foraging for food. In total I designed 4 treatments to investigate this.

1. The trail pheromone of *L. flavus* was extracted from worker hindgut by dissection and then immersion in hexane. This extract was then applied to the paper substrate on the maze providing a pheromone trail to the food source. A forager was then allowed onto the maze where it followed the trail to the food. Once an ant had found the food, the pheromone trail was removed and the ant's return journey observed.

2. A forager was allowed onto the maze without any trail pheromone to lead it to the food. This forager then explored the maze until it found the food, and its return to the nest was observed.

3. As in Treatment 1, individual foragers were led to the food source with pheromone, but this trail was left in place for the return journey.

4. As in Treatments 1 and 3 foragers were led to the food with a pheromone trail, but once at the feeder the pheromone trail was replaced with paper conditioned with home-range markings lining the route back to the nest.

These four treatments allowed me to test the ability of ants to form a route memory under different conditions. They also allowed me to examine the ability of foragers to use home-range markings to navigate back to the nest.

In addition to these four treatments, I also performed an experiment to check if *L. flavus* workers could indeed detect CHCs on paper conditioned by the method described for Treatment 4. This involved allowing naïve ants onto a T-maze where they were presented with the choice between plain unmarked paper and paper that had been conditioned with CHCs.

3.2 Materials & Methods

3.2.1 Housing and maintenance of the ants

All colonies were collected from meadowland in the South Downs near the University of Sussex campus. They were stored in 30 cm by 30 cm plastic boxes with some of the soil they were collected in. The walls of the nest boxes were covered with Fluon to prevent escape. All colonies were queenless with ≈1000 workers and a small amount of brood. Colonies were fed twice a week with honey water and protein jelly. Colonies were starved for 4 days prior to testing to ensure high motivation for foraging and recruitment. Colonies had continuous access to water.

3.2.2 Conditioning paper with CHCs for T-maze experiment and Treatment 4

Colonies were connected to a foraging platform 1 cm wide and 20 cm long that had been covered with filter paper. The foraging platform was orientated perpendicular to the nest box, resulting in higher traffic and CHC concentration at the nest box end, and a decreasing gradient in CHC concentration with distance from the nest. Ants were then allowed to explore the platform, which was not rewarded with a food source, and condition the paper for 24 hours prior to testing.

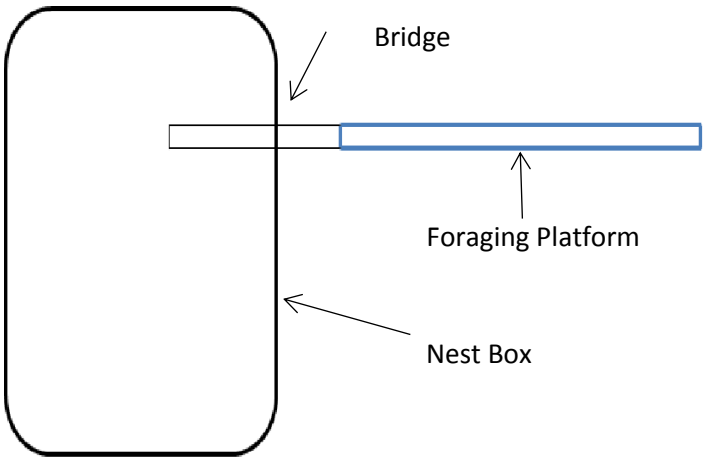

Figure 3.1: Setup for conditioning of home range-marked filter paper. The foraging platform was 1 cm wide and 20 cm long, drawn to approximate scale.

3.2.3 Experimental setup for T-maze: detection of CHC home range markings

A T-maze with a stem of 9 cm and a head of 15 cm (Figure 3.2), was covered in clean filter paper. Decision lines were drawn 2 cm from the junction on each arm. Once conditioned, the middle section of the paper from the foraging platform was cut to size (8 cm) and placed along one arm of the T-maze with a decision line drawn 3 cm from one end. Ants were then allowed access to the maze one at a time, where they would be presented with the choice between a CHC marked and the unmarked branch. A decision was considered to have been made once an ant had crossed a decision line. Once an ant had made a decision, it was removed and the marked paper moved to the other branch of the T-maze for the next experimental run. The unmarked paper on the non-conditioned side was replaced with clean filter paper every two runs. A control was performed by presenting ants with plain paper on each branch.

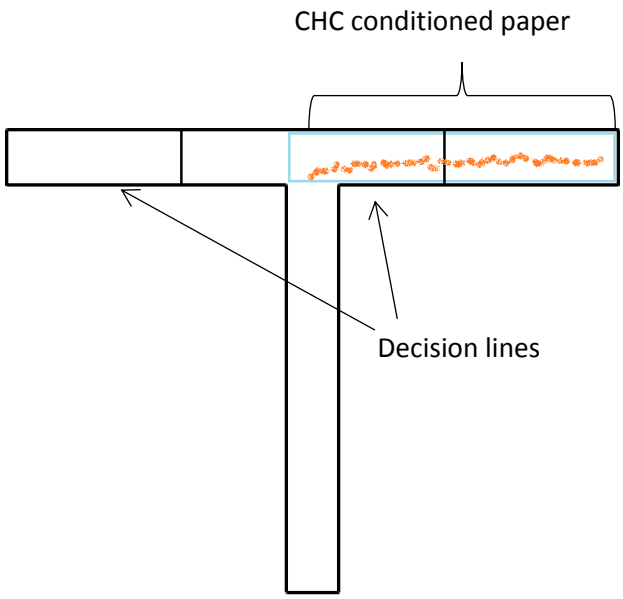

Figure 3.2: The T-maze setup for determining whether ants can detect home-range markings on paper. In this case the right branch of the T-maze has been covered with home-range marked paper, shown here with a blue outline and orange markings.

3.2.4 Experimental setup for H-maze: Treatments 1-4

Test colonies were allowed access to an acrylic H-shaped maze (stem 9 cm long, head 11 cm, arms 11 cm, width 1 cm, Figure 3) via a cardboard bridge (25 cm long, 1 cm wide where connected to the maze, 3cm wide from the nest box). The H-maze was covered with clean filter paper, which was replaced after each experimental run. To allow a consistent judgement on whether an ant had made a decision when encountering a turn, decision lines were drawn onto the maze 2 cm from each turn. An ant was considered to have made a decision once it had crossed two decision lines e.g. an ant leaves the feeder and crosses the first decision line, it then turns at the junction and crosses the next decision line; this combination was judged 'correct'. Alternatively an ant could leave the feeder crossing the first decision line and then go straight on at the junction and cross the next decision line; this was scored as an 'incorrect' decision. A decision was also adjudged to have been made if an ant crossed the first decision line, performed a U-turn and then crossed the same decision line in the opposite direction; this was also scored as 'incorrect'.

All experiments took place in the same location with 2 ceiling lights and one skylight visible as obvious landmarks, as well as many other landmarks in the room that could be used to form a route memory. A drop of 1 molar sucrose solution was placed on a small sheet of plastic (roughly 2 cm by 2 cm) to act as a feeder. This feeder was then placed at the terminus of one of the maze arms marked with a yellow spot in Figure 3.3. The maze was completed when the ant stepped back onto the bridge. Once an ant had completed the maze it was removed from the colony.

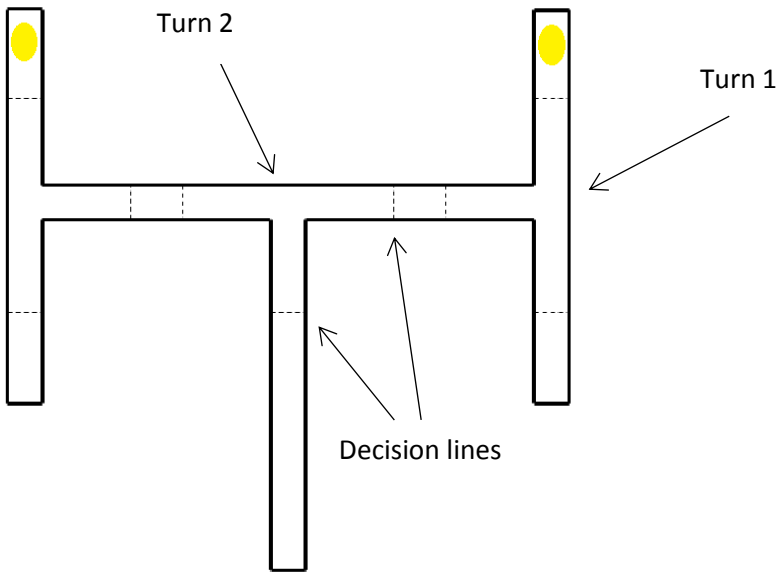

Figure 3.3: H-shaped maze. Dashed lines show decision lines for judging decisions made by returning ants. The yellow circles mark the two possible positions that the feeder could be placed. As return journeys were observed, turn 1 is the first turn encountered on the return journey and turn 2 is the second.

3.2.5 Method for rectal gland extraction and preparation of trail-pheromone extract

A total of 10 *L. flavus* workers were collected from a colony at random and killed by placing in a freezer at −20 ˚C for 30 minutes. Rectal glands, containing trail pheromone, were then removed under a dissecting microscope using forceps and placed in 200 µL of hexane to extract trail pheromone. This extract was then applied to the substrate using a Hamilton syringe.

3.2.6 Encouraging ants to enter the maze

A feeder was placed at the top of the bridge and a few ants were allowed to feed and return to the nest to recruit other foragers and to establish a pheromone trail on the bridge. This subsequently encouraged foraging ants to enter the experimental set-up while denying ants experience of the maze. The bridge was then raised and the feeder and any remaining ants were removed.

3.2.7 Method Treatment 1: ants led to feeder with pheromone

A feeder was placed at the terminus of one of the maze branches (Figure 3.3). 10 µL of pheromone extract was then applied along the length of a route template (Figure 3.4) which then was placed on the maze to guide ants to the sucrose solution. The bridge was then lowered and ants were allowed onto the experimental setup one at a time. Once an ant had followed the pheromone trail to the feeder, the route template was removed while the ant was feeding. The ant's return journey was then observed to measure the decisions made at each junction. The number of decisions made at each junction was recorded, as well as the time taken to complete the maze. Ants were allowed a maximum of 10 minutes to complete the maze, at which point the trial was abandoned if the ant had not navigated back to the nest.

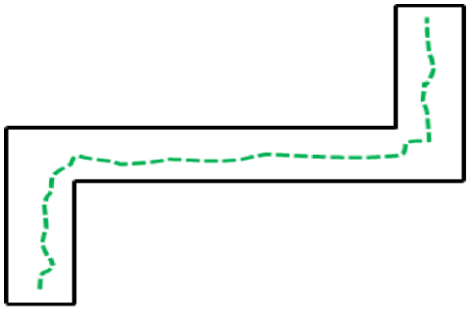

Figure 3.4: Route template. A single piece of filter paper marked with pheromone extract (green dashed line), placed onto the maze to guide foragers to the feeder.

3.2.8 Method Treatment 2: ants allowed to explore the maze to find the feeder

Ants were let onto the experimental setup one at a time and allowed to explore the maze until they found the feeder. Once the ant had fed, its return journey was observed and decisions recorded as in Treatment 1.

3.2.9 Method Treatment 3: ants provided with pheromone trail to and from the food

A dose of 10µl of pheromone extract was applied to the paper route template (Figure 3.4) which was placed on the maze branch leading to the feeder. Ants were allowed onto the maze individually. After they had followed the pheromone extract trail to the feeder, they were then allowed to follow the pheromone trail back to complete the maze. Decision making was recorded as for previous Treatments and ants were removed from the colony once the maze had been completed.

3.2.10 Method Treatment 4: ants led to feeder with pheromone, return journey marked with home-range markings

Once the paper had been conditioned with CHCs, it was cut to fit the shape of the route template, with paper from the nest end of the foraging platform (Figure 3.1) that had been more heavily trampled attached to the nest end of the template, and paper less trampled placed at the food end. This provided a gradient in the concentration of CHC markings on the route template (CHC concentration increased as the ant follows the route template back to the nest). Ants were then led to the feeder with a pheromone-extract marked route template. Once an ant had found the feeder, the pheromone-marked route template was removed and replaced with a CHC marked route template. The return journey was then observed as in all other treatments.

3.2.11 Statistical analysis method

All data were analysed in SPSS version 22. T-maze data were analysed with a Chi-square test. Comparisons across Treatments were performed using a Kruskall-Wallis test. Comparisons between Treatments were performed using a post hoc Mann-Whitney U test with a Bonferroni correction

3.3 Results

3.3.1 *L. flavus* foragers can detect paper marked with CHCs

When presented with a binary choice between CHC marked paper prepared in the same way as in Treatment 4, and plain unmarked paper, foraging *L. flavus* workers choose paper marked with CHC home-range markings.

	(n)	Choice%	Choice%	p
CHC vs unmarked	(54)	65% CHC	35% unmarked	0.0295
Control	(50)	54% left	46% right	0.5716

Table 3.1: Significantly more ants choose the branch marked with CHCs over unmarked paper when presented with a binary choice on a T-maze. The control showed no statistically significant preference for turning left or right.

When tested, 65% of foragers chose the paper that had been conditioned with CHCs, significantly more than would be expected if plain paper was on both branches of the T-maze (Chi-square test, X^2 (1) = 4.74, p=0.0295). The control showed no significant bias towards left or right with 54% of ants choosing the branch on the left and 46% choosing the branch on the right (Chi-square test, X^2 (1) = 0.32, p=0.5716). This suggests that *L. flavus* workers are able to detect, and are also attracted to, CHC marked paper over plain unmarked paper in a binary T-maze assay. It also suggests that my method of conditioning paper with CHCs is effective.

3.3.2 Total number of attempts taken to complete the maze

The way in which food is discovered significantly effects how many attempts are needed (and therefore how many errors are made) to successfully navigate each of the turns on an ant's return journey (Kruskall-Wallis test, H (3) = 118.6, p = <0.001). Ants provided with a pheromone trail to and from the food (Treatment 3) take the fewest

attempts to complete the maze (Figure 3.5). The median number of attempts for this group is two, which as there are two turns is the lowest number of attempts possible (no errors made). This is significantly fewer than in all other treatments ($p=<0.001$ post hoc Mann-Whitney U against all other treatments). Ants that had been allowed to explore the maze independently, (Treatment 2), take the second fewest attempts to complete the maze, taking a median of three attempts across both turns to complete the maze. Again, this is significantly different from all other treatments ($p=<0.001$). Ants that had been led to the food with pheromone, but not provided with pheromone for the return journey (Treatment 1), take twice as many attempts to complete the maze as those in Treatment 2, and three times as many as Treatment 3. This is the same for ants that had been led to the food, and provided with home-range markings for the return journey (Treatment 4). Treatments 1 and 4 are the only treatments that are not significantly different from one another (Mann-Whitney U, $U = 1080.5$, $z = -0.169$, $p=0.866$). This suggests that ants returning to the nest after following a pheromone trail to a food source are unable to use home-range markings as a replacement for pheromone trails in a foraging scenario.

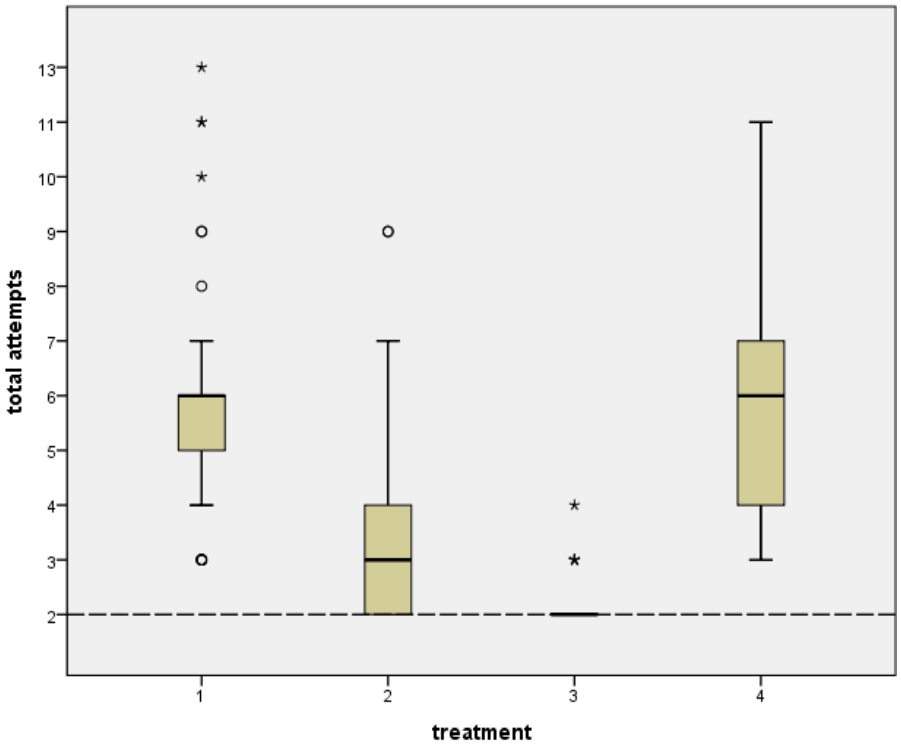

Figure 3.5: A box and whisker plot showing that the different methods of food discovery affect the number of errors made on the return journey. Ants in Treatment 3 made the fewest errors with Treatment 2 showing the second lowest number of errors. Treatments 1 and 4 made the most errors and were not significantly different from each other (Mann-Whitney U p=0.866). The median number of attempts for each treatment is represented by the thick black line in each box. The box indicates the interquartile range and the whiskers extend to the maximum and minimum number of attempts. Asterisks and circles denote outliers. The dashed line represents zero errors made, which could be described as 'perfect behaviour'.

3.3.3 Time taken to complete maze

The time taken to complete the maze is strongly influenced by the method of food discovery (Kruskall-Wallace test, H (3) = 123.7, p = <0.001)

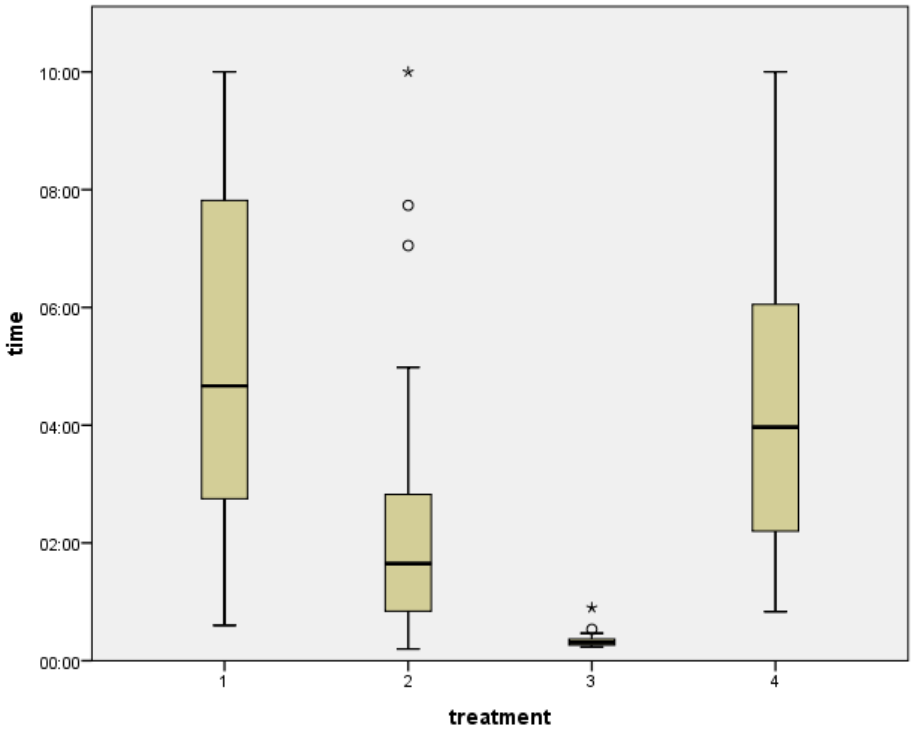

Figure 3.6: A box and whisker plot showing that across all treatments how food is discovered significantly impacts the time taken to navigate home (Kruskall-Wallace p=<0.001). Median time for completion is shown by the thick black line inside each box, with Treatment 3 being the fastest, Treatment 2 the second fastest, and Treatments 1 and 4 the slowest and not significantly different from one another (Mann-Whitney U p=0.153). Boxes show the interquartile range with whiskers extending to the minimum and maximum time. Asterisks and circles represent outliers.

The time taken directly reflects the number of errors made by returning ants in each treatment (Figure 3.6). This can be seen in the observation that the treatment with the fewest attempts, Treatment 3, is also the treatment that produced the fastest return times (median of 18 seconds). Treatment 2 produced the second fastest time for return (median 1minute 39 seconds), again reflecting the number of attempts made at each turn. These two median times are significantly different from one another (Mann-Whitney U, U = 67, z = -8, p = <0.001). These two treatments are also significantly different from Treatments 1 (median time 4 minutes 40 seconds) and Treatment 4 (median time 3 minutes 58 seconds). Again, the time taken seems to be

directly influenced by errors made as all treatments differ significantly from one another except Treatments 1 and 4 (Mann-Whitney U, U = 914, z = -1.43, p=0.153). Unsurprisingly, this reflects the statistical difference between treatments when analysing the number of errors made on the return journey.

3.3.4 Turns encountered earlier on the outward journey are remembered more accurately on the return journey

My results indicate that the order in which trail bifurcations are encountered on the outward journey influences how well they are remembered when returning. Significantly more errors were made at turn 1 than at turn 2 in Treatments 1, 2 and 4 (Figure 3.7).

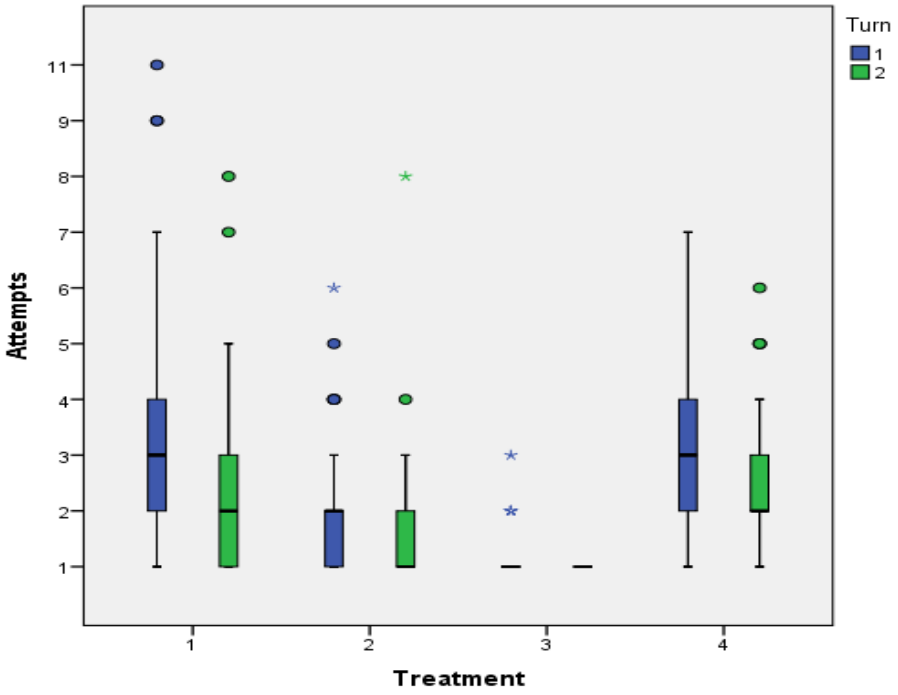

Figure 3.7: Box and whisker plot showing that ants made fewer errors at turn two (green data) than at turn one (blue data). This was the case for all treatments except the perfect performance in Treatment 3. The median is represented by the thicker black line contained within the box, the boxes represent the interquartile range, asterisks and circles represent outliers.

In Treatment 1, the median number of attempts at turn one was three compared to the median number of attempts at turn 2 which was two. This is a significant difference (Mann-Whitney U, U = 812, z = -2.84, p = 0.004). This result was also seen in Treatment 2 where the median number of attempts at each turn was two and one respectively (Mann-Whitney U, U = 708.5, z = -3.32 p < 0.001); so for both Treatments 1 and 2 the order in which turns are encountered on the outward journey seems to affect how well they are remembered. This is despite the fact that they had reached the food via different methods (led vs found).

This pattern was also seen in Treatment 4. The median number of attempts at turn one was three and the median number of attempts at turn 2 was two (Mann-Whitney U, U = 695.5, z = -2.63, p = 0.009). While we may have not have expected this pattern to be the same in Treatments 1 and 2, due to the difference in errors made and time taken to complete the maze, it is not surprising to see the same pattern in Treatments 1 and 4 as these two treatments were not significantly different from one another in errors made or time taken.

3.4 Discussion

These results show that the method of food discovery has a significant impact on the ability of *L. flavus* workers to navigate their return to the nest after visiting a food source. Naïve ants that had followed a pheromone trail to a food source made more errors and took longer on their return to the nest when compared to naïve ants that had been allowed to explore the maze independently to locate food or when compared to ants that had been provided with a pheromone trail between the food and the nest in both directions. I also found that on a trail with two bifurcations, the number of navigation errors was not evenly spread across the two turns, with more errors made at turn 1 than at turn 2. Finally, I found that the presence of home-range

marking did not improve homeward navigation for foragers that had been led to the feeder with trail pheromone, indicating that *L. flavus* workers were unable to replace one chemical cue with another in the context of foraging. The results of this study were not what I had expected when first devising this experiment. One particular feature of the results was the relatively poor navigational performance of ants returning to the nest after being led to the feeder with pheromone.

The complexity of the maze may be one of the reasons for the apparent lack of ability of the ants to quickly form an accurate memory. Previous studies have found that *L. niger* foragers, trained on one trip to a feeder on a simple one bifurcation T-maze, make the correct subsequent decision 75% of the time. This rises to correct decisions 97% of the time after three trips to a feeder (Gruter et al 2011). The maze used in our experiment was more complex with two trail bifurcations, and this extra complexity has been shown to reduce the ability of foragers to form an accurate route memory. When tested on a more complex maze with two trail bifurcations, accuracy of decision making of *L. niger* foragers on a subsequent outward journey dropped to 56% at the first bifurcation and only 79% for the second bifurcation, after three visits to a feeder (Czaczkes et al 2013). This same study also found that routes involving two bifurcations of alternate direction (e.g. left, right versus left, left) produced more errors. In my study, the route always involved alternating turns and so this could be an added explanation for the relatively poor performance in decision making under some of the experimental conditions described here. Unlike the previous study on complex route navigation, I found that it was the first turn encountered on the outward journey that was more accurately remembered. This could be explained by the fact that in this study, the first return journey from the food to the nest was the journey that was recorded. Czaczkes et al (2013) recorded the second journey out to the food. In both studies, it was the turn at the first trail bifurcation on their most recent journey before decisions were recorded, which the foragers remembered most accurately.

One of the more interesting results of this study was that ants led to the feeder with a pheromone trail made significantly more errors on their return to the nest than foragers that had been allowed to explore the maze independently to find the feeder. One explanation for this could be that in the presence of trail pheromone, foragers are 'reassured' that they are on the correct path and so need to invest less time in error checking and establishing route memory. Czaczkes et al (2011) found that U turns increased when ants moved from a path marked with trail pheromone to one with no pheromone. This matches my observation of ant behaviour when foraging on the maze under different treatment conditions. Ants that were following a pheromone trail showed great fidelity to it and performed few if any U-turns, whereas ants without pheromone guidance performed regular U-turns. From my observations, and results from Czaczkes et al (2011), I infer that trail pheromone decreases the number of U turns in foraging ants, which play an important role in forager orientation, memory formation and error checking. This is not unexpected as all animals face a trade-off between speed and accuracy when performing tasks such as foraging (Chittka et al 2009). Normally this behaviour of reducing error checking in the presence of trail pheromone allows foragers to increase speed without reduction in accuracy, and so allows greater exploitation of resources. This may go some way to explaining the apparent lack of route memory in foragers led to the feeder with trail pheromone, and my personal observations of foragers following trail pheromone supports this. During the experiment it was noticeable that workers showed a high level of fidelity when following the trail pheromone, walking with a higher speed and with fewer pauses to investigate the environment than foragers that were exploring the maze without a pheromone trail to follow. As a result, ants in these experiments may have failed to build up the mental picture of the environment required to navigate to the nest using memory alone.

The initial aim of this experiment was to find out if home-range markings could act as a substitute for pheromone trails in ants returning to the nest after finding food. To do this I led ants to a feeder with a pheromone trail and then removed it once the ant had found the food, either leaving just clean paper or replacing the pheromone trail with a route marked with home-range markings. I found that ants provided with this home-range marking guide were not significantly better at decision making than ants that were not provided with this cue, suggesting that foragers were unable to use these home-range markings in navigation. This is a surprising result, given that I have shown that *L. flavus* foragers will choose home-range marked paper over unmarked paper in a binary choice assay. Home-range markings can have a number of influences on behaviour. When presented with a binary choice between home-range marked and plain paper, *L. niger* workers will choose home-range marked paper, even when the markings originate from an alien colony (Devigne & de Biseau 2012). To see if *L. flavus* workers showed the same behaviour, I tested workers on a T-maze with one branch of the maze marked with CHCs and the other covered with unmarked paper. In my experiment, *L. flavus* workers chose the CHC marked paper 65% of the time, showing that *L. flavus* workers are able to perceive CHCs in the environment. This is a weaker effect than was found in *L. niger*, where workers chose the route marked by their own colony 86% of the time (Devigne & de Biseau 2012). This could be due to a difference in behavioural response of the different species or due to the difference in the way the CHC marked paper was conditioned. In the Devigne & de Biseau (2012) paper, the filter paper used in the behavioural assay was conditioned by 20 ants for 40 hours. In my experiment the paper was conditioned for 24 hours and the number of ants on the paper was not constant. This could have resulted in a lower concentration of CHCs being deposited on the conditioned paper which in turn may have led to a lower response in my study. This leads to the question of whether a greater concentration of CHCs may have provided a better chemical cue for returning ants in Treatment 4 of my experiment. Another explanation for the lack of response to the presence of CHCs could be the motivational condition of the ants themselves. Previous work has shown

that whether ants are in 'scouting mode' or have been recruited, can affect the response to chemical cues (Czaczkes et al 2014). This different response to olfactory cues in different circumstances has also been seen in the desert ant *Cataglyphis fortis*. This desert ant will respond to olfactory signals associated with their nest site only when other navigational cues indicate proximity to their home nest (Buehlmann et al 2012). In addition, this species changes its reliance on these olfactory signals, depending on whether the ant is leaving or returning to the nest (Buehlmann et al 2013).

One final explanation for the apparent lack of navigational memory in Treatment 2 (ants led to the feeder with trail pheromone) and Treatment 4 (ants led to feeder with pheromone, then provided CHCs for the return) could be that the ants are reacting to an environmental change and as such are adjusting their level of confidence. Previous studies have shown that foraging ants are able to modify their behaviour when confronted with environmental change. Czaczkes & Heinze (2015) found that when a previously discovered food source was moved, it resulted in a change in trail pheromone deposition behaviour. Most interestingly, foragers that had experienced the environmental change of having the feeder relocated, and went on to make errors on their return to the feeder, exhibited a decrease in pheromone deposition. It has also been shown that when forced to forage in darkness, and as a result being deprived of visual cues, *L. flavus* foragers delay pheromone deposition on their return to the nest after finding food (Jones et al in preparation). Pheromone deposition is then increased on their second journey out to the food once greater certainty has been achieved. These results suggest that individual foragers are able to assess their own confidence in relation to route memory, and that uncertainty can result in changes in behaviour. My study showed a similar response to increased uncertainty in Treatments 2 and 4. Along with a greater number of errors at trail bifurcations, I noticed that ants returning from the nest from the feeder appeared to delay laying

trail pheromone until both turns had been navigated. Unfortunately, I did not record this as part of the experiment so I do not have data to confirm this as a consistent behaviour, but it is possible that my results reflect a decrease in route confidence rather than a total absence of route memory.

If I were to repeat this experiment I would record trail pheromone deposition specifically, as this can act as a proxy for route confidence, but I would also include extra treatments. I did not examine the effect of providing home range markings for navigation towards the food, which could then have been left in place for the return journey, removed and replaced with a pheromone trail, or removed with no substitute cue supplied. The reason for this is that the treatments used in this experiment were a result of my observations of poor navigation when ants were lead to a feeder with a pheromone trail which was subsequently removed. The treatments I designed were a natural progression from this original observation, but with hindsight it is clear that a greater crossing of provided cues would have given greater understanding of the effect of different chemical cues on navigation.

4. Kairomone communication between Brazilian predator ants and their predated termites

Abstract

Kairomones are transpecific chemical signals, the adaptive benefits of which fall on the recipient rather than the emitter. *Nasutitermes corniger* is a common species of arboreal termite found in Central and South America, which build conspicuous nests in trees, as well as extensive networks of tunnels on the tree surface, often reaching ground level. These structures play an important role in colony defence, and as such are well maintained by *N. corniger. Camponotus arborious* is a common ant species often found in the same environment as *N. corniger,* and is a member of a genus that opportunistically predates on termites. This study, undertaken during field work in Brazil, examines the possibility of ant cuticular hydrocarbons (CHCs) acting as a kairomone, and the role they may play in mediating the relationship between two social insect groups. CHCs were extracted from *C. arborious* workers, and applied to the surface of experimentally opened *N. corniger* tunnels. The rebuilding behaviour of the termites was then observed.

Applying ant CHCs to the surface of experimentally opened termite tunnels resulted in 4 of the 7 colonies tested blocking rather than repairing opened tunnels, and across the whole data set, termites were less likely to move across an area marked with ant CHCs, when compared to a hexane control. This provides evidence that ant CHCs can be detected by termites, and that ant CHCs may act as a kairomone by allowing termites to avoid areas frequented by predatory ants.

Kairomone communication between Brazilian predator ants and their predated termites

4.1 Background

Chemical cues are vital in eusocial insects for communication. These chemical cues can have varying functions, for example trail pheromones allow nestmates to direct each other to food sources, and alarm pheromones alert nestmates to threats. Other chemical cues such as cuticular hydrocarbons (CHCs) allow for nestmate recognition (Martin & Drijfhout 2009) and task allocation (Green & Gordon 2003). These cues provide reliable information to the nestmates of the emitter but they can also be used by heterospecific organisms in a form of 'chemical eavesdropping'; these chemical signals are called kairomones, and have been defined in Brown et al (1970) as *transpecific chemical messengers the adaptive benefit of which falls on the recipient rather than on the emitter*. Kairomones play an important role in mediating predator-prey interactions, and can lead to changes in morphology and behaviour, especially in prey species. Kairomones are particularly important in aquatic environments and it has been suggested they are the most important chemical cues in mediating ecological interactions in such ecosystems (Burks & Lodge 2002). An example of this is the change in morphology of the grey tree frog, *Hyla versicolor*, tadpole in the presence of dragonfly nymphs. The presence of this predator species leads to tadpoles developing with an increase in tail depth and decrease in body length, morphological adaptations that increase the chances of a tadpole escaping an attack by a predator (Schoeppner & Relyea 2005). This response to predator cues was also observed in the tadpoles of another species, *Rana pipiens* (Shoeppner & Relyea 2009). Another example of morphological adaptation in response to predator kairomones can be found in the waterflea *Daphnia pulex*. When exposed to kairomones from predatory larvae of the midge *Chaoborus flavicans,* juvenile *D. pulex* develop 'neck teeth', jagged protrusions on the head that have been shown to reduce vulnerability to predation by *C. flavicans*. They also develop larger bodies and delay reproduction (Tollrian 1995).

The effect of kairomones on behaviour has also been well described. The Giant Asian Honeybee *Apis dorsata* will avoid foraging on flowers when the weaver ant *Oecophylla smaragdina* is present because this ant species attacks and kills foraging bees. As well as avoiding the ants, *A. dorsata* also avoided foraging on flowers that were treated with an extract *of O. smaragdina* trail pheromone; this shows that the presence of this kairomone alone is enough to prompt avoidance (Li et al 2014). The mosquito *Culiseta longiareolata* uses temporary pools as nurseries for its larvae; these are vulnerable to predation by the backswimmer *Notonecta maculata,* and it has been shown that female mosquitos avoid oviposition in the presence of hydrocarbons released by *N. maculata* (Silberbush et al 2010).

The previous example shows predator produced chemical cues being used as kairomones by a prey species, but there is also evidence of hydrocarbons being used by predators and parasite species. The European Beewolf *Philanthus triangulum* excavates its nests in sandy soil, and is parasitized by a specialist Cuckoo Wasp *Hedychrum rutilans*. During excavation of nests, the cuticular hydrocarbons of female *P. triangulum* rub off onto soil excavated from the nest. This chemical cue can then be used by *H. rutilans* to distinguish between nests of their host *P. triangulum,* and other non-host species' excavated nest material (Kroiss et al 2008). In the termite raiding ant *Pachycondyla analis,* it has been shown that hydrocarbons produced by termites and found on gallery soil are an important chemical cue which attracts *P. analis* and allows it to detect its termite prey (Yusuf et al 2014).

Ants and termites are two of the ecologically dominant groups in terrestrial ecosystems, especially in the tropics. Their interactions have been well studied with ants being considered the main predators of termites (Hollldobler & Wilson 1990).

Interactions between ants and termites are often more complex than simple predator-prey relationships. Ants and termites have been shown to cohabit and there are even examples of true mutualism between ant and termite species. In cases where there is temporary or permanent cohabitation, it tends to be ant species that take advantage of pre-existing termite structures. In these instances ants tend to occupy distinct areas of termite nests so it is reasonable to assume that there is some mechanism by which the nest space is partitioned. One way this could happen would be through deposition of cuticular hydrocarbons by walking ants. To mediate this, CHCs could be laid passively to form home-range markings. Home-range markings have been shown to have various effects on ant behaviour in foraging and conflict scenarios, so it would be interesting to find out if they can mediate interspecies relationships between two different ecologically dominant social insect groups.

Nasutitermes corniger is an arboreal termite that is endemic to the neo-tropics and is widely distributed throughout South America. The species is conspicuous because *Nasutitermes corniger* builds large nests in trees, as well as carton tunnels to provide protection to foraging termites on the surface of host trees (Figure 1). Maintenance of these tunnels forms a vital part of colony defence. Like most social insects, *N. corniger* are territorial and will aggressively defend foraging territory against intra and interspecific threats. Both workers and soldiers of *N. corniger* will react aggressively when presented with termites from another colony (Levings & Adams 1984).

Figure 4.1: Left: the termite *Nasutitermes corniger* builds large earth and wood nests (large white arrow) in trees from which protected galleries extend to other sections of the tree (small white arrow), and down to ground level. Right: protective galleries of *N. corniger* extending to ground level, being studied by Professor Jonathan Bacon and an MPhil student (me). It was the protective galleries extending to ground level that were opened in my experiments.

As well as the protection provided by carton tunnels, *Nasutitermes corniger* also have a highly specialised soldier caste known as nasutes (Figure 4.2). This caste lacks mandibles and specialises purely in colony defence. As an adaptation to their role, these nasutes have a pointed rostrum on their heads through which they squirt a sticky secretion which is produced in the cephalic gland. This secretion is made up of volatile terpenes and other high molecular weight non-volatile components which act as a glue which binds and traps potential arthropod predators. It has also been suggested that nasute defensive secretions are effective at deterring large vertebrate predators such as ant eaters, which will choose to attack foraging sites rather than potentially more rewarding nest sites because foraging sites are less vigorously defended by nasute soldiers. Ant eaters have also been shown to reject recently killed nasute soldiers while accepting workers and reproductives of the same species as food (Lubin & Montgomery 1981).

Figure 4.2: *N. corniger* nasutes on an uncovered section of carton tunnel. These individuals are approximately 3 mm in length; their black heads with pointed rostrums specialised for colony defence are clearly visible.

The aim of this study was to investigate the possible kairomone effect of ant CHCs on *N. corniger*. To do this I chose a species of ant that I found commonly on the same sites where I found colonies of *N. corniger*. For this study, I chose the ant species *Camponotus arborious* (Figure 4.3). Ants in the genus *Camponotus* are often considered opportunistic predators of termites (Holldobler & Wilson 1990), and my own personal observations confirmed this when I witnessed a *C. arborious* worker snatching a termite from a gallery that I had opened.

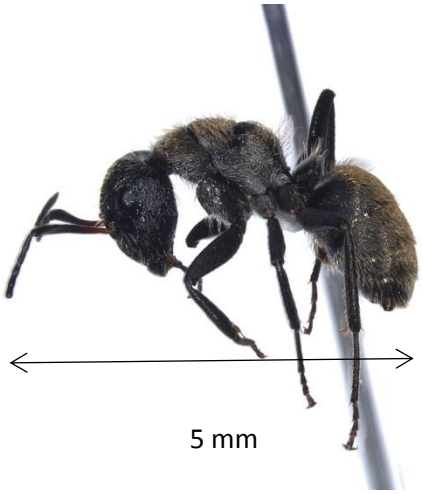

Figure 4.3: *C. arborious* worker collected from USP Riberao Preto campus, identified by Professor Fabio Nascimento.

To investigate the ability of termites to detect ant CHCs, I extracted CHCs from *C. arborious* workers and applied them to the surface of freshly opened termite carton tunnels. Under normal conditions, *N. corniger* will quickly repair any damage to carton tunnels as they form a vital part of colony defence. It was my expectation that if *N. corniger* could detect *C. arborious* CHCs, then the repairing behaviour would be affected, causing the tunnel to be blocked or to be diverted so as to avoid any perceived threat. I went on to find that indeed the presence of *C. arboriuous* CHCs could prevent termites from repairing broken carton tunnels.

4.2 Materials & Methods

4.2.1 Extraction of ant cuticular hydrocarbons

All ants were collected from the same colony of *Camponotus arboreus* on the University of Sao Paulo (USP) campus in Ribeirao Preto, Brazil, as needed. Extractions were performed in a 5 mL glass screw top vial. 10 workers were killed by freezing at -20 ˚C for 30 minutes. Cuticular hydrocarbons were then extracted by submerging 10 ants in 500 µL of hexane for 5 minutes. Hexane was used as a solvent as it is non-

polar; as such CHCs which are also non-polar dissolve easily in hexane. The hexane was then allowed to evaporate to dryness. Cuticular hydrocarbons were then re-suspended in 200 µL of clean hexane giving a CHC concentration of 1 ant equivalent per 20 µL extract. Extracts were stored in a freezer at -20 ˚C in the laboratory and transported in a polystyrene box lined with ice packs in the field.

4.2.2 Termites detecting ant cuticular hydrocarbons

Seven termite colonies located on 7 different trees on the USP Ribeirao Preto campus were used in this study. Experiments were filmed with a Sony Handycam (models HDR-CX115E and HDR-XR520). Once a suitable colony had been selected, marks 2 cm apart were made on the tree to guide the removal of the section of carton tunnel and for filming (Figure 4.4). Once these marks had been established, a video camera was set up to record the experiment. A 2 cm section of the termite carton tunnel was then removed using a razor blade. The gap in the carton tunnel was then cleared of any debris and 20 µL of *Camponotus arboreus* cuticular hydrocarbon extract or clean hexane (as a control) was evenly applied to the tree surface across the whole gap using a Gilson Pipette.

Hexane evaporated immediately after being applied to the surface of the tree leaving only the less volatile CHCs. The experiment was then filmed for 45 minutes, after which I recorded whether the tunnel had been repaired or blocked. Any attempt to rebuild the gallery was recorded as a repair. If a repair had been attempted, I measured the progress of the rebuild, from the centre of the upper and lower tunnel ends, and along each side of the gap. After the experiment, I analysed the video and counted the number and caste of termites crossing the gap in 5 minute periods during the 45 minutes after application of the CHC extract or clean hexane. Only complete crossing of the gap by termite workers or soldiers was recorded.

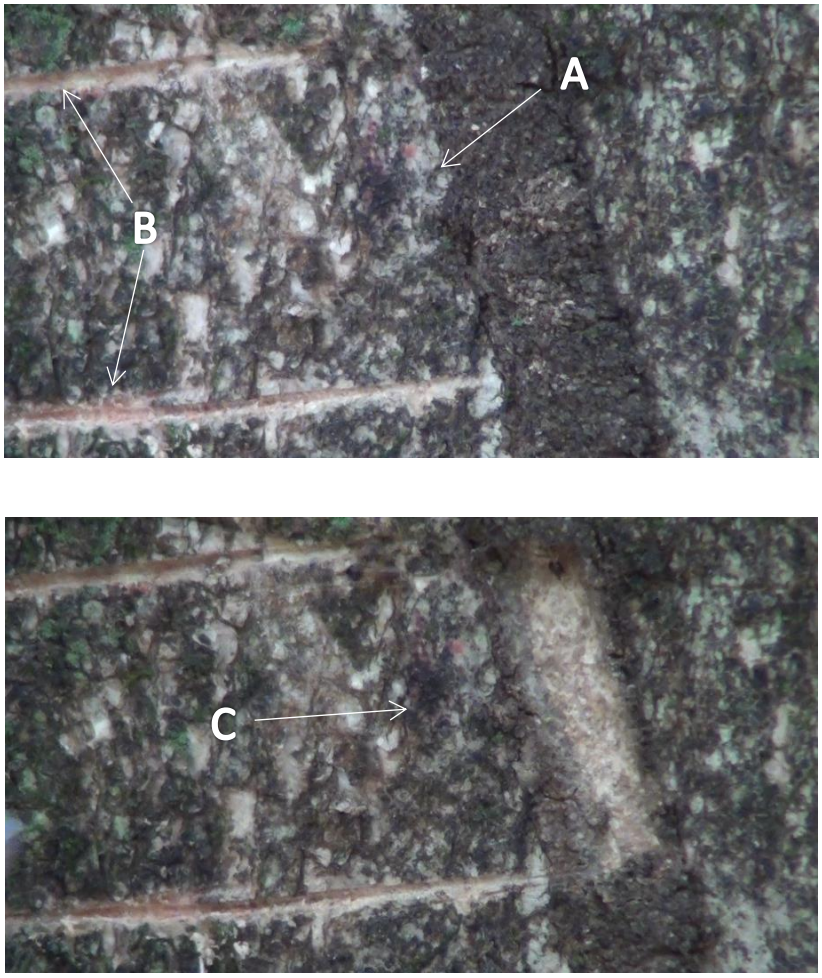

Figure 4.4: Stills taken from a video showing experimental setup.

Upper Panel, termite carton tunnel before opening (A), with guidance lines for tunnel opening 2cm apart (B).

Lower Panel, the same section of tunnel immediately after opening, ready for application of CHC extract or clean hexane control (C). CHC extract or clean hexane was applied evenly across the entire gap between tunnel ends.

4.2.3 Statistical analysis

Statistical analysis on the repair vs blocking response to CHC application was performed using a one-tailed Fishers Exact test. This is appropriate for a small sample size. For statistical analysis of termite crossing data, a Generalised Linear model with a Poisson error structure was used as these data were non-normally distributed. This test was performed using SPSS version 22.

4.3 Results

4.3.1 Preliminary Observations

Under normal conditions, the initial response to a break in the carton tunnel is that nasutes inside the nest are attracted to the damaged area. Initially nasutes will cross the broken section and use their antennae to check for any threat. If no threat is detected after a period of around 5 to 10 minutes, nasutes line up along either side of the break facing outward and more workers will start to cross the gap and repair the gallery from either end. This was the behaviour that I observed in control conditions, and it was quite different from the behaviour of the colonies that blocked up rather than repaired damaged galleries (Table 4.1).

4.3.2 Repair vs Blocking

The application of *C. arborious* CHCs has a clear effect on termites' willingness to repair damaged galleries (Table 4.1). Of the 7 sites, all colonies in the control treatment repaired the carton tunnel. In the treatment with CHCs, 4 of the 7 colonies did not repair the damaged gallery, blocking each end instead. This showed a significant difference in behavioural response (one tailed Fishers exact test $p=0.035$) to the presence of ant CHCs. In the colonies that blocked in response to the CHC treatment, nasutes were recruited to the damaged section but often did not cross the CHC

marked area. Nasutes investigated the marked area with their antennae but mostly stayed within the tunnel. Workers started to appear but rather than cross and rebuild the broken section they avoided leaving the cover of the tunnel and blocked off the damaged end of the gallery. The colonies that did repair the tunnel in CHC treatment conditions showed a range of behaviour. In one of the colonies, there did appear to be some suppression of nasute and worker crossing, whereas the two other colonies did not appear to react any differently from the control. Unfortunately the small sample size meant that analysing these different reactions to the CHC treatment was not possible.

	Repair	Block
control	7	0
treatment	3	4

Table 4.1: The application of ant CHCs prevents some termite colonies from repairing damaged carton tunnels. In the treatment, 4 of the 7 colonies blocked up damaged carton tunnels which contrasts significantly (p=0.035 (one tailed Fishers Exact test)) with the control trials in which all colonies repaired damaged carton tunnels.

4.3.3 Application of *C. arborious* CHCs suppresses the number of termites crossing a damaged section of carton tunnel

To further examine the behavioural response of termites to ant CHCs, I analysed the number and caste of termites crossing the gap opened in the carton tunnel. Previous unpublished observations by our group have shown that in normal conditions termites will repair any breaks in the tunnel. These repairs are characterised initially by a large number of nasutes responding to the break in the tunnel, followed by workers attending the break, increasing over time as more are recruited to repair the damage.

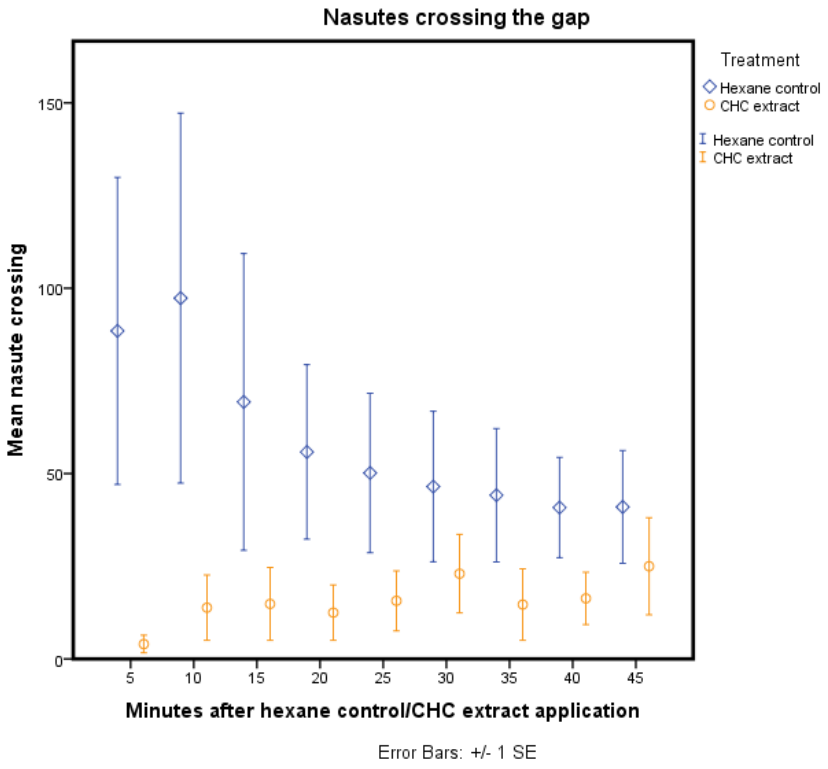

Figure 4.5: the number of nasutes crossing the gap in each 5 minute interval of my experiment; the application of ant CHCs suppresses the number of nasutes crossing the gap in the treatment (orange) relative to the control (blue). Data points represent the mean number of crossings per 5 minute interval, error bars show +/- 1SE.

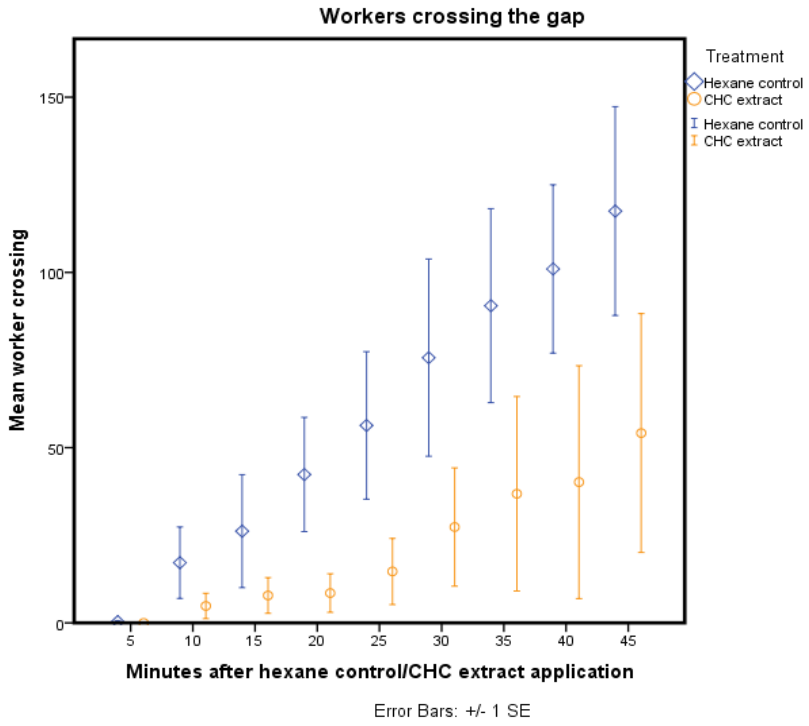

Figure 4.6: number of workers crossing the gap in each 5 minute interval of my experiment, showing that as time passes the number of workers crossing the gap increases but that this number is suppressed in treatment conditions (orange) relative to control conditions (blue). Data points represent the mean number of crossings per 5 minute interval, error bars show +/- 1SE.

The application of *C. arborious* CHCs to a damaged section of carton tunnel significantly reduced the number of *N. corniger* nasutes (X^2 (1) = 1192.5, p =<0.001) and workers (X^2 (1) =847.8, p =<0.001) crossing the damaged section of carton tunnel during the 45 minute period of observation (Figures 4.5 & 4.6). In control conditions, in all the colonies, the response of nasutes to a break in the carton tunnel results in high numbers of nasutes crossing in the first five minutes. This increases slightly between 5 and 10 minutes and then the number of nasutes crossing starts to decrease as nasutes take up guard positions rather than crossing the gap. Nasute crossing then flattens out during the final minutes of observation. In the CHC treatment, the pattern

of the response is quite different. The application of ant CHCs suppresses the number of nasutes crossing the gap, especially in the first 10 minutes of observation. After the first 10 minutes, there is a slow increase in the number of nasutes crossing; however the number of nasutes crossing the gap in treatment conditions never reaches even the lowest level of nasute crossing for the control conditions. In the control and in the CHC treatment, workers do not cross the gap during the first five minutes of observation. In both the control and the CHC treatment workers numbers then steadily increase during each 5 minute period of observation but in the treatment conditions the number of crossings by workers is lower and the rate of increase is slower, especially for the first 30 minutes of observation. The main effect of the application of ant CHCs is to initially delay, and then supress the number of *N. corniger* workers responding to the damaged carton tunnel.

4.3.4 Proportion of nasutes crossing to total termite crossing

A response that could indicate the detection of predatory ant CHCs would be an increased proportion of nasutes crossing the break in the tunnel, as their defensive role in the colony is to attack predators with sticky secretions fired from their specialised rostra.

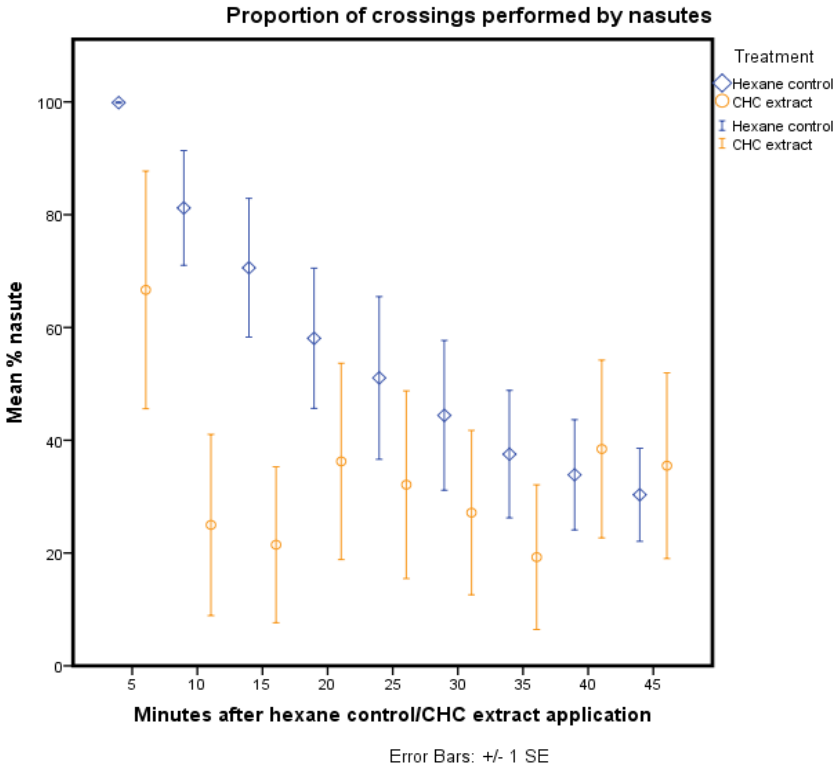

Figure 4.7: the proportion of termites attending the gap in the tunnel that are nasutes, show that in treatment (orange) the proportion of nasutes is initially lower than in control conditions (blue).

Figure 7 shows the proportion of nasutes crossing over time. This is significantly different when comparing the control and the CHC treatment (X^2 (1) = 801.5, p =<0.001). In the control the proportion of termite crossings performed by nasutes starts at 100% as the colony reacts to the opening of the gallery. We then see a steady decrease in the proportion of nasutes crossing as more workers are recruited to repair the damaged carton tunnel and nasutes take up stationary positions guarding the repair. In the CHC treatment, we actually see a lower proportion of nasutes crossing.

4.4 Discussion

These results demonstrate that *Nasutitermes corniger* are capable of detecting the cuticular hydrocarbons of a predatory ant species *Camponotus arborious*. This resulted in four of the seven colonies tested responding to the application of *C. arborious* CHCs by blocking up damaged carton tunnels, in contrast to all seven colonies repairing damaged tunnels in the control. It was also observed that across the whole data set, the application of ant CHCs suppressed the number of both workers and nasutes that crossed the gap made in the carton tunnel during the experiment, but this result was influenced by the fact that those colonies that blocked rather than repaired made far fewer termite crossings. A greater number of trials would have allowed me to examine the effect of ant CHCs on those colonies that did manage to repair their damaged galleries in CHC treatment conditions, and to see if termite crossing was suppressed in those cases.

These results can be interpreted as a response to a perceived threat, and this could be described as a form of chemical eavesdropping that would allow vulnerable termites to build their vital colony defence structures in such a way as to avoid areas frequented by potential predators.

I had expected the application of *C. arborious* CHCs to negatively impact the gallery repairing behaviour of *N. corniger*, but was surprised that the proportion of nasutes crossing the damaged tunnel was initially lower after the application of CHCs compared to the control. The sole role of nasutes within the colony is to provide defence against predators so it was unexpected that the presence of ant CHCs would result in a lower proportion of nasutes crossing than in the control. I had expected to see an increase in the proportion of nasutes if the behaviour recorded was a defensive response to a threat. However, the first thing to note when examining this result is

that the largest divergence in the proportion of nasutes crossing the gap in control and treatment conditions occurs early on in the 45 minute experiment. As this period sees the fewest termite crossings generally, the proportion of each caste crossing the gap can be greatly influenced by a small number of crossings from the other caste. It is also possible that a lower proportion of nasutes cross the gap in treatment conditions due to nasutes being more sensitive to perceived threat than workers. It has been shown in other social insect groups that sensory ability can be linked to caste (Mysore et al 2010). If nasutes are more sensitive to threats than workers it could make them less likely to cross the gap, and more likely to take up a guarding position at the tunnel break, rather than exhibit normal behaviour as seen in control conditions. This combination of a lower number of crossings in general, and potential greater threat sensitivity of nasutes, may account for a lower proportion of nasutes crossing in treatment conditions. This unexpected result does not however change the overall conclusion of this study.

Another piece of evidence that ant CHCs could be detected and interpreted as a threat was some personal observations of behaviour that was uncommon, but quite striking. At one site, the application of ant CHCs to the surface of the tree resulted in a nasute attacking the area marked with CHCs with its defensive secretion (Figure 4.8). As this was simply a piece of bark, no different to any other, except for the presence of the applied CHCs, it seems reasonable to conclude that it was the presence of the CHCs that prompted this response. If this was the case then it would strongly support the hypothesis that termites could detect ant CHCs and could interpret their presence as a threat.

Figure 4.8: Trigger happy termite. The red arrow shows a termite attacking an area of bark marked with ant CHCs, just visible is the white secretion itself (white arrow) as it is fired at, and sticks to the surface of the tree.

As this experiment was undertaken as field work during a relatively short time period of two weeks, there are many areas that I would investigate further if time constraints were not a factor. The first thing I would do to improve this experiment would be to increase the sample size. This would give me a better idea of the response of termites to ant CHCs and make any results far more statistically robust. I would add an extra control to my experiment by running each site in the same assay but without applying anything to the gap between each end of the gallery. My suspicion is that there would be no difference between the response to clean hexane application and zero application, as once applied the hexane visibly evaporated within 2 to 3 seconds. However, this extra control would add greater robustness to my results.

Further experiments would increase the scope of my investigation. The first thing would be to investigate the persistence of the CHC effect over time. Cuticular hydrocarbons in ants are predominantly made up of long-chain hydrocarbons of a

chain length between C_{19} and C_{33} (Martin & Drijfhout 2009). These long chain hydrocarbons are non-volatile and as such should persist in the environment, but how long do these effects of the CHCs last?

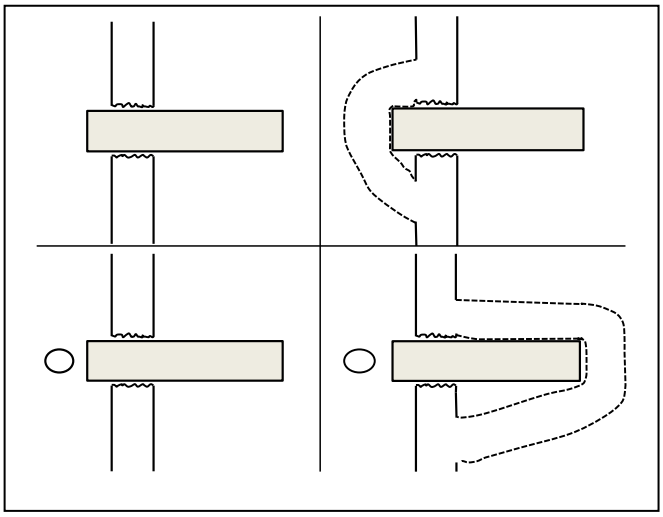

Figure 4.9: The modified experimental set-up. Top left: by placing clean masking tape between the two ends of a broken termite gallery, termites can be forced to repair around the obstacle, generally the repair will be around the shorter route (top right). In my improved experiment some CHC extract could be applied to the surface of the tree next to the gallery, the position of this deposit is shown here by the circle in this diagram. This could be applied at any time interval *before* the gallery is opened. Once the predetermined time interval has elapsed, the gallery is opened and clean masking tape placed so that the shorter route is marked with CHC extract (bottom left). If the termites then repaired around the longer side of the tape obstruction (bottom right), I could say that the CHC extract is being avoided. The same would be true if the gallery was blocked and no repair attempted.

When breaking open a termite carton tunnel, it is possible to force the termites to rebuild around an obstacle, in this case some white masking tape. If this tape is placed in the gap between the two ends of the opened carton tunnel, the termites will repair around the edge of the tape rather than across it, and if the tape is placed across the gap asymmetrically then the termites will generally repair along the shorter route which avoids going directly across the tape (Figures 4.9 & 4.10).

In my modified experiment, the CHC extract would be applied to the surface of the tree, adjacent to where I planned to open the carton tunnel. When I then open the carton tunnel, I would place the tape obstacle in such a way that the shorter route around the obstacle was the side previously marked with CHC extract. If termites repaired over the longer route, or didn't repair at all, I would conclude that they were avoiding the ant CHCs. As this protocol would allow me to apply the extract before opening the tunnel, I would be able to leave different amounts of time between applying the extract and opening the tunnel e.g. 1, 2, 4, 6 hours, which in turn would give me an idea of how long any effect of the CHCs persisted.

Figure 4.10: Photograph showing a potential way of investigating the persistence of CHCs in the environment. Normally termites will not walk over the tape, but will generally build a repair around the shorter side of the obstruction, though the stochastic nature of decision-making in choosing a side means that some repairs will take place around the longer route, as can be seen in the repair lower down in this photograph. This experiment, by my supervisor Professor Jonathan Bacon, was done on a farm building wall.

The protocol described above could also be used when examining other factors. By changing the concentration of the CHC extract, I could determine how sensitive termites are to ant hydrocarbons. I could also use this set up to investigate the reaction of termites to different ant species. *C. corniger* has been shown to share nest

space with the ant *Crematogaster brevispinosa rochai* (Quinet et al 2005). How would the termites react to a CHC extract from this species compared to one of a more predatory species? Presumably termites would be more likely to avoid areas marked with predatory ant CHCs than those areas marked with non-predatory ant CHCs. An ability to differentiate between more and less threatening signals would provide important information to avoid using the extra time and resources that a longer repair would require.

Finally, the result of this experiment, namely that *N. corniger* gallery-repair behaviour is disrupted by the presence of predatory ant CHCs, has one major potential application. Termites are a major pest globally; in fact one estimate from 2005 puts the global cost of termite damage at US$50 billion (Korb 2007). *Nasutitermes* species are a major pest in Brazil, causing 50% of all insect related structural damage, with *N. corniger* being the most important (Constantino 2002). Current pesticides are becoming less effective as insects evolve resistance to them (Deletre et al 2016), and as such novel ways of combatting pests, such as utilising CHCs from their natural predators could be an effective method of pest control. One method of using predator CHCs to protect from structural damage would be to suspend CHCs in another liquid, which could then be applied directly onto the surface in need of protection, using only a brush or paint roller. There are already a number of products available which use borates suspended in liquid to protect against termite damage, so it is reasonable to assume that this would be a viable way of utilising predator CHCs in pest control.

5. Final Discussion and New Directions

This thesis describes my attempts to investigate some of the vital components of successful social-insect societies, namely, hygienic behaviour, navigation, and the ability of social insects to read chemical cues in the environment to reduce the risk of predation.

In Chapter 2, I found that the antimicrobial agent micromolide is present on the cuticle of *Lasius flavus,* and that it could also be found deposited on a substrate that had been walked on by workers of this species. This chapter perhaps has the greatest scope of any of my studies for future work.

Antibiotic resistant bacteria are a widespread and increasing problem (Levy 2005, French 2010). Given this, novel antibiotic compounds are increasingly in demand, and compounds produced by social insects, or by bacteria that have formed exosymbiotic relationships with them, provide a possible avenue for the discovery of important new antibiotics. Termites, for example, build their nests, and their vulnerable brood incubation and nursery areas from clays, some of which have been shown to host exosymbiotic *Streptomyces* bacteria that help protect nest material from infestation with pathogens (Chouvenc et al 2013, Zhang et al 2013). These insect-earth bacteria are already envisaged as a valuable source of novel natural products of pharmaceutical interest (Choi & Oh 2015), and compounds produced directly by social insects are another possible source, micromolide (Bergström & Lofqvist 1970) being one of them.

If I were to extend the study presented in Chapter 2, I would first repeat, with a greater number of samples, my examination of the distribution of micromolide across the cuticle of the ants. I found no significant difference in concentrations on different

body parts of *L. flavus* workers, but as I mentioned in my discussion of that chapter, I suspect that was due to the small sample size in my experiment. Next I would attempt to extract micromolide directly from *L. flavus* nest material. Finding how this could be done without sampling too much soil may prove difficult. *L. flavus* nest mounds are large and contain a lot of soil. The tunnels and chambers of the nest probably have a small surface area relative to the total size of the nest, so only a small amount of the soil in the nest will have been walked on by micromolide depositing ants. Boots et al (2012) used a narrow corer to take small samples of soil from *L. flavus* nests for an analysis of nest soil microbial assemblages, which may be one way of avoiding excessive excavation of soil which might make detection of micromolide difficult. Finally I would determine that micromolide, derived from *L. flavus,* is an efficacious antibiotic using a bacterial growth assay. Organisms such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus sp.,* and *Staphylococcus aureus,* which are commonly associated with human infections, can be ordered as reference strains from the Public Health England NCTC collection. Assays would be culture based, with strains grown at different concentrations, and exposed to micromolide at different concentrations to assess the presence of any antibacterial activity, and determine the Minimum Inhibitory Concentration (MIC) of micromolide. Research into insect produced antibiotics represents an exciting opportunity to discover novel antibiotic compounds that could become increasingly more important in the fight against antibiotic resistant disease.

In Chapter 3 of this thesis I found that the method of food discovery affects the number of errors made by ants returning from the food to the nest. This was, at least for me an unexpected and interesting finding, and I want to repeat the experiment with a different species, such as *Lasius niger*, *Myrmica rubra* or *Monomorium pharaonis*, to determine the generality of this phenomenon.

For example *L, niger* is an above-ground foraging species, and so the environment in which *L. niger* workers forage is very different to the environment in which *L. flavus* workers forage. Previous work has shown that these species differ in their navigational strategy when using social and private cues. Grüter et al (2011) showed that when facing a conflict of social and private information (trail pheromone vs route memory), *L. niger* favours private route memory. The same study also found that increasing trail pheromone strength did not increase the chance of a naïve forager choosing to follow that pheromone trail. Jones et al (in preparation) repeated this study with *L. flavus*, and found that *L. flavus* were significantly less likely to choose their own private route memory over trail pheromone, and that increasing the strength of trail pheromone did significantly increase trail pheromone following behaviour in naïve foragers. These results provide evidence that *L. niger* and *L. flavus* possess differing navigational strategies that reflects their ecology. By repeating the experiments presented in Chapter 3 with *L. niger,* I could ascertain whether the method of food discovery influences memory formation, in a species that favours visual cues over chemical cues, in the same way as it does in *L. flavus.*

I would also be interested to revisit the question of how *L. flavus* navigates underground. To replicate the darkness of an underground ant nest, the experiments could be performed under red light as Hymenoptera are not capable of seeing light of this wavelength (Pietsch et al 1992). I would modify this experiment in a few ways, for example the maze would be simplified to a T-maze, I would allow ants to visit the feeder more times with a pheromone trail as guidance to and from the nest before testing, and I would increase the strength of the home-range markings provided as a guide in the absence of trail pheromone. I would also add a treatment that would provide home-range markings to guide ants towards the feeder, rather than only supplying home-range markings for ants returning to the nest.

Chapter 4 of this thesis investigated the possibility of ant CHCs acting as a kairomone when detected by termites repairing a damaged tunnel. I found that the presence of ant CHCs did affect rebuilding behaviour in termites, causing colonies to block damaged tunnels rather than repair them.

The project presented in Chapter 4 took place during field work in Brazil, and the biggest limiting factor on this study was the time available (two weeks). The first issue to address is the sample size used, and as such increasing the number of colonies sampled would greatly improve the interpretation of the experimental results. As already outlined in the discussion in Chapter 4, I would modify this experiment in a number of ways. By applying ant CHCs to the surface of the tree at varying time intervals before opening the tunnel, and then subsequently forcing termites to repair around an obstacle which directed the termites over the CHC marked area, I could examine how long these CHCs persist in the environment. By changing the species of ant CHCs used, I could see whether termites can distinguish between predatory and non-predatory ant CHCs. I could also apply CHCs from other termite colonies. *Nasutitermes corniger* is territorial, and will fight non-nestmates when confronted by them (Levings & Adams 1984). Would CHCs from non-nestmate conspecifics elicit the same response as those from ant predators? Or would this lead to a more aggressive reaction? By investigating possible ways of disrupting termite nest and tunnel building, I might be able to shed light on a possible method for controlling the behaviour of a major pest species.

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