Chemical Based Communication and its Role in Decision Making Within the Social Insects

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This thesis, whether in the same or different form, has not been previously submitted to this or any other University for a degree

Abstract

This thesis investigates chemical communication and decision making in a stingless bee (*Tetragonisca angustula*) and two species of ants (*Lasius flavus* and *L. niger*). Complex chemical signalling and seemingly elaborate behavioural patterns based upon decisions made by individuals of a colony have facilitated the evolution of social living in these insects. This thesis investigates two important features of social living that involve these features: nest mate recognition and navigation. The first part of this thesis (Chapter 3 and Appendix 3) investigates nestmate recognition and nest defence in the Neotropical stingless bee *T. angustula*. In Chapter 3, two mechanisms are investigated which could potentially facilitate the extremely efficient nest mate recognition system, previously demonstrated in this bee species. Both are found to play no role which will enable further work to focus on the few remaining possibilities.

The second part of this thesis (chapters 4-6) focuses on navigational decision making in two common British ant species with contrasting ecologies. Chapter 4 investigates how *L. niger* foragers adapt to foraging at night when the visual cues, so important to these ants for diurnal foraging, are unavailable. This study showed that nocturnal foraging is achieved in these ants by increasing trail pheromone deposition while concomitantly switching to a greater reliance on these cues to navigate. Chapter 5 contrasts the navigational strategies and capabilities of *L. niger* with another *Lasius* ant species, *L. flavus,* and demonstrates how these species can flexibly switch dependency between available navigational cues to cope with foraging within a fluxional ecological environment. Finally, Chapter 6 focuses on the glandular components and trail pheromone of *L. flavus* by measuring behavioural responses to glandular constituents

and identifying the glandular source of the trail pheromone. The aim was to also identify the trail pheromone(s) but due to time constraints this was not possible. However, a new methodology that simplifies the process of identifying trail pheromone components was developed and is described. Furthermore, this study has laid the foundations for further work to establish if the compound prevalent in the Dufour glands' of *L. flavus* does indeed serve as an antibacterial agent within the humid nest environment.

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Publications

Publications and manuscripts that arose from this thesis

Jones, S.M., Zweden, J.S. van, Grüter, C., Menezes, C., Alves, D.A., Nunes-Silva, P., Czaczkes, T., Imperatriz-Fonseca, V.L. & Ratnieks, F.L.W. (2012) The role of wax and resin in the nestmate recognition system of a stingless bee, *Tetragonisca angustula*. *Behavioral Ecology and Sociobiology*, **66**, 1–12. **(Chapter 3)**

Van Zweden, J.S., Grüter, C., Jones, S.M. & Ratnieks, F.L.W. (2011) Hovering guards of the stingless bee *Tetragonisca angustula* increase colony defensive perimeter as shown by intra- and inter-specific comparisons. *Behavioral Ecology and Sociobiology*, **65**, 1277–1282 **(Appendix 4)**

Jones, S.M., Gallagher, A., Gourlay, E. & Bacon, J.P. (to be submitted) Sensory information use and trail laying in the ant *Lasius niger* is determined by light intensity **(Chapter 4)**

Jones, S.M., Gallagher A. & Bacon, J.P (to be submitted) Navigational decision making in two congeneric ant species is directly related to their contrasting ecological environments. **(Chapter 5)**

Publications and manuscripts from work not included in this thesis

Czaczkes, T.J., Grüter, C., Jones, S.M. & Ratnieks, F.L.W. (2011) Synergy between social and private information increases foraging efficiency in ants. *Biology Letters*.

Czaczkes, T.J., Grüter, C., Jones, S.M. & Ratnieks, F.L.W. (2012) Uncovering the complexity of ant foraging trails. *Communicative & Integrative Biology*, **5**, 78–80.

Grüter, C., Schürch, R., Czaczkes, T.J., Taylor, K., Durance, T., Jones, S.M. & Ratnieks, F.L.W. (2012) Negative Feedback Enables Fast and Flexible Collective Decision-Making in Ants. *PLoS ONE*, **7**, e44501.

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1

Background

This chapter presents the relevant background information which gave rise to the experimental studies that are included in this thesis.

1.1 Eusocial Insects

Within the insects, eusociality is only displayed in five orders of insects: the Hymenoptera (all ants except some parasitic species, some bees and wasps), Isoptera (all termites), Hemiptera (43 aphid species; see for e.g. Aoki 1982), Coleoptera (a bark beetle; Kent & Simpson 1992) and the Thysanoptera (6 thrip species; see for e.g. Crespi 1992). Eusociality is most prevalent within the Isoptera and Hymenoptera and it is members of the Hymenoptera that are the subjects of this thesis. Truly eusocial organisms exhibit three important traits: cooperative brood care, an overlap of generations contributing to colony labour with offspring serving parents for some part of their life, and reproductive division of labour involving sterile or near sterile individuals working on behalf of other fecund individuals (Wilson 1971). Within the Hymenoptera eusociality has evolved independently nine times (Hughes *et al.* 2008), and despite a controversial paper by Nowak et al. (2010) most evolutionary biologists agree that high relatedness was essential for its evolution (Hughes *et al.* 2008; Abbot *et al.* 2011; Boomsma *et al.* 2011).

But why study social insects? Their immense success is certainly one important reason. Two particularly useful surrogates of ecological success are species richness and biomass (Strassmann & Queller 2007). Ants are highly speciose with over 12,500 species identified to date (Agosti & Johnson 2005; Bolton *et al.* 2006). While ants are omnipresent and well represented in temperate regions, it is the species richness and total biomass measured in the tropics that is most illuminating. Ants alone comprised 94 % of all arthropod species and 86 % of the total biomass taken from fogging samples in tropical rainforests (Davidson 1997; Davidson *et al.* 2003) and as many as 61 ant species have been identified from a single tree in Borneo (Floren & Linsenmair 2000).

Equally as important as their evolutionary success is the role social insects play within their ecological environment. Social insects carry out a range of important ecological services which include amongst others predation, pollination, seed dispersal, soil aeration and decomposition. Their importance as flower pollinators is governed by the large numbers of visiting individuals rather than actual numbers of species. Of all the bees counted visiting flowers within three habitats in Costa Rica eusocial bees only comprised a mere 5 % of all species recorded but almost 50% of all visiting individuals (Heithaus 1979). The role ants play as seed dispersers is often underestimated; the seeds of 35 % of all herbaceous plants are estimated to be dispersed by ants (Beattie 1985) while in the South Eastern United States, ants are ranked as one of the principal granivores (Davidson, Brown & Inouye 1980).

Another fascinating behaviour exhibited by social insects, thereby making them very attractive study organisms, is the group-like behaviour or colony response such as hive

thermoregulation in bees or mound construction in ants, in which individuals appear to work together to achieve a common goal. The colony response that we see is a result of self-organisation. That is, the pattern we see at the colony level is a property that emerges from actions by a vast number of individuals responding to local information. In this way a complex emergent pattern can arise at the colony level that is purely a result of simple behavioural rules followed by individual ants. This gave rise to the term 'superorganism', a concept first coined by Wheeler (1911) in the early twentieth century. After losing favour within the scientific community for nearly four decades the concept has rapidly returned to prominence in recent years (Hölldobler & Wilson 2009).

1.2. Decision making in Social Insects

In this thesis I focus on decision making made by individuals and how they are shaped by the local information that is available to them, rather than on any collective response that may or may not result from these decisions. Many decisions that a social insect makes may either influence or be influenced by decisions that are made by other colony members (Sumpter 2010). For example, the decision by a foraging ant to deposit a pheromone trail on finding a rewarding food source may guide subsequent naive foragers to the same location. If these guided ants also deposit pheromone on their way back to the nest a stronger trail is established, resulting in a higher probability that other ants will follow this trail (Beckers, Deneubourg & Goss 1993). What emerges from this positive feedback mechanism is the majority of ants following this reinforced trail, as a result of the amplification of an initially small bias (Sumpter & Beekman 2003; Sumpter 2010). Thus, at the root of pheromone trail development is a

decision or decisions made by a single or small group of ants, solely based upon local information, such as the quality of a food source or the complexity or length of a route.

However, many decisions made by social insects are not influenced by the decisions of others. Furthermore, these decisions do not always elicit a positive feedback mechanism, a requirement necessary for the establishment of a collective response. One such example that I investigate in Chapter 3 is guarding behaviour at the nest entrance. A more detailed account of this behaviour is given below in section 2.3 but suffice to say a guard's decision on whether to accept or reject an individual is not influenced by the decisions of other guards and the decision it makes does not result in a collective response. That does not mean that this decision is any less important; for example, in bees robbing by conspecifics can be very harmful to the colony (Downs & Ratnieks 2000; Couvillon *et al.* 2008).

It is decisions made at the individual level and how they are affected by various local cues that I focus on in this thesis. In the first part of this thesis I investigate nestmate recognition (Chapter 3) and colony defence in the Neotropical stingless bee *Tetragonisca angustula*. In the second part I then switch to studying how local cues affect the decision making behaviour in navigating ants (Chapters 4 and 5) and finally in the third section I concentrate on one important local cue used by the yellow meadow ant *Lasius flavus* during navigation, namely its trail pheromone. In this chapter (6) I analyse some of the behavioural properties of the pheromonal components and begin the technically complex process of identifying the key compounds that elicit the trail following response in this ant.

1.3. Nestmate Recognition and Nest Defence in Social Insects

A variety of recognition systems are employed across the animal kingdom which include the recognition of kin from non-kin (Cheney & Seyfarth 1982; Carlin & Hölldobler 1986; Fletcher & Michener 1987), recognition of potential mates (Higgie, Chenoweth & Blows 2000; Ryan *et al.* 2003) and the ability to determine friend from foe (Kelley & Magurran 2003; Akira, Uematsu & Takeuchi 2006; Guerrieri *et al.* 2009). Incorrect recognition decisions are likely to incur fitness costs (Sherman, Reeve & Pfennig 1997; Keller 1997) and this is most apparent when we consider the importance of distinguishing between intruders and nestmates in social insects.

Social insect colonies are prone to robbing of brood (Stamps & Terrell 1991; Foitzik *et al.* 2001), food stores (Hölldobler 1986; Wittmann *et al.* 1990), nest material (Corbara & Dejean 2002) and parasitism (Roubik 1989; Wittmann *et al.* 1990; Lenoir *et al.* 2001) by both conspecific and allospecific intruders, which in some cases can result in the loss of the colony (Downs & Ratnieks 2000). In order to reduce these harmful occurrences, entrances to the nest are guarded, usually by specific guard worker castes or subcastes. This is most impressively demonstrated in the ants and termites where morphologically distinct guard castes have evolved solely for the purpose of protecting the colony. For example, guards of the ant *Pheidole morrisi* possess buttress shaped heads that close the entrance hole to the nest (Gregg 1942), termite soldiers of the sub-family Nasutitermitinae have nozzle shaped heads that can fire a sticky liquid terpenoid polymer (Prestwich 1982), and guards of many species of ants and termites possess large armoured heads with strong muscular mandibles (Hare 1937; Hölldobler & Wilson 1990). Until recently, morphologically specialised worker castes were thought not to exist within the social bees and wasps. However, a recent study by

Grüter et al (2012) clearly demonstrated that guards of the stingless bee *Tetragonisca angustula* are significantly larger and have better developed legs than the foragers, thus giving them a grappling advantage when defending the nest against often larger intruders.

It is the guard's task to decide if the chemical odour profile carried on the cuticle of an incoming individual at the entrance, which we call the label, closely resembles its own learned neural representation of the odour of its own colony, which we call the template (van Zweden & d' Ettorre 2010). The guard achieves this by antennating (ants and bees) and licking (bees only) the cuticle of the incomer. If the guard deems that the label of an incomer satisfactorily resembles the template then the individual is accepted and permitted to enter the nest, otherwise it is aggressively rejected (Hölldobler & Michener 1980). The template which the guard uses as a reference reflects the odour of the colony and appears to be constantly updated as the colony odour changes (Buckle & Greenberg 1981, Couvillon *et al.* 2007).

The hydrocarbons present on the cuticle of social insects, whose primary role is to prevent dehydration, appear to serve a secondary role as cues for recognition (Howard & Blomquist 1982). These genetically derived compounds can be used by guards to discriminate degrees of genetic relatedness, a process essential for kin recognition within the colony (Greenberg 1979, Greenberg & Grafen 1990). For example, in the sweat bee, *Lasioglossum zephyrum* acceptance rates of sister bees are significantly greater than acceptance rates of aunts or nieces, which are in turn greater than those for cousins (Greenberg 1979). When these hydrocarbons are supplemented with compounds from the nest environment an encompassing colony odour is achieved

through homogenisation of these cues across the colony inhabitants. In bees and wasps the source of this odour blend is the wax comb or paper nest (Gamboa *et al.* 1986; Breed *et al.* 1995), and in most ant species it is the post-pharyngeal gland (PPG)(Soroker *et al.* 1994). Contact with the wax combs in the nest environment ensures honey bees acquire an odour that originates both from genetically derived hydrocarbons within the wax and prominent environmentally derived compounds also present (Breed *et al.* 1995). In ants, compounds detected via antennation with other colony members are mixed with glandular components in the PPG and these contents are sporadically smeared over the cuticle, providing a homogenised Gestalt or colony odour (Soroker *et al.* 1994). The important hydrocarbons present on the cuticle of insects that serve as recognition cues tend to be a wide variety of branched alkanes (van Zweden & d' Ettorre 2010), but fatty acids and alkenes have also been shown to play a role in honey bees (Breed 1998; Dani *et al.* 2005).

There is always likely to be some overlap between the chemical profiles of different colonies of the same species, particularly as the differences are mostly quantitative variation between the same set of chemical compounds, and this therefore increases the recognition challenge for a guard. As a result of this, recognition errors occur where either non-nestmates are accepted (acceptance errors) or nestmates are rejected (rejection errors). Figure 1.1 below shows how this can occur.

This process becomes a little more complicated when we consider that the threshold of similarity which the label of an incoming individual must meet with the guard's template is dynamic. That is, as certain conditions change the guards adapt by either increasing or decreasing this threshold. This idea was first proposed by Reeve (1989) in

which he suggested that the acceptance threshold dynamically shifts depending on the costs of accepting non-nestmates or rejecting nestmates and also on the frequency that these interactions occur. Evidence supporting these ideas has since been found in honey bees and ants. In honey bees accepting non-nestmates into the nest is less costly when nectar is abundant and food stores are plentiful but during late summer a scarcity of nectar results in increased robbing (Seeley 1985) and the costs of making acceptance errors rise (Downs & Ratnieks 2000). Colonies counteract this by increasing

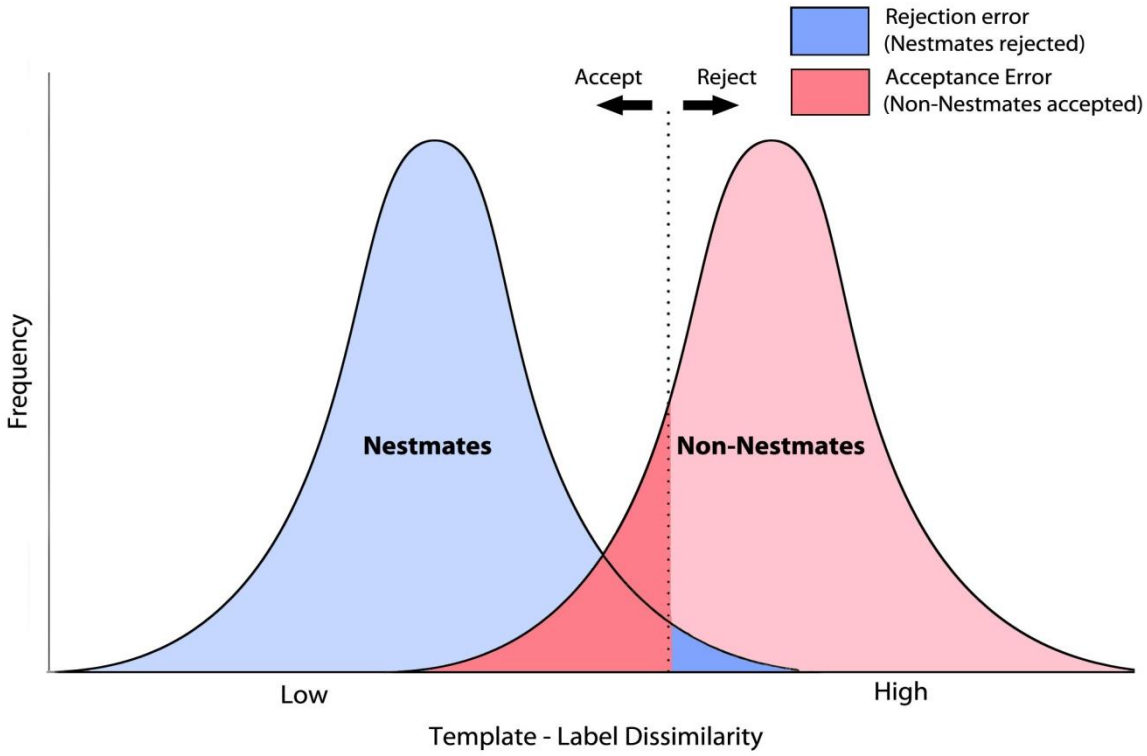


Figure 1.1. Acceptance threshold model showing that when the odour profile of non-nestmates overlaps that of a guard's template, recognition errors occur. The ratio of acceptance errors to rejection errors depends upon the permissiveness of the threshold (shown as the dotted line)(Reeve 1989).

the number of guards stationed at the nest entrance (Couvillon *et al.* 2008) and more importantly by reducing the acceptance threshold so that acceptance errors are reduced, at the cost of an increase in rejection errors (Downs & Ratnieks 2000). Increasing the numbers of guards at a nest entrance improves the decision making process because there are then multiple opportunities for guards to make the correct

decision, a further example of collective decsio making (Johnson *et al.* 2011). Evidence supporting the idea that guards will shift an acceptance threshold in response to the frequency of interactions with non-nestmates has been found in both *Apis mellifera* and the ant *Formica rufibarbis*. In both cases an increase in the frequency of interactions with non-nestmates resulted in a reduction in the acceptance threshold to minimise acceptance errors (D' Ettorre *et al.* 2004; Couvillon *et al.* 2008). Similarly, an adaptive shift in tolerance also occurs in response to next invasion. When colonies of *Melipona panamica* are artificially disturbed, to simulate an attack by a cleptoparasite such as *Lestrimelitta limao*, the tolerance of introduced callow workers significantly drops with greater numbers being rejected and/or killed (Inoue *et al.* 1999).

1.3.1. Nestmate Recognition & Nest Defence in Tetragonisca angustula (Chapter 3)

The Neotropical stingless bee *Tetragonisca angustula,* local name Jataí*,* is a common species whose range stretches from Veracruz in Mexico down to Misiones in Argentina (Michener 1974). It is a relatively small (4-5 mm) yellow/brown bee living in colonies of up to 10,000 bees (van Veen & Sommeijer 2000). *T. angustula* is a particularly attractive species for the study of nestmate recognition and nest defence. This is because its nestmate recognition capabilities are particularly proficient when compared with the honey bee and other studied stingless bees and also because it possesses a unique complement of two types of entrance guard: standing and hovering.

In a study by Kãrcher and Ratnieks (2009), the overall recognition error rate (acceptance and rejection errors combined) made by guards of *T.angustula* was only 8 %. This strongly contrasts with 52 % found for *A. mellifera* (mean taken from following studies: Breed 1983; Downs & Ratnieks 1999, 2000; Couvillon *et al.* 2007, 2008;

Couvillon, Roy & Ratnieks 2009; Couvillon *et al.* 2010) and 26 – 86 % found for five other stingless bees (Breed & Page 1991; Buchwald & Breed 2005; Couvillon & Ratnieks 2008; see Johnson *et al.* 2011 for a review of acceptance errors in social insects). Knowing that the recognition system of *T. angustula* is particularly proficient, we sought to understand how this might be achieved in Chapter 3.

Tetragonisca angustula is the only social bee yet discovered that possesses two types of entrance guard (Grüter, Kärcher & Ratnieks 2011b). Like other stingless bees and cavity nesting honey bees there are standing guards that patrol the entrance tube and check incoming individuals (Michener 1946; Kärcher & Ratnieks 2009), but in addition to these there are hovering guards that hover in front and to the sides of the nest (Wittmann 1985; Grüter *et al.* 2011b). The hovering guards are able to efficiently recognise bees of a different colour (many robber bees are black; Bowden, Garry & Breed 1994) and the standing guards use chemoreception to discriminate non-nestmate conspecifics from nestmates at the entrance (Kärcher & Ratnieks 2009). Hovering guards may also be able to utilise chemical cues to discriminate nestmates but this has yet to be investigated thoroughly.

Wittman (1985) proposed that the function of the hovering guards is to protect the nest against the cleptoparasitic bee *Lestrimelitta limao* by incapacitating scouts of this species before they recruit a robbing swarm. Another not mutually-exclusive hypothesis is that hovering guards increase the defensive perimeter of the nest so that any intruders are more rapidly discriminated (Grüter *et al.* 2011b). We investigated these hypotheses and this study is included as Appendix 4.

1.4. Navigational Decision Making in Ants (Chapters 4 & 5)

A key factor that has contributed to the evolutionary and ecological success of ants is their uncanny ability to efficiently locate and retrieve important resources for the developing colony. Ants, like most bees and wasps, are central place foragers; this requires that an outgoing forager be able to relocate its nest, which often consists of a single entrance, often after travelling distances of up to 200 metres (Carroll & Janzen 1973) from the nest. To achieve this feat, foraging ants have evolved a variety of different strategies enabling them to relocate and retrieve food resources in an often heterogeneous landscape.

1.4.1. Path Integration

Without the benefit of a memory or pheromone trail a naive ant on its first foraging foray must rely on a process of dead reckoning much like that used by ancient mariners to navigate the world's oceans. In ants and other animals this strategy is called path integration (Mittelstaedt & Mittelstaedt 1982). Three separate features are required in order for path integration to successfully guide an ant between its nest and a foraging location (Collett & Collett 2000). Of most importance is an accumulator which enables an ant to update its position as it leaves the nest (Collett & Collett 2000). Once an ant reaches a foraging site, a second feature is required to record the accumulator state at this position and finally a comparator is utilised to subtract the current position of the ant from the recorded state, such that on reaching the nest the accumulator state returns to zero.

To achieve this, ants, just like the ancient mariners, require some form of compass to measure direction together with a means of measuring distance (an odometer). A skylight compass utilising celestial cues such as polarised e-vectors (for more

information see: Wehner 2003; Wehner & Müller 2006) is used by many ant species in addition to a vast number of other animals including honeybees (Rossel 1993), spiders (Dacke *et al.* 1999), shrimp (Goddard & Forward 1991) and lizards (Freake 1999). A method of measuring distance has been demonstrated in the desert ant. Although it was known for some time that desert ants must be able to measure distance by some proprioceptive means (Ronacher & Wehner 1995; Labhart & Meyer 2002), it was not until 2006 that the exact method was finally resolved by an ingenious, albeit slightly macabre, experiment involving the shortening and lengthening (via pig bristle stilts) of the legs of ants (Wittlinger, Wehner & Wolf 2006). It was elegantly demonstrated that desert ants possess an internal pedometer capable of measuring both distance, by counting strides, and walking speed, with those ants possessing manipulated leg lengths misgauging travel distances (Wittlinger *et al.* 2006; Wittlinger, Wehner & Wolf 2007).

1.4.2. Navigational Strategies

In most ant species experienced foragers are able to use cues from their environment to aid navigation rather than relying on path integration alone. Often a foraging ant will want to return to a persistent resource such as seeds beneath a tree or a population of honeydew producing aphids on a plant. Ants have evolved a diverse and elaborate range of strategies to navigate between their nest and rewarding resources, relying on cues from a number of different sensory modalities. These include among others trail pheromones (Attygalle & Morgan 1985; Morgan 2009), geomagnetic (Anderson & Meer 1993; Camlitepe & Stradling 1995), thigmotactic (Klotz & Reid 1993) and memorising a route using visual landmarks (Graham & Collett 2002; Graham & Cheng 2009) or the forest canopy (Hölldobler 1980).

1.4.3. Navigational Cues and Decision Making

Decisions made by foraging ants are thought to be based upon simple behavioural rules. In the case of pheromone deposition behaviour an ant must decide, for example, whether to lay a trail or not, how much to deposit and which pheromone to use if a choice is available. Until fairly recently it was believed that an ant had very few decisions to make during its foraging excursions. However, it is now becoming evident that a whole range of factors influence the behavioural decision making of foraging ants; rules such as 'follow the trail with the greater pheromone concentration' (Beckers *et al.* 1990) and 'deposit pheromone if food is found' have been complemented with others such as 'deposit less frequently if traffic on the trail is high' (Czaczkes *et al.* 2013) or 'deposit more pheromone the closer you get to the food source' (Beckers, Deneubourg & Goss 1992). Thus behavioural decision- making is clearly more complex than originally thought.

Alternative strategies to pheromone laying are also available to foraging ants. Most ant species are able to navigate using a number of methods, often using cues from different sensory modalities (Hölldobler & Wilson 1990). The acuity and reliability of a particular cue utilised by an ant is rarely consistent over time due to the fluctuating nature of the environment. To accommodate this, ants are capable of using more than one strategy based upon the salience of different navigational cues. These strategies together constitute an ant's 'navigational toolbox' and which strategy is used is thought to depend upon its position within a hierarchy (Hölldobler & Wilson 1990; Wehner 2003). For example, foragers of *Lasius niger* and *Paraponera clavata* predominantly utilise a route memory based upon visual cues over trail pheromones in daylight (Harrison *et al.* 1989; Grüter, Czaczkes & Ratnieks 2011a). Rarely are

navigational cues in true isolation, which enables ants to utilise overlapping cues from different sensory modalities in tandem, making navigation more efficient and reliable. For example, *Lasius niger* ants are able to increase their speed of travel when their memory of a route is accompanied by a pheromone trail (Czaczkes *et al.* 2011). Similarly, in moths (Balkenius, Rosén & Kelber 2005), bumble bees (Kulahci, Dornhaus & Papaj 2008), the colorado beetle, *Leptinotarsa decemlineata* (Otálora-Luna & Dickens 2011) and dolphins (Pack & Herman 1995) decision making has been enhanced by multimodal processing. In Chapter 4, I investigate further the flexible use of different navigational strategies by the ant *Lasius niger*; in particular I seek to determine what strategy is used when visual cues are unavailable and is this accompanied by any behavioural adaptations?

The presence of particular environmental cues and therefore which strategy is available to a foraging ant is dictated by the ecological environment the colony inhabits. If we consider the desert for example, the hot and windswept sand is a poor substrate for pheromone deposition while the barren shapeless landscape is often bereft of landmarks and contrast, a prerequisite for visual development of a route memory (Collett, Graham & Durier 2003; Graham & Cheng 2009). *Cataglyphis* desert ants overcome these obstacles by utilising strategies that rely on cues which are available to them. They successfully navigate using path integration (see above) using polarised e-vectors from the brightly lit skies as a compass (Wehner 2003; Wehner & Müller 2006) and measuring distances by counting strides (Wittlinger *et al.* 2006). Remarkably, these ants are quite capable of also using visual landmarks to develop a route memory and olfactory cues to locate their nest when these cues are available to them (Collett, Collett & Wehner 2001; Akesson & Wehner 2002; Steck 2012). We see

similar hierarchical flexibility in strategy use in other environments such as canopy orientation by the ant *Pachycondyla* (*Paltothyreus*) *tarsata* in tropical forests (Hölldobler 1980), and pheromone reliance by the subterranean ant *Acanthomyops interjectus* (Hangartner 1969). In Chapter 5, I investigate how the ecological environment has shaped the navigational abilities and decision making of two different *Lasius* ant species with contrasting ecologies. Two common strategies used by ants in navigation, which I investigate in this thesis, are the use of pheromone trails and a route memory, based upon visual cues.

1.4.4. Trail Pheromones

Pheromone trails are predominantly used by ants to aid nest migration (Franks *et al.* 1991; Blatrix *et al.* 2002; Schöning, Njagi & Franks 2005) and foraging (Carroll & Janzen 1973; Traniello 1989; Traniello & Robson 1995). An ant leaving the nest to forage does not lay a pheromone trail. Only once an ant has located a rewarding food source and is returning to the nest will an ant begin to lay a pheromone trail (Beckers *et al*. 1992).

As the ant returns to the nest the amount of pheromone deposited along the route is governed by a number of factors, such as quality of the resource and complexity of the route. For example, *Lasius niger* ants deposit more pheromone when returning from a higher quality carbohydrate source or when the route back to the nest is more complex (Beckers *et al.* 1993, Czaczkes *et al*. 2013). On reaching the nest the ant will recruit nestmates to forage and these ants will follow the trail to locate the food source. After feeding, these ants reinforce the route by depositing pheromone themselves. This positive feedback ensures that a colony is able to rapidly exploit a rewarding resource (Deneubourg *et al*. 1990). Outgoing foragers will always follow the trail with the greatest pheromone, as this indicates a superior reward (Beckers *et al*.

1993, Couzin & Franks 2003). To ensure that rewarding trails are followed, ants actively deposit a greater amount of pheromone along weakly marked but rewarding trails (Beckers *et al*. 1992). As traffic increases along a trail, collective organisation occurs to maximise trail speeds (Dussutour *et al*. 2009, Couzin & Franks 2003). This behaviour is most suitably demonstrated in the immense trunk trails of the army ant *Eciton burchelli*. A three column structure is fomed with returning ants using the central lane, flanked by outgoing ants on either side, thus reducing high speed collisions in bidirectional traffic (Couzin & Franks 2003).

These chemicals may be a single compound as has been found in eight species of *Myrmica* (Morgan 1984). Alternatively, they can consist of multiple chemical components that may originate from the same gland (e.g. Attygalle & Morgan 1983; Vander Meer, Alvarez & Lofgren 1988; Hölldobler *et al.* 1995) or occasionally from different glands (e.g. Attygalle *et al.* 1988; Hölldobler *et al.* 1994; Kohl, Hölldobler & Bestmann 2001; see also; Hölldobler 1995. For a review of the glandular sources of trail pheromones see: Attygalle & Morgan 1985; Billen & Morgan 1996, Morgan 2008). Furthermore, the ratio of the chemical components is often important for the pheromone to function efficiently. In the ant *Tetramorium caespitum*, for example, the highest trail following response is found when the ratio of the two components 2,5-dimethyl pyrazine and 3-ethyl-2,5-dimethyl pyrazine are in a ratio of 7:3 (Attygalle & Morgan 1983).

Ants are often very sensitive to the relative concentrations of the trail pheromone, sometimes to the extent that at a low concentration the pheromone components elicit a trail following response while at higher concentrations alarm or repellent behaviour

is induced (Moser, Brownlee & Silverstein 1968; Riley *et al.* 1974; Simon & Hefetz 1991).The potency of trail pheromones can be extraordinary. For example, methyl 4-methylpyrrole-2-carboxylate (MMPC; see fig 2), the trail pheromone of the leaf cutting ants *Atta texana* and *A. cephalotes*, induces a strong following response at concentrations as low as 0.4 ng/µl; thus theoretically just 1mg of this compound would be sufficient to form a trail three times around the planets circumference (Riley *et al.* 1974).

Figure 1.2. *Lasius niger* ants 'farming' aphids. Honeydew from aphids provides ants with a rich source of carbohydrates (photo courtesy of Christoph Grüter)

In addition to orientating an ant to a food source, it is also important that nestmates are quickly recruited, thus enabling the colony to monopolise a large or persistent food source (Traniello 1989; de Biseau *et al.* 1997). This is especially important when, as is usually the case, competition for food resources is high. Behaviours such as antennation, regurgitation of the food source and jerking movements induce recruitment in many ant species (Hölldobler & Wilson 1978; Liefke, Hölldobler & Maschwitz 2001; Morgan 2009). However, these behaviours are, in many cases, unlikely to be as efficient as chemical communication, especially in larger colonies, where one would expect volatile odours to reach a greater audience in a shorter time

span. Recruitment using chemical cues is generally achieved by one of two means. One option, seen in many *Myrmica* species, is to use a single component which serves both to orientate and attract other colony members (e.g. Evershed, Morgan & Cammaerts 1982; Attygalle *et al.* 1986; Morgan & Ollett 1987). This strategy may be enhanced by ants also utilising the behavioural mechanisms mentioned above. More commonly seen is the use of a separate recruitment pheromone or inducer component that can sometimes be released separately or otherwise as part of the multicomponent mixture laid down by the ant (e.g. Vander Meer, Lofgren & Alvarez 1990; Attygalle *et al.* 1991; Kohl, Hölldobler & Bestmann 2003). For example, in the ant *Megaponera foetens,* poison gland secretions provide orientation cues while components added from the pygidial gland aid in recruitment (Hölldobler *et al.* 1994). In some cases an inducer component must be present before ants will even follow the trail (Vander Meer *et al.* 1990; Oldham *et al.* 1994a).

Complexity is further increased in many ant species by the use of multiple trail pheromones, each conveying different properties, governed by chemical structure. Multiple trail pheromones have been found in many ants, including the invasive species *Monomorium pharaonis* (Jeanson, Ratnieks & Deneubourg 2003; Jackson *et al.* 2006), *Solenopsis invicta* (Vander Meer, Williams & Lofgren 1981; Vander Meer *et al.* 1990) and *Pheidole megacephala* (Dussutour *et al.* 2009), where their dual purpose is thought to be rapid mobilisation of large numbers of foragers to a food source, while also enabling the colony to react sensitively to environmental changes. Volatile, less persistent pheromones are used when a forager locates food, rapidly recruiting nestmates to the resource while longer lasting pheromones are used for exploration and to provide a structural network within the colony's territory. A different strategy is

used by the red Myrmicine ant, *Myrmica sabuleti,* which utilises two pheromones originating from different glands to distinguish between the food types it locates (Cammaerts & Cammaerts 1980). On finding a protein source in the form of a large dead insect prey item, a foraging *M. sabuleti* worker marks the trail to the nest with a secretion from its dufours gland. However, when the resource is a carbohydrate (sugar water) the foraging ant only marks the route with its trail pheromone, originating this time from the poison gland.

1.4.5. Route Learning

Visually acute ant species such as *L. niger* are capable of using contrast in the surrounding landscape together with any prominent landmarks to form a memory of a rewarding route which they can subsequently use to return to the same location. Ants have an innate attraction to distinct vertical objects, aiming at them when this does not divert too far from their initial goal (Graham, Fauria & Collett 2003). This behaviour enables ants to reliably maintain a correct route during the learning process. During learning ants learn other available visual cues so that if the prominent visual feature they want to reach is subsequently removed the ants were still able to able to reach the site, even when displaced from their normal starting position (Graham *et al.* 2003). Similarly ants are also innately attracted to edges such as barriers; however, in these situations their choice of route is determined by the physical properties of the barrier, such as its height (Pratt, Brooks & Franks 2001; Graham & Collett 2002). By manipulating the height of a barrier, experiments have shown that ants use the retinal elevation of the top of the barrier to guide their route such that a perceived increase in height indicates that the ant should move further away and vice versa if the height is perceived to be shorter (Pratt *et al.* 2001; Graham & Collett 2002).

In addition to these forms of innate navigation, ants, like many insects, can use snapshots of surrounding landmarks taken at a particular locality, such as rewarding food source, and a number of stages along the route (Collett & Collett 2002; Collett *et al.* 2003). These retinotopic views can then be sequentially recalled during the return journey and compared with the ant's current view. Alignment of the direction, so that any discrepancy between the retinotopic and current views is removed, ensures that the ant remains on the correct path (Judd & Collett 1998; Harris, Graham & Collett 2007). A single snapshot is insufficient to guide an ant back to a rewarding location, so retinotopic views (visual snapshots) are recorded at a number of vantage points as the ant returns to the nest (Judd & Collett 1998). In order to accurately navigate back to a particular locality the appropriate memorised snapshot needs to be recalled at the correct stage of the journey. Initially the correct set of snapshots must be recalled as this determines which journey the ant takes. Both feeding state (Harris *et al.* 2005) and time of day (Harrison & Breed 1987) have been found to prime the particular sequence of memories that need to be used. A full gaster, for example, has been found to initiate the sequence of snapshots that guides the ant back to the nest (Harris *et al.* 2005). Once on the route the sequential position assures that the correct snapshot is recalled (Judd & Collett 1998).

Many ants are able to rapidly acquire a reliable route memory, often after just a single visit to a food source (*Leptothorax unifasciatus*, Aron, Deneubourg, & Pasteels 1988; *Lasius niger*, Grüter, Czaczkes, & Ratnieks 2011) and even the highly pheromone dependent Argentine ant *Linepithema (Iridomyrmex) humile* is able to depend on this strategy when necessary (Aron *et al.* 1993). Navigation via a route memory is generally regarded to be a more efficient method than relying solely on trail pheromones, as it

results in fewer deviations from the route and faster travel speeds (Collett *et al.* 2003). In these situations pheromones still have a role to play, serving as an initial structural guide for naive foragers, after which they switch to their newly acquired route memory. In addition the presence of pheromone can also increase the travel speed and reduce the sinuosity of foraging ants when used in conjunction with a route memory (Czaczkes *et al.* 2011).

1.5. Analysis of Trail Pheromones (Chapter 6)

The first trail pheromone to be isolated and identified belonged, in fact, to a termite. Neocembrene A (now (E)-6-Cembrene A) was isolated from the termite *Nasutitermes exitiosus* in 1966 (Moore 1966) and it wasn't until 1971 that the first trail pheromone was discovered in an ant species. Tumlinson and colleagues (1971) required 3.7 Kg of

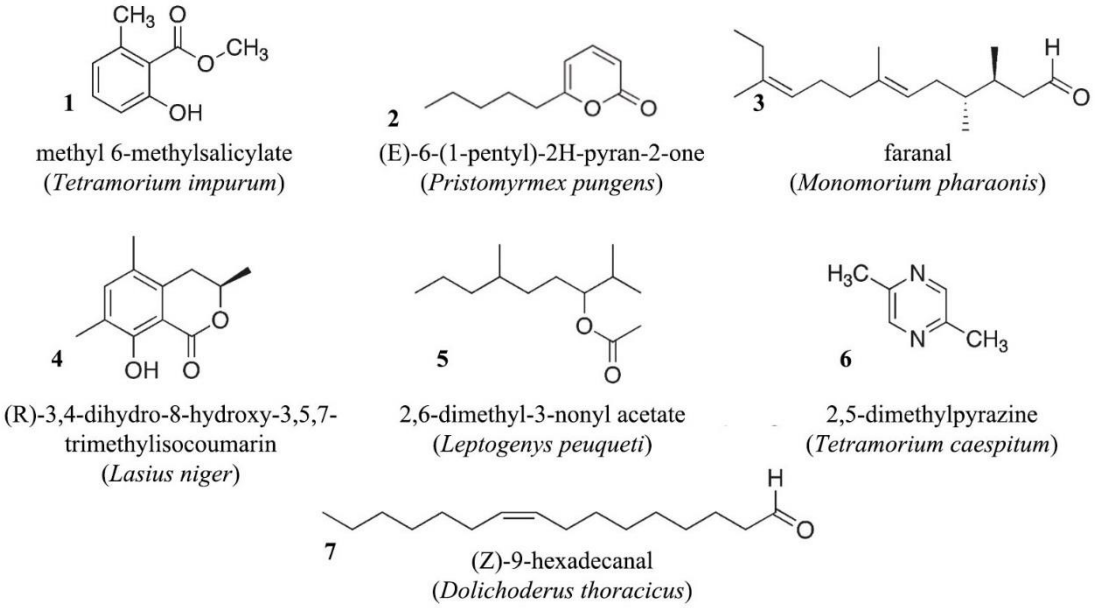


Figure 1.3. Range of compounds utilised by ants as trail pheromones which include: salicylates (**1**, ester of salicylic acid)(Morgan & Ollett 1987), lactones (**2**, Janssen et al. 1997a), terpenoids (**3**, Ritter et al. 1977b), coumarins (**4**, Bestmann et al. 1992), aliphatic esters (**5**, Janssen et al. 1997b), nitrogen heterocycles such as pyrazines (**6**, Attygalle & Morgan 1983) and aldehydes (**7**, Attygalle et al. 1998).

dried *Atta texana* leafcutter ants to identify the pheromone, methyl 4-methylpyrrole-2-carboxylate, found in the venom gland.

Since this discovery a whole host of different trail pheromones have been identified, originating from various exocrine glands. The variety of structures are bewildering and include terpenoids (e.g. Ritter *et al.* 1977b; Vander Meer *et al.* 1981), pyrazines (e.g. Cross *et al.* 1979; Jackson *et al.* 1990), aldehydes (e.g. Cavill, Davies & McDonald 1980; Attygalle *et al.* 1998), aliphatic and cyclic esters (Morgan & Ollett 1987; Bestmann *et al.* 1995a; Blatrix *et al.* 2002) and alcohols (e.g. Attygalle *et al.* 1988; Morgan *et al.* 2004)(For reviews see: Attygalle & Morgan 1985; Morgan 2009, see figure 1.3)

The standard protocol used to identify the chemical components that elicit a trail following response generally involves separation of the glandular extract into fractions followed by a bioassay to determine the response of the insects to each fraction. Analysis of the active fraction using a technique such as gas chromatography coupled with mass spectroscopy (GCMS) subsequently enables identification of the pheromonal component(s). Initial separation is usually achieved using techniques such as high performance liquid chromatography (HPLC), liquid chromatography (LC) and gas chromatography (GC). Three simple bioassay methods are commonly used today: measurement of the response of an ant to extract intersecting a straight trail, provision of a choice test on a Y or T maze where ants can choose either between two extracts or extract and a solvent control and the distance that ants follow a circumference of evenly applied extract (Morgan 2009). In a few cases electroantennograms have also been used to measure a neurophysiological response to extracts in ants (e.g. Kern & Bestmann 1994; Janssen *et al.* 1997a).

Due to the extremely small volumes involved (Morgan 2009), analysis of crude extracts or fractions is usually carried out using gas chromatography coupled with mass spectroscopy (GCMS). This method initially involves separation of chemical compounds, primarily based upon differences in vapour pressures and is followed by fragmentation of each separated constituent into a particular set of molecular ions. This fragmentation pattern can be used to identify unknown compounds because the fragments produced are indicative of structural patterns in the molecule, are predictable and can be compared to databases of fragmentation patterns of known compounds. Internal standards (known amount of a compound, different from the unknown, which is added to analytical samples) can be used to quantify the concentration of each unknown constituent. Comparison between the chromatographic peaks of the internal standard and the unknown enables the concentration to be derived. Identification of the chemical structure requires inspection of the mass spectrum for diagnostic fragmentation peaks and comparison of fragmentation patterns with those of known compounds held in large databases.

In Chapter 6, I investigate the glandular source of the trail pheromone of *Lasius flavus* and determine whether multiple components originating from separate glands could play a role in trail orientation. Once the glandular source(s) of the trail pheromone components were determined the process of identifying them could begin. One of the goals in this study was to develop a sensitive method that would allow us to extract and identify the compounds deposited by trail laying ants. If we can positively match these structures with compounds found in extracts taken from the gland, then we can be sure that the chemical in question is part of the chemical trail. A method such as

this is hugely beneficial as it saves the huge amount of effort and time that is normally taken to test every glandular component for trail following activity.

2

How This Thesis Evolved

2.1. How I arrived at LASI

Friends and family can often be the harshest critics and it did not take them long, upon finding that I had been successful in obtaining a PhD position at LASI, to point out that as an "endless student" it was high time I got a "proper job"! In my defence I pointed out that I had eight years of working experience and this exciting opportunity was a necessary step for my future employment. The insults continued but all agreed that I am at my happiest in an environment where constant learning, development and research are core, which this PhD position certainly offered. The jests from my friends and family stemmed from the considerable amount of time I had already committed to higher education. After completing my A levels I moved north to Leeds to undertake a BSc degree in Zoology. Animals have fascinated me from an early age and it was a logical step to pursue the study of them as a career. The faculty member who had the greatest influence on me during my time at Leeds was the legendary Professor Robert McNeill Alexander. His genial nature, incredibly rounded knowledge and insightful teaching style had a great impact on my learning experience. He increased further in my esteem when I discovered that he was consulted as an expert for the dinosaur locomotion in Jurassic Park and was also voted Britain's craziest scientist by the Daily Telegraph, following a study involving harassment of tigers by pulling their tails.

A year after completing my BSc in Zoology I joined a voluntary excursion to Zambia where I was involved in an ongoing biological survey of a fairly remote National Park for three months. Once the three months was finished I managed to secure an exciting job as a co-manager of a safari lodge in South Luangwa National Park, Zambia's flagship reserve. Despite being surrounded by large, impressive mammals, colourful and graceful birds, and dangerous reptiles, my fondest memories are of the insects; in particular the ants. Ants were ubiquitous: everywhere you looked an interesting behaviour could be observed. On those rare occasions when your mind might be elsewhere you were rapidly jolted back into their world as a sadistic individual sank its jaws or sting into some other part of your anatomy. The most charismatic were the driver ants (*Dorylus* spp). Immense raiding parties of these army ants often appeared in the lodge grounds and on a few occasions I had to evacuate customers from chalets after swarms of ants appeared in a search for tasty morsels. The sheer aggressiveness of these ants needs to be seen to be believed; locals told me that elephants have been known to succumb to their attacks. If large numbers of these ants manage to enter the sensitive trunk the elephant reacts to their persistent biting by beating its trunk against trees, which can on occasion damage the trunk and thus result in a slow death.

Following my 26 month excursion to Zambia I found myself back in cold dreary England working as an account assistant for Leicester City Council. I enjoyed the social aspects of office work and having money to spend but within a couple of years I was itching to return to Science. In 2005 I made the decision to return to university, and this time, with the wanton student drinking games already under my belt (and the results of these activities hanging over my belt), I intended to prove to myself that I could achieve the highest grades. I undertook a BSc in Chemistry at the University of

Leicester thinking that it would offer a new challenge while also complementing my zoological knowledge. My favourite aspects were organic and analytical chemistry. I quickly realised that knowledge of organic chemistry could offer alternative insights into biological and natural phenomena. One such example was chemical communication in insects. The challenge of analytical chemistry appealed to me and I really enjoy the detective work involved in resolving the structure and identity of chemical compounds using information from the three spectroscopy methods: infra-red (IR), Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy (MS).

By the time I had completed my BSc degree in Chemistry I was sure that I wanted to work with insects in my future career. Therefore, when I discovered that Imperial College in London offered a Masters course in entomology, I jumped at the chance. I thoroughly enjoyed my year at Silwood Park and found the course hugely rewarding involving an intensive six month period devoted to lectures and a further six months assigned to a research project. As my time at Silwood came to an end I decided that I would like to continue in academia and find a PhD that really interested me. A PhD not only would provide me with greater credentials when applying for a future job but would also enable me to pursue a career in academia if I found this prospect appealing later on. Moreover, being called Dr Jones appealed to me and I still look forward to buying a fedora hat in the future. So with seven years of higher education already behind me I arrived at LASI to study behavioural and chemical ecology in ants and stingless bees, with Professors Francis Ratnieks and Jonathan Bacon.

2.2. Chapter 3

I began my tenure at LASI by thoroughly reviewing the available literature on nestmate recognition in the attractive little stingless bee, *Tetragonisca angustula*, with a view to undertaking a study on them during an upcoming trip to Brazil. The timing of this trip to Brazil was ideal as it allowed us to escape the cold dull winter months of January and February and instead bask in temperatures above 30 ^{o}C. We were going to spend four weeks based at a farm named Fazenda Aretuzina near the town of São Simão, São Paulo State, Brazil. This farm housed a large number of hives containing various stingless bee species and is owned by Dr. Paulo Nogeira-Neto, a genial host who is interested in stingless bees and the conservation of Brazilian wildlife. I was particularly looking forward to seeing a diverse array of wildlife and in particular insects, birds and reptiles. My four weeks in Brazil did not disappoint with particular highlights being the leafcutter, trap jaw and velvet ants, numerous beautiful butterflies, the stingless bees of course, a toco toucan, geckos and surprisingly a mammal in the guise of Dr Paulo's potentially man eating peccaries.

Following previous work by members of Francis's lab we were aware that *T. angustula* was highly proficient in determining nestmates from non-nestmates, making very few recognition errors. This contrasted strongly with results using exactly the same protocol for honeybees and another stingless bee species and we wondered what enabled this stingless bee species to achieve so few recognition errors. One difference between *T. angustula* and honeybees was the greater use of propolis; resin collected from various trees and large shrubs. Honeybees do use propolis to block up holes in the hive but in stingless bees propolis plays a much more important role.

Figure 2.1. The author Sam Jones hard at work in Brazil (photo courtesy of Francis Ratnieks).

Tetragonisca angustula workers store piles of resin randomly throughout the nest and use it in the construction of the nest comb as well as in defence. Guards carry small amounts in their corbiculae which they can then use to incapacitate nest intruders by sticking wings and limbs together. Interestingly, wood ants (*Formica* species) also make use of resin, by storing solidified pieces in their nest. In this case the ants use the antibacterial and antifungal properties of the resin to protect themselves against harmful pathogens (Chapuisat *et al*. 2007).

Another difference that separates *T. angustula* from not only honeybees but also other stingless bees is the construction of a wax entrance tube to the nest on which guards stand and patrol. Using the well designed and constructed hives of Dr Paulo we could easily manipulate entrance tubes and resin stores between hives enabling us to

discover whether cues from propolis or the wax entrance tube allow these bees to distinguish nestmates from non-nestmates.

Figure 2.2. The author holding a dead centipede attached to transparent thread. This provided us with entertainment at dinner, during which it appeared from beneath Francis Ratniek's plate of food (photo courtesy of Tomer Czaczkes)

2.3. Chapter 4

The trip to Brazil was a yearly event for the lab, and during the previous year my colleague Christoph Gruter collected some interesting data which suggested that hovering guards increased the defensive perimeter of the nest in the stingless bee *T. angustula. Tetragonisca angustula* are unique amongst bees in possessing two types of guards: standing guards that patrol the nest entrance and hovering guards that hover around the entrance and when housed in hives, to the sides also. Thus, during our visit to Fazenda Aretuzina in 2010 Christoph enlisted the help of Jelle Van Zweden and myself to investigate this hypothesis further. Our fun experimental design involved hanging a freshly killed *Lestrilemitta limao* bee from some fishing wire and then dangling this in front of the hive, initially at a considerable distance from the entrance before gradually reducing this. The distance at which a guard attacked the hanging bee was recorded for hives from a number of different stingless bee species. *L. limao* bees are obligatory robbers of many other stingless bee species and produce a

characteristic smelling semiochemical called citral, which in this situation is acting as a kairomone (chemical signal that benefits a receiving species but not the emitter). On hearing the roar of bees within the hives of some of the larger bee species I was ready to shove Jelle in front of the hive and run. One has to look after one's self in these situations.

2.4. Chapter 5

Shortly after joining the LASI lab Francis encouraged me to get actively involved in some new ant experiments being run in the lab by Christoph and Tomer Czaczkes. This, as Francis hoped, allowed me to quickly gain excellent hands on experience in working with ants in a lab setting, alongside undergraduate students and my lab colleagues. Indeed, the experimental work I was involved in during the end of 2009 and early 2010 led to two published papers, both of which included me as a named author. This then prepared me for my own projects working with ants that came later.

During the first term of the new academic year in 2010, Jonathan asked me to help supervise two undergraduate students with ant projects. As the timetables of the two students were notably different it made sense to give them both different projects to work on. These studies gave rise to both this chapter and Chapter 6. The student who played a significant role in the study that forms this chapter was a very capable medical student called Ewan Gourlay. An odd subject for a medical student maybe, but Ewan showed absolutely no lack of interest or effort throughout. Apart from the knowledge that they had six legs and sadistically enjoyed dispensing pain, Ewan arrived with no other prior knowledge of ants. But with a considerable amount of reading

Ewan quickly showed a good grasp of the subject and it really wasn't long before he was asking searching questions and making excellent suggestions.

The idea for this project ultimately came from Christoph and I am particularly grateful to him for his excellent suggestion as this developed into a very interesting project. Together with Tommy, Christoph had recently showed that foraging *Lasius niger* ants preferably utilise their own memory rather than a pheromone trail when returning to a food source, in daylight. The question was would this remain the case when the ants foraged at night. One study had suggested that pheromone trails become more important in these cases but there was no quantitative data available to demonstrate this. I was well aware of the nightly escapades of *L. niger* ants after they brazenly gorged themselves on the icing and glacé cherries of my cherry bakewells on one fateful evening while camping on the Isles of Scilly. This dastardly act deprived me of the only treat I possessed, which I had carefully stowed away for a special occasion and very nearly ended my love affair with ants.

The experimental design for this experiment gave us a few headaches early on. Somehow we needed to be able to observe the ants foraging in the dark, focusing particularly on a select few individuals that had been paint marked at the feeder. Paint marking allowed us to follow those ants that had been to the food already and thus observe their decision on returning towards the food source. Our first idea was to use a glow in the dark fluorescent acrylic paint to mark the ants with. However for the fluorescence to work it needed activating under intense light for a few minutes and once activated, would rapidly degrade in a number of minutes. This was impractical for our needs and we eventually settled upon the use of high pass IR filters which provided

us with infra-red light sufficient for experimental work while being undetectable to the foraging ants.

2.5. Chapter 6

I received invaluable assistance throughout this long project from Jonathan's second student Alan Gallagher. Alan was an environmental science student, who as far as I am aware had at this time no real fascination with ants. However, that was all to change with this project as he became as equally enamoured, as I was, with them and his ideas and probing questions were paramount to this projects success. Alan also came back to help me on this project after graduating, a testament to just how much he enjoyed research projects and working with ants.

I also owe a great deal of gratitude to Martyn Stenning, the long serving and immensely helpful Life Sciences technician, for introducing *Lasius flavus* to the LASI lab. I became inordinately fond of these ants during my work with them over this and the following study. Both Alan and I quickly realised how amenable to experimentation these colourful ants were. Despite being smaller than *L. niger* they were easy to paint mark, largely due to their docile nature. We found *L. flavus* to be far more relaxed than their manic and escape prone congeners *L. niger*. I discovered that I could keep them happily constrained within large square plastic tubs, as long as sufficient dampened soil was available. Some of the architecture these little creatures produced would have had Antoni Gaudi salivating. The impressive structures these ants created provided yet another example of how simple rules governed by local cues, once followed by large numbers of individuals, can result in a remarkable collective response.

Lasius flavus is a predominantly subterranean ant species, rarely observed above ground. As a result little is known about the foraging behaviour and navigational abilities of these evasive ants. The well-studied lifestyle of the surface foraging *L. niger* is markedly different, making a comparative study an obvious and interesting study choice. We knew foraging *L. niger* ants predominantly use a memory developed from visual cues but these are quite clearly unavailable to *L. flavus* ants in their dark subterranean environment. Amongst the many questions we sought answer were: what navigational cues are utilised by *L. flavus* ants and do we see a switch in decision making as environmental variables, such as light change?

2.6. Chapter 7

From the beginning of my PhD it had always been my plan to involve some chemistry in my studies as I wanted to utilise and improve upon the analytical skills I had developed in my earlier Chemistry degree. *Lasius flavus* offered a great opportunity for this because the trail pheromone used by this ant species remains unknown. My earlier studies had shown me that these ants use such a pheromone and, more importantly, that trail pheromones were the predominant cue used to navigate in their underground environment. Unfortunately, the organic chemist who had initially been keen on the idea failed to commit to the study and then took early retirement. My luck changed when I met Professor Elizabeth Hill, while demonstrating on a practical course she convened. Liz was very keen to be involved in a project and had the expertise and facilities for such an undertaking, running a busy and successful trace analysis lab with all the equipment I would need. Like Jonathan, Liz liked to be regularly informed about my progress and I found this really helpful in focussing me on the task, while ensuring any mistakes I made were quickly rectified (and there were many!).

The plan for this study was simple. Firstly, identify the source(s) of the trail pheromone component(s) and then identify what these components actually are. Unfortunately, the study itself proved to be quite the opposite. We decided to tackle the problem from two angles, both by attempting to collect deposited trail pheromone from a surface and also by analysing crude extracts from the glands themselves.

Collection of pheromone proved far more difficult than I had anticipated, largely due to its high volatility. Initially I designed an ingenious tubular glass T maze with detachable arms. I knew pheromone was being deposited along the arm that led to the food, because when I switched the arm to the opposite side, the ants obediently followed this arm, leading them away from the food, on their return. However, when I attempted to collect the pheromone by washing the tube with solvent (pentane), the extract, when reapplied to a glass tube, failed to elicit a trail following response. This, I decided was due to a very low concentration, caused by either evaporation of much of the pheromone, or not enough being deposited by the ants in the thin glass tube. I switched to a glass microscope slide which provided a greater surface for ants to deposit pheromone on. Again this proved unsuccessful and it was only when I switched to a more absorbent surface in the guise of filter paper that I had any success, proving that the volatility of the pheromone was the main problem.

Glandular analysis was somewhat more successful. The main issue with this part of the study was the vast numbers of ants that had to be dissected in order to analyse behaviourally and chemically. I spent innumerable days dissecting these small ants and I cannot imagine just how many of these poor creatures perished under my scalpel. I know it was enough for me to regularly have ants missing various body parts appear in

my dreams. I comfort myself by thinking that all these deaths were for the betterment of science and their species (it was the best I could come up with). I should also mention here that Alan yet again was of great help during this study, participating in the ant mutilation and helping with the collection and bioassays. Just as in the previous study his help was invaluable.

3

The Role of Wax and Resin in the Nestmate Recognition System of a Stingless Bee, *Tetragonisca angustula*

3.1. Abstract

Recent research has shown that entrance guards of the stingless bee *Tetragonisca angustula* make less errors in distinguishing nestmates from non-nestmates than all other bee species studied to date, but how they achieve this is unknown. We performed four experiments to investigate nestmate recognition by entrance guards in *T. angustula*. The first experiment investigated the effect of colony odours on acceptance. Nestmates that acquired odour from non-nestmate workers were 63% more likely to be rejected while the acceptance rate of non-nestmates treated with nestmate odour increased by only 7%. We further hypothesised that guards standing on the wax entrance tube might use the tube as an odour referent. However, experiment 2 showed that there was no difference in the acceptance of non-nestmates by guards standing on their own colony's entrance tubes versus the intruder's entrance tube. Experiment 3 sought to determine if wax or resin derived odours played a role as nestmate recognition cues. Introduction of bees treated with nestmate and non-nestmate resin or wax saw a drop in acceptance rates of up to 65%, regardless of the origin of the wax or resin. The role of resin as a source of recognition

cues was further investigated in experiment 4 by unidirectionally transferring resin stores between colonies. Acceptance rates of nestmates declined by 37% for hives that donated resin, contrasting with resin donor hives where instead acceptance of non-nestmates increased by 21%. Overall our results confirm the accuracy of nestmate recognition in *T. angustula* while eliminating wax entrance tubes as the mechanism involved. The lack of consistency among colonies plus the complex results of experiments 3 and 4 highlight the need for further research on the role of nest materials and cuticular profiles in understanding nestmate recognition in *T. angustula*.

3.2. Introduction

Recognition of self versus non-self is ubiquitous among organisms, operating at several different levels and involving a variety of mechanisms (e.g. Beale 1990; Janeway and Medzhitov 2002; Nasrallah 2002; Glass and Kaneko 2003). Eusocial insects demonstrate self versus non-self recognition predominantly at the group level (but see Tibbetts 2002; d'Ettorre and Heinze 2005). In most species the nest entrance is defended by guards who deter both allospecific and conspecific intruders (Butler and Free 1952; Bell et al. 1974; Wittmann et al. 1990). Conspecific recognition requires the matching of a set of cues carried on the cuticle of an encountered individual (the label) with a previously acquired representation of colony odour (the template) of an evaluating individual, often a guard (van Zweden and d'Ettorre 2010). Depending on the degree of similarity/dissimilarity the encountered conspecific is accepted or rejected (Lacy and Sherman 1983; Vander Meer et al. 1998). Ideally nestmate recognition should categorise all incoming individuals without error (Sherman et al. 1997), but mistakes are made: nestmates may be rejected (rejection errors) or non-nestmates admitted (acceptance errors). Which of these two errors are made may vary

adaptively, within and between species, via adjustment of the acceptance threshold, so that, for example, increased rejection errors are traded off for decreased acceptance errors when the frequency of intruders or the cost of admitting them is higher (Reeve 1989; Downs and Ratnieks 2000; Couvillon et al. 2009).

In the honeybee, *Apis mellifera,* the number of entrance guards and the permissiveness of the acceptance threshold change adaptively, depending on nectar availability and robbing intensity (Downs and Ratnieks 2000; Couvillon et al. 2008). Overall, the recognition error rates are surprisingly high, with means of approximately 25% (range: 9 – 62%) for rejection errors and 29% (range: 0 – 52%) for conspecific acceptance errors (Downs and Ratnieks 1999, 2000; Wood and Ratnieks 2004; Couvillon et al. 2007, 2008, 2009, 2010). This gives a total error mid-way between the two extremes of perfect (0%) and zero information (100%, Ratnieks 1991). This is in stark contrast to recent results for the Neotropical stingless bee, *Tetragonisca angustula*. Guards of *T. angustula* made few errors in discriminating nestmate workers from non-nestmate conspecifics, accepting all nestmate workers (0% rejection errors) while rejecting 92% of conspecific non-nestmate workers, giving a total error of only 8% (Kärcher and Ratnieks 2009). This is also considerably lower than error rates reported for five other Neotropical stingless bees (Table 3.1). An exception is *Trigona minangkabau* with a rejection rate of 100 % for non-nestmates (Takeshi & Tamiji 1993), but it is difficult to draw conclusions from this because the sample size used was extremely small.

This raises the question of what the underlying mechanisms are that allow *T. angustula* to have lower recognition error rates than honeybees or other stingless bees. One

obvious difference between *T. angustula* and the six other bee species is that *T.angustula* is the only one of these species that constructs wax entrance tubes for their nests. Wax is important in honey bee recognition, functioning as the primary source of colony odour cues and a wax entrance tube might provide guards with a more direct template with which to compare incoming bees (Breed et al. 2004; Couvillon et al. 2007).

Table 3.1. Error rates for *T. angustula* and six other bee species. * designates average error rates from studies given in references column.

Bee species	Rejection error rate (%)	Acceptance error rate (%)	Total error rate (%)	Reference(s)
Apis mellifera	25*	29*	54	(Downs and Ratnieks 1999, 2000; Wood and Ratnieks 2004; Couvillon, Caple, et al. 2007; Couvillon et al. 2008, 2009, 2010)
Frieseomelitta varia	11	27	38	(Couvillon and Ratnieks 2008)
Melipona quadrifasciata	0	26	26	(Breed and Page 1991)
M. rufiventris	0	86	86	(Breed and Page 1991)
M. scutellaris	0	40	40	(Breed and Page 1991)
Tetragonisca angustula	**0**	**8**	**8**	(Kärcher and Ratnieks 2009)
Trigona fulviventris	24	24	48	(Buchwald and Breed 2005)

This might allow guards to update their template more frequently to allow peripheral sensory detection via desensitization (c.f. Ozaki et al. 2005), or to simply enable a direct comparison.

Figure 3.1. A wax entrance tube of a *Tetragonisca angustula* colony with standing guards patrolling the edge (photo courtesy of Alex Wild [myrmecos.net])

A further difference between *T. angustula* and *A. mellifera* is the former's greater use of plant resins. Leonhardt et al (2009) recently demonstrated that terpenoid profiles, derived from resin, extracted from the cuticles of seven Paleotropical stingless bee species varied quantitatively between colonies of the same species, leading them to suggest that terpenoids may serve as recognition cues in stingless bees. This is entirely feasible because nestmate recognition within a given eusocial species relies on quantitative differences between the same set of compounds (vander Meer et al. 1989; Espelie et al. 1990; Martin et al. 2008; van Zweden and d'Ettorre 2010). Nests of *T. angustula* contain substantial amounts of resin stored in piles throughout the nest (Fig. 3.2).

Resin can be seen in a layer on the legs, head and thorax of foragers (J.S. van Zweden, unpublished data) and is also mixed with wax to form cerumen, which is used to construct the combs and surrounding involucrum (pers. obs; Wille 1983). Thus the ubiquitous presence of resin within the nest, either in its pure form as piles or as cerumen, is sufficient for acquisition of a colony encompassing odour profile. Indeed, this would be synonymous to the combs of honeybees (Breed et al. 1995; d'Ettorre et

al. 2006).

Figure 3.2. *Tetragonisca angustula* workers on a resin pile within the hive (photo courtesy of Christoph Grüter)

The aim of this study was to investigate conspecific recognition in *T. angustula*, with the emphasis on the roles of odours derived from wax entrance tubes, plant resins and worker bees. This was achieved by investigating whether the acceptance of introduced nestmates and non-nestmates by guards standing on the entrance tube was influenced by: i) the acquisition of cuticular odours derived from nestmates and non-nestmates onto the cuticle, ii) swapping wax entrance tubes between colonies, iii) the acquisition of resin and wax derived from nestmates and non-nestmates onto the cuticle and iv) the unidirectional swap of entire resin stores between hives.

3.3. Materials and Methods

3.3.1. Study site & organism

Data were collected in February 2009 (Experiments 1 and 2) and 2010 (Experiments 3 and 4) at Fazenda Aretuzina, São Simão, São Paulo State, Brazil. During both study periods the weather was hot, with daytime high temperatures of ca. 24-32 °C, and periodic heavy rain. Data were only collected on non-rainy days during active foraging (between 9.00 and 17.00 hrs).

We studied five colonies of *Tetragonisca angustula*, local name Jatai, in 2009 and six in 2010. Each colony was housed in a wooden hive box (ca. 50 high x 20 x 30 cm), with a circular entrance hole 1.8 cm in diameter. Each colony had built a wax entrance tube from this hole. Entrance tubes were ca. 1-3 cm long and had a circular opening at the tip ca. 0.6 cm in diameter (see also figures in Wittmann 1985; Couvillon et al. 2007; for more detail Grüter et al. 2011). The entrance tubes on the study colonies appeared identical to those of unmanaged colonies nesting in walls. Hives were raised ca. 1m above ground on hive stands or attached to the walls of buildings. The study colonies were queenright and thriving, with the hive nearly full of combs, honey pots and covering involucrum, with a population of many thousands of workers. Mature colonies of *T. angustula* in Costa Rica were estimated to have approximately 10,000 workers (van Veen and Sommeijer 2000).

3.3.2. Introduction of worker bees to guards and their acceptance or rejection

The acceptance or rejection of conspecific workers by guards standing on the entrance tube was determined using a standard bioassay (Downs and Ratnieks 2000) developed for studying honey bees, *Apis mellifera*, and modified for use with *T. angustula* (Kärcher and Ratnieks 2009). Returning foragers were collected at the hive entrance, placed in a tube and immediately chilled in an ice chest for 10-20 min, then removed one at a time and allowed to warm to ambient temperature. Once warmed, these workers walked actively but were less likely to fly than previously unchilled workers. A worker was taken from the ice chest, allowed to grasp a clean wooden toothpick and walk onto the outer surface of the tip of the entrance tube of a discriminator hive. On contact with the guards standing on the entrance tube, behaviour was observed for up to 2 min. The introduced worker was considered “rejected” if it was bitten and tugged

for the duration of the observation period or fell off the tube while grappling with a guard (Kärcher and Ratnieks 2009). The worker was considered "accepted'" if she was subjected only to licking and antennal contact, or bitten and tugged for a few seconds and then left alone. The observer was unaware of the treatment group of the introduced workers and introduction order was randomised. Numbers of standing guards present on the entrance tube were recorded before introductions commenced (mean ± SD = 15.43 ± 4.38).

Figure 3.3. The author Sam Jones introducing bees to the entrance of a *T. angustula* colony, while co-author Jelle van Zweden records the results (photo courtesy of Francis Ratnieks)

3.3.3. Experiment 1: The effect of bee derived odours on acceptance rates of worker bees.

The aim of this experiment was to determine how the transfer of native and alien odours onto worker bees affected the acceptance of nestmate and non-nestmates. Four hives (A-D) were used both as discriminator and donor colonies. These were grouped into 2 pairs (A&B, C&D) to serve as donors and discriminators to each other (Fig. 3.4a). A fifth hive (E) was used as an additional source of non-nestmates. Worker bees (n = 20 ± 3) were collected at hive entrances and placed in a 6 ml clear plastic vial for 60 min to transfer odours to the vial, two vials per hive per study day. The bees were then released. Odours deposited on the inside of the tubes by these bees were

then indirectly transferred to returning foragers by placing 12 individuals into a prepared vial for 15 min. Each vial was used only once. Vials were prepared fresh on each study day and used within 4 hours.

The acceptance rate of the following seven treatments of workers were compared (Fig. 3.4a): 1) Nestmates, untreated; 2) Nestmates, treated with nestmate odour using the vial; 3) Nestmates, treated with non-nestmate odour from the paired hive using the vial; 4) Non-nestmates from the paired hive, untreated; 5) Non-nestmates from hive E, untreated 6) Non-nestmates, treated with non-nestmate odour from the paired hive; 7) Non-nestmates, treated with nestmate odour.

3.3.4. Experiment 2: Is the wax entrance tube used as a referent?

The aim of this experiment was to determine whether *T. angustula* guards use the wax entrance tube as a template or referent for colony odour. To achieve this we swapped entrance tubes between paired colonies using the same pairings as in experiment 1. The entrance tube was gently cut away from the hive entrance hole using a penknife. By using the natural stickiness of the wax the entrance tube could be attached to the end of a 1.5 cm length of plastic tube that exactly fitted into the hive entrance hole. The plastic tube was then placed into the hive's entrance hole and the colony was given 1-3 days to attach the entrance tube firmly to the plastic tube using additional wax. Entrance tubes could then be swapped between hives in minutes, without physical damage and with minimal disturbance. Following tube swapping, guards appeared to behave normally on the entrance tube.

The experimental design was the same as used for experiment 1, with the exception that the acceptance rate was determined only for the following treatments: 1)

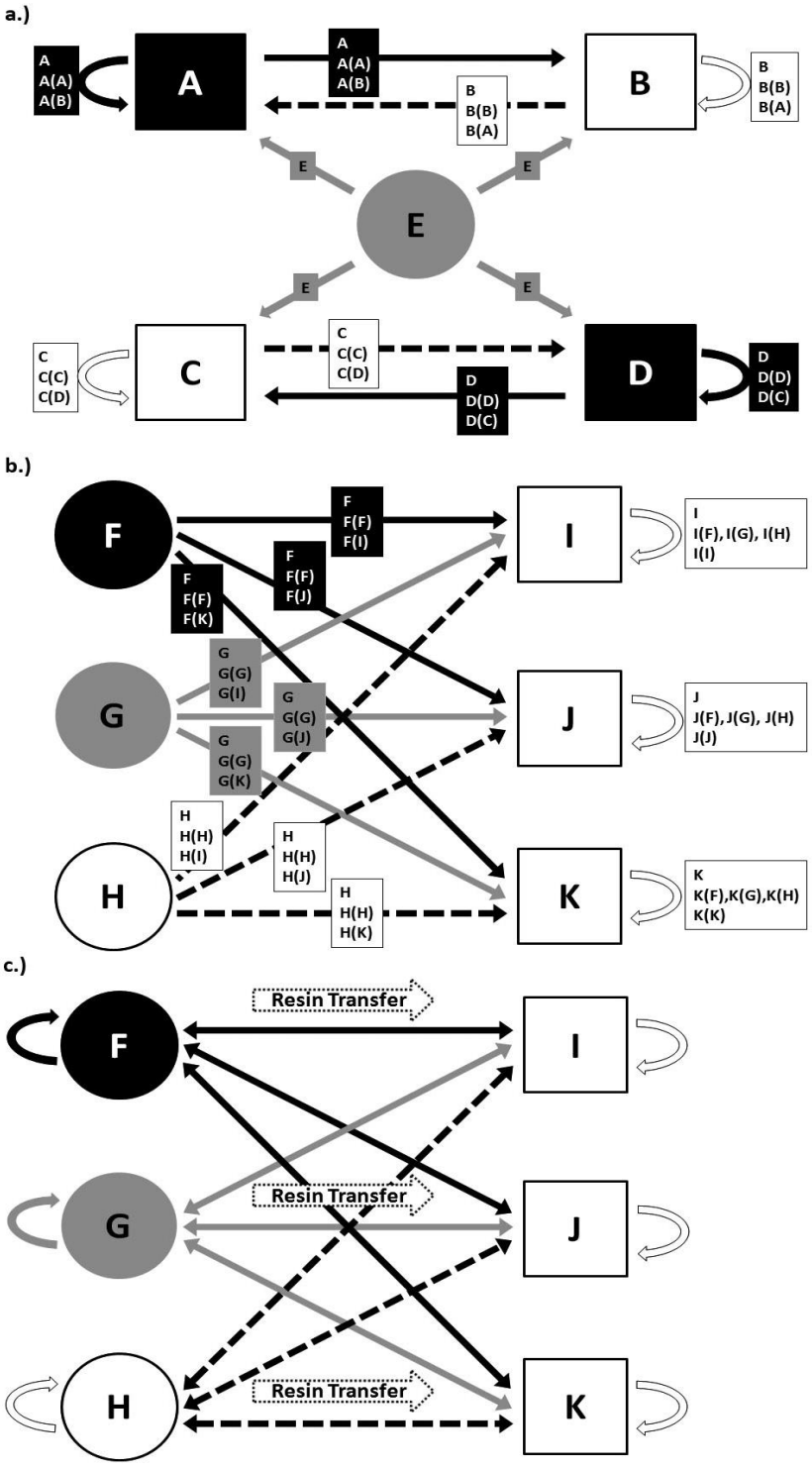


Figure 3.4. a) Experimental design of experiments 1 & 2. Treated and untreated worker bees were introduced to the entrance tubes of each discriminator hive. There were two pairs of discriminator hives (one black and one white). Hive E served as an additional source of non-nestmates, common to all four colonies. In the boxes on the arrow, unbracketed capital letters refer to the colony the introduced bee is from and bracketed letters refer to the colony that the odour treatment, if any, originates from. **b.)** Experimental design of experiment 3. Treated and untreated worker bees were introduced to the three discriminator hives, I, J and K, from the source hives, F, G and H. **c.)** Experimental design of experiment 4. Untreated worker bees were introduced to the entrance tubes of six discriminator hives F-K. Hives F-H acted as "resin donors" and hives I-K as "resin recipients". Resin was swapped unidirectionally from the donor hives to the recipient hives. Worker bees were subsequently introduced to all hives 24 hours afterwards for a period of seven days

Nestmates, 4) Paired-hive non nestmates, 5) Hive-E non-nestmates. These were compared before and 1, 5 and 24 hours after the swap (Fig. 3.4a).

3.3.5. Experiment 3: Transfer of wax and resin odours to workers

The aim of this experiment was to determine if resin or wax derived recognition cues were utilised by *T. angustula* for the purpose of nestmate recognition. Six hives (F-K) were used; three discriminator colonies and three source colonies for wax, resin and non-nestmates (Fig. 3.4b). Each discriminator colony thus received workers from all of the source colonies in addition to their own nestmates. This change to the design used in experiments 1 and 2 diminishes any possible colony-specific effects as these can be dealt with statistically. Each discriminator hive received bees from its own hive and each of the three source hives (i.e. a full factorial design) treated as follows: 1) Nestmates untreated; 2) Nestmates treated with nestmate resin/wax; 3) Nestmates treated in non-nestmate resin/wax; 4) Non-nestmates untreated; 5) Non-nestmates treated with nestmate resin/wax, 6) Non-nestmates treated with non-nestmate resin/wax.

Resin was collected from resin piles within each colony and white wax was collected from newly constructed entrance tubes. Each 4ml glass vial was treated with 0.5 ml of hexane containing 2.5 mg of either wax or resin. Evaporation left a thin, barely visible coating within each vial. Up to four workers were transferred to a treated vial and left for at least 15 min to allow indirect transfer. The bees were then chilled and introduced individually to the entrance tube of a discriminator colony as in Experiment1. Each vial was used up to three times to treat a maximum of 10 bees.

3.3.6. Experiment 4: One way transfer of resin between hives

The aim of this experiment was to investigate the effect on the nestmate recognition label and/or template of unidirectional transfer of resin between *T. angustula* hives. Hive inspections showed that all the *T. angustula* nests had resin piles of varying sizes, all dark brown in colour, which were distributed throughout the nest. The mean weight of the entire resin reserves for the six colonies was 7.79 ± 2.01 g (mean ± 1 s.e., range = 2.04 - 16.05g).

Entire resin stores were removed from a source hive, weighed and distributed as new piles within a receiving hive that had been cleared of existing resin piles the day before. Six colonies were used (F-K), paired up as three groups containing a 'resin donor' and 'resin acceptor' (F & I, G & J, H & K, Fig. 3.4c). Bees were introduced to all hives prior to and following the swap. Each donor hive received nestmates and non-nestmates from each of the three resin acceptor hives and vice versa for the receiving hives. Introductions were undertaken at four different time periods: between 12 and 96 hours before the resin swap (control) and then at 12, 60 and 84 hrs after.

3.3.7. Statistical analyses

For data analysis we used generalized linear mixed-effect models (GLMM) with binomial errors in R 2.9 (R Development Core Team 2009). We fitted the models using the lmer function (Bates 2007). Colony was included as a random effect throughout to control for the non-independence of data points from the same colony (Bolker et al. 2009; Zuur et al. 2009). For model selection we used the protocol proposed by Zuur et al. (2009). We first explored the optimal structure of the random components by comparing random intercept models with random intercept and slope models. Then

we explored the optimal fixed component structure. Wald-tests were used to determine the significance of the fixed effects (Bolker et al. 2009).

For all cases the dependent variable was the response of the guards (accept or reject). The random variable was "discriminator colony" in all experiments. Fixed variables were "treatment" in experiment 1, "time (time following entrance tube swap)" in experiment 2, "treatment" and "origin" of bee (nestmate or non-nestmate) for experiment 3 and "treatment", "time" (before or after swap) and "origin" of bee for experiment 4.

3.4. Results

3.4.1. Experiment 1: The effect of odour transfer on acceptance rates of worker bees.

Guards standing on the entrance tubes made few recognition errors with untreated introduced bees, accepting significantly more nestmates than non-nestmates, as expected (Fig. 3.5a; 94.6% vs. 4.5%, GLMM, Wald's $z = -14.15$, $p < 0.001$). The strongest effect of odour treatment came from treating nestmates with non-nestmate odour, which resulted in an acceptance 54.3% lower than for nestmates treated with nestmate odour (85.6 % vs. 31.3 %, $z = -7.41$, $p < 0.001$). Conversely, the acceptance rate of non-nestmates was not significantly affected by treatment; only 6.8 % more non-nestmates were accepted when treated with nestmate odour than when treated with non-nestmate odour (9.6% vs. 2.8%, $z = 1.92$, $p = 0.054$). A small but significant effect of the vial treatment itself could be seen on acceptance rates of bees treated with their own odour (94.6% vs. 85.6%, $z = -2.03$, $p = 0.04$).

3.4.2. Experiment 2: Is the wax entrance tube used as a referent?

Swapping entrance tubes did not affect the acceptance of either nestmates or non-nestmates (Fig. 3.5b). There was no significant difference between the acceptance rates of nestmates for the four different time periods individually (0h vs. 1h: $z = -0.46$, $p = 0.65$; 0h vs. 5h: $z = -1.83$, $p = 0.067$; 0h vs. 24h: $z = 0.14$, $p = 0.91$) and combined (0h vs. 1/5/24h: 94.6 % vs. 90.8 %, $z = -1.05$, $p = 0.29$). Similarly, there was no significant difference among the acceptance rates of paired hive non-nestmates for the different time periods, both individually (0h vs. 1h: $z = 0.79$, $p = 0.43$; 0h vs. 5h: $z = 0.79$, $p = 0.43$; 0h vs. 24h; $z = 1.32$, $p = 0.19$) and combined (0h vs. 1/5/24h: 4.5 % vs. 8.3 %, $z = 1.27$, $p = 0.21$). As expected there was also no change in the acceptance of non-nestmates from hive E post-swap (0h vs. 1/5/24h: 10.9 % vs. 9.2 %, $z = 1.26$, $p = 0.21$). In addition there is no indication that tube swapping affected the acceptance of non-nestmates from the paired colony any differently than non nestmates from the control, hive E, with no significant interaction between treatment and tube swapping (pre-swap vs. post-swap; $z = 1.27$, $p = 0.21$).

3.4.3. Experiment 3: Transfer of wax and resin odours to workers

Treatment of nestmate bees with either wax or resin lowered their acceptance rates significantly to that of non-nestmates, irrespective of whether the wax/resin originated from a nestmate or non-nestmate hive (Fig. 3.5c, 84.1% vs. 33.3%, $z = 4.78$, $p < 0.001$; 84.1% vs. 42.0%, $z = 4.71$, $p < 0.001$; 84.1% vs. 25.7%, $z = 5.27$, $p < 0.001$; 84.1% vs. 18.6%, $z = 6.81$, $p < 0.001$). Acceptance of non nestmates remained low regardless of treatment (16.7% vs. 14.3%, $z = 0.41$, $p = 0.67$; 16.7% vs. 18.6%, $z = -0.33$, $p = 0.73$; 16.7% vs. 18.2%, $z = -0.26$, $p = 0.79$; 16.7 % vs. 5.7 %, $z = 2.05$, $p = 0.040$).

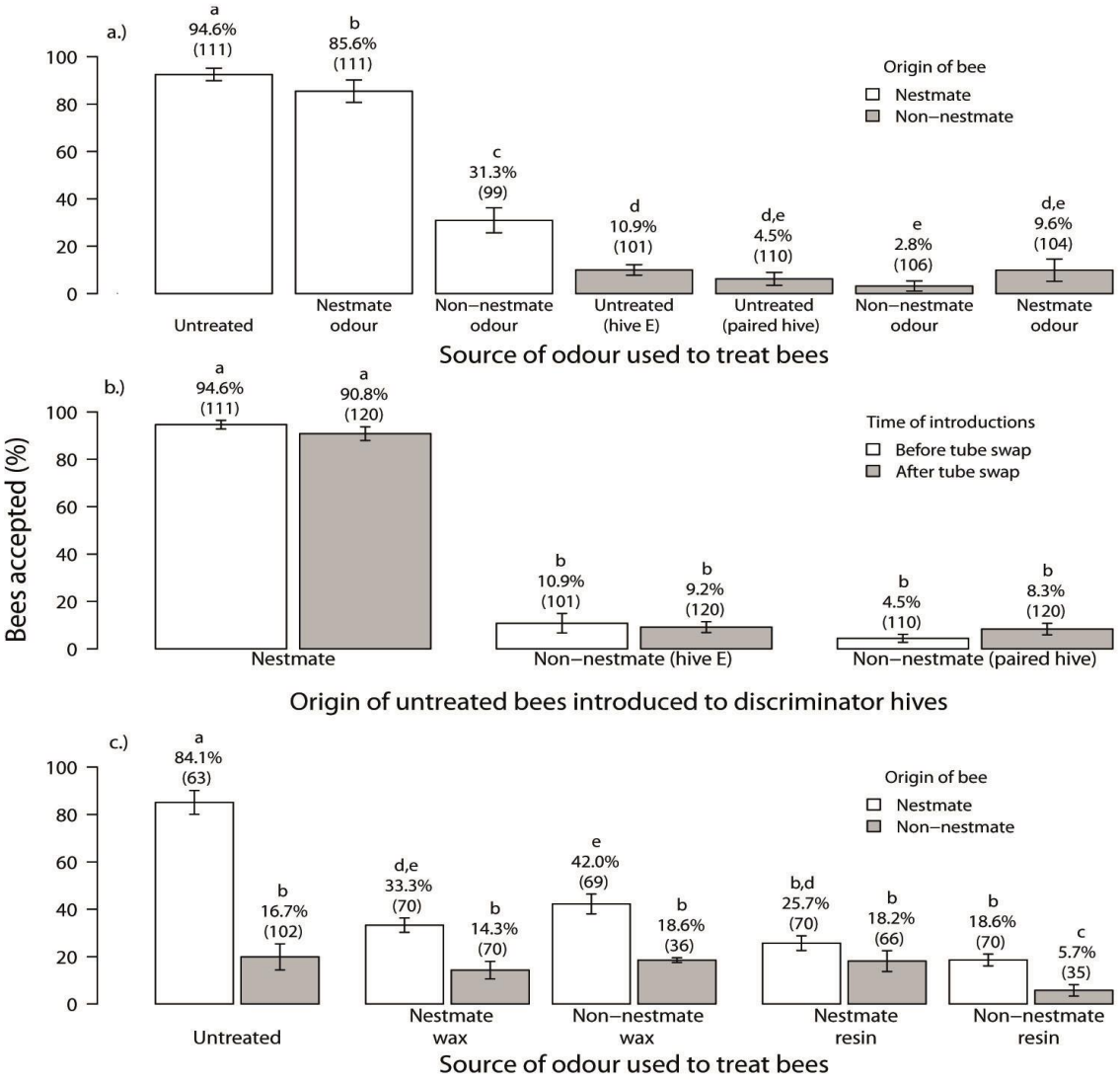


Figure 3.5. Acceptance rates of introduced bees in experiments 1, 2, and 3. **a.)** Experiment 1. Treated and untreated worker bees were introduced to four discriminator hives, A, B, C and D. Non nestmates introduced to discriminator hives originated from the paired hive and a fifth colony, colony E (untreated only), which served as a control. **b.)** Experiment 2. Nestmate workers and non-nestmate workers originating from both the paired hive and hive E (control) were introduced to four discriminator hives, both before and after swapping the wax entrance tubes. **c.)** Experiment 3. Nestmates and non-nestmate workers, either untreated or treated were introduced to three discriminator hives. Treated bees bore wax- or resin-derived odours from either their own hive or a foreign hive. Exact percentage acceptance rates and sample sizes are given above the bars. Statistically significant differences are denoted by different letters.

Interestingly, there were no pronounced differences between resin or wax sourced from nestmate and non-nestmate hives. The acceptance rates of nestmates treated

with nestmate or non-nestmate wax did not differ significantly (33.3% vs. 42.0 %, z = -0.86, p = 0.39), reflecting what we found for non-nestmates with the same treatments (14.3% vs. 18.6%, z = -0.68, p = 0.49). Similarly, the acceptance rates of nestmates treated with nestmate or non-nestmate resin did not differ significantly (18.6 % vs. 25.7 %, z = 0.84, p = 0.39). However, non-nestmates treated with non-nestmate resin were rejected to a greater extent than nestmates treated with non-nestmate resin (5.7 % vs. 18.2 %, z = 2.14, p = 0.03).

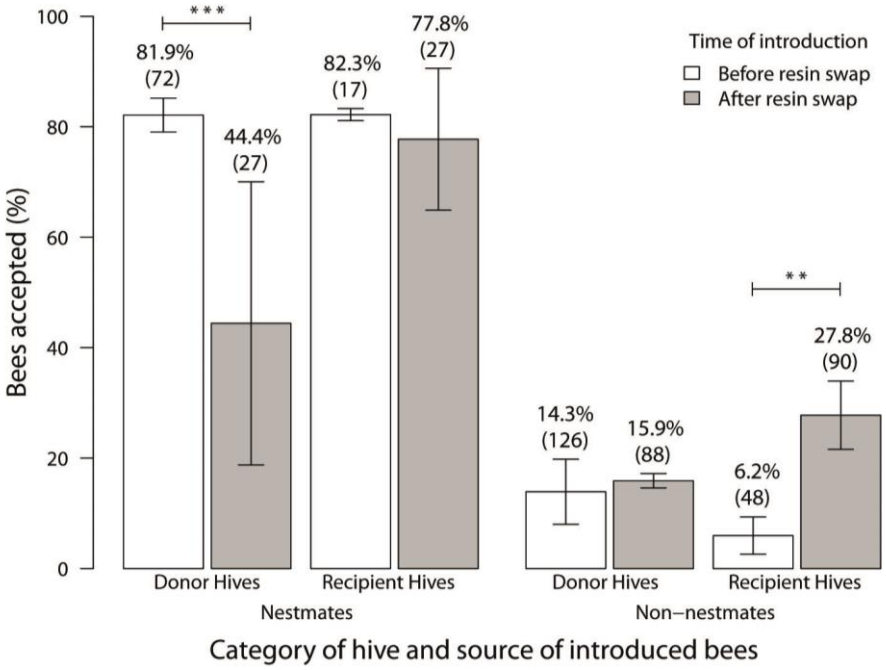


Figure 3.6. Results from Experiment 4. Nestmate workers and non-nestmate workers were introduced to six discriminator hives, both before and after a unidirectional resin transfer. Three discriminator hives were resin donors and three were resin recipients, forming three pairs. Exact percentage acceptance rates and sample sizes are given above the bars. Statistically significant differences are indicated (** <0.01, *** <0.001).

3.4.4. Experiment 4: One-way transfer of resin between hives

After unidirectional transfer of resin the acceptance rate of nestmates dropped by 37.5% for resin donor hives (from 81.9 % to 44.4 %, z = -3.51, p <0.001, Fig. 3.6), while only a decline of 4.5% was seen for resin recipient hives (from 82.3% to 77.8%, z = -0.36, p = 0.71). Conversely, for non-nestmates, a non-significant increase of 1.6% in

acceptance rates was seen in donor hives (from 14.3% to 15.9%, z = 0.35, p = 0.72), while a significant rise of 21.6 % was observed for recipient hives (from 6.2% to 27.8%, z = 2.75, p = 0.005). This effect was independent of the resin source; that is, acceptance rates did not differ between non-nestmates from the paired hive and non-nestmates from other hives (z = -1.03, p = 0.30). The overall trends were somewhat inconsistent amongst the hives with notable variation apparent (see Appendix 1).

3.5. Discussion

The results of experiment 1 confirm the exceptional recognition abilities of *T. angustula* compared to other studied bee species, with combined recognition errors of 10 %. Acceptance rates of nestmates treated with non-nestmate odour were greatly reduced while the positive effect of treating non-nestmates with nestmate odour was non-significant. Together with recent studies on the honeybee (Ratnieks *et al.* 2011), the stingless bee *F. varia* (Couvillon & Ratnieks 2008) and *Camponotus* ants (Guerrieri *et al.* 2009) these results support the idea that the odour space of a particular colony odour utilised in nestmate recognition is complex and multidimensional. In particular, a multidimensional odour space helps to explain why it is far easier for a nestmate odour to diverge from the colony odour, rather than a non-nestmate odour to converge (Ratnieks *et al.* 2011).

In experiment 2, swapping of entrance tubes had no effect on acceptance and clearly rejects the hypothesis that accurate nestmate recognition in *T. angustula* is due to the wax entrance tube serving as a convenient or immediate template for colony odour. Our data show that guards standing on the entrance tube taken from the same colony as the introduced non-nestmate bees accepted 8% of the non-nestmates compared to

5% before tube swapping. This 3% increase is non-significant and although in the predicted direction is only a very small fraction ($^{1}/_{30th}$) of the 90% difference seen between the acceptance of untreated nestmates (94.6%) and untreated non-nestmates (4.5%) observed in experiment 1. Our results also show no significant trend with time, from 1 to 24 hours post tube swap. We would expect to see an effect in this time frame as swapping of wax combs in honeybees leads to a change in behaviour within hours (Couvillon *et al.* 2007).

While experiments 1 and 2 both provided very clear results, the results of experiment 3 and 4 were less clear. Experiment 3 found no difference between acceptance rates of bees treated with nestmate or non-nestmate wax or resin while held in treated vials. If wax or resin serves as a source of colony odour we would have expected to see a disparity between nestmates treated with their own wax or resin versus non-nestmate wax or resin, as was seen in experiment 1 where the odours in the vial were derived from live bees. Instead, acceptance rates of nestmates dramatically dropped (by 50.8% & 42.1% for wax; by 58.4% & 65.5% for resin) regardless of whether the wax or resin originated from a nestmate or a non-nestmate hive, respectively. The absence of any difference between the bees' responses to the different odour sources suggest that neither resin nor wax (from entrance tubes) serve any function in the recognition process of *T. angustula*. However, the negative effect on overall acceptance may have been an artefact of the vial coating procedure (e.g. a smothering effect).

Transfer of resin stores in experiment 4 used a different method of causing worker bees to acquire odours from foreign colony resin. In hives that had donated resin, acceptance rates of non-nestmates remained the same following resin transfer, while

acceptance rates of nestmates declined by over 37% (Figure 3.6). Conversely, hives that had received resin accepted a significantly greater number of non-nestmates (an increase of 21%), while the acceptance rate of nestmates remained the same. We had expected to see a trade off in which an increase in acceptance errors and a simultaneous decrease in rejection errors both occurred (Reeve 1989; Couvillon *et al.* 2009), and vice versa, but this negative correlation was not observed. If the template of the guards had been updated following the introduction of resin, then we would have expected to see a rise in the acceptance rate of non-nestmates from the partnered donor hive (c.f. Couvillon *et al.* 2007). Although this effect is apparent, the acceptance rate of non-nestmates from non-partnered hives also increases to the same degree. At face value, it appears that guards were unable to distinguish between non-nestmates introduced from their partnered hive and other non-nestmate hives.

The behaviour shown by guards of resin donor hives in experiment 4 is also puzzling. It may be a response to the loss of the colony's entire resin store, but if this were the case we would predict a simultaneous increase in rejection rates of non-nestmates, which was not seen. The high variation in acceptance rates evident within both the donor and recipient colonies is perhaps indicative of guard confusion. Indeed, this was conspicuous with guards exhibiting frequent and intense antennation with greater periods of time preceding rejection (pers obs.). This lack of consistency in changes in acceptance rates among the discriminator colonies was also apparent in experiment 3 and is notably different from the consistent changes seen in experiments 1 and 2. Our findings appear to show that *T. angustula* do not use pure resin as a source of cues for nestmate recognition. Several studies have failed to identify the presence of terpenoids on the wings of various Neotropical stingless bees (Abdalla *et al.* 2003;

Jungnickel *et al.* 2004; Kerr, Jungnickel & Morgan 2004; Nunes *et al.* 2008). To our knowledge no study has yet analysed the cuticular chemical profiles of *T. angustula* but it would be surprising if terpenoids were absent when we know that resin, which is a rich source of terpenoids (Velikova *et al.* 2000; Sawaya *et al.* 2006), is found on the thorax of many foragers (J.S. van Zweden, unpublished data) and is universally present within the hive both in resin piles or mixed with wax as cerumen (pers. obs; Michener 1974).

It is possible that the inconsistent results may have arisen because the terpenoid composition of the pure resin we collected from the resin piles does not reflect the terpenoid profile present on the cuticles of the bees. Leonhardt et al (2011) showed that terpenoids present on the cuticles of six different Paleotropical stingless bees differed from those found on nest material. Leonhardt et al (2010) were also able to show that from a total of 1117 terpenoids available in stored resin, only 10 % (105) were actually present on the cuticle of the Paleotropical stingless bee, *Tetragonilla collina*. To explain this Leonhardt et al (2011) proposed a hypothesis whereby stingless bees are able to perform some form of post collection manipulation of resin terpenoids to ensure odour constancy. Resin stored by colonies of *T. angustula* from across Brazil was found to have a remarkably consistent composition, regardless of location (Sawaya *et al.* 2006). Therefore, for terpenoids to function as cues for nestmate recognition quantitative differences between a discrete set of these compounds must be apparent among colonies and this would have to be achieved by some form of post collection manipulation. However, the idea of selectively manipulating and distributing specific terpenoids is somewhat *ad hoc* and in order to function would require a number of complex and unknown mechanisms. A more

parsimonious explanation is that resin simply does not function as a primary source of recognition cues. An inherent problem with using collected materials, such as resin or food, as odour cues is the likelihood that availability of the sources will change with time (Downs & Ratnieks 2000; Downs *et al.* 2001). Once a bee collects new material which is not consistent with its colony odour there is a strong possibility that it will be rejected. For example floral odours, most of which are terpenoids, were found to have no function in honey bee nestmate recognition (Downs & Ratnieks 2000).

Although our results showed that swapping entrance tubes had no effect and also that there was no effect of vial transfer of wax onto individual workers, wax may still be used in other contexts. For example the wax used to construct brood combs. Waxes are secreted by the bees and therefore would be expected to be fairly consistent to a colony whereas resin may be more changeable with time, thus presenting a less consistent basis for a colony odour. Cerumen, formed by bees from a mixture of wax and resin, is universally present within the hive architecture and involucrum and thus may serve a role in colony odour acquisition. Callow workers could acquire a colony odour profile through contact with the comb and surrounding involucrum, analogous to young honeybees on wax combs (Breed *et al.* 1995; Breed 1998). Indeed, the results of a recent study by Nunes et al. (2011) suggest that cerumen may be a source of recognition cues, used by colony members of the stingless bee *Frieseomelitta varia*.

Overall, our results confirm the accuracy of the nestmate recognition system in *T. angustula*. When results of all experiments were combined, a typical average of 10 % was observed for both acceptance and rejection errors, giving a total error rate of 20%. Although not as low as that found by Karcher and Ratnieks (2009) this remains

considerably lower than error rates reported for honey bees (Downs & Ratnieks 1999, 2000; Couvillon *et al.* 2007, 2008, 2009, 2010) and lower than all stingless bee species studied to date (Breed & Page 1991; Buchwald & Breed 2005; Couvillon & Ratnieks 2008). Although our results do not show how *T. angustula* achieves this accuracy, we have ruled out one strong contender: the wax entrance tubes of *T. angustula* nests appear to play no role in nestmate recognition. Our results from experiments 3 and 4 suggest that odours acquired directly from resin also serve no function as nestmate recognition cues, although the observed shifts in the acceptance threshold seen in experiment 4 suggest a possible secondary role. However, the variation and inconsistency of our results in experiments 3 and 4 together highlight the need for future chemical analysis of resin stores, cerumen and the cuticular profiles of worker bees. Moreover, it also remains to be seen whether this proficient recognition system has evolved as a result of low genetic variability or high parasite pressure (Martin *et al.* 2011). Maybe then we will be able to identify the underlying mechanisms that permit such a proficient nestmate recognition system with so few errors

4

Sensory Information Use and Trail Laying in the Ant *Lasius niger* is Determined by Light Intensity

4.1. Abstract

Foragers of the black garden ant, *Lasius niger,* are able to navigate between their nest and a food source by using both olfactory (pheromonal) cues and by memorising visual cues from their surroundings. When a conflict between these two information sources is presented to *L. niger* foragers at a trail bifurcation, under daylight conditions, they predominantly choose their visual route memory over a pheromone trail. Given that this species forages at night as well as during the day, the present study seeks to determine whether pheromone deposition increases with reduced visual acuity, and if this is the case, whether there is a concomitant shift in dependency from visual to olfactory cues. Both pheromone deposition and the visual/olfactory conflict were evaluated at three different light levels: bright (3,100 lux), intermediate (10 lux) and near darkness (0.0007 lux). Pheromone deposition, overall, was found to increase as light intensity decreased, however, the rate of deposition for each journey, between food source and nest, differed significantly between the three light treatments.

When presented with a conflict between a strong pheromone trail and a visual route memory the response to pheromone differed significantly between the bright (27 %) and near darkness (61 %) light treatments. Foraging ants preferentially chose route

memory in bright light conditions and trail pheromone in darker light conditions. We also tested if foraging workers of *L. niger* were able to memorise a route in the absence of any visual stimuli, perhaps using idiothetic cues. In near darkness the proportion of ants choosing the rewarding route after one visit did not differ from that of naive ants, suggesting that this species cannot navigate idiothetically. Overall, our results demonstrate that while pheromone deposition does indeed increase with reduced visibility, individual decisions to lay pheromone are also governed by route familiarity. We also show that foraging ants flexibly respond to available light by increasing their dependence on these olfactory cues as light levels decrease, thus enabling efficient exploitation of resources under different environmental conditions.

4.2. Introduction

The ability of social insects to efficiently exploit available resources is a primary reason for their ecological success. Eusocial insect species have evolved a variety of communicational methods, enabling many of them to thrive beyond that of their solitary counterparts (Wilson 1971; Oster & Wilson 1978). For example, mass recruitment, aided by trail and recruitment pheromones (Hölldobler 1976; Traniello 1989; Beckers *et al.* 1992), and stridulation (Roces, Tautz & Hölldobler 1993; Pavan *et al.* 1997), are two contrasting foraging strategies utilised by ants to mediate efficient resource retrieval.

Navigation aided by the use of pheromone trails is common in ants (Hölldobler & Wilson 1990; Traniello & Robson 1995) and has also been found in termites (Howard, Matsumura & Coppel 1976; Traniello 1982) and stingless bees (Jarau *et al.* 2006; Barth, Hrncir & Jarau 2008). In ants the use of trail pheromones is often complex with multi

component pheromonal blends which allow foraging ants to adjust behaviour according to changing ecological conditions (Dussutour *et al.* 2009) and to optimise food exploitation (Jackson *et al.* 2007). The rules that govern this often sophisticated trail laying behaviour are diverse: the direction of travel (Czaczkes *et al.* 2012) proximity to a food source (Beckers *et al.* 1992), quality of a food source (Beckers *et al.* 1993), and presence of home-range markings or trail pheromone (Devigne & Detrain 2006; Czaczkes *et al.* 2012), are all thought to play a role in determining the pheromone laying behaviour of foraging ants (Czaczkes *et al.* 2012).

Navigation is by no means restricted to olfactory cues; for example, some ant species are also able to utilise celestial (Wehner & Duelli 1971; Wehner 1984), thigmotactic, (Klotz & Reid 1993) and geomagnetic (Anderson & Meer 1993; Camlitepe & Stradling 1995) cues to aid orientation. While foraging, ant workers are also able to use visual cues to develop a route memory between one or more food resources and the nest (Collett *et al.* 2003; Kohler & Wehner 2005; Harris *et al.* 2007). Memories of a particular resource location can last for weeks or even months (Rosengren & Fortelius 1986; Quinet & Pasteels 1996), and development of a dependable route memory may only require a single visit. For example, 77 % of *Leptothorax unifasciatus* workers (Aron *et al.* 1988), and 75 % of *Lasius niger* workers (Grüter *et al.* 2011a), chose the correct branch of a 2 branched maze after one visit to a food source, in the absence of any trail pheromone.

Which cue an ant species relies upon is often governed by its position within a hierarchy. In this way, one sensory modality, such as visual, is utilised until it becomes ineffective after which a switch is made to another sensory modality, e.g. olfaction and

so on (Hölldobler & Wilson 1990). Ants relying on visually based cues may be forced to rely on a more accessible source of cues if foraging nocturnally, when light becomes limiting. After sunset foraging *Catglyphis* ants switch to using anemomenotaxis (air currents) and tropotaxis (light) towards the azimuth of the moon (Wehner & Duelli 1971), while black carpenter ants, *Camponotus pennsylvanicus*, orientate thigmotactically (using touch) using structural heterogeneity in their terrestrial environment (Klotz & Reid 1993). Trail pheromone may also aid route navigation when a primary cue becomes unreliable or unavailable. Cammaerts et al. (1980) observed *Myrmica sabuleti* foragers switching to pheromone laying during nocturnal foraging when access to visual cues was reduced. While this behavioural switch makes intuitive sense it has yet to be thoroughly investigated and quantified

Because ants are not restricted to a single sensory modality, one would expect situations to occur in nature, where experienced foragers face a conflict between two different information sources. One such example is a conflict between an ant's own route memory and a pheromone trail at a route bifurcation. This has been studied in a number of ant species with some species relying more heavily on memory and others on olfactory cues (*Formica sp.*, Rosengren & Fortelius 1986; *Paraponera clavata*, Harrison et al. 1989; *Lasius niger & Iridomyrmex humilis*, Aron et al. 1993; *Lasius niger*, Grüter, Czaczkes, & Ratnieks 2011). Utilisation of these sensory modalities are not always mutually exclusive; bimodal sensory input acquired by combining the input of visual and olfactory cues appeared to increase perception and learning in the desert ant *Cataglyphis fortis* (Steck, Hansson & Knaden 2011), and aid navigation of a simple maze in the *Myrmica ruginodis* (Cammaerts *et al.* 2012).

The common garden ant *Lasius niger* uses both an acquired route memory and trail pheromones to navigate between nest and food resource (Beckers *et al.* 1992; Aron *et al.* 1993; Evison *et al.* 2008). A route memory is established through the use of surrounding visual landmarks (Evison *et al.* 2008), and its development is rapid, with often only a single visit required (Grüter *et al.* 2011a). Recently Grüter et al (2011a) demonstrated that, in daylight conditions, *L. niger* foragers predominantly choose their own route memory over a pheromone trail, irrespective of trail pheromone strength. Because *L. niger* ants forage both diurnally and nocturnally (S Jones & E Gourlay pers. Obs; Wilson 1955) we would predict that trail laying would increase to aid nocturnal navigation, as was observed in *M. sabuleti* by Cammaerts et al. (1980). If this is the case we would further expect to see a shift in dependence on these now readily available olfactory cues, as visual ones become unavailable. While this switch in cue dependence has been studied in both *Formica* ants (Beugnon & Fourcassie 1988) and *C. pennsylvanicus* (Klotz & Reid 1993) in the field a more rigorous laboratory based procedure is needed to directly compare the conflict between the two information sources at distinctly different light levels.

In this chapter we show that pheromone deposition does indeed increase as available light diminishes but with significant differences in laying behaviour between the outward and return journeys under the three different lighting regimes. Concomitant with this I also show that as light levels are reduced, foraging ants switch to a greater reliance on olfactory cues when facing a conflict between trail pheromone and route memory. Finally I demonstrate that *L. niger* foragers are unable to navigate idiothetically in the absence of visual cues.

4.3. Material and Methods

4.3.1. Study Species

We studied nine colonies of *Lasius niger* ants, all collected from Falmer in East Sussex, UK. Each colony was housed in a plastic container (30 × 30 ×10 cm high) with a plaster of Paris base containing a circular nest cavity constructed from plaster of Paris (13.5 cm diameter × 1.5 cm high) and covered by a disc of dark card. All colonies were queenless with 1,000 – 3,000 workers and small numbers of brood. Queenless colonies readily forage, produce trails and are commonly used in behavioural experiments (Evison *et al.* 2008; e.g. Grüter *et al.* 2011a), remaining viable for 18 months or more. The ants were fed three times a week on a mixture of agar, honey, raw egg and vitamins (see Hölldobler & Wilson 1990 p. 632) with *ad libitum* access to water. To ensure motivation, feeding was stopped 4 days prior to an experiment.

4.3.2. Experimental Design

Following the method of Grüter et al. (2011a), we constructed a foraging trail as shown in Figure 4.1b. A white cardboard bridge (20 × 2 cm) connected the colony container to a transparent polycarbonate plastic T-shaped trail covered with white paper (plain paper 90 g/m^2). The stem of the T was 15 cm long and each branch was 11 cm long, with a consistent width of 2cm. Experiment 1 was run in a small windowless room with temperature control. The ambient temperature of the room was maintained at 22 ^{o}C. Experiments 2 and 3 were carried out in a small room containing various items of lab equipment and furniture which served as visual landmarks for the foraging ants. During the intermediate and near darkness treatments the window in this room was covered using black sugar paper. A portable halogen work light (IP 44; model NXS-500P) with a 500 w halogen bulb, positioned 1.5 m from the trail was used to provide

high intensity illumination for the high light treatment. At this distance heat produced by the lamps was negligible. To provide a medium light treatment, a 1.8 m tall floor lamp with a 230 w linear halogen bulb and dimmer switch (Dar; model OPU 4946), again positioned 1.5 m from the trail, was used. The In the medium and low light experiments infrared light was used to provide illumination for experimental working and behavioural observations, but this long wavelength illumination was not detectable by the ants. Like us, most insects have trichromatic vision (UV, blue and green in the case of insects; Briscoe & Chittka 2001), but their visible spectrum is shifted towards shorter wavelengths than ours (Menzel 1979): for example the spectral sensitivity maxima (λ_{max}) for the ants *Atta sexdens* and *Cataglyphis bicolor* are 500 nm (Martinoya *et al.* 1975) and 570 nm (Kretz 1979), considerably lower than 700 nm found in humans (Autrum 1968). To provide infrared light a sleeve was created from 2 ply corrugated cardboard to fit tightly over the hood of an angle poised lamp with a 60 w bulb. Two 50 mm square 665 nm long pass (IR) filters (Schott; model FRG-66550) were slotted tightly together into a hole cut in the centre of the cardboard hood so that when switched on the lamp only provided infra red light. A photometer (LI-COR inc; model LI-188B) was used to ensure illumination was consistent within treatment replicates. Luminances were 0.0007, 10 and 3,100 lux (conversion from photosynthetic photon flux as per Thimijan & Heins 1983) for the three treatments. These luminances were chosen to reflect a moonless night, crepuscular light and normal daylight respectively.

4.3.3. Experiment 1: Does the frequency of pheromone deposition change with illuminance?

Six colonies were used to investigate the frequency of pheromone deposition at different light levels. Ants were allowed to locate and feed on 1M sugar water solution, randomly allocated to the end of the left or right branch of the T maze (Fig. 4.1). A 5 cm long section of paper, located just before the branches of the T, was marked by lines at either end and a video camera (Sony; model HDR-XR520) was positioned to record, from the side, all pheromone laying behaviour of ants walking along this designated section (Fig. 4.1). This section was chosen because ants were observed to regularly deposit pheromone near the junction and it was also easier to monitor using the camera. The low lux camera setting was used for the medium and low light levels. Depending on foraging activity of the colony, the first 12-15 ants that reached the food source and began to feed were marked with a dot of grey acrylic paint (the most discernable colour under IR light), using a wooden cocktail stick. If done carefully the ants ignored the procedure or, if disturbed, rapidly resumed feeding. Any ants that did not settle were removed from the experiment. All unmarked ants were removed from the bridge and T maze. The marked ants were allowed to: find their way back to the nest, return to the food source and navigate towards the nest once more. Marked ants were removed after passing through the observation section on this final trip. Thus, a maximum of 3 journeys were recorded for each ant. When analysing the videos we assumed that an ant deposited a drop of pheromone each time we saw it clearly curve and dip its gaster to the surface (Aron *et al.* 1993; Grüter *et al.* 2011a). The experiment was carried out under the three different lighting regimes.

4.3.4. Experiment 2: Effect of illumination on pheromone dependency

To test if reliance on trail pheromones increases at lower light levels, foraging ants were offered a choice between their own route memory and a pheromone trail at a T

junction. A pheromone trail was created by allowing ants to freely forage on a solution of 1M sugar water situated on the T maze before the bifurcation (Fig. 4.2a). A piece of paper (Segment X; 10 × 2 cm) was placed directly before the food source with a section of it (4 × 2 cm) covered by an additional piece of paper (segment Y). This ensured that the covered section beneath segment Y remained free from pheromone deposited by ants leaving and returning to the food source. A consistent trail strength was achieved by ending foraging once 35-40 deposits had been recorded. The maximum time we allowed for trail establishment was 20 min; if the minimum number of deposits was not reached in this time the experiment was terminated.

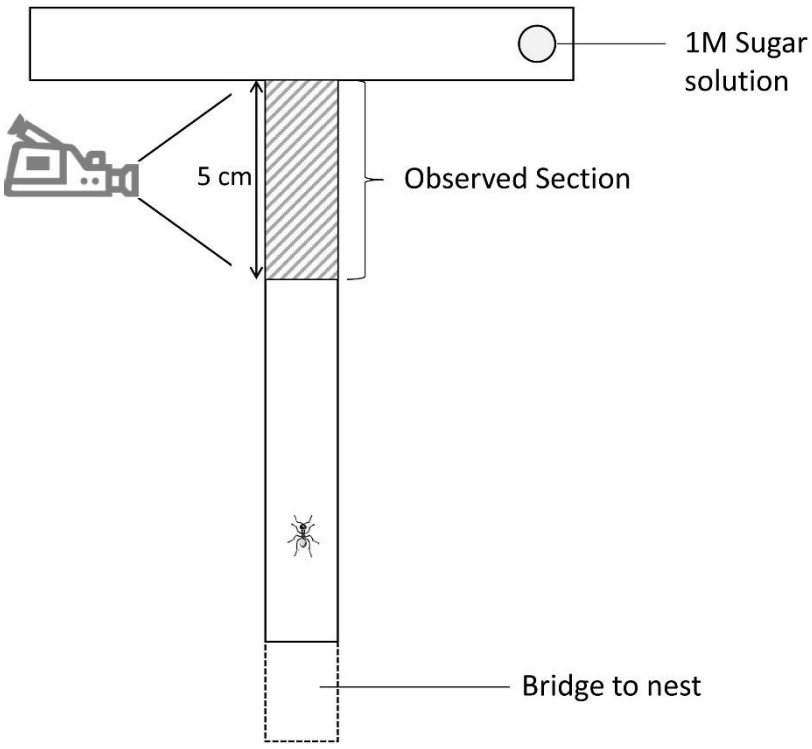


Figure 4.1. Experimental design used to measure trail pheromone deposition frequency by foraging ants under three different lighting regimes (near darkness, intermediate and bright). For each treatment frequency of deposition was recorded for the observed section (hashed area) for three journeys: the first journey to the nest, the first return to the food source and the second return to the nest. Each experiment involved 8-12 ants that were marked with paint when feeding on the sugar solution for the first time.

Memory was then established by placing a 1M sugar solution source on the end of a randomly selected branch of the T maze and allowing the ants to find the food source via the bridge. Feeding ants were marked with a dot of grey acrylic paint applied using a wooden cocktail stick and allowed to return to the nest. Before these marked ants

left the nest to relocate the food, section X was transferred to the bifurcation of the T maze (Fig 4.2b) with the pheromone marked side placed on the branch opposite to where the food source had initially been situated. The covering piece Y was removed so that the bifurcation now had two new arms, only one of which was marked with pheromone. The decisions of the returning marked ants were then recorded. The maximum time allowed for memory development and subsequent decisions by the ants was 30 mins, giving a total maximum experimental time of 50 min, when including

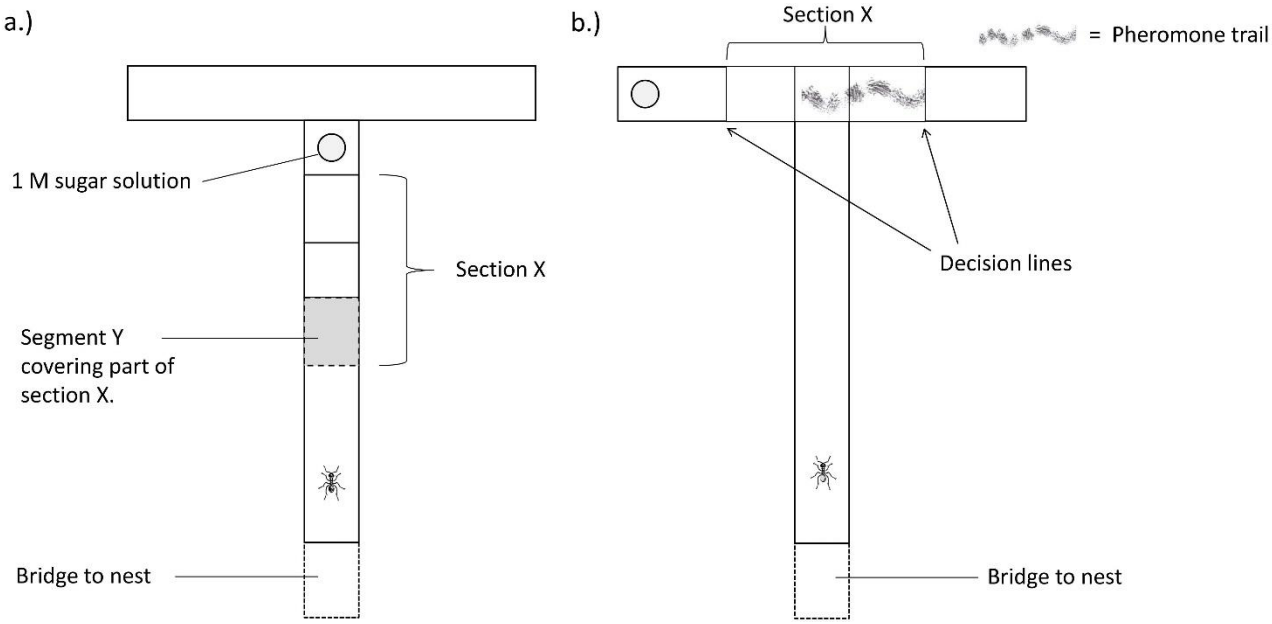


Figure 4.2. a.) Experimental design used to acquire trail pheromone for subsequent conflict situations. A section of paper (X) was partially covered by a segment (Y) and ants were allowed to forage on a 1M sugar solution. Pheromone deposition on the uncovered part of section X was monitored until 35-40 deposits were reached, after which foraging was stopped and section X, minus segment Y, was transferred to a second T maze for experiment 2**. b.)** Experimental design used for experiment 2. Ants were allowed to locate a 1M sugar solution on a randomly chosen branch. While feeding ants were marked with a paint dot and allowed to return to the nest. Section X was then transferred to the bifurcation so that marked returning ants were faced with a conflict between their route memory and trail pheromone. Decisions were recorded once an ant passed either of the two decision lines. Naive ants with no memory were also tested to determine their response to pheromone alone, and as a control for any side bias.

trail establishment, which corresponds to the mean trail lifetime reported for *Lasius*

niger (Beckers *et al.* 1993; Evison *et al.* 2008). The same procedure was repeated using naive ants with no prior visit to the food source to control for any side bias. Decisions were recorded for ants from nine colonies, tested under the three different lighting regimes.

4.3.5. Experiment 3: Is memory based solely upon visual cues?

Ten colonies were used to investigate whether ants could develop a route memory in the absence of visual cues. As in experiment 2 the nest was connected to the T maze by a cardboard bridge and a one Molar sugar solution was placed at the end of a randomly assigned branch. Under near darkness (0.0007 lux), foraging ants were allowed to locate the food source and were subsequently marked with grey acrylic paint while feeding. Unmarked ants were removed from the maze and marked ants allowed to return to the nest. Fresh paper was placed on the T maze to remove any pheromone present and the binary choices made by returning marked ants at the T junction were recorded and compared to those of naive ants.

4.3.6. Statistical analysis

Data for the pheromone deposition frequency was found to be zero-inflated so we consequently chose to use the MCMCglmm package (Hadfield 2010) implemented in R v. 2.14.2 (R Development Core Team 2012) using the “zipoisson” family function. Uninformative prior distributions were used for fixed effect parameters with a mean of 0 and a large variance of 10^8. Priors for the variance components were inverse-Wishart distributed with the degree of belief parameter (n) set at ¼ 0.01 and variance (V) limited to 1. Each model was run for 120,000 Markov chain Monte Carlo (MCMC) simulation iterations with a burn-in of 40,000 iterations and a thinning interval of 10 iterations. Autocorrelation between successive iterations was low (<0.05). Maximal

models were created and non-significant fixed effects were sequentially removed from the model. Models were compared using the deviance information criterion (DIC). The fixed effects included light treatment (levels of bright, intermediate and near darkness) and journey (levels of towards nest (1 & 2) & towards food source) while colony and date were used as independent random effects. Mean parameter estimates and 95% credible intervals were constructed and are reported in the results; where estimates do not range over zero, the parameter is deemed to be significant.

Data from experiments 2 and 3 were analysed using generalised linear mixed-effect models (GLMM) with binomial errors in R v.2.14.2 (R Development Core Team 2012). Models were fitted using the lmer function (Bates, Maechler & Bolker 2011). For model selection we used the protocol proposed by Zuur et al. (2009). We first explored the optimal structure of the random components by comparing random intercept models with random intercept and slope models. Colony was included as a random effect throughout while date was dropped from both models following model comparison. The optimal fixed component structure was then explored whereby any non-significant fixed effects were dropped from the model. Wald tests were used to determine the significance of the fixed effects and models were fitted using the Laplace approximation (Bolker *et al.* 2009; Bates *et al.* 2011). Fixed effects included in the optimal model were light treatment (levels = bright, near darkness & intermediate) in experiment 2 and experience (levels = experienced & naive) in experiment 3.

4.4. Results

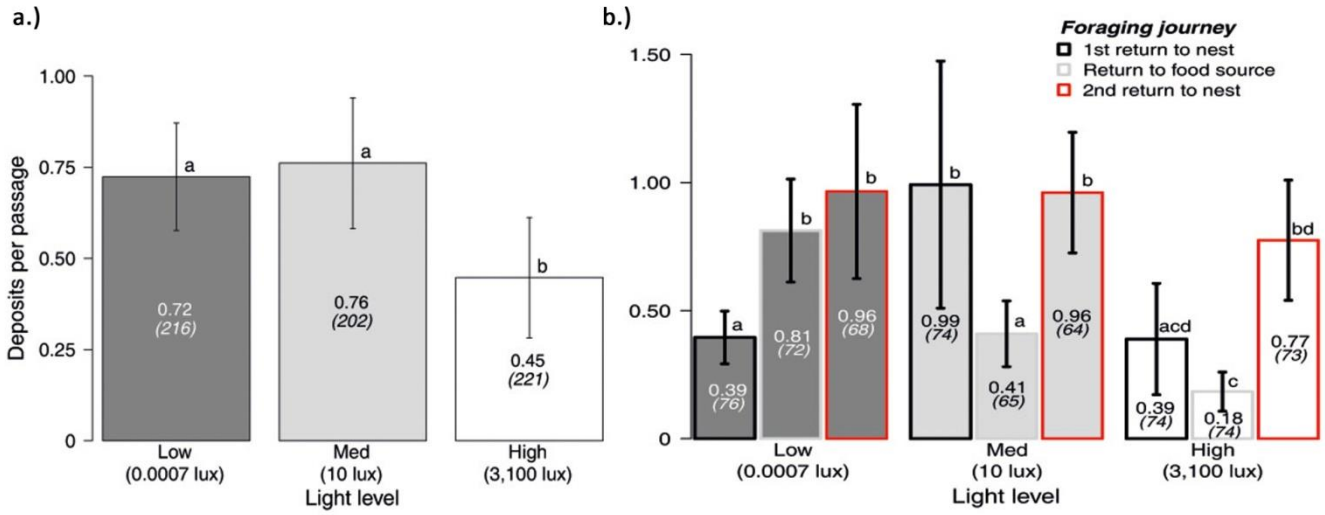
4.4.1. Experiment 1: Does the frequency of pheromone deposition change with luminance?

As luminance dropped to 10 lux, pheromone deposition significantly increased from 0.45 deposits per passage to 0.76 [intermediate vs bright; parameter estimate = -1.477, 95% CI = (-2.769,-0.122); near darkness vs bright; parameter estimate = -1.206, 95% CI = (-2.267,-0.167), Fig. 4.3a]. However, pheromone deposition did not continue to increase when lumination was further reduced from 10 lux to 0.0007 lux [Med vs Low; parameter estimate = -0.488, 95% CI = (-1.784, 0.884)]

However, when the frequency of pheromone laying was analysed over each of the three foraging journeys made by the ants, significant differences become apparent. Significantly more deposits were made on the first journey back to the nest under the intermediate light level compared to what was found for the other two light treatments [Intermediate vs Bright; 0.99 vs 0.39, parameter estimate = 2.175, 95% CI = (0.605, 3.713), Intermediate vs Near Darkness; 0.99 vs 0.39, parameter estimate = 2.309, 95% CI = (1.048, 3.602), Fig. 4.3b]. Of particular note is the significantly greater number of deposits on the return journey from the nest to the food source in the dark, when compared to either intermediate or bright light levels [Dark vs Intermediate; 0.81 vs 0.41, parameter estimate = -2.249, 95% CI = (-3.509, -0.880), Dark vs Bright; 0.81 vs 0.18, parameter estimate = -2.184, 95% CI = (-3.973, -0.600)]. On the second return journey to the nest, pheromone deposition was almost one deposit per passage in both darkness and bright light, but in each case was found not to differ significantly from the0.77 deposits per passage, seen in high light conditions [Dark vs Bright; 0.96 vs 0.77, parameter estimate = -0.462, 95% CI = (-2.210, 1.158), Intermediate vs Bright; 0.96 vs 0.77, parameter estimate = -0.834, 95% CI = (-2.373, 0.884)]. There was no significant difference in the rate of pheromone deposition between the two return journeys to the nest in either the bright or intermediate light conditions [Bright; 0.39

vs 0.77; parameter estimate = 0.565, 95% CI = (-0.842, 1.730), Intermediate; 0.99 vs 0.96, parameter estimate = -0.174, 95% CI = (-1.351, 1.019)], but in the dark, pheromone deposition significantly increased on the second return journey [Low; 0.39 vs 0.96, parameter estimate = 1.066, 95% CI = (0.085, 1.929)].

Figure 4.3. a.) The mean number of pheromone deposits per passage is for the 3 different light levels (dark, intermediate and bright) for the three journeys combined. The deposition rate



significantly increased when light intensity was reduced to 10 lux, but no further increase was seen with a further reduction in light intensity. **b.)** The mean number of pheromone deposits per passage for the three separate journeys for each of the three light levels. During the first inward journey to the nest deposition frequency is greatest at the intermediate level, while for the subsequent outward journey the deposition frequency is significantly greater at the lowest light level. For the second inward journey there is no significant difference between the deposition rates for three lighting regimes. Within each bar is given the true bar height (mean) and in brackets, the sample size (number of passages). Different letters (given above each bar) denote statistically significant differences and error bars represent standard deviations of the mean. Passage refers to the 5cm long observed section along the T maze.

4.4.2. Experiment 2: Effect of illumination on pheromone dependency

Response of ant foragers to pheromone increased with decreasing light intensity (Fig. 4.4a). Under bright light only 27 % of ants chose the pheromone treated branch, significantly less than the 61 % seen in near darkness ($z = -3.50$, $P < 0.001$). While an

obvious gradient in pheromone response is visible in Figure 4.4a, the effect of changing light from intermediate to near darkness is insignificant (43 vs. 61; z = 1.28, P = 0.202). The increase in response to pheromone from 27 % to 43 %, however, is significant (H vs. M; z = 2.31, P = 0.021). The random effect of colony contributed a significant amount of the overall variance (10.1 %).

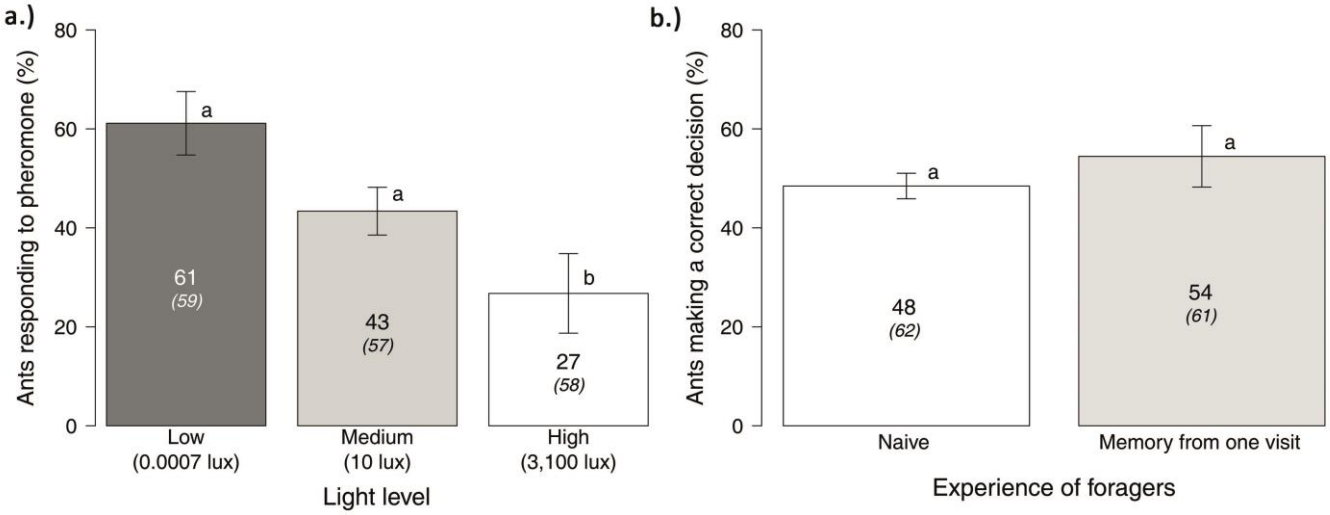


Figure 4.4 a.) Mean percentage of ants choosing the branch of a T maze treated with trail pheromone when presented with a conflict between their memory of the route (acquired from 1 visit) and trail pheromone, under three different lighting regimes (bright, intermediate and dark). Response to pheromone significantly increased as light dropped from bright to intermediate. However, mean responses did not differ significantly between the intermediate and dark light levels, despite there being a visible difference. **b.)** Mean percentage of ants choosing the branch of a T maze that leads to a food source under a low light level of 0.0007 lux. The grey bar represents ants that had visited the food source once before and the white bar represents naive ants with no memory. Ants with experience of one visit were no better than naive ants at choosing the correct branch, suggesting that navigation by idiothetic means is not occurring. Within each bar is given the true bar height (mean) and in brackets, the sample size (number of ants). Different letters (given above each bar) denote significant differences and error bars represent the standard error of the mean.

4.4.3. Experiment 3: Is memory based solely upon visual cues?

Ants with experience of visiting a food resource, based upon a single visit under 0.0007 lux, did not perform better than naive ants when choosing a branch at the bifurcation

(Figure 4.4b). While 48 % of naive ants chose the branch to the food, only 54 % of ants with a memory made the correct decision ($z = 0.99$, $P = 0.31$). The random effect of colony contributed to over 9 % of the overall variance.

4.5. Discussion

Foraging *L. niger* ants increased the rate of pheromone deposition as light intensity dropped from 3,100 to 10 lux. On the first return journey to the nest, when pheromone was absent from the substrate, the ants' deposition behaviour was very different at the three light levels, with a lower frequency of deposition in the dark and bright light treatments, but significantly greater deposition under the intermediate level. My interpretation of this observation is that a fed ant is reluctant to lay a trail on its first journey back to the nest when it is unsure of its location in the darkness, but does deposit more frequently at intermediate light levels when adequate visual information enables it to correctly determine the route back to the nest. Ants can measure ambient light levels and use these cues to dictate when to forage (Narendra, Reid & Hemmi 2010), so it is entirely possible that these cues are also used to initiate the behavioural change of increasing pheromone deposition. In bright light, readily accessible visual cues enable foraging ants to navigate back to the nest with little need for olfactory confirmation. On the return to the food source in darkness, on what was evidently a rewarding route, the trail was reinforced by an increased rate of pheromone deposition. In contrast, at intermediate and high light levels, frequency of deposition was low, presumably because the propensity of an ant to lay is significantly lower when the gaster is empty (Czaczkes *et al.* 2013). On the second return to the nest, with a full gaster, these rewarding trails were reinforced at a similar rate for all three light levels. The increase in the deposition frequency for this journey in daylight

conditions, despite being statistically non-significant, is still suggestive of growing route familiarity, especially when we know route memory is well established after two visits to a food source in this species under these lighting conditions (Grüter *et al.* 2011a). Therefore, rather than aiding the navigation of both itself and its nestmates, deposition by an ant in these conditions is likely to be solely for the benefit of inexperienced foragers from the colony.

In darkness, the increased frequency of deposition over subsequent journeys supports the idea of foraging ants developing route confidence or familiarity. Just as navigational difficulties in darkness increase the frequency of laying, so does route complexity; when forced to navigate a doubly bifurcating maze (thus providing four possible options instead of two) deposition was found to increase (Czaczkes *et al.* 2013). This lends credence to the hypothesis whereby navigational difficulties resulting from a lack of visual acuity result in increases in individual deposition rates.

Concomitant with *L. niger* ants' investing more in pheromone deposition as light levels decline, our data clearly show that ants switch from utilising their previously acquired route memory to using olfactory cues for navigation at these lower light levels. During the day *L. niger*, like many epigeal ant species, use visual features of the surrounding landscape to form a route memory (Harris *et al.* 2007; Evison *et al.* 2008). As light levels decrease these visual cues become less reliable and therefore other available cues, such as olfactory, become more important. This shift in cue reliance has been previously reported in field observations on two *Formica* species, *F. polyctena* (Beugnon & Fourcassie 1988) and *F. nigricans* (Rosengren 1977), but our study is the first to demonstrate this using a behaviourally relevant choice test under stringently

controlled laboratory conditions. Both Beugnon and Fourcassie's (1988) and Rosengren's (1977) studies were undertaken in the field, where extraneous environmental effects and navigational cues such as temperature (Traniello, Fujita & Bowen 1984; van Oudenhove *et al.* 2011), wind (Wolf & Wehner 2000), substrate (Detrain & Deneubourg 2002; Jeanson *et al.* 2003) and light sources (Wehner 1984; Klotz & Reid 1993) may have affected the behaviour of foraging ants. Furthermore, we are sure that the shift in cue reliance we report, in response to changing light levels, is governed by the salience of visual cues and not pheromone concentration because the amount of pheromone present at the test junction was assumed to be constant in all our experiments. There is a possibility that impurities in the paper used in this experiment, such as bleach compounds, may have affected the chemical properties of the pheromone. However, the pheromone still appeared to induce trail following behaviour throughout the experimental trials. A further caveat that could affect our results, involves the possibility that pheromone could penetrate the overlaying paper (section X) at the junction of the T maze, thus contaminating the underlying layer. While this is possible, I believe the size of the pheromone droplets, likely to be in the realm of picolitre volumes (Czaczkes T. Pers comm.), are unlikely to penetrate very far through the fibrous substrate.

A potentially confounding factor that could influence our findings in this study would be the possession of an idiothetic memory (memory based upon internal cues derived from self motion) by *L. niger* foragers, as this could provide an alternative means of navigating in darkness. However, our results show that *L. niger* ants are unable to develop a route memory after a single visit to a food source, when under complete darkness. We found that only 54 % of ants chose the correct branch after a single visit

which contrasts strongly with 75 % found by Grüter et al (2011a) for *L. niger* foragers after a single visit in full light, using an identical experimental protocol. *Lasius niger* foragers, therefore, appear to be unable to navigate by idiothetic means and this may explain why their response to trail pheromones increases so markedly in reduced light. A pheromone trail of high concentration is a fairly reliable indicator of a rewarding route due to the positive feedback mechanism of repeated laying by successful foragers (Goss *et al.* 1989; Beckers *et al.* 1992), while idiothetic navigation is prone to cumulative errors (Etienne *et al.* 2004) and less efficient than an internal or polarisation compass (Cheung *et al.* 2007; Lebhardt, Koch & Ronacher 2012), which are used by some ant species to aid orientation (Fourcassie, Dahbi & Cerdá 2000; Wehner 2003).

We have shown that changes in a physical parameter, namely light, elicit behavioural changes in foraging *L. niger* ants. While the frequency of pheromone deposition does increase as light levels decrease, it is more complex than a simple response to a reduction in visual cues. The increase in available olfactory cues that results from this behavioural change helps facilitate a shift in response from a visual modality to an olfactory one, and provide ants with the flexibility to adapt to local environmental changes (Detrain & Deneubourg 2002). As far I am aware, this is the first study to analyse quantitatively the change in trail-laying behaviour under different light levels, and show the graded shift in stimuli response that accompanies it. Our findings highlight the complexity of decision making in the trail laying behaviour of ants in which a combination of factors determine an ants propensity to lay; Moreover, the particular combination of factors involved is dependent upon the prevailing environmental conditions.

The fluctuating nature of the environment requires that ants are able to navigate using more than one sensory modality. Multimodal processing has been shown to enhance decision making in ants (Czaczkes *et al.* 2011; Steck *et al.* 2011), bumble bees (Kulahci *et al.* 2008), moths (Balkenius *et al.* 2005) and the Colorado potato beetle *Leptinotarsa decemlineata* (Otálora-Luna & Dickens 2011). Despite this, *Lasius niger* commonly forage at night, where we have shown, they must rely solely on olfactory orientation, and it remains to be seen whether mistakes are more frequent during cloudy nights when only cues from a single modality are likely to be available.

5

Navigational Decision Making in Two Congeneric Ant Species is Directly Related to Their Contrasting Ecological Environments

5.1. Abstract

The foraging and navigational strategies of the subterranean yellow meadow ant *Lasius flavus* remain largely unstudied. In this chapter I investigate the navigational and behavioural strategies of *L. flavus* and contrast them with those of the well-studied epigaeic ant *Lasius niger*. In each species I examine: the ability to develop a route memory in both dark light and darkness, trail pheromone laying behaviour and reliance on this pheromone, both when it is the only information cue available and when in conflict with memory. The results clearly show that both *Lasius* species are adept at using either olfactory or visual based cues to navigate but environmental constraints determine the primary strategy utilised by each species. Reliance on memory developed from visual snapshots of their surroundings is a more efficient method for the epigaeic (surface foraging) ant *L. niger* while for the hypogaeic (subterranean foraging) ant *L. flavus* trail pheromones serve as the primary source of navigation. I also demonstrate that *L. flavus* are able to increase the efficiency of this navigational strategy by both increasing the pheromone deposition rate per ant and utilising these cues to a greater degree when the trail concentration is greater. This

study has significantly furthered our knowledge of the foraging behaviour and navigational strategies of this common but rarely seen ant species.

5.2. Introduction

The ability to navigate in a complex environment is particularly important for central place foragers such as bees (Menzel *et al.* 1998; Goulson & Stout 2001), ants (Akesson & Wehner 2002; Harris *et al.* 2007) and rodents (Siegrist *et al.* 2003; Etienne & Jeffery 2004). Ants have evolved a diverse range of navigational strategies to locate and retrieve widely scattered resources, in often spatially heterogeneous environments. To achieve this, ants use specific environmental cues, from a number of different sensory modalities. For example, wood ants, *Formica rufa,* use visual cues to form snapshots of a prominent landmark with which they depend on to relocate a resource (Judd & Collett 1998) while pharaoh ants, *Monomorium pharaonis,* instead use chemical cues in the form of trail pheromones, deposited by nestmates (Jackson *et al.* 2006). Furthermore, a foraging ant is rarely restricted to a single strategy but instead chooses the most pertinent sensory cues in a given situation, or even utilises two modalities in tandem (Czaczkes *et al.* 2011; Steck *et al.* 2011). Together these methods that are available to an ant species comprise its 'navigational toolkit' (Wehner 2003).

Which particular strategy is adopted by a foraging ant often depends upon what cues are available and/or are the most reliable, and the salience of these cues is governed by the surrounding ecological environment. For example, prominent landmarks that are readily available to aid navigation in wood ants are rarely present in the barren landscape of a desert, rendering this strategy impractical for a desert dwelling ant such as *Cataglyphis fortis*. Foraging *Cataglyphis* ants solve this issue by utilising celestial

cues in the form of polarised e-vectors together with an internal step-counting odometer, to measure distance (Wehner 2003). In the understory of a forest, where celestial cues are largely unavailable, African stink ants *Pachycondyla* (*Paltothyreus*) *tarsata* use visual cues provided by contrast in the overlying canopy to navigate (Hölldobler 1980). An ant will adapt to utilising a more reliable secondary sensory modality or cue once the primary option becomes unavailable or unreliable. Thus, ants that utilise visually acquired cues to navigate in daylight are forced to switch to alternative olfactory (Jones et al. unpubl, Beugnon & Fourcassie 1988) or tactile (Klotz & Reid 1993) cues under low light conditions. This sensory flexibility is not only restricted to central-place foragers like ants but is also prevalent in hawkmoths (e.g. *Manduca sexta*; Raguso 2005), bats (e.g. *Macrotus californicus* Bell 1985) and dolphins (e.g. *Tursiops truncatus*; Pack & Herman 1995).

Most ant species are able to use trail pheromones to aid relocation of a food source or a new nest site, both for themselves and other colony members (Attygalle & Morgan 1985). The rules that govern trail laying behaviour are sophisticated and diverse; quality of a food source (Beckers *et al.* 1993), proximity to a food source (Beckers *et al.* 1992), complexity of the route between food and nest (Czaczkes *et al*. 2013) and presence of home range markings or a trail pheromone (Devigne & Detrain 2006; Czaczkes *et al.* 2012) are some of the factors thought to play a role in determining trail laying behaviour of ants. Furthermore, multicomponent blends in which different constituents convey different information can also occur. For example, in the pharaoh's ant *Monomorium pharaonis,* workers can lay short lived pheromones lasting approximately 20 minutes, persistent components that can last over 48 hours or even a repellent constituent used to mark an unrewarding path (Robinson *et al.* 2005).

Alternatively many ants are able to use visual cues to develop a route memory. Visual snapshots stored at stages along a foraging route by an ant can later be retrieved from its memory and the correct orientation achieved by aligning particular landmarks in its retina with those stored in memory (Harris *et al.* 2007). This method allows ants to develop a reliable route memory as the same journey is repeated and is an efficient form of navigation when focusing on an enduring resource (Collett *et al.* 2003). In these situations the chemical trail acts as a structural guide enabling inexperienced ants to rapidly memorise the correct route, after which trail pheromone commonly acts as a backup (Collett *et al.* 2003). This shift to a memory dependent strategy often results in faster travel speeds, while reducing the likelihood of losing the trail (Harrison *et al.* 1989; Collett *et al.* 2003). A route memory can be acquired after a single visit to a food source by some species (*Leptothorax unifasciatus*, Aron, Deneubourg, & Pasteels 1988; *Lasius niger*, Grüter, Czaczkes, & Ratnieks 2011) and has even been demonstrated in the highly pheromone dependent Argentine ant *Linepithema (Iridomyrmex) humile* (Aron *et al.* 1993).

The black garden ant *Lasius niger* forages predominantly above ground for arthropod prey and aphid honeydew (Portha, Deneubourg & Detrain 2004). In this species, route navigation is achieved using both visual cues and trail pheromones (Beckers *et al.* 1993; Evison *et al.* 2008). During daylight hours when visual cues, such as landmark beacons, are readily accessible *L. niger* foragers favour these cues over olfactory ones. When faced with a conflict between the two information sources, significantly more ants, with an experience of one visit to a food source, chose the same branch over the alternative branch which was heavily marked with their trail pheromone (Aron *et al.* 1993; Grüter *et al.* 2011a). This effect increases as experience, and thus memory

development, increases (Grüter *et al.* 2011a). However, Chapter 5 of this thesis shows that the outcome of this decision based conflict changes when light intensity is reduced. As light intensity drops to a level that corresponds to a moonlit or cloudy night, foraging *L. niger* ants respond both by increasing the rate of trail pheromone deposition and simultaneously switching reliance to these now readily available olfactory cues. This sensory flexibility enables *L. niger* to successfully forage both diurnally and nocturnally.

While foraging in *L. niger* has been widely studied, the foraging ecology of the yellow meadow ant *Lasius flavus* has been neglected to date. This is most likely due to its elusive subterranean lifestyle; *Lasius flavus* is a true hypogaeic ant, being rarely encountered above ground (Pontin 1961; King 1977) and is able to obtain both carbohydrates and protein from its symbiotic subterranean aphid partners, supplemented by other soil living invertebrates (Pontin 1978). The subterranean environment differs considerably from the exposed surface climate primarily inhabited by *L. niger*; it has a stable humidity and temperature with no light or air currents (Kimchi & Terkel 2002). These contrasting environments are likely to pose different navigational challenges to foragers of these two ant species and as such we would expect to see differences in the navigational strategies and foraging behaviour of these ants. For example, do pheromones play a more substantial role in the subterranean environment, scarce of visual cues, as we see in termites (Reinhard & Kaib 2001). In addition, are *L. flavus* ants able to develop an efficient route memory in brightly lit conditions like their epigaeic congener *L. niger*?

The work in this chapter seeks both to investigate the navigational foraging strategies and abilities of *L. flavus* and to compare these with those of *L. niger,* and by doing so, will provide answers to the questions raised above. This is the first study to investigate the foraging ecology of *L. flavus* and compare the foraging strategies and abilities of two congeneric ant species that inhabit very different ecological environments. I seek to determine and contrast in both *Lasius* species: the route memory forming ability both in light and darkness, trail pheromone laying behaviour and reliance on this pheromone, both when it is the only information cue available and when in conflict with memory. Contrary to what I had expected, *L. flavus foragers* are equally as adept as *L. niger* at forming a reliable route memory in daylight conditions but neither species was able to form a route memory in the absence of light. Additionally I found that only when the pheromone concentration was strong did significantly more *L. flavus* ants respond to trail pheromone than did *L. niger* and the pheromone laying frequency was considerably greater in *L. flavus* than *L. niger*, both in daylight and darkness. Finally, when faced with a conflict between the two information sources, significantly more foragers of *L. flavus* rely on pheromonal cues than *L. niger* foragers. The results are discussed in the context of the foraging ecology of each species and the impact of relevant environmental constraints.

5.3. Materials and Methods

5.3.1. Study Species

Ten colonies of *Lasius flavus* and eleven colonies of *Lasius niger* were used over the five different experiments. All colonies were collected from Falmer in East Sussex and housed in square plastic containers (30 x 30 x 10 cm high) with a plaster of Paris base to control humidity. Large plastic petri dishes, covered by dark card, were provided

(13.5 cm diameter x 1.5 cm high) containing either plaster of Paris (for *L. niger*) or damp soil (for *L. flavus*). All colonies were queenless and contained 1,000 - 3,000 workers and a small number of brood. Queenless colonies readily forage, produce trails and are commonly used in behavioural experiments (Evison *et al.* 2008; Grüter *et al.* 2011a). Colonies of both species were fed a mixture of agar, honey, raw egg and vitamins and were provided with water ad libitum. To ensure motivation for experiments, feeding was stopped three days prior to an experiment.

5.3.2. Experimental Design

A similar experimental set up was used for all five experiments. Following the method of Grüter et al. (2011a) a simple foraging trail was constructed using a white cardboard bridge (20 × 2 cm) to connect a colony container to a transparent polycarbonate plastic T piece covered with white filter paper (Whatman No. 1). The stem of the T used for *Lasius niger* ants was 15 cm long and each branch was 11 cm long, with a consistent width of 2 cm. This was scaled down to 1.2 cm (60 %) for the smaller *Lasius flavus* ants.

Experiments 1, 2 and 3 were carried out in a room with plenty of large pieces of furniture and lab equipment that have been shown to serve as visual landmarks for foraging ants (Evison *et al*. 2008). Experiments 4 and 5 were run in a small, windowless, temperature controlled room. For all experiments the ambient temperature was maintained at 22 ^{o}C. No windows were present so darkness was easily achieved in this room and a well maintained temperature was essential for direct and realistic comparisons to be made about pheromone laying behaviour. Light for all other experiments was provided by fluorescent ceiling lighting and an angle poise lamp with a 60 W bulb. During the near darkness treatment adequate visibility for working and observing behaviour was provided by infra-red light, which is not

perceived by ants (Menzel 1979). To provide infrared light a sleeve was created from 2 ply corrugated cardboard to fit tightly over the hood of an angle poised lamp with a 60 W bulb. Two 50 mm square 665 nm long pass (IR) filters (Schott; model FRG-66550) were slotted tightly together into a hole cut in the centre of the cardboard hood so that when switched on the lamp only provided infra-red light.

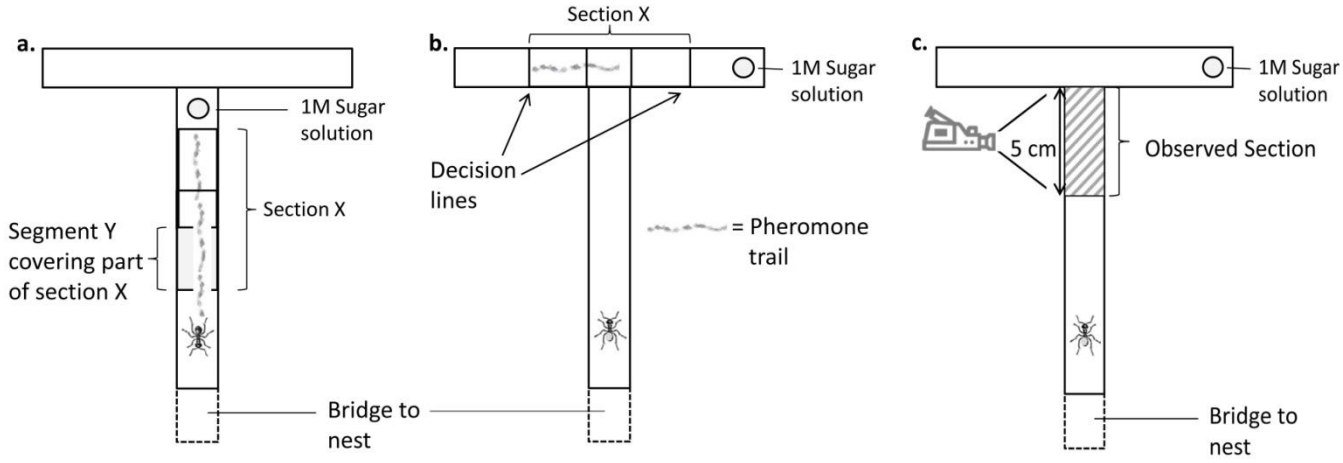


Figure 5.1 a.) Experimental design used to establish a weak/strong pheromone trail which was subsequently used to determine pheromone response of naive workers in experiment 2 and provide a conflict with an ants own route memory in experiment 3. A section of filter paper(X) was partially covered by a segment (Y) and ants were allowed to forage on a 1M sugar solution. Pheromone deposition on the uncovered part of section X was monitored until 5-7 (weak trail) or 35-40 (strong trail) deposits were reached, after which foraging was stopped and section X, minus segment Y, was transferred to be used in experiments 2 or 3**. b.)** Experimental design used for experiments 2 and 3. In experiment 2 naive ants were faced with a choice between a pheromone trail on one branch and a clear and unmarked section on the other branch. Weak (5-7 deposits) and strong (35-40 deposits) pheromone strengths were tested. For experiment 3 ants were allowed to locate a 1M sugar solution on a randomly chosen branch. While feeding ants were marked with a paint dot and allowed to return to the nest. Section X was then transferred to the bifurcation so that marked returning ants were faced with a conflict between their route memory and trail pheromone. Decisions were recorded once an ant passed either of the two decision lines. Three conflict types were tested: Strong pheromone (SP) vs Weak Memory (WM), SP vs SM and WP vs SM. **c.)** Experimental design used to ascertain trail pheromone deposition frequency of both species of ant under near darkness and normal light. For both treatments the deposition frequency was recorded for the observed section (hashed area) for three journeys: two returns to the nest and one trip to the food source.

5.3.3. Experiment 1: How rapidly is a route memory formed in both species?

Using the experimental set up described above, ants were allowed to locate a 1M sucrose solution randomly allocated to the end of one of the branches. The first eight ants to begin feeding were each marked with a different coloured dot of acrylic paint, using a wooden cocktail stick. If done carefully the ants ignored the procedure or, if disturbed, rapidly resumed feeding. Any ants that did not settle were removed from the experiment. All unmarked ants were then removed from the bridge and T maze. Marked ants were allowed to forage between the nest and food source and the decisions made at the junction on the return to the food source were recorded. Each ant was judged to have chosen a particular branch once its antenna passed one of the two decision lines (Fig. 5.1b).

Figure 5.2. *Lasius niger* ants that have been individually paint marked, feeding at the end of a branch of a T maze (photo courtesy of Christoph Grüter)

To ensure that the decision was based upon a route memory and not through the utilisation of olfactory cues (trail pheromones or home range markings) a clean section of filter paper was placed at the junction once an ant had crossed it. Decisions were recorded for 3 subsequent returns to the food source (a total of 4 visits to the food) for each marked ant. A control was also run using

naive ants to identify any side bias and provide a baseline value for branch choice without route memory.

5.3.4. Experiment 2: Do the two species differ in their response to trail pheromone?

To establish the response of foraging ants to trail pheromone ants were given a choice of a clean unmarked branch and one treated with trail pheromone. A pheromone trail was created by allowing ants to freely forage on a solution of 1M sugar water situated on the T maze before the bifurcation (Fig. 5.1a). A piece of filter paper (X; 10 × 2 cm) was placed directly before the food source with a section of it (Y; 4 × 2 cm) covered by an additional piece of filter paper. This ensured that this area of the paper remained free from pheromone deposited by ants leaving and returning to the food source. Two trail strengths were tested: strong resulted from 35-40 deposits on the paper section, while weak consisted of 5-7 deposits only. I assumed that an ant deposited a drop of pheromone each time we saw it clearly curve and dip its gaster to the surface (Beckers *et al.* 1992). This behaviour is easy to observe in both *Lasius* species. The maximum time allowed for trail development was 20 minutes for *Lasius niger* and 10 minutes for *Lasius flavus.* Previous observations showed that the trail pheromone of *L. flavus* was less persistent than the 45 minutes reported for *L. niger* (Beckers *et al.* 1993; Evison *et al.* 2008), so the shorter time period was used.

Once the desired trail strength was established section X was transferred to the junction of a new T maze with fresh filter paper (Fig. 5.1b). Once section Y was removed to reveal the unmarked section, ants would be faced with the choice of a pheromone marked branch and a clean unmarked branch. Ants from the same colony were then allowed to forage on the trail and decisions were recorded once an ant

passed a marked decision line. A control was also run in which ants were faced with clean unmarked branches on both sides to identify any side bias.

5.3.5. Experiment 3: Do the two species respond differently when facing conflicting information sources?

Each ant species was faced with a choice between its own route memory and trail pheromone to establish which information source was preferred. Three different conflicts were tested: strong pheromone (SP) vs weak memory (WM), SP vs strong memory (SM) and weak pheromone (WP) vs SM. The other possible combination of WM vs WP was not investigated as we were interested in decision making when at least one cue was readily available and reliable. Pheromone strength was established as described for experiment 2 above. Memory was established as described for experiment 1 above with strong memory represented as 3 consecutive trips to the food source and weak memory as a single visit. Section X was then positioned at the junction once the marked ants had established the required experience (1 or 3 trips to the food source), with the pheromone marked branch placed opposite to the branch with the food source. The returning marked ants were thus faced with the dilemma of trail pheromone on one branch and their memory of the food source on the other (Fig 5.1b). Decisions were recorded once an ant's antenna passed either decision line, marked on each branch.

5.3.6. Experiment 4: Do the two species differ in their ability to navigate in the absence of any visual or olfactory cues?

To test whether either of the ant species was able to navigate in the absence of any visual or olfactory cues, ants were allowed to forage on a T maze and locate a 1M sucrose solution, randomly allocated to a branch in almost complete darkness (0.0007 lux). A photometer (LI-COR inc; model LI-188B) was used to measure illumination. The

first ten ants to begin feeding were marked with a dot of grey acrylic paint (the most discernable colour under IR light) using a wooden cocktail stick. All other ants were removed from the T and the marked ants were allowed to return to the nest. Once all marked ants had returned to the nest a clean piece of filter paper was placed at the junction to prevent navigation using olfactory cues. Decisions made by marked ants returning to the food source were recorded, once an ant's antenna touched either marked decision line. A control was also run using naive ants with no memory.

5.3.7. Experiment 5: Does the pheromone laying behaviour differ between the two Lasius species.

Pheromone laying frequency was measured for both species under two contrasting light levels: normal daylight and near darkness. Ants were allowed to locate and feed on 1M sugar water solution, randomly allocated to one of the branches of the T maze. A 5 cm long section of filter paper, located just before the branches of the T, was marked by lines at either end and a video camera (Sony; model HDR-XR520) was positioned to record, from the side, all pheromone laying behaviour of ants walking along this designated section for all journeys combined (Fig. 5.1c). This section was chosen because ants were observed to regularly deposit pheromone near the junction and it was also easier to monitor using the camera. The low lux camera setting was used to record behaviour under IR light. The first 10-12 ants that reached the food source and began to feed were marked with a dot of grey acrylic paint using a wooden cocktail stick and all unmarked ants were removed from the T maze. Marked ants were allowed to return to the nest, navigate back to the food source and then return to the nest once more before being removed. Thus the laying behaviour of each ant was recorded for a maximum of three journeys. The experiment was carried out under

near darkness and artificial light for both species. When analysing the videos, I assumed that an ant deposited a drop of pheromone each time I saw it clearly curve and dip its gaster to the surface (Beckers *et al.* 1992).

5.3.8. Statistical analysis

Data from the first four experiments were analysed using generalised linear mixed-effect models (GLMM) with binomial errors in R v.2.14.2 (R Development Core Team 2012). Models were fitted using the lmer function (Bates *et al.* 2011). For model selection the protocol proposed by Zuur et al. (2009) was used. I first explored the optimal structure of the random components by comparing random intercept models with random intercept and slope models. Colony was included as a random effect throughout while the variable date was dropped from all models after model comparison. The optimal fixed component structure was then explored and any fixed effects found to be non-significant were dropped from the model. Wald Z tests were used to determine the significance of the fixed effects and models were fitted using the Laplace approximation (Bolker *et al.* 2009; Bates *et al.* 2011). The optimal model included the fixed effects: species (levels included *L. flavus* and *L. niger*) for all experiments, journey (levels included 1-4) for experiments 1 and 4, pheromone strength (levels included high and low) for experiment 2, conflict type (levels included SP/WM, SP/SM and WP/SM) for experiment 3 and light treatment (levels included light and dark) for experiment 4.

Initial analysis of the data for the fifth experiment revealed it to be zero inflated, so to deal with this I chose to use the MCMCglmm package (Hadfield 2010) implemented in R v. 2.14.2 (R Development Core Team 2012) using the “zipoisson” family function. Uninformative prior distributions were used for fixed effect parameters with a mean of

0 and a large variance of 10^8. Priors for the variance components were inverse-Wishart distributed with the degree of belief parameter (n) set at ¼ 0.01 and variance (V) limited to 1. Each model was run for 100,000 Markov chain Monte Carlo (MCMC) simulation iterations with a burn-in of 35,000 iterations and a thinning interval of 10 iterations, giving a low autocorrelation between successive iterations (<0.025). An optimal model was created by removing non-significant fixed effects sequentially from the model. Models were compared using the deviance information criterion (DIC). The fixed effects included in the optimal model were species (levels being the two *Lasius* species) and light treatment (levels of dark and light) while both colony and date were included as independent random effects. Mean parameter estimates and 95% credible intervals were constructed and are reported in the results. When estimates did not range over zero, the parameter was deemed to be significant.

5.4. Results

5.4.1. Experiment 1: How rapidly is a route memory formed in both species?

As expected, on the first journey on the T maze the number of ants choosing the rewarding branch did not differ significantly from what we would expect at random (50 %). However, route memory is significantly developed after a single visit to the food source in both species (*L. flavus*: 89 vs 52 %, z = 4.41, P <0.001; *L. niger*: 73 vs 54 %, z = 4.54, p <0.001, Fig. 5.3a). Route memory establishment after one visit is particularly high in *L. flavus* with 37% more ants making the correct decision compared to 19% in *L. niger* (1 visit 89 vs 73 %; z = 2.84, P = 0.004). Memory development appears to increase as more journeys are completed by *L. niger,* with a maximum of 91% of ants making the correct decision after three visits to the food source; a significant improvement on the 73% seen after one visit (91 vs 73 %; z = 2.51, P = 0.012). Overall there is no

significant difference between the two species (*L. flavus* vs *L. niger*; z = 1.26, P = 0.21) and colony effects are relatively small, contributing only 4.3 % of the overall variance.

5.4.2. Experiment 2: Do the two species differ in their response to trail pheromone?

The response to a low pheromone strength (5-7 deposits) is exactly the same in each *Lasius* species (Low: 65 vs 65%, z = -0.21, P = 0.83), with a greater number of ants choosing the branch treated with pheromone rather than the untreated branch (Fig. 5.3b). The number of ants choosing the treated branch is significantly greater than the untreated control in both species (*L. flavus*: 65 vs 54 %, z = 2.76, P = 0.0058; *L. niger*: 65 vs 54 %, z = 2.39, P = 0.017). When the pheromone strength is increased (35-40 deposits) there is no corresponding increase in response for *L. niger* foragers (66 vs 65 %; z = -0.68, P = 0.496). In contrast, *L. flavus* show a small but significant positive shift in response with 12% more ants choosing the pheromone treated branch (77 vs 65 %; z = -2.31, P = 0.025)(Fig. 5.3b).

5.4.3. Experiment 3: Do the two species respond differently when facing conflicting information sources?

The two *Lasius* species differ significantly in their responses to the navigational cues in two of the three experimental conflict scenarios. When trail pheromone strength is strong almost half of foraging *L. flavus* ants prefer these cues to a strong route memory and this increases, though not significantly, to 61 % when route memory is weaker (Fig. 5.4a). These responses are significantly greater than those seen for *L. niger* (SP vs WM: z = -4.08, P <0.001; SP vs SM: z = -3.06, P = 0.002) in which the route memory predominates over trail pheromone, irrespective of pheromone strength, for all three scenarios. When pheromone strength is weak route memory is strongly preferred in *L. flavus* (WP vs SM: z = 0.38, P = 0.702), similar to that seen for *L. niger*

(Fig. 5.4a).

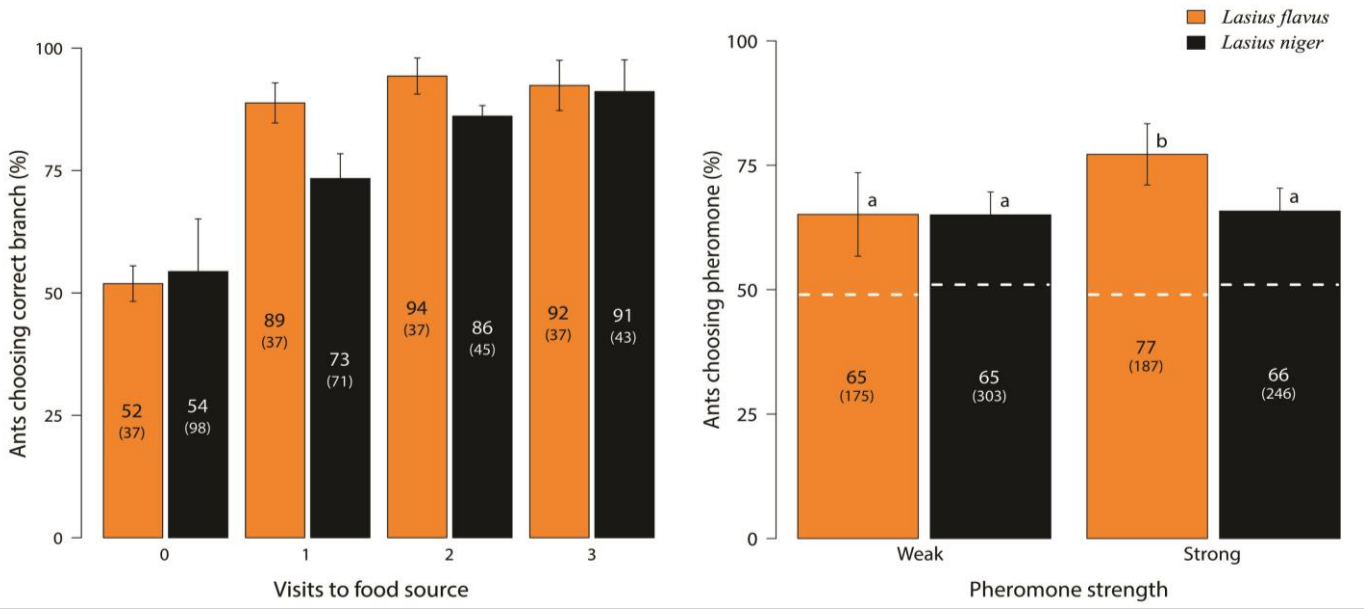


Figure 5.3 a.) Development of a route memory in both *Lasius* species. On the first journey to locate the food source the number of ants choosing the rewarding branch did not significantly differ from a random expectation (50 %). After a single visit significantly more ants choose the branch that led to a reward on the previous visit. **b.)** Response of naive ants to a pheromone trail. Significantly more ants chose the branch treated with trail pheromone over the untreated branch in both species and for both weak (5-7 deposits) and strong concentrations (35-40 deposits). While increasing pheromone strength has no effect on *L. niger* foragers, a significantly greater number of *L. flavus* ants respond to an increased pheromone concentration. White dotted lines indicate results for the control (no trail pheromone on either branch). Within each bar is given the mean and in brackets, the sample size (number of ants). Different letters (given above each bar) denote significant differences and error bars represent the standard error of the mean.

5.4.4. Experiment 4: Do the two species differ in their ability to navigate in the absence of any visual or olfactory cues?

After a single visit to a food source located on one branch of a T maze, in the light, a significantly greater number of ants choose the correct branch on their return to the food source, compared to ants with no prior experience. This was observed for both species (*L. flavus*: 89 vs 56 %; Z = 4.45, P <0.001; *L. niger*: 73 vs 58 %, Z = 2.53, P <0.001, Fig. 5.4b). This contrasts strongly with the results under dark conditions; significantly less foragers chose the correct branch on their return from the nest (*L. flavus*: 56 vs 89

%, Z = 3.47 , P <0.001 ; *L. niger*: 58 vs 73 %, Z = 2.09, P = 0.03), and importantly, in each species the numbers of ants that chose the previously rewarding branch did not significantly differ from the control (*L. flavus*: 56 vs 50 %, Z = -0.71 , P = 0.47; *L. niger*: 58 vs 51 %, Z = -0.99 , P = 0.32).

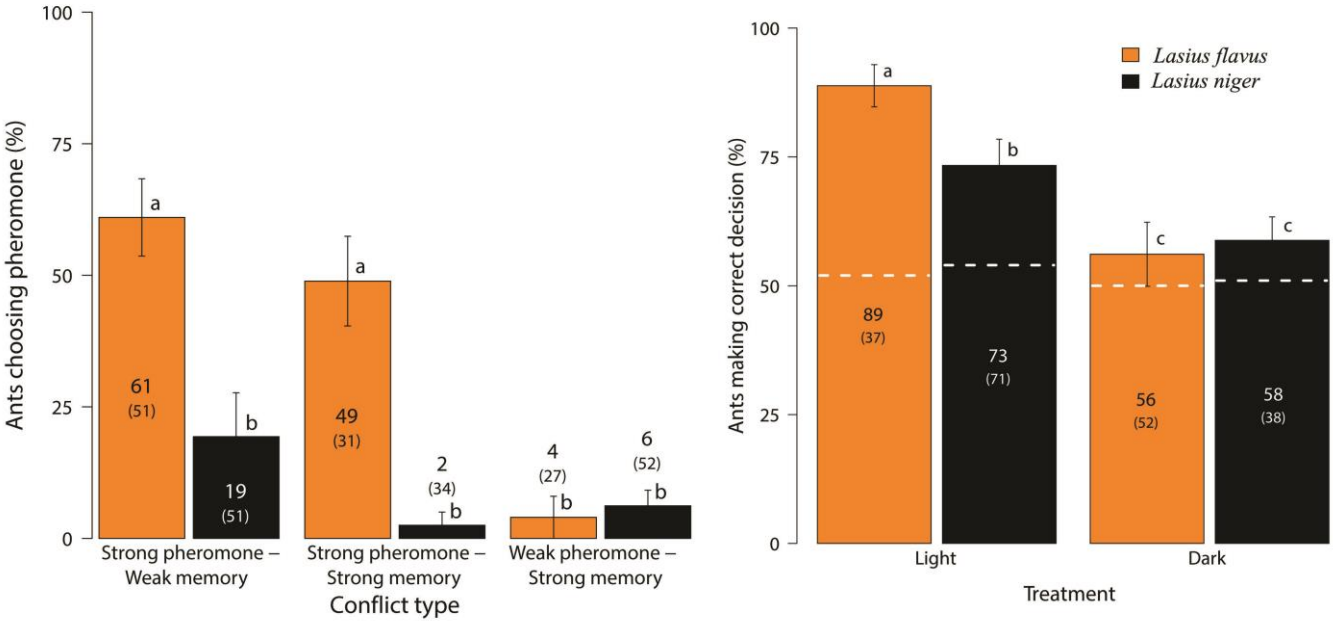


Figure 5.4 a.) Decision making by ants when facing a conflict between their own memory and a pheromone trail. Strong pheromone constituted 35-40 deposits and weak 5-7 deposits; strong memory involved 4 visits to a food source while weak memory was based upon a single visit. Memory is the predominant choice by *L. niger* foragers, irrespective of pheromone strength, while a strong pheromone trail has equal or greater importance than memory, depending on memory strength, in *L. flavus*. **b.)** Comparison of memory development after a single visit to a food source in light and darkness. In light both *Lasius* species are able to rapidly acquire a route memory. However, in the darkness the number of ants choosing the branch that was rewarding on their previous visit does not differ from the control in both species. White dotted lines signify the control results (decisions by naive ants). Within each bar is given the mean and in brackets the sample size (number of ants). Different letters (given above each bar) denote significant differences and error bars represent the standard error of the mean.

5.4.5. Experiment 5: Does the pheromone laying behaviour differ between the two Lasius species.

The frequency of pheromone deposition is greater for *L. flavus* with significantly more deposits made over the three trips in both dark (*L. flavus* vs *L. niger*: 1.48 vs 0.72; parameter estimate = 1.534, 95% CI = (1.033, 2.001)) and light (*L. flavus* vs *L .niger*:

1.75 vs 0.45; parameter estimate = -1.506, 95% CI = (-1.967, -1.038), Fig. 5.5) conditions. When luminance drops from daylight to near darkness there is no significant change in the frequency of deposition for *L. flavus* (high vs low: 1.75 vs 1.48; parameter estimate = -0.116, 95% CI = (-0.478, 0.244)), but *L. niger* shows a significant increase in pheromone deposition as light level declines [high vs low: 0.45 vs 0.72, parameter estimate = 0.842, 95% CI = (0.339, 1.318)] (Fig. 5.5).

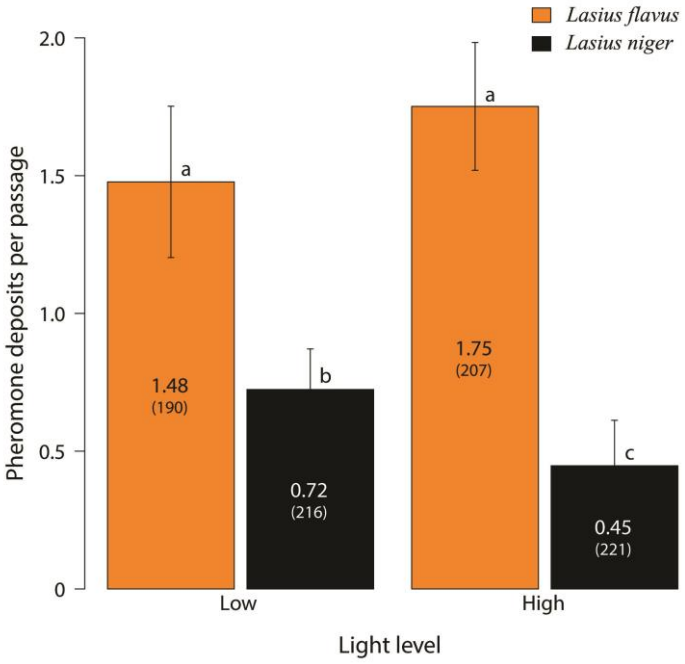


Figure 5.5. Pheromone deposits per passage in both light and darkness. A passage is represented by a 5cm section of filter paper located just before the junction of a T maze. Depositions were recorded for a maximum of three journeys per ant beginning on the first return to the nest after locating the food. The frequency of pheromone deposition is significantly greater in *L. flavus* than *L. niger* in both light treatments. The deposition rate does not vary with light intensity in *L. flavus*, but the number of deposits made per passage significantly increases as light levels decline in *L. niger*. Within each bar is given the mean and in brackets the sample size (number of passages). Different letters (given above each bar) denote significant differences and error bars represent the standard error of the mean.

5.5. Discussion

Despite predominantly residing underground *Lasius flavus* ants are able to memorise a route in daylight equally as proficiently as their surface dwelling congeners *Lasius niger*. Eyes are costly organs to develop and maintain (Land & Nilsson 2012), and as in most animals that exist in an environment void of light the eyes of subterranean ants

are far simpler (Klotz, Reid & Gordon 1992; Depickère, Fresneau & Deneubourg 2004). In addition to interommatidial angles and rhabdom dimensions, visual resolution is also influenced by ommatidia numbers (Wilson 1971; Land 1997). The simplified eyes of *L. flavus* contain on average 44 ommatidia (Seifert 1983) while the eyes of a predominantly surface foraging ant, *Formica rufa* consist of 600 or more (Wilson 1971). Despite my a priori expectation, *L. flavus* was found to be able to detect sufficient contrast in the surrounding landscape to form a reliable route memory. Indeed, both *Lasius* species demonstrated the continuous development of a route memory as more rewarding journeys were completed.

Both species were unable to form a route memory in the absence of light, suggesting that in the absence of visual cues neither species is able to navigate using idiothetic (internally derived) cues. Idiothetic navigation has been demonstrated in the ant *Pachycondyla tesserinoda* (Jessen & Maschwitz 1986), the termite *Coptotermes formosanus* (Bardunias & Su 2010) and some spiders (Seyfarth *et al.* 1982; Nentwig 1987), but it is prone to cumulative errors (Etienne *et al.* 2004), unlike more efficient alternative methods such as an internal or celestial compass (Cheung *et al.* 2007; Lebhardt *et al.* 2012), utilised by many other ant species (e.g. *Cataglyphis iberica*: Fourcassie *et al.* 2000; *Cataglyphis* spp: Wehner 2003; *Melophorus bagoti*: Schwarz *et al.* 2011). However, in the confines of a dark subterranean environment reliance on an idiothetic method is by *L. flavus* seemed entirely feasible and warranted this investigation.

Both *Lasius* species positively responded to a low trail pheromone concentration in a similar fashion with significantly more ants choosing a pheromone marked branch than

an unmarked one. However, when the trail pheromone concentration is increased the responses differ between the two species. Unlike *L. flavus,* the numbers of *L. niger* ants choosing a pheromone treated branch did not increase with a corresponding increase in pheromone, much like what was found by Grüter *et al*. (2011a). But why is there a difference between the two species? We know that when choosing between two different trail strengths *L. niger* foragers will preferentially choose the more strongly marked branch (Beckers *et al.* 1990) but when faced with a choice between pheromone or no pheromone there appears to be an implicit response with a high error rate, irrespective of trail concentration. Could it be due to the relative importance given to the available cues such that the reliance on an available cue may be dependent upon its position within each ant species navigational cue hierarchy. In *L. flavus* the choice of which tunnel to follow is likely to be strongly governed by pheromone concentration while in *L. niger* the preferential use of visual cues may alter the response level to pheromone. A possible caveat that could potentially influence our results, involves the pheromone penetrating the overlaying paper section at the junction of the T maze (section X) and contaminating the underlaying layer. This would influence the decisions of later ants when reaching the junction. However, I believe the possibility of this occurring is unlikely due to the very small size of the pheromone droplets deposited (likely to be in the realm of picolitre volumes, (Czaczkes T. pers comm.) and the absorbancy of the filter paper used in these experiments.

Clear differences in the preferential choice of navigational cues exist between the two *Lasius* ant species. In *L. niger* memory dominates irrespective of trail pheromone concentration, particularly when route memory is strong. Despite being very capable of navigating using route memory in light, 50% of *L. flavus* workers preferentially

follow trail pheromones with this response increasing when their route memory is weak. While each species convincingly demonstrates an ability to switch flexibly between these two informational sources when the need arises, there is a striking difference in each species strategy preference with visual based route memory predominating over trail pheromones when it is strong in *L. niger* and olfactory based cues predominating when strong in *L. flavus*. Memory dependent strategies are thought to be more efficient than chemical trails in ant navigation because searching for a chemical trail tends to be slower (Collett *et al.* 2003). This appears to be the case for diurnal foraging in eigaeic ants such as *L. niger* and *Paraponera clavata* (Harrison *et al.* 1989) but for subterranean ant species like *L. flavus* a strategy based upon olfactory cues appears to be a more reliable option.

Both *Lasius* species feed on persistent food sources: honeydew produced by shrub based aphids in the case of *L. niger* (Portha *et al.* 2004) and root aphids by *L. flavus* (Pontin 1978). Root aphid colonies are a truly persistent food source for *L. flavus* colonies with workers creating specially made cavernous chambers to house their mutualist homopterans (Pontin 1978). Between these resident aphid populations and the colony's nest chambers lies a web of intricate tunnels which are likely to limit directional options of foraging ants (Hangartner 1969). In addition to acting as guides, tunnels may also convey extra information to foraging ants. Termites can make directional choices based upon the width of the tunnel; in the absence of pheromones foragers of the termite *Coptotermes formosanus* choose the widest option available when at a tunnel intersection (Ku, Su & Lee 2010). Wider tunnels imply greater traffic use and are therefore more likely to be rewarding. One would expect that decision making is further simplified, with concomitant gains in navigational efficiency when

only one or two tunnels are marked with a strong pheromone trail. A strong pheromone trail signifies a recently rewarding route because the combination of deposition cessation and trail decay quickly signals the end of a rewarding resource. It remains to be seen whether other cues such as geomagnetic (Anderson & Meer 1993; Camlitepe & Stradling 1995) also play a navigational role in *L. flavus*.

As is seen in the strongly pheromone dependent Argentine ant *L. humile* (Aron *et al.* 1993) the importance of trail pheromones to *L. flavus* is demonstrated by the high rate of pheromone deposition (number of gaster dips on substrate). Not only was the frequency of deposition significantly greater in *L. flavus* than *L. niger* in both light and near darkness but also the frequency did not change between the two light levels. This suggests that pheromonal cues are as important to these ants during rare forays above ground as they are below ground. This contrasts with *L. niger* where deposition significantly increases when light levels drop. Visual cues are unavailable in near darkness so when foraging nocturnally *L. niger* flexibly switch to a reliance on olfactory cues instead (see Chapter 4). These results may also explain why I see an increase in response as pheromone concentration is increased in *L. flavus* but not in *L. niger*. Because trail pheromone appears to the primary cue used for navigation by *L. flavus* it is important that these ants are sensitive to pheromone concentration. Therefore, mistakes by ants pursuing unrewarding routes are minimised. As my results show, one ant can make a considerable number of deposits over a short distance (equivalent to our weak pheromone trail); it is therefore more important to follow a stronger trail which has been marked by a number of ants and as such has a higher probability of being rewarding when other cues are unavailable.

This raises the question: why does *L. flavus* possess such a proficient memory under daylight conditions if this ant is truly hypogeaic? One possibility could involve the need for occasional surface foraging, although as yet there is no evidence for this. Although in most cases root aphids provide *L. flavus* colonies with almost all of their required nutrition (Pontin 1978) there may be times when foraging above ground becomes necessary. Destruction of the nest by livestock or birds (Pontin 1963; pers obs.), extreme climatic conditions, intraspecific competition or interspecific competition with other ant species such as *L. niger* (Pontin 1961) may all increase the need to forage on the surface. Another possible reason could be explained by the observed high volatility of the trail pheromone of *L. flavus*, observed during experiments. The subterranean environment with its stable temperature and humidity requires a shorter lived pheromone due to the reduced trail decay rate. However, above ground and exposed to light, air currents and fluctuating temperatures the perdurance of this pheromone will be considerably reduced and therefore the ability to flexibly utilise other strategies above ground is particularly important.

To conclude, our results show that each *Lasius* species is adept at using either visual or olfactory information to navigate but environmental constraints appear to have shaped the primary strategy utilised by each species. Reliance on memory developed from visual snapshots of their surroundings is a more efficient method for the predominantly surface foraging ant *L. niger* while for the hypogaeic ant *L. flavus* trail pheromones serve as the primary source of navigation. I also demonstrate that *L. flavus* exhibits two behavioural adaptations that further increase the efficiency of this navigational strategy: an increased pheromone deposition rate, relative to *L. niger*, and an enhanced response to a strong pheromone concentration. It is entirely feasible that

more strategies are available in the 'navigational toolkit' of the common yellow meadow ant, *L. flavus*, but this study has furthered our knowledge of the foraging behaviour and navigational strategies of this secretive species.

6

Behavioural and Chemical Analysis of the Glandular Pheromonal Components of the Yellow Meadow Ant *Lasius flavus*

6.1. Abstract

Previous work (Chapter 5) has shown that the yellow meadow ant *Lasius flavus* utilises trail pheromones to aid navigation. The identity of the component(s) present in the trail pheromone of this ant species are unknown, and therefore this study was undertaken with the aim of identifying the compound(s) and determining the glandular source. In addition, properties of the pheromone and other glandular constituents were investigated. The results demonstrated that, as with other members of the Formicinae, the trail pheromone originates from the hindgut. Furthermore, the findings show that the trail pheromone is not colony specific and component(s) within the hindgut do not act synergistically with components from the Dufour gland. Due to time constraints the identity of the pheromonal component(s) was not elucidated, however, an adaptable method of extracting the active chemical components deposited during trail formation was developed, thus reducing the workload that comes with testing every major glandular component in a behavioural bioassay. The presence of a unique lactone in the Dufour gland, that appears not to play a role in trail formation, is discussed and a potential alternative role is suggested. This study lays the foundations for future work, both to determine the trail pheromone

component(s) of *L. flavus* and identify the role of a lactone compound present in the Dufour gland of this ant species.

6.2. Introduction

The use of trail pheromones to navigate between a nest and a rewarding resource is widespread in the ants (Hölldobler & Wilson 1990). This elaborate form of chemical communication was known to exist as far back as 200 years ago (Bonnet 1779) and is now one of the most extensively studied forms of biological organisation. The general notion that an ant will lay a trail of pheromones back to the nest on finding food is now known to be far too simplistic, as is the assumption that all nestmates will choose to follow the trail to the resource. In reality, the chemical nature of trail pheromones and the decision rules that govern both an ant's response to these glandular products and its trail laying behaviour are diverse, thus greatly increasing the complexity of the process. For example, ants may increase pheromone deposition when other navigational cues become unreliable (Chapter 5), when the quality of the food source is greater (Beckers *et al.* 1993), or if the colony is starving (Mailleux, Detrain & Deneubourg 2006).

In addition to orientating an ant towards a particular location, most trail pheromones also act as an attractant, recruiting ants to the trail. To achieve this, ants have evolved a diverse range of chemical compounds to serve this role which include pyrazines (e.g. Cross *et al.* 1979; Jackson *et al.* 1990), alcohols (Attygalle *et al.* 1988; Morgan *et al.* 2004), ketones (Hölldobler *et al.* 1995; Morgan 2009), esters (Morgan & Ollett 1987; Bestmann *et al.* 1995a) and terpenoids (e.g. Ritter *et al.* 1977b; Vander Meer *et al.* 1981). While some ants rely on a single chemical component to elicit trail following,

the majority of species use a combination of several components which are often only efficient when released in a specific ratio (Billen, Beeckman & Morgan 1992; Oldham *et al.* 1994b; Hölldobler *et al.* 1995). For example, *Tetramorium caespitum* responds most strongly to a mixture of 3-ethyl-2,5-dimethylpyrazine and 2,5-dimethylpyrazine when present in a ratio of 7:3 (Attygalle & Morgan 1983). Many ants also use a number of different pheromones which can convey different information and thus increase a colony's foraging efficiency (Dussutour *et al.* 2009). The Pharaoh ant *Monomorium pharaonis* uses at least three different pheromones during foraging (Jackson *et al.* 2006; Robinson *et al.* 2008): a short-lived but highly attractive pheromone, a longer lasting pheromone, acting as a route memory (Jeanson *et al.* 2003), and a repellent pheromone that is used to mark unrewarding routes (Robinson *et al.* 2005). While the identity of the attractive pheromones have been identified as Monomorine I-V and faranal (Ritter *et al.* 1977a; b), the exact function of each has yet to be confirmed. The repellent "stop pheromone" is yet to be identified.

By definition, a pheromone is an evolved species-wide signal (Wyatt 2010), meaning that all colonies of a given species should utilise and respond to the same pheromone. While this is the case for the vast majority of ants studied, in one species of *Lasius* ant, *Lasius neoniger*, it has been shown that foragers only respond to a colony specific trail pheromone (Traniello 1980). Therefore, it is entirely possible that other species of ant use colony specific trail pheromones, like *L. neoniger*, to demarcate territory in addition to orientating nestmates to a food location.

Ants possess a bewildering variety of exocrine glands, from which at least 9 of these are involved in the production of trail pheromones (Billen & Morgan 1996). Which

glands are involved depends on the genus or subfamily. For example, the trail pheromones of all *Atta* (Billen & Morgan 1996) and *Myrmica* (Cammaerts-Tricot, Morgan & Tyler 1977; Evershed *et al.* 1982) ant species are derived from the poison gland while the hindgut is the source of all trail pheromones of the sub-family Formicinae (Hölldobler & Wilson 1990). In some species the chemical components of the trail pheromone are derived from different glandular sources, making detection and analysis of these pheromones particularly challenging for the chemical ecologist. In the Ponerine ant, *Megaponera foetens*, for example, components from both the pygidial and poison glands are required for recruitment and trail following (Hölldobler *et al.* 1994).

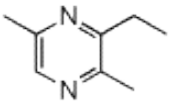
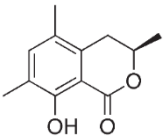
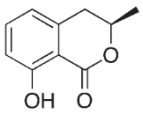
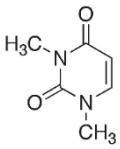
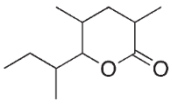
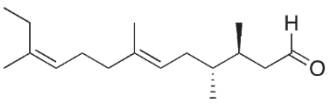
The extremely small volumes of pheromone involved in trail laying (ng quantities deposited) increases the difficulty of detection and analysis because it rules out the use of a number of analytical techniques such as Nuclear Magnetic Resonance (NMR) and Fourier Transform Infra-Red Spectroscopy (FT-IR). Both proton (^{1}H-NMR) and carbon-13 (^{13}C-NMR) NMR are powerful analytical techniques that provide detailed information about the structural framework of hydrocarbons and the position of functional groups. However, these techniques usually require reasonable quantities of a pure substance within a 500 µl sample: usually 1 mg for ^{1}H-NMR and 5 mg for ^{13}C-NMR, although lower concentrations have been reported in the literature (Attygalle & Morgan 1988). Mass Spectroscopy (MS) is the most suitable choice for working with nanogram volumes, with chemists now being able to detect quantities as low as 0.3 femtograms (3×10^{-16} grammes) (Kim *et al*. 2013).

The standard protocol for identifying an unknown trail pheromone first involves establishment of the glandular source, followed by the identification of the active compound or compounds (e.g. Jackson *et al.* 1990; Attygalle *et al.* 1991; Janssen *et al.* 1997b). To identify the glandular source, the major exocrine glands are usually dissected from ants and crushed and mixed in a solvent. Measuring the response of ants to the extract compared to a solvent control determines the glandular source (Hölldobler *et al.* 1995). Trail pheromones of many ant species are derived from multiple sources, so to ascertain if this is the case, ant responses to a particular gland should be compared with responses to a mixture of glands or an entire gaster extract (Attygalle *et al.* 1991; Blatrix *et al.* 2002). To identify the active compounds in an extract the procedure involves chromatographic separation linked to mass spectroscopy (GC-MS) to initially identify the mixture of chemical components present, followed by a bioassay to measures the ant's response to the extract or its components. Final confirmation is then achieved by running a further bioassay using readily available or synthetically prepared compounds that match the suspected pheromonal component(s).

In this Chapter I investigate the, as yet unknown, trail pheromone of the common subterranean ant species *Lasius flavus*. The trail pheromones of two other *Lasius* species (*L. niger*: Bestmann *et al.* 1992; *L. fuliginosus*; Kern *et al.* 1997) and members of other genera in the subfamily Formicinae have now been identified (e.g. *Formica rufa*; Bestmann *et al.* 1992; *Camponotus inaequalis*: Bestmann, Übler & Hölldobler 1997; *C. silvicola*: Ubler *et al.* 1995; *C. atriceps*: Haak *et al.* 1996; *Polergus rufescens*: Visicchio *et al.* 2001) and are all mono or bi-cyclic 2-pyranone based structures (see Table 6.1). Moreover, in all these cases the trail pheromones have been located in the same

organ, the hindgut, where they are produced by the rectal glands (Hölldobler & Wilson 1990; Morgan 2009).

Table 6.1. Glandular sources, names and structures of major trail pheromone components from a range of ant species

Species	Major Trail Pheromone component	Glandular Source	Structure	Reference
Tetramorium caespatum	3-ethyl-2,5-dimethylpyrazine 2,5-dimethylpyrazine	poison gland		Attygalle & Morgan, 1983
Lasius niger *Camponotus inaequalis* *C. silvicola*	3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin	hind gut		Bestmann *et al*., 1992, 1997 Ubler *et al.* 1995
Lasius fuliginosus *Formica rufa*	(R)-3,4-dihydro-8-hydroxy-3-methylisocoumarin	hind gut		Kern *et al*., 1997 Bestmann *et al*., 1992
Megaponera foetens	N,N-dimethyluracil	poison gland		Hölldobler *et al.* 1994
Camponotus atriceps	3,5-dimethyl-6-(2 ¢-butyl)tetrahydro-2H-pyran-2-one	hind gut		Haak *et al*., 1996
Monomorium pharaonis	Faranal	Dufour gland		Ritter *et al*., 1977

In the first section I investigated the source of the trail pheromone and found, as in other *Lasius* species, that the glandular source was the hindgut. Further to this, I investigated the properties of both the trail pheromone and the constituents of a number of prominent exocrine glands located in the gaster. The results showed that the trail pheromone is not colony specific and there was no synergistic effect from

constituents of other glands. In the second section I discuss a methodology which should successfully enable the collection of volatile pheromone components for analysis. Unfortunately due to time constraints the identity of the pheromonal component(s) was not elucidated but the analytical process and results are reported to aid future work in identifying the active compound(s).

Figure 6.1. *Lasius flavus* ants following a pheromone trail between a food source on the branch of a T maze and the nest (further along the stem to the right).

6.3. Material and Methods

6.3.1. Study Species

Seven colonies of *Lasius flavus* were used for all behavioural experiments and bioassays. All colonies were collected from meadow managed land on the campus of the University of Sussex. The colonies were housed in fluon coated square plastic containers (30 x 30 x 10 cm high) containing a large card covered petri dish (13.5 cm diameter x 1.5 cm high) filled with damp soil. All colonies were queenless and consisted of 1000-3,000 workers and a small number of brood. Queenless colonies readily forage, produce trails and are commonly used in behavioural experiments (Evison *et al.* 2008; Grüter *et al.* 2011a). Colonies were fed a mixture of laboratory grade nutrient agar, honey, raw egg and mixed vitamins (Hölldobler & Wilson 1990)

and were also provided with water *ad libitum*. To ensure motivation for foraging, feeding was stopped four days prior to an experiment.

6.3.2. Chemical Analysis

Extracts of hindguts, Dufour, sternal and poison glands were obtained by dissection of frozen ants under a stereo microscope. The appropriate glands were crushed in pentane filled chromacol vials using a heat sealed glass capillary rod to give 0.1 glandular equivalents per µl of solvent. Dissection was carried out in aqueous ethanol (70 %) apart from when the contents were to be derivatised, in which case pure ethanol was used (water impedes the reaction). For chemical analyses pentane extracts were concentrated by blowing down with pure nitrogen gas. Derivatisation of glandular extracts was carried out using BSTFA (40 µl) with pyridine (40 µl). A Thermo GC Ultra gas chromatograph was linked to a mass spectrometer (Thermo ITQ1100). Helium was used as the carrier gas with a flow rate of 1.3 ml/min, using a 30 m x 0.25-mm-DB, 0.25 µm film fused silica column (Agilent DB-5ms). Oven temperature was programmed from 60 (4 min hold) to 300 ^{o}C (4 min hold) at 10 ^{o}C/min. The Mass spectrometer was operated in EI mode (at 70 eV) and the scanning range was m/z 40 to 500. All solvents were HPLC grade, purchased from Sigma Aldrich, UK.

6.3.3. Bioassays

A plastic T maze, accessed via a white cardboard bridge, was used for all bioassays and behavioural analyses. The T measured 15 cm along the stem, with each branch being 11 cm long and 1.2 cm wide. Unbleached filter paper was used to cover the entire base of the plastic T and was changed after each experiment. A pheromone trail was developed along the straight stem by allowing ants to feed on a 1 Molar aqueous sucrose source at the end of the stem for 5 minutes. After 5 minutes the ants were

quickly removed from the T maze. At this junction, returning ants would then be faced with a choice of a pentane only control on one branch and a glandular extract in pentane on the other branch. This was achieved by applying a line of 15 µl of pentane to one half of a section of filter paper (section X; 8.2 cm long) and a line of 15 µl of the glandular extract (0.1 glandular equivalents per µl of pentane) to the other half (Fig. 6.2). Section X was then centrally positioned at the junction of the T with the direction of the glandular extract and pentane randomly chosen to account for any directional bias. The ants were then allowed to return to the T maze and their decisions were recorded. A decision was deemed to have been made once the antennae of the ant touched either end of section X. All decisions made in a 5 minute period following placement of section X were recorded. Experiments were run in a temperature controlled room, maintained at 20 ^{o}C.

6.3.4. Experiment 1 – What is the glandular source(s) of the trail pheromone?

Extracts from the hindgut, poison, sternal and Dufour glands (Fig. 6.3) were prepared as described above. Each extract was tested against a control using our standard bioassay protocol (see above) for a total of six ant colonies. To identify whether trail pheromone components emanated from both the Dufour gland and the hindgut, ant responses to extracts from each gland were compared with the response to the two glands combined. Each prepared glandular extract was only tested with ants from the same colony.

6.3.5. Experiment 2 – Is the trail pheromone of Lasius flavus colony specific?

Once the hindgut had been identified as the source of the trail pheromone, extracts from these glands were tested against the source colony and two other colonies. Extracts from 5 colonies were used to determine if responses to the trail pheromone

differed between the source colony and other unrelated ant colonies, using the bioassay described above.

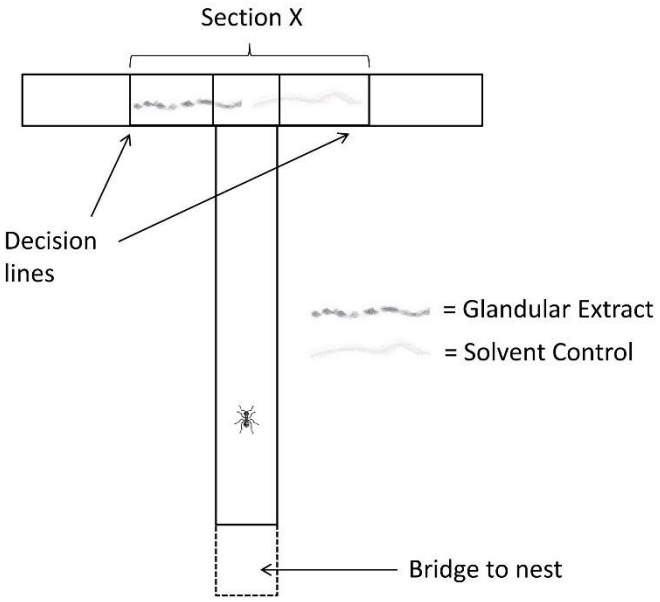


Figure 6.2: Experimental design used to measure trail choices of *Lasius flavus* ants. Ants were allowed to form a trail to the end of the straight stem by feeding on a sugar solution placed at the junction. After 5 minutes ants were removed from the T and a piece of filter paper (section X) was placed at the junction. On one half of section X was a trail consisting of 15 µl pentane (control) and on the other half was a trail of 15 µl a glandular extract dissolved in pentane. Ants were allowed to return to the T and their decisions were recorded once they passed a decision line from either branch.

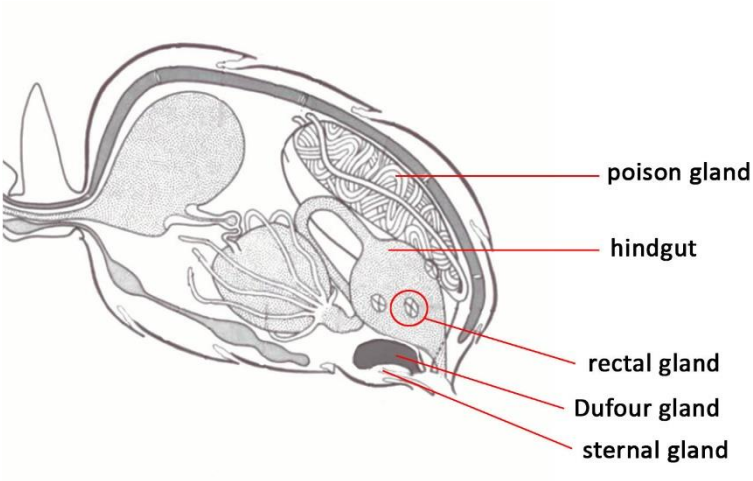


Figure 6.3. Representation of the gaster of a *Lasius flavus* ant, highlighting the relative position and sizes of the different endocrine glands.The hindgut is the source of the trail pheromone for all studied members of Formicinae to date, where it is produced by the rectal glands.

6.3.6. Analysis of the glandular constituents of the Dufour gland and hindgut

Prior to the analysis of the contents of the Dufour gland and hindgut a standard mixture was prepared containing eleven structurally different compounds previously found in the glands of *Lasius* ants: Undecane (*L. niger* & *L. flavus*: Bergström & Löfqvist 1970; *L. fuliginosus*: Akino & Yamaoka 1996), tridecane (*L. niger* & *L. flavus*: Bergström

& Löfqvist; *L. fuliginosus*: Hayashi & Komae 1980), octan-3-one (*L. flavus*: Cammaerts 1973), tridecan-2-one (*L. niger*: Hayashi & Komae 1980), octan-3-ol (*L. flavus*: Cammaerts 1973), dodecan-1-ol (*L. niger*: Bergström & Löfqvist 1970), dodecyl acetate (*L. niger*: Attygalle *et al.* 1987), formic acid (*L. alienus & L. flavus*: Stumper 1953), citronellal (3,7-dimethyloct-6-en-1-al) (*L. fuliginosus*: Hayashi & Komae 1980; *L. umbratus*: Blum 1969), nerol((Z)-3,7-dimethyl-2,6-octadien-1-ol) and geraniol ((E)-3,7-dimethyl-2,6-octadien-1-ol) (*L. neoniger* & *L. alienus*: Law *et al*. 1965; *L. fuliginosus*: Hayashi & Komae 1980). In addition, two coumarin compounds, 4-methylumbelliferone (7-hydroxy-4-methylchromen-2-one) and 4-hydroxycoumarin (2-hydroxychromen-4-one), which were structurally similar to mellein (a dihydroisocoumarin), found in the hindgut of *L. fuliginosus* (Kern *et al.* 1997) and 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin found in the hindgut of *L. niger* (Bestmann *et al.* 1992) were included in the standard mixture. All the constituents of the standard were present at a concentration of 1ng μl^{-1} in pentane. This allowed the determination of the approximate retention times of the different chemical groups, while also identifying compounds that would require derivatisation to elute from the column. All compounds were sourced from Sigma Aldrich, UK. The standard mixture was also derivatised using BSTFA and pyridine to identify the retention times of any non-volatile compounds that did not elute from the column when analysed as the underivatised compounds. The active hydrogen of less labile functional groups such as a hydroxide of the analyte is replaced by the more stable trimethylsilyl group (from BSTFA). The presence of a trimethylsilyl group increases the volatility of the analyte, allowing it to elute from the GC column.

Dufour glands and hindguts were then carefully extracted separately and added to pentane. Seven glands were added to 70 µl of pentane, crushed with a glass rod and the solvent extract was gently evaporated to dryness using pure nitrogen. The extract was then redissolved in 50 µl of pentane and divided into two halves. Half of the extract was derivatised using BSTFA and pyridine, before being analysed and the other half was analysed directly by GCMS. Prior to analysis, a small volume of the underivatised extract was tested using the standard bioassay described above, to confirm pheromone activity.

6.3.7. Collection of trail pheromone from substrate

As expected, analysis of the hindgut contents identified a considerable number of different compounds, most of which were unlikely to be constituents of the trail pheromone. To help determine the active trail components a straight foraging trail was constructed in which the ants walked over a glass slide to reach a 1M sugar solution food source. Ants leaving the food source to return to the nest walked on a 1.5cm section of filter paper before reaching the glass slide to reduce contamination with sugar solution carried on the ant's tarsi. After 5 minutes of busy foraging the glass slide was washed with 250 µl pentane and the trail collection repeated several times. Because the deposition behaviour involving the ant dropping its gaster to the surface was clearly observable, it was ascertained that five minutes of busy foraging traffic corresponded to ca. 40-70 deposits.

It was found that the concentrated pentane solution extracted from glass slides elicited a very weak trail-following response when using the classical bioassay method (see Fig 6.2), so an alternative method using filter paper instead of glass slides was developed. Two short pieces of filter paper (ca. 4 cm long) were washed in pentane,

dried and then placed on the foraging trail. After 5 minutes of busy foraging traffic the filter paper was folded using clean forceps and soaked and agitated in 3 ml of pentane contained in a small glass vial on ice. In this way 12 – 14 segments of filter paper marked with pheromone could be soaked with a loss through evaporation from the procedure of ca. 2ml of pentane. The extract resulted in an initial weak trail following response in the bioassay but this was significantly increased once the solvent extract was concentrated to between 50-75 µl using nitrogen gas. Half of this concentrated extract was derivatised using BSTFA and pyridine before being analysed with GCMS and the other half was directly analysed using GCMS. Controls used in the analysis consisted of a pentane solvent blank in addition to a sample of pentane from which the filter paper had been washed prior to placing on the ant foraging trail. Comparison of the GCMS analyses of these extracts with the gross glandular extract results from earlier analyses, identified the compounds from the rectal gland which areactually deposited during trail formation.

6.3.8. Statistical Analysis

Data from the bioassay experiments were analysed using generalised linear mixed effect models (GLMM) with binomial errors in R v.2.15.1 (R Development Core Team 2012). These models take into account the fact that due to their close genetic relatedness, ants from the same colony cannot be treated as independent from one another. Binomial errors were used in the model because the proportion of ants that chose one branch and the proportion that did not were both known. Generalised linear mixed effect models were fitted using the lmer function (Bates *et al.* 2011). For model selection I used the protocol proposed by Zuur et al (2009). Initially this involves investigating the optimal structure of the random factors and comparing random

intercept models with random intercept and slope models. Comparisons between models was based upon Akayike Information Criterion (AIC) and Bayesian Information Criterion (BIC) values, with lower AIC and BIC scores signifying a model with a superior fit to the data. As a result of model comparison the random effect of "date" was dropped from the model while "colony" was included. The optimal fixed component structure was then determined by sequentially dropping any non-significant fixed effects from the model. Wald tests were used to determine the significance of the fixed effects and models were fitted using the Laplace approximation, which provides accurate estimates when used with binary data (Bolker *et al.* 2009; Bates *et al.* 2011). Fixed effects used in the optimal models were gland (6 levels: 5 glands & control) and side (2 levels: left & right) in experiment 1 and pheromone source in experiment 2 (2 levels: nestmates & non-nestmates).

6.4. Results

6.4.1. Experiment 1 – What is the glandular source(s) of the trail pheromone?

Ant responses to extracts differed from the control for all treatments except that from the Dufour gland (Fig. 3). The extract eliciting the greatest trail following behaviour was seen for the hind gut which was significantly greater than all other extracts, including the gaster (80 vs 73%, Z = 2.33, P = 0.02). Extract from the sternal gland elicited a greater trail following response than the control (62 vs 50%, Z = 2.55, P = 0.011), but not from the Dufour (62 vs 52%,Z = 1.67, P = 0.095). The only extract that evoked a negative trail following response was the poison gland, with significantly fewer ants following this treatment than for the control (35 vs 50%, Z = -3.81, P <0.001).

6.4.2. Experiment 2 – Is the trail pheromone of Lasius flavus colony specific?

No significant difference was found between the response of ants to trail pheromone extracted from the hindgut of their nestmates and hindgut extracts from ants of other colonies (nestmate vs non-nestmate: 90.2 vs 92.3 %, Z = 0.584, P = 0.56, Fig 6.4). Variation in colony responses was unexpectedly greater when presented with nestmate trail pheromone rather than when provided with a trail derived from non-nestmate pheromone.

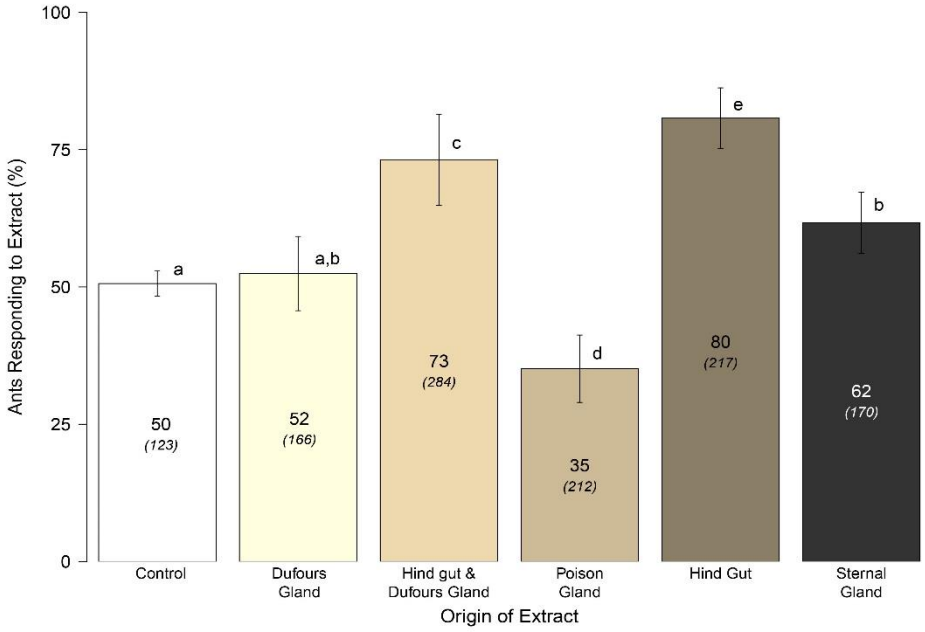


Figure 6.3: Results for Experiment 1. While foraging on a T maze ants were faced with a choice between a branch marked with glandular extract in solvent and an unmarked branch (solvent only). Extracts consisted of contents from one of four glands or the entire gaster crushed in pentane (0.1 gland equivalents in 1 µl pentane). With the exception of the Dufour gland, the ants' responses to all extracts differed from the control. Within each bar is given the mean and in brackets the sample size (number of ants). Different letters (given above each bar) denote significant differences between treatments, while error bars represent the standard error of the mean.

6.4.3. Analysis of the glandular constituents of the Dufour gland and hind gut

6.4.3.a. Standards

The gas chromatogram in Figure 6.5 shows the retention times of the underivatised individual components of the standard mixture. All compounds were present except for the highly volatile formic acid and the coumarins, 4-hydroxycoumarin and 4-methylumbelliferone. The smaller hydrocarbons were the first

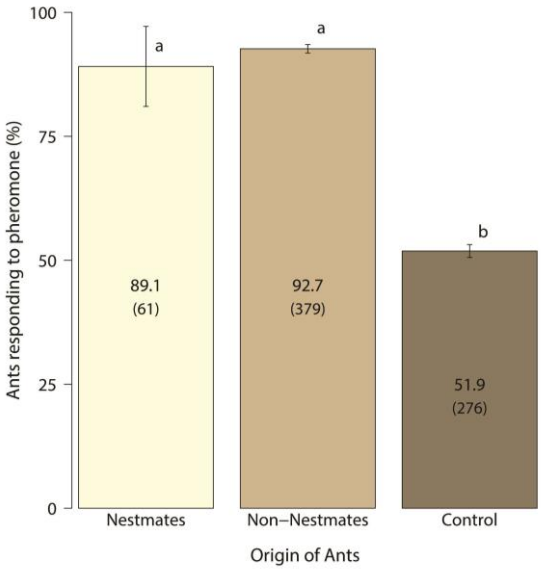


Figure 6.4: Results for Experiment 2. At the junction of a T maze foraging ants were given a choice of following a pheromone trail (crushed hindguts in pentane) on one side and a control (pentane only) on the other. Significantly more ants chose the branch with the pheromone trail, irrespective of the origin of the pheromone. Thus, ants from one colony were equally as likely to follow the trail produced by another colony, as their own. Within each bar is given the mean and in brackets the sample size (number of ants). The same letters above each bar denotes no significant differences between the two treatments, while error bars represent the standard error of the mean.

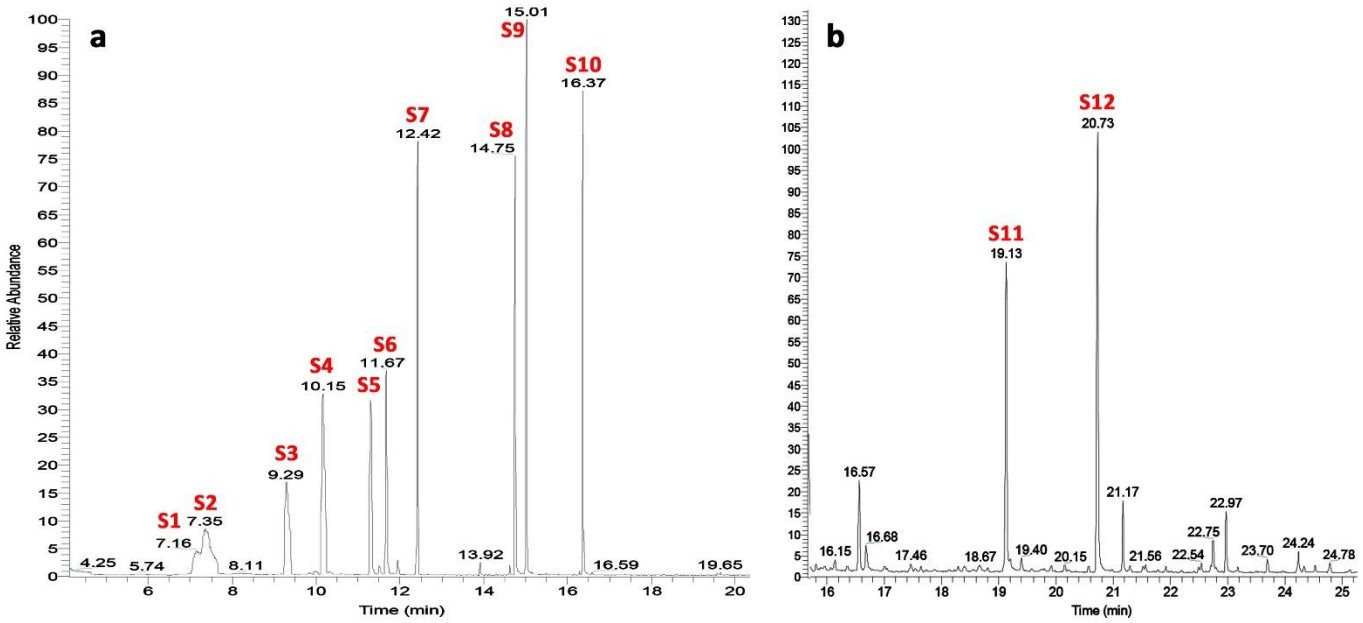


Figure 6.5: a.) Gas chromatograph of the underivatised standards prepared at 1 ngµl^{-1}. All 10 compounds have been found in glands of *Lasius* ants. Numbered peaks refer to: 3-octanone (**S1**), octan-3-ol (**S2**), undecane (**S3**), citronellal (**S4**), nerol (**S5**), geraniol (**S6**), tridecane (**S7**), dodecyl acetate (**S8**), tridecan-2-one (**S9**) and dodecan-1-ol (**S10**). Formic acid was also present in the standard mixture but was too volatile to appear here. **b.)** Gas chromatograph of the derivatised standards prepared at 1 ngµl^{-1}. Numbered peaks refer to 4-hydroxycoumarin (**S11**) and 4-methylumbelliferone (**S12**).

to elute while the longer chained, more polar compounds such as the ketone, Tridecan-2-one (**9**), were slower to elute from the column (see Fig. 6.5a).Both coumarins could be seen in chromatogram of the derivatised sample, with retention times of 16.56 for 4-hydroxycoumarin and 20.71 for 4-methylumbelliferone (Fig. 6.5b).

6.4.3.b. Dufour Gland

Two peaks were particularly distinct in the Dufour gland extract at retention times of 9.23-9.25 and 23.52 (**D1** & **D5**; Fig. 6.6). Compound **D1** was identified as undecane, with the same retention time as the standard (see **S3** in Fig. 6.5a) and a very similar mass spectrum, while compound **D5,** which was not present in the database, was more difficult to assign. The most likely candidate is (9E)-octadec-9-en-4-olide, which has been identified in the gaster extracts of *Lasius flavus* ants (Bergström & Löfqvist 1970).

Comparison of the mass spectra of **D5** with that of the Z isomer of the lactone found by Bergstrom & Löfqvist (1970) suggests that these may be the same compound (Fig. 6.7a & b). The molecular mass of both compounds is 280 amu and the peaks of 262 and 220 in both spectra correspond to the loss of water and subsequent fragmentation of the lactone ring, via cleavage of two bonds. The ion 67 is likely to correspond to a double allylic fragment (C_5H_7); one of which is likely to be the double bond in the acyclic chain of the lactone. The intense fragment ions that follow 67 (81, 95, 109, 123) are 14 amu apart indicating the presence of the acyclic chain with each lengthened by a carbon and two associated hydrogens.

This suggests the ring structure in both compounds appear to be the same. While the intensities differ slightly between the two spectra, the major peaks occurring from fragmentation of the aliphatic chain are present in both. However, the characteristic

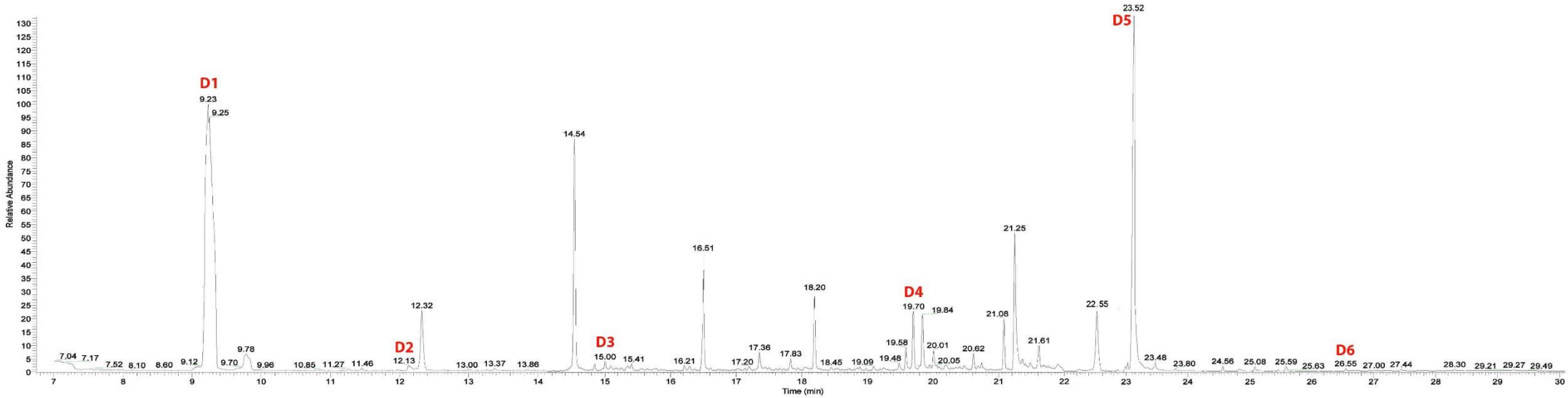


Figure 6.6. Gas chromatogram of the underivatised Dufour gland extract in pentane. Two compounds, undecane (**D1**) and a likely diastereoisomer of (4R,9Z)-octadec-9-en-4-olide (**D5**) are present in significantly greater volumes than the other four unidentified compounds (**D2, D3, D4, D6**).

peak of a γ-lactone, 85, corresponding to the ring after loss of the aliphatic chain, is notably weak in our mass spectrum (Fig. 6.7b). This peak is usually strong for γ-lactones (Honkanen, Moisio & Karvonen 1965; McFadden, Day & Diamond 1965) due to the stability of the 5 membered ring. For example, in the lactone 4-octadecanolide (the same structure as (4R,9Z)-octadec-9-en-4-olide but without the double bond) 85 is the base peak (McFadden *et al.* 1965). In micromolide the peak at 85 amu is also less intense (Fig. 6.7a) but is still considerably stronger than the peak seen in my spectrum. ..

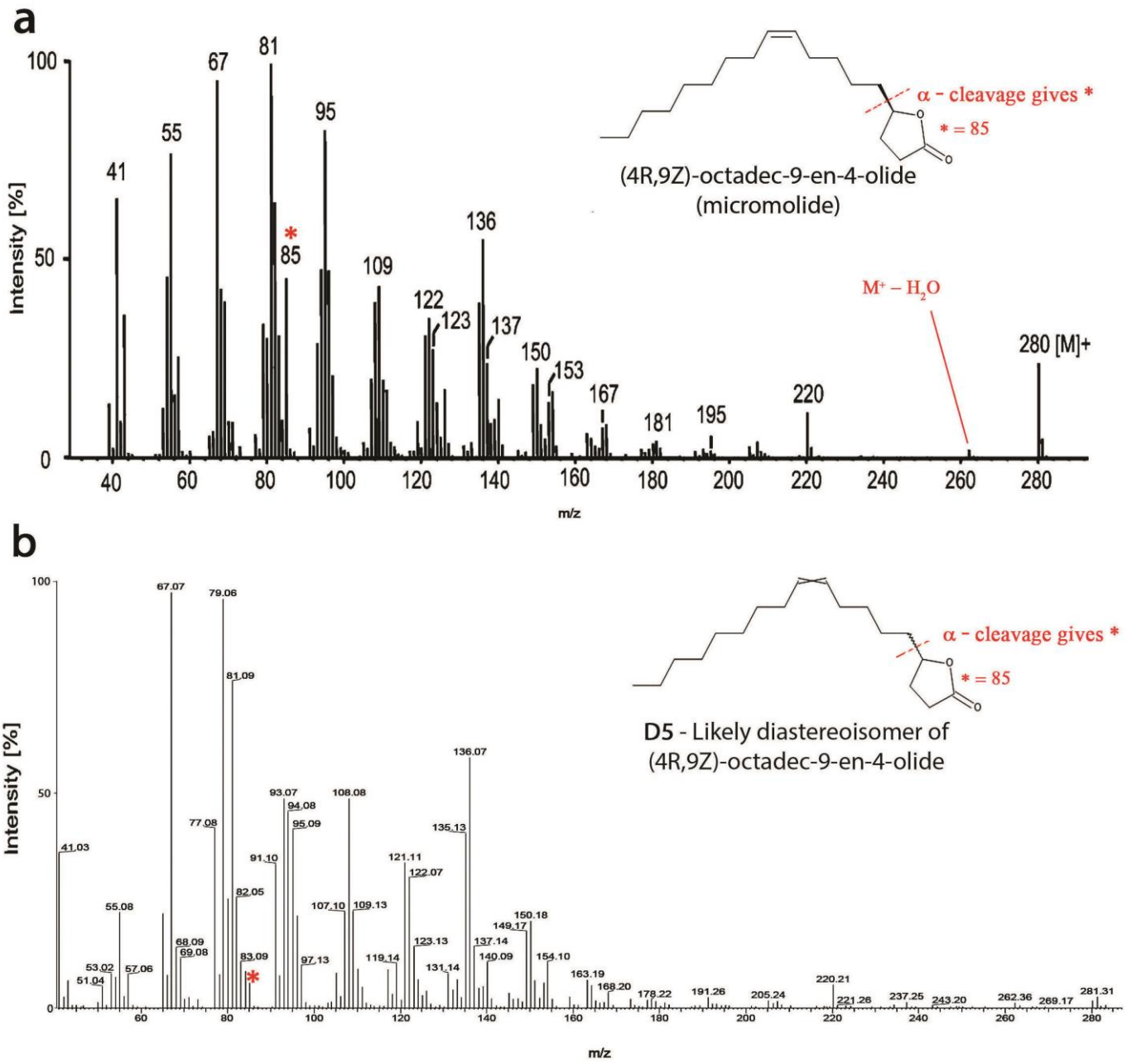


Figure 6.7. a) Mass spectrum of (4R,9Z)-octadec-9-en-4-olide, a γ-lactone known for its antibacterial properties. Adapted from Herzner et al. (2013) **b)** Mass spectrum of **D5**, a likely diastereoisomer of (4R,9Z)-octadec-9-en-4-olide, based upon the similarity of the fragmentation patterns**.**

The low intensity we see for the peak at 85 amu suggests that ring stability is somewhat reduced in compound **D5**, derived from *L. flavus*. Whether this could be a result of different stereochemistry at C4 or due to differences in the GC-MS sources used remains to be seen and warrants further investigation. Analysis of a pure sample

of **D5** using NMR would help to elucidate the stereochemistry at C4 and identify the position of the double bond within the aliphatic chain. Subsequent derivatisation would also prove useful in determining the stereochemistry of the double bond. NMR (^{1}H) requires a minimum sample of 1 mg so extraction and separation of Dufour gland extracts from a large number of ants (ca. 1000) would be necessary.

Four other peaks, significantly weaker than **D5**, were present in the extract but absent in the control. Unfortunately, due to time constraints these compounds were not identified.

6.4.3.c. Hind Gut (containing rectal glands)

Nine separate compounds were present in the underivatised hindgut extract but absent in the pentane control (**H1** – **H9**; see Fig. 6.8a). Of these, one peak, labelled **H9**, was particularly strong at a R_t of 27.55. The fragmentation pattern suggests a large terpenoid. Strong peaks at 136, 121, 95 and 81 m/z are typical of terpenoid fragmentation patterns. A long alkyl chain is present, signified by characteristic peaks 14 daltons apart. Many of the other compounds appeared to be esters (**H4**, **H5**) or long chain hydrocarbons (**H7**, **H8**) as these were the closest compounds found in the Wiley and NIST spectra databases. Compound **H4** has a high probability of being isopropyl palmitate (the methyl, ethyl ester of hexadecanoic acid) and **H5** is similar in structure to a methyl ester of octadecanoic acid. Only carbohydrates appeared to be present in the derivatised hindgut extract.

6.4.3.d. Filter Paper

Strong peaks arising from impurities in the filter paper reduced the size of any peaks of interest. Despite this, two compounds of interest which were also present in the hindgut extract but absent in the control, were determined (**P1** & **P2**)[Fig. 6.8b]. By

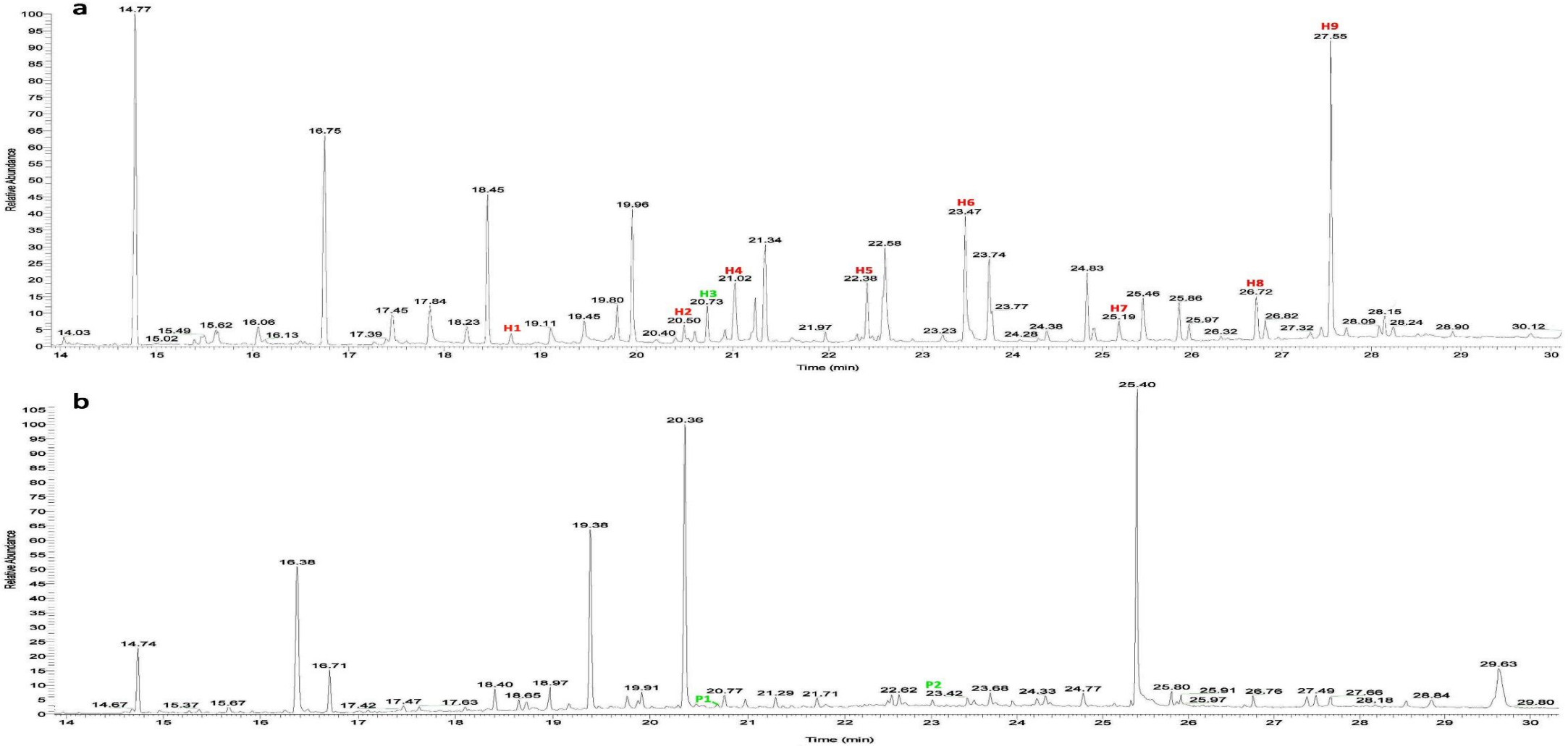


Figure 6.8. **a.)** Gas chromatogram of the underivatised hindgut extract in pentane. The nine compounds labelled (**H1** – **H9**) were not present in the pentane control. Of these nine compounds, a single compound (**H3**) was found to be also present in the extract taken from filter paper removed from a trail of ants depositing pheromone (**H3** = **P1**). **b.)** Gas chromatogram of filter paper removed from a trail of pheromone depositing *Lasius flavus* ants in pentane. Two compounds (**P1** & **P2**) were found to be present in the rectal gland extract and not in the filter paper/pentane work-up control. The large peaks refer to a number of different phthalate contaminants arising from the filter paper.

analysing particular ion fragments specific to the compound of interest the presence of the compound can be determined in other hind gut extracts and the work-up control samples. In Figure 6.9 two ions specific to **P1** (157^+ & 284^+) were selected and their presence determined in the hindgut and filter paper extracts, together with relevant controls, to ascertain if this compound originated from the hindgut. The compound containing these ions was present in both the filter paper and hindgut extracts but not in the controls (Fig. 6.9).

The fragmentation pattern of **P1** is suggestive of an ethyl ester (Fig. 6.10). There is a notable peak at 88 m/z which is most likely the product of a McLafferty rearrangement of an ethyl ester (see Fig. 6.11 for fragmentation mechanisms). This is supported by the fragment at 73 amu which is likely to be the resonance stabilised acylium ion, possessing an ethyl group. If we assume that the molecular mass of this ester is 284 amu, which these spectra strongly suggest, this indicates that there is no methyl group present as a branch on the hydrocarbon chain as there is no peak at 269 (M^+- CH3). The drop from the molecular ion peak of 284 to the next significant peak of 255 amu gives a difference of 29 amu, suggesting an ethyl group. This may arise from the loss of the ethyl group from the ester end of the compound or by loss of an ethyl branch along the hydrocarbon chain. The ester contains a long saturated hydrocarbon chain with a minimum length of 13 carbon atoms. Together, the evidence suggests compound **P1** has a likely molecular formula of $C_{18}H_{36}O_2$ and is likely to be similar in structure to ethyl hexadecanoate.

The other compound present on the paper extract (**P2**) appears to be the same γ-lactone as that discovered in the Dufour gland in high concentrations (**D5**, Fig. 6.6) after comparison between the mass spectra (Fig. 6.7b & Fig. 6.10b). Analysis of selected key fragment ions confirmed this compound as **H6**, present in the hindgut extract, in addition to being present

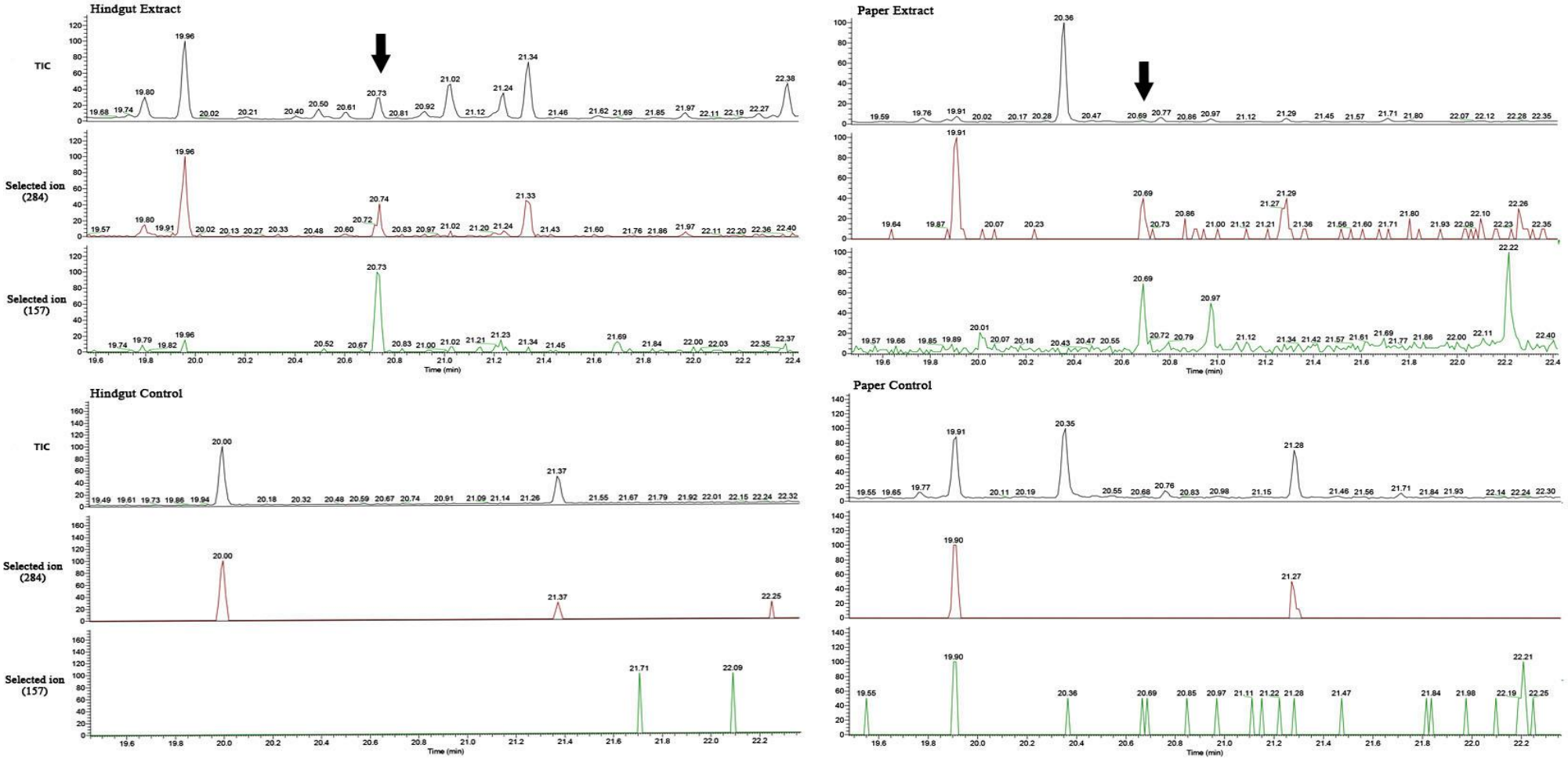


Figure 6.9 Selected ion chromatograms for hindgut extract, filter paper extract removed from an ant trail and associated controls. The compounds of interest (**H3**/**P1**) are visible as peaks at a retention time of 20.73-20.74 in the hindgut extract and 20.69 in the paper extract. For each selected ion (284+ & 157+) a peak is also present at these retention times indicating that these key ions are present in our compounds of interest and therefore suggesting that these compounds are the same. Peak intensity on the y axis corresponds to the amount of each compound in the Total Ion Chromatogram (TIC) and the amount of a particular ion (284+ & 157+) for the two other chromatograms. The hindgut control consisted of the solvent only while the paper control comprised of solvent in which the filter paper had been soaked in.

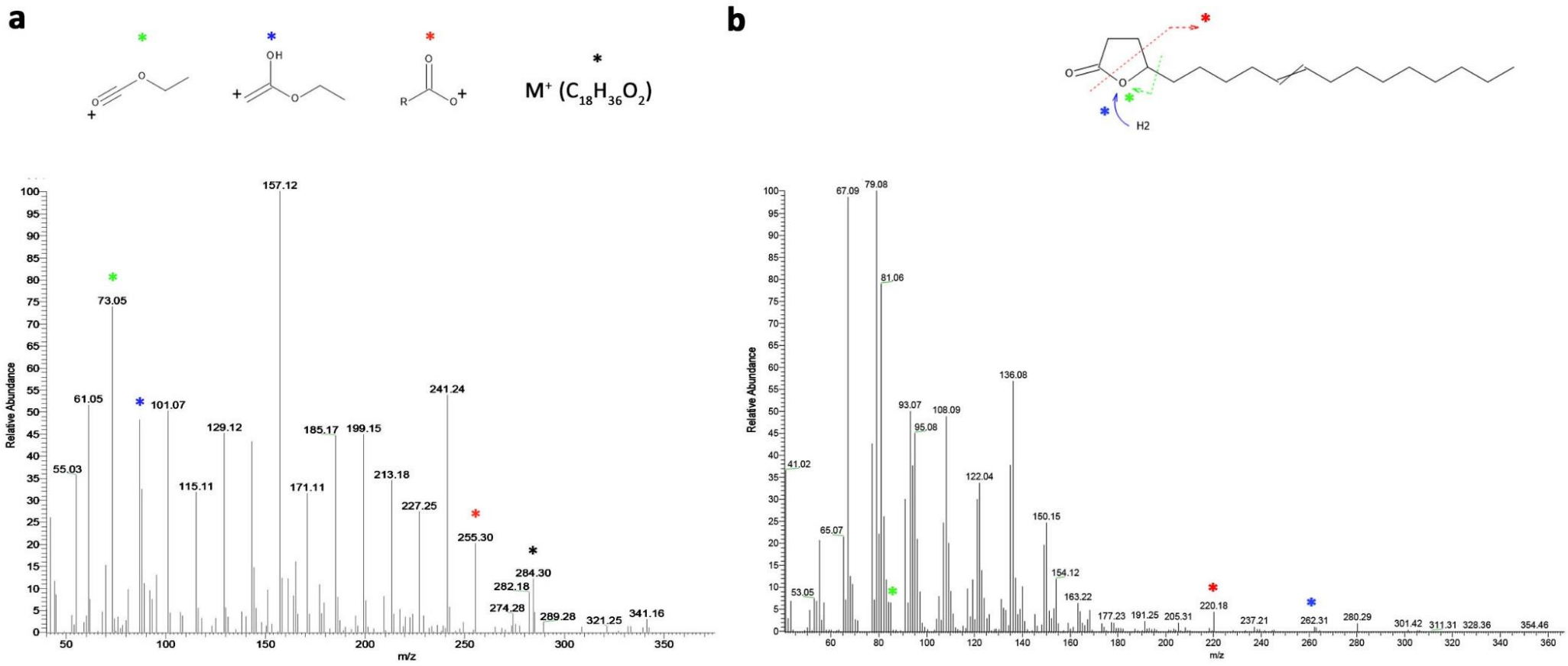
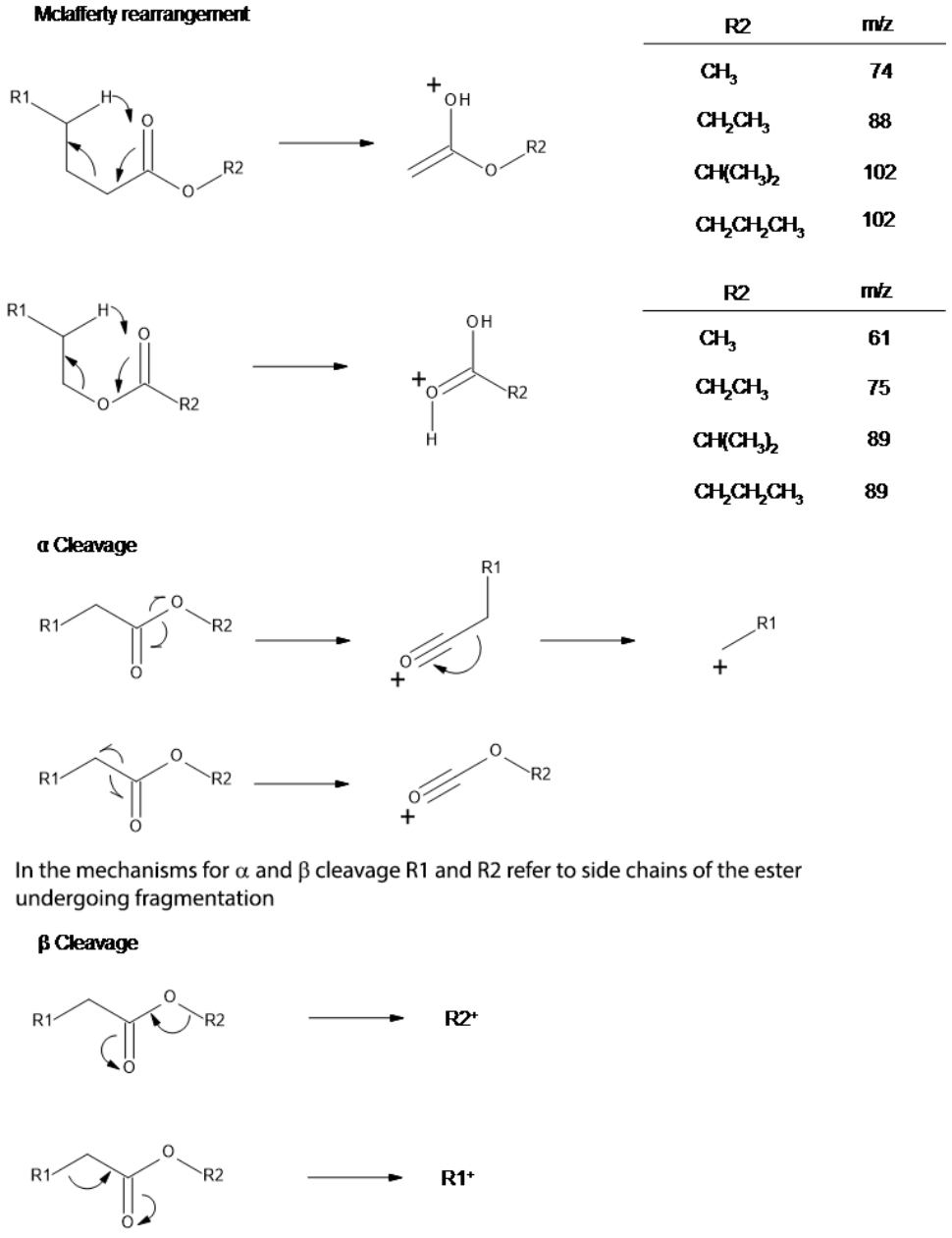


Figure 6.10. a.) Mass spectrum of unknown compound **P1**, extracted from filter paper removed from a pheromone trail. The fragments shown for the peaks indicated suggest that the compound is an ethyl ester. **b.)** Mass spectrum of compound **P2**. This is likely to be the γ-lactone identified in the Dufour gland extract earlier (see Fig. 6c). The fragments that help determine parts of the structure are shown..

in the Dufour and the trail laid by *L. flavus* ants (Fig. 6.12). However, analysis of other hindgut and Dufour gland extracts showed that **P2** was always present in significant concentrations in the Dufour, but often absent and always lower in samples taken from the hindgut. Contamination of hindgut samples with contents from the Dufour gland is entirely feasible as these fragile glands were occasionally ruptured during dissection. Undecane, the other compound present in a large volume in the Dufour gland was absent from the paper extract.



R2	m/z
CH_3	74
CH_2CH_3	88
$CH(CH_3)_2$	102
$CH_2CH_2CH_3$	102

R2	m/z
CH_3	61
CH_2CH_3	75
$CH(CH_3)_2$	89
$CH_2CH_2CH_3$	89

Figure 6.11. Examples of common fragmentation patterns of esters in electron impact mass spectroscopy. Mclafferty rearrangements, α and β cleavage are common fragmentation processes and the resulting peaks are usually fairly prominent (McLafferty 1980).

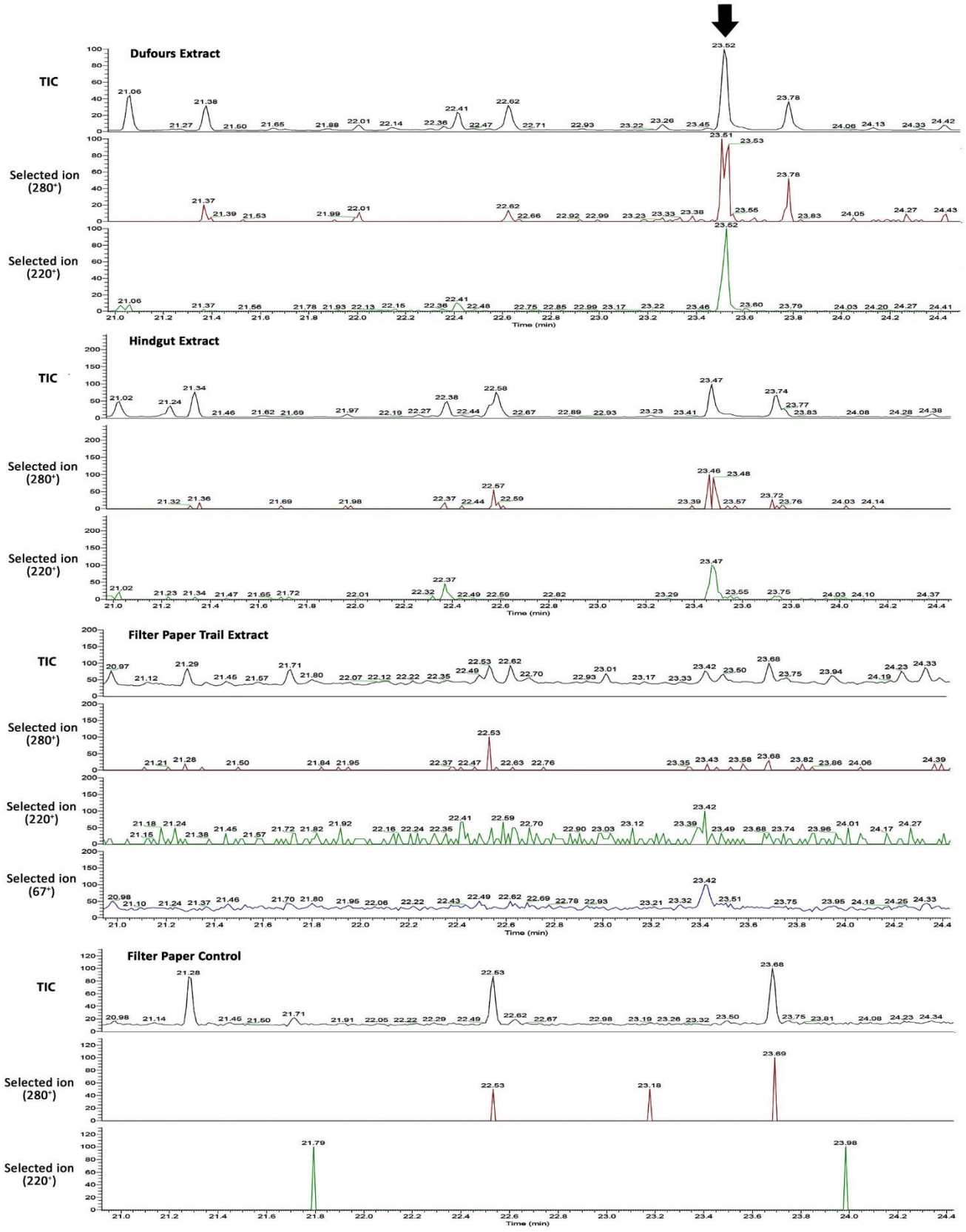


Figure 6.12. Selected ion chromatograms for the Dufour gland extract, hindgut extract, filter paper extract removed from an ant trail and its relevant control. The compound of interest appears as a visible peak in the Total Ion Chromatogram (TIC) at a retention time between 23.42-23.64 and corresponds to the compound **D5** for the Dufour gland (Fig. 6.6), **H6** for the

hindgut (Fig. 6.8a) or **P1** for the paper extract (Fig. 6.8b) respectively. Below the Total Ion chromatogram are selected ion chromatograms (280^+, 220^+ & 67^+) that also show peaks at the same retention time, indicating that the compounds of interest found in the Dufour gland (**D5**), hindgut (**H6**) and paper extract (**P2**) are very likely to be the same compound. Peak intensity on the y axis corresponds to the amount of the each compound in the Total Ion Chromatogram and the amount of a particular selected ion (280+, 220+ & 67+) for the other chromatograms. The extra selected ion of 67+ (present in the Dufour, filter paper and hindgut extracts) was included for the filter paper extract, specifically to provide further evidence as the noise within the other selected ion chromatograms was substantial. The work-up paper control comprised of solvent in which the filter paper had been soaked in.

6.5. Discussion

The results from the glandular bioassays clearly demonstrate that, as in all other Formicine ants studied to date, the hindgut is the source of the trail pheromone ((e.g. *Lasius niger*; Bestmann *et al.* 1992, *Camponotus herculaneus*; 1995a; *L. fuliginosus*; Kern *et al.* 1997; C. balzani; Kohl *et al.* 2003). The Dufour gland contents on their own fail to elicit any attractive properties and equally do not increase the trail following response in combination with the hindgut contents. In contrast, the repellent effect observed for the poison gland exudate is considerable and unlikely to be an alarm response. Responses to alarm pheromones in ants usually involve a degree of attraction towards the source of the pheromone, together with aggressive stances and behaviour (Blum 1969; Bradshaw, Baker & Howse 1975), rather than the repellent reaction seen here.

In many Formicine species, Dufour contents are released together with poison gland constituents (Maschwitz 1964; Regnier & Wilson 1968; Blum 1969) thus producing an alarm response in conjunction with the release of defensive compounds. This is facilitated by the dual and independent release of poison and Dufour gland contents through the same sting reservoir (Billen 1987). However, if this was the case in *L. flavus*

we would expect to see some form of response to the Dufour extracts, which is clearly not the case in our bioassay results. Furthermore, ants of the genus *Lasius* have previously been shown to respond indifferently to formic acid (Maschwitz 1964) so it may be another constituent within the poison gland that is inducing the repellent response in *L. flavus*. Unlike in the termites, there appears to be scant evidence of either trail (Billen & Morgan 1996; Morgan 2009) or alarm pheromones (Hölldobler & Wilson 1990; Morgan 2008) originating from sternal gland exudates so the weakly attractive effect seen in our results is likely to be an artifact of contamination.

The novel method of extracting a trail pheromone directly from filter paper, developed during this study successfully enabled the identification of a potential candidate very similar to ethyl hexadecanoate, which may function as a trail pheromone component of the yellow meadow ant *Lasius flavus*. A comparison of chromatographs and spectra of the hindgut extract with those of filter paper removed from a trail of pheromone depositing ants quickly identified compounds peculiar to both samples. The obvious advantage of this method is the time saved by the removal of "noise" from the hindgut extract, allowing one to focus only on those compounds that are actually deposited by the ants, as they form a trail between a food source and their nest.

Esters are used as trail pheromones by a number of ant species spanning the subfamilies Ponerinae, (*Ectatomma ruidum*: Bestmann *et al.* 1995b; *Leptogenys peuqueti*: Janssen *et al.* 1997b; *Gnamptogenys striatula*: Blatrix *et al.* 2002), Myrmicinae (*Tetramorium impurum*; Morgan & Ollett 1987) and the Formicinae (*Camponotus atriceps*: Haak *et al.* 1996; *C. ligniperda*: Bestmann *et al.* 1997). However, if this compound is identified as a trail pheromone it will be the first reported case of

an ester serving as a pheromone within the *Lasius* genus. This possibility is entirely feasible, especially when we consider that the unique lactone (cyclic ester), **D5**, is already biosynthesised in the Dufour gland. Undecane (**D1**), one of the two compounds present within the Dufour gland in substantial quantities, serves as an alarm pheromone in a number of Formicine species (e.g. *Lasius alienus*: Regnier & Wilson 1969; *L. fuliginosus*: Dumpert 1972; *Formica rufa*: Löfqvist 1976; *F. argentea*: Lenz, Krasnec & Breed 2013), but our results show that this is not the case for *L. flavus*. Our analysis shows that while undecane is present in high concentrations within the Dufour gland no attractive (or aggressive) response was observed when ants were presented with Dufour gland extract. Thus, undecane may in fact function more as a solvent or spreading agent for a constituent within the same gland such as the γ-lactone.

The other compound also present in large quantities was the γ-lactone (**D5**), a compound unique to ants. Its distinct structure and omnipresence suggests an important role but what this actually is has yet to be demonstrated. It elicits no response from ants when presented in the Dufour extract so it may play a role if mixed with the trail pheromone component(s) from the hindgut or with defensive compounds from the poison gland. The presence of the lactone in the filter paper extract suggests that this maybe the case. However, it is surprising that undecane (**D1**) was not found in the filter paper extract while the γ-lactone was, when both originate from the same gland, in similar volumes.

A feasible alternative function for the lactone, **D5**, is to serve as a specific anti-microbial agent. Some species of arboreal ants have lost the need for a metapleural gland, possibly because the diversity of pathogens present in the tree canopy is

reduced (Walker & Hughes 2011). One might expect the pathogen pressure to be quite the opposite in the humid subterranean environment and therefore glandular production of an anti-microbial agent that could be smeared on the brood and/or within the nest environment would be beneficial. If this is the case, it would help to explain the presence of the γ-lactone on the filter paper extract. If present within the nest environment, the lactone is likely to be carried on the tarsi of ants and then transferred to the filter paper during foraging. This could easily be determined by analysing filter paper that had formed part of a non-rewarding and therefore pheromone free ant trail.

In further support of this idea, a variety of compounds have been demonstrated to serve as social immunity agents in ants. For example, venom alkaloids of the fire ant *Solenopsis invicta* (Storey *et al.* 1991) and proteins of the Ponerine ant, *Pachycondyla goeldii* (Orivel *et al.* 2001), both act against harmful pathogens. Furthermore, the γ-lactone, micromolide ((4*R*, 9*Z*)-octadec-9-en-4-olide), has proven antibacterial activity (Yuan *et al.* 2008) and has recently been found to function as a parasitoid's preserving agent. Herzner et al. (2013) identified micromolide as an antimicrobial agent that is produced by the larvae of the parasitic wasp, *Ampulex compressa*, and secreted within the cavity of its cockroach host to keep it fresh while it is devoured. Interestingly, another antimicrobial compound produced by the larvae of *A. compressa* is mellein ((*R*)-(-)-3, 4-diydro-8-hydroxy-3-methylisocoumarin) which is also found in the hindguts of a considerable number of formicine ants (e.g. *L. fuliginosus*; Kern *et al.* 1997; *Camponotus* spp.; Payne, Blum & Duffield 1975; Ubler *et al.* 1995; Brand, Mabinya & Morgan 1999; Voegtle *et al.* 2008).

Our results clearly show that the trail pheromone of *L. flavus* is not colony specific with non-colony members exhibiting an equally strong trail following response as colony members to a given pheromone trail. The only example of a colony specific trail pheromone in ants, discovered to date, is seen in *Lasius neoniger*, where this property is likely to reduce territorial confrontations in populations as dense as 5 colonies per square metre (Traniello 1980). Thus, this trail pheromone is probably acting in much the same way as the other colony specific pheromone found in ants, a territorial pheromone deposited by the ant *Oecophylla longinoda* (Beugnon & Dejean 1992). Densities of *L. flavus* nests can reach as much as 2 colonies per square metre (Pontin 1961) so territorial cues such as a colony specific pheromone would, in principle, be advantageous. However, there appears to be little evidence of fighting between *L. flavus* colonies (no heads attached to legs of workers for example) (Pontin 1963), which suggests that this species either avoids territorial disputes, thus demonstrating the "dear enemy" phenomenon (Heinze *et al.* 1996; Langen, Tripet & Nonacs 2000; Dimarco, Farji-Brener & Premoli 2010), or alternatively other glandular compounds serve as territorial cues.

To conclude, the work in this chapter demonstrates that the trail pheromone of *L. flavus* originates from the hindgut, does not act synergistically with components from the Dufour gland and is not colony specific. Furthermore, a very useful method of extracting the active chemical components deposited during trail formation was developed, reducing the workload that comes with testing every major glandular component in a behavioural bioassay. While I did not manage to positively identify the compounds that are deposited in a trail by *L. flavus* during this study, the foundations have been laid for the future identification of the trail pheromone. Moreover, the

demonstration that the lactone present in the Dufour gland does not play an obvious role in trail following suggests that its possible role could be sanitation of the nest through antimicrobial activity.

7

Final Discussion and New Directions

7.1. So how does Tetragonisca angustula discriminate friend from foe with such accuracy?

While our study in Chapter 3 fails to conclusively answer the question of how the bee *Tetragonisca angustula* is able to accomplish such accurate nest mate recognition with so few recognition errors, it does take us one important step closer. We can now rule out two possible mechanisms; we know guards are not using the wax entrance tubes as template referents, and resin, stored in hives, is not on its own serving as the source of colony odour. So what exactly is enabling these successful little bees to recognise friend from foe with such accuracy? If our chemical samples taken from bees, wax tubes and resin piles from each hive had survived the storage and transit we may have been in a better situation to address this question. However, one or a combination of two, not necessarily mutually exclusive mechanisms are the most likely factors governing this bee species highly efficient nestmate recognition system, and should I believe, become the focus of future research.

One of these mechanisms is the lower genetic variability present in stingless bee colonies as a result of monandry. Unlike the polyandrous honeybees virtually all workers in the stingless bee colony share the same mother and father (current queen may have superseded a previous queen). We can therefore postulate that the

glandular produced hydrocarbons, being a product of the genes possessed by the workers, are likely to be significantly less diverse across the colony than in honeybees, in which queens may mate with up to 20 males. This in theory should reduce recognition errors by making the guards' job easier when assessing the 'label' of incoming bees.

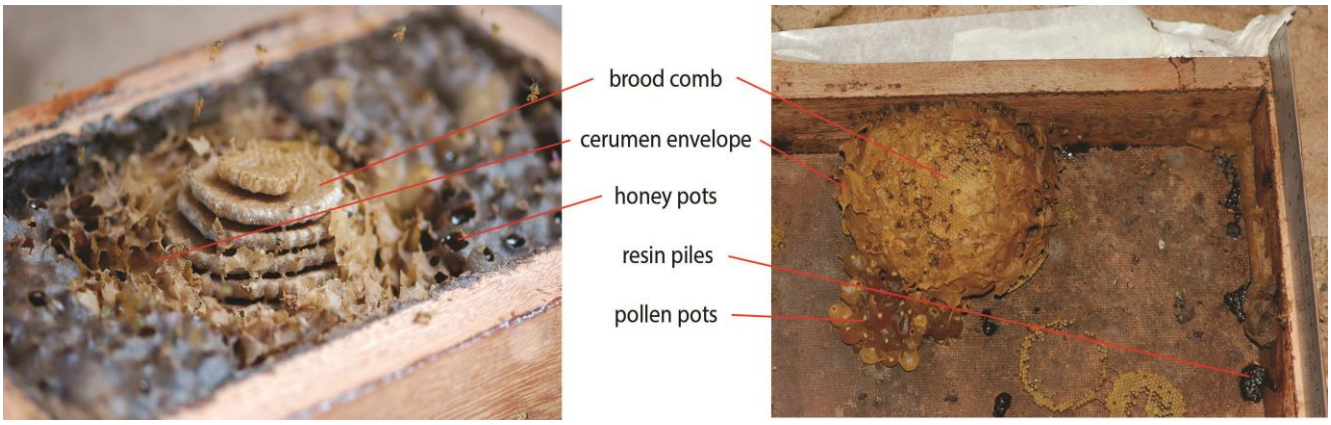


Figure 7.1. These photos show the circular horizontally arranged brood combs with the cerumen envelope peeled away. Other nest features are also highlighted (photos courtesy of Tomer Czaczkes).

However, this mechanism alone cannot explain the difference we see between the recognition system of *T. angustula* and other stingless bees. All stingless bees are monandrous so there must be additional influential factors that separate *T. angustula* from the other bees studied to date. This brings me to the second potential mechanism that I believe warrants further research; this involves. *T. angustula* workers acquiring a complex odour blend from the nest environment which combines both genetic and environmentally derived chemical cues. The combs and the involucrum, a surrounding envelope, within the nest of *T. angustula* are created using a substance called cerumen. This structural material is composed of a mixture of wax and resin, giving it a caramel like colour. Like honeybees, newly emerged *T.angustula* workers could then passively acquire the odour of the colony via contact with the substrate

surface and fellow nestmates. However, in the case of *T. angustula* the newly emerged workers would be in contact with a comb consisting of cerumen, rather than one created from pure wax. This means that in addition to the genetically derived chemical cues present in the wax, the cuticle of workers would also be in contact with environmentally derived odours, such as terpenoids, emanating from the resin. Thus, it may be this mixture of wax and terpenoids within the cerumen that provides *T. angustala* workers with their colony odour. Further research is now needed to investigate this.

7.2. The unique dual defensive system in *Tetragonisca angustula* – what have we learnt?

A further peculiarity that makes *Tetragonisca angustula* such an attractive model organism is the utilisation of two guard sub-castes to defend the colony. The presence of both standing and hovering guards is so far unique in stingless bees and indeed within the Apidae as a whole. Our study clearly shows that possession of hovering guards, in addition to standing guards, provides *T. angustula* with a greater perimeter of defence, especially useful when dealing with the robbing cleptoparasite bee *Lestrimellita limao*. Detection of *L. limao* scouts before they can recruit a raiding party offers *T. angustula* an obvious advantage over other species that are targeted. So why do we not see two different guard sub-castes in other stingless bee species? One possibility is that *T. angustula* and *L. limao* have a long co-evolutionary history in which increased robbing pressure resulted in defensive countermeasures, in the form of a second guard sub-caste specialised in identifying the parasite by visual and olfactory means. This suggests that either robbing pressure from *L. limao* was less intense or the costs involved in evolving this specialised division of labour were too high for other

stingless bees which are targeted by *L. limao*. The next step following our findings, therefore, would be to investigate defensive strategies used by other stingless bee species targeted by *L. limao* and scrutinize further the behaviours and strategies involved in the non-mutual relationship between *T. angustula* and *L. limao*.

Figure 7.2. The standing guards can be seen on the entrance tube while the hovering guards, which can be seen, patrol the nest vicinity (photo courtesy of Christoph Grüter).

7.3. Decision making within a changeable ecological environment - future directions

Synergistic use of information sources derived from different sensory modalities has been shown to benefit decision making in a variety of animals (e.g. beetles (Otálora-luna & Dickens 2011), moths (Raguso 2005), bumble bees (Kulahci & Dornhaus 2008), birds (Able & Bingman 1987), bats (Bell 1985) & dolphins (Pack & Herman 1995)), so it is no surprise that we also see this in ants (e.g. Czaczkes *et al*. 2011, Steck *et al.* 2011), one of the most successful insect families on the planet. Rapid and efficient decision making is vitally important when both intra and interspecific competition for patchy and often ephemeral resources is intense. To cope with this, ants have evolved a range of navigational strategies that enable them to rapidly relocate and monopolise resources within an often highly heterogeneous environment (e.g. visual (Akesson & Wehner 2002, Collett *et al*. 2001), olfactory (Morgan 2009, Steck 2012), geomagnetic

(Camlitepe & Stradling 1995)). In chapters 4 and 5 I demonstrate how the ecological environment shapes which strategy an ant chooses to use and how behavioural switches are made by individual ants based upon local information. For example, by increasing the frequency of pheromone deposition as visual acuity begins to diminish, *Lasius niger* foragers are able to exploit resources nocturnally as well as diurnally. In contrast we see that *Lasius flavus,* an ant inhabiting a perpetually dark environment, maintains a high frequency of pheromone deposition, irrespective of the level of light intensity.

My findings, as is always the case in science, lead to further questions. For example, why are subterranean *L. flavus* equally as proficient, if not better than *L. niger* at forming a route memory in daylight conditions? And, does the width of a tunnel provide a thigmotactic cue to inform *L. flavus* foragers of traffic levels (an indirect measurement of route profitability)? Or, are either of these two ant species able to navigate within an illuminated arena completely bereft of visual cues? These are just some of the questions that have arisen from my navigational studies of *L. niger* and *L. flavus* and could be easily answered using carefully designed manipulative experiments, within a laboratory setting. My studies of *L. niger* and *L. flavus* have demonstrated the importance of having access to a number of available navigational strategies, which surely has to be one of the reasons that ants have grown to be so successful on the world stage.

7.4. How might the difficulties encountered in the analytical study of a trail pheromone be overcome?

Contrary to the observations of Meier (Hangartner 1969) and Hangardtner (1967) my work with *L. flavus* conclusively proves that these subterranean ants do in fact use trail

pheromones and that these indeed originate from the hindgut as in other Formicines. While I have proven that these pheromones do exist, I was unable to identify the active components in the time available. However, my journey to elucidate these active compounds has resulted in the development of a novel strategy to identify trail pheromone components and also identified a potential anti-microbial compound produced in the Dufour gland - so clearly this study could not be labelled unproductive.

While the method of extracting trail pheromones from filter paper showed excellent potential, future work could be assigned to developing it further. Two issues remain with this method which could potentially pose a problem when dealing with minute quantities of an unknown substance: firstly, the filter paper increases the noise in a sample by eluting a range of impurities into the sample and secondly, a large volume of solvent is needed to wash the filter paper. Washing the filter paper in pentane prior to trail formation reduces this noise but certainly does not remove it completely. Software such as SIEVE can selectively search for ions found in one sample but not in another, thereby reducing this problem, however, the sensitivity of this process would be greatly increased if the noise was considerably reduced. One option to achieve this would be to wash the filter paper in a polar solvent such as dichloromethane, in addition to pentane. This is likely to reduce the number of impurities further, but I would expect the noise to still remain, just at lower intensities. This method also does not address the issue of excess solvent, an important issue if the trail pheromone is particularly volatile.

This brings us back to glass which proved unsuccessful during earlier trials with *L. flavus*. Glass has the advantage of being free from impurities (when properly cleaned)

and also offers a potentially useful way of minimising the volume of solvent used for extraction. If warm solvent could be fogged onto the glass tube containing the trail pheromone, it could be collected as it condenses as a very low volume (John Pickett, pers comm). Using glass as a collection substrate failed with *L. flavus* due to the ants' reluctance to forage on the smooth surface and the rapid evaporation from the substrate. It would be useful to see if evaporation from a glass surface could be reduced by keeping the surface cool, which could be achieved by using a form of apparatus like a condenser, where water from a tap circulates within a sleeve surrounding an inner glass tube. While glass is unquestionably an ideal substrate for trail pheromone collection, working with *L. flavus* has demonstrated that there can be issues, and the continuing work to identify the trail pheromone(s) of this species may demonstrate that the filter paper method remains the most suitable strategy.

7.5. An interesting but unrelated study for the future arising from this thesis

Another promising avenue for future research arising from this PhD concerns the chemical compound, a γ-lactone, which is stored in the Dufour gland of *Lasius flavus* in large quantities. While its existence as a compound unique to ants has been known since 1970 (Bergström & Löfqvist 1970) it is only now that we can rule it out as a component that elicits trail following or alarm in nest mates. The unique structure and large volume suggests this γ-lactone plays an important role in *L. flavus's* subterranean society. A highly feasible role for this compound emerges when we consider that a number of potentially very similar γ-lactone's have been demonstrated to be very efficient antibacterial agents (Yuan *et al*. 2008, Herzner *et al*. 2013). In the dark and humid subterranean environment inhabited by hypogaeic ants one would expect there

to be a far greater pathogen pressure than that faced by their epigeic counterparts, living primarily above ground (Walker & Hughes 2011). Future work, therefore, needs to establish the exact structure of the γ-lactone produced in the Dufour gland and demonstrate its antimicrobial activity against pathogens likely to be present in the nest environment. If this compound is proven to be a potent antimicrobial agent then it raises further tantalising questions. For example, is use of the compound restricted to the eggs and brood or do ants inoculate the entire nest environment and is exactly the same lactone found in *Lasius flavus* populations across the globe or do we see variations in order to cope with different microbial strains.

7.6. Final thoughts

This thesis sought to address a number of questions arising from previous research and in doing so has given rise to a plethora of new questions to be answered by future investigations. This clearly demonstrates that there remains a huge amount still to learn about chemical communication and decision making within the social insects. Ants and stingless bees have proved to be excellent subjects for scientific examination, being highly amenable to experimental manipulations and easy to keep. This gives me great confidence that the fascinating questions arising from this thesis will be answered in the near future.

8

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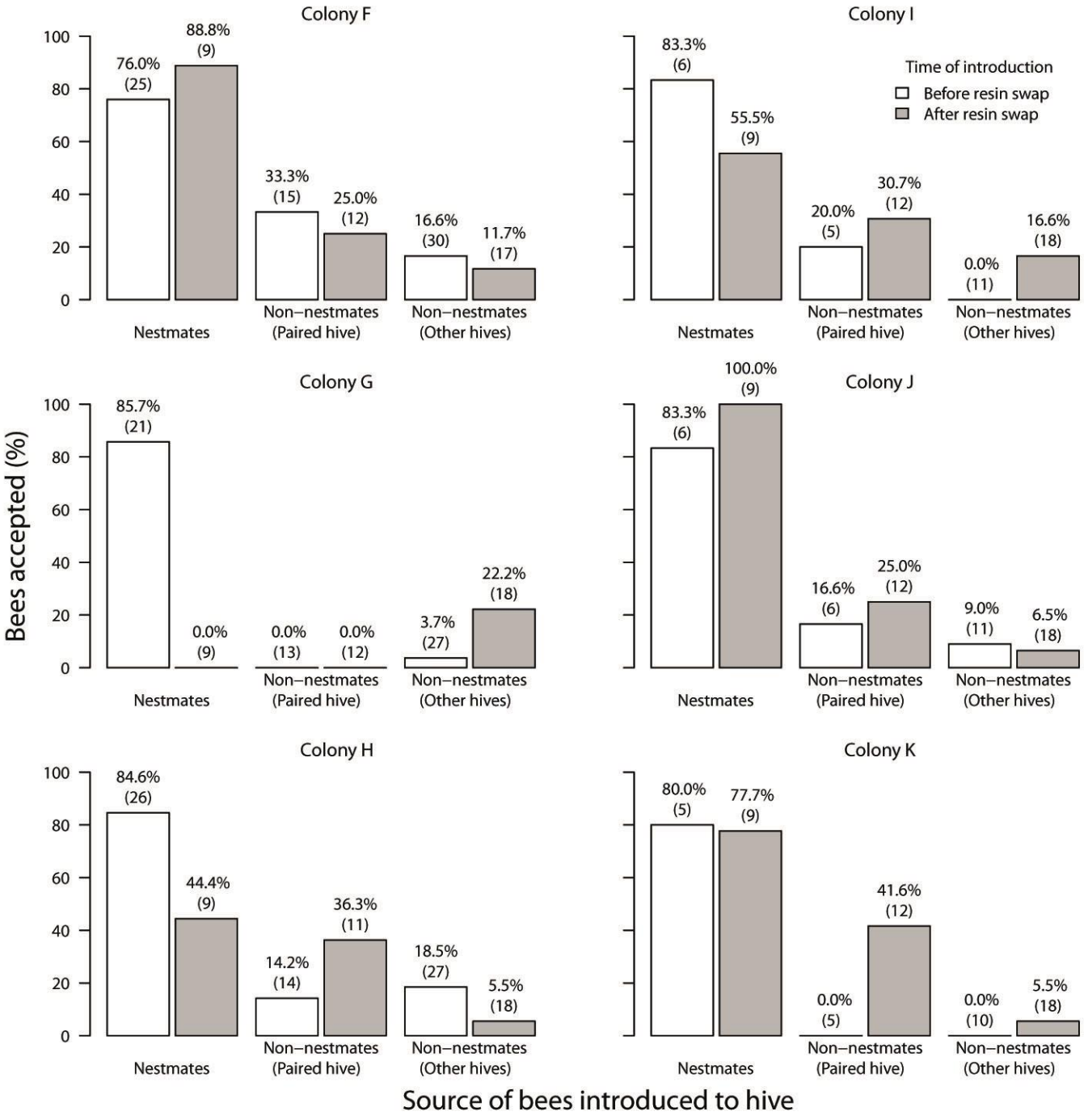
Appendices

Appendix 1 – Extra data for Chapter 3

Acceptance rates for individual hives from Experiment 4.



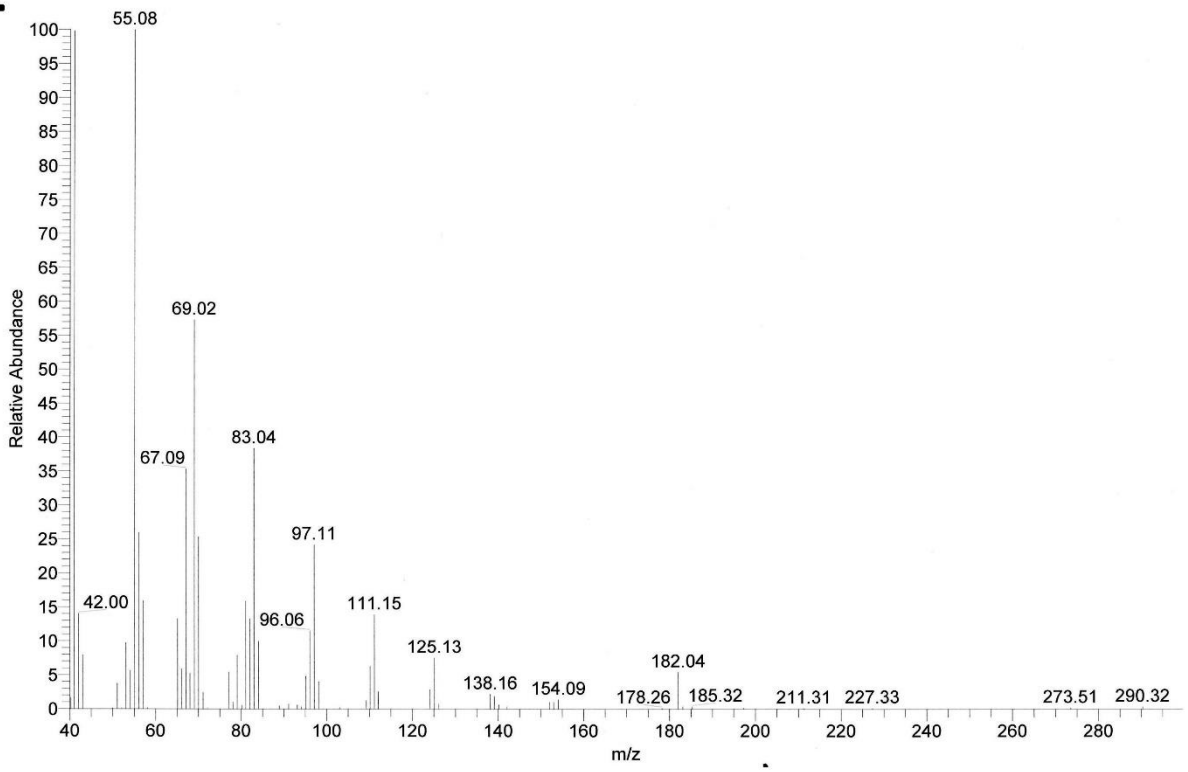
Appendix 1. Experiment 4. Acceptance rates for all 6 discriminator colonies individually. Colonies F, G and H were the resin donors and colonies I, J and K were the resin recipients. Each colony on the left was paired with the colony shown to its right. Acceptance rates are given for nestmates, non-nestmates from the paired hive and non-nestmates from other hives. Exact percentage acceptance rates are given above the bars.

Appendix 2 – Extra data for Chapter 6

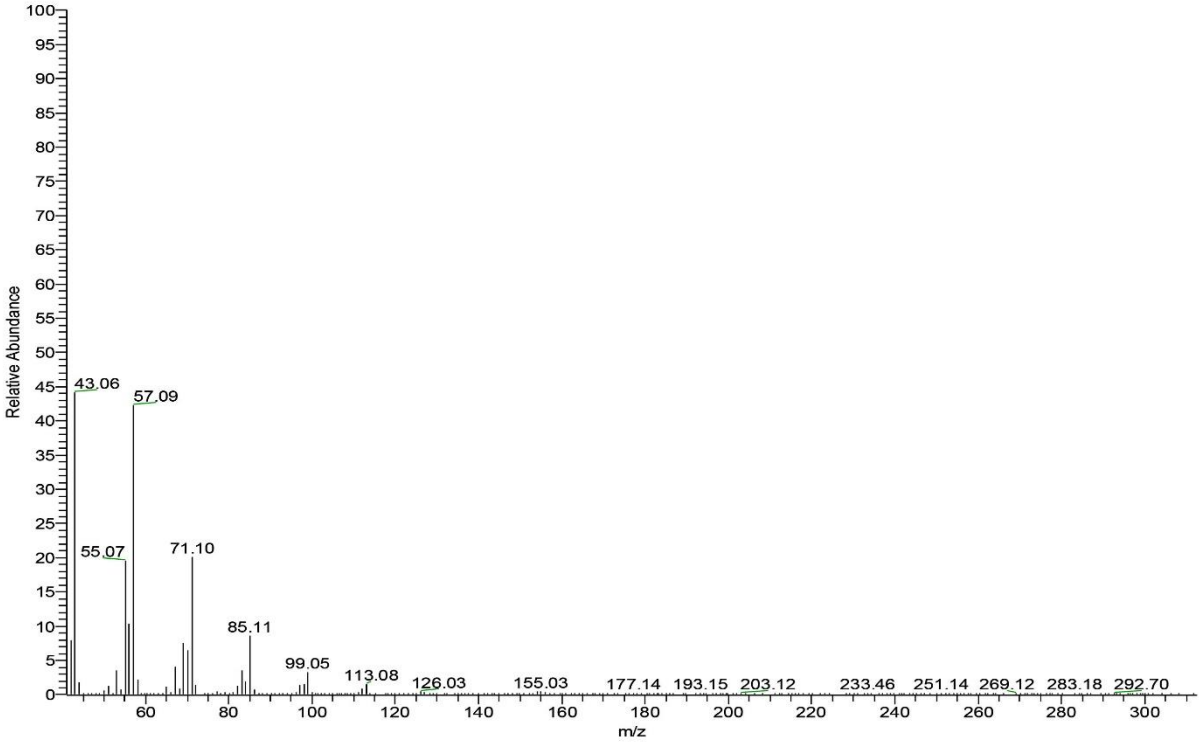
Mass Spectra from glandular extracts.

Mass Spectra for compounds found in the Dufour gland (see Fig 6.6)

D1

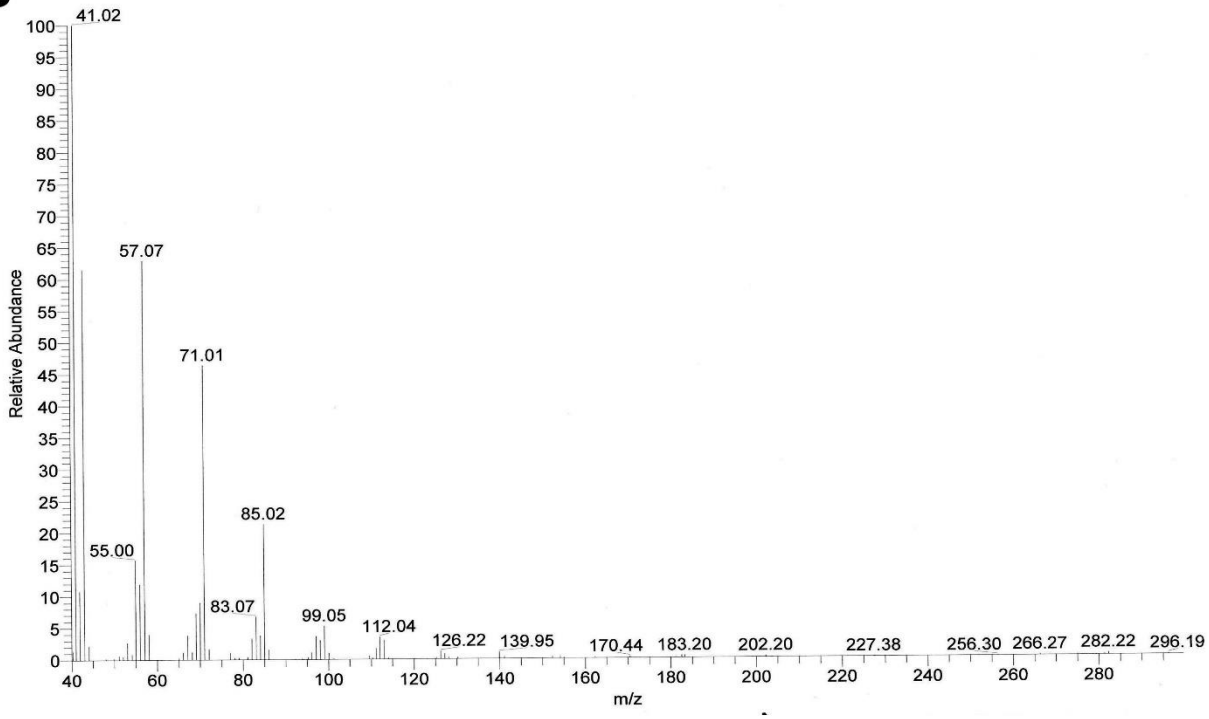


D2

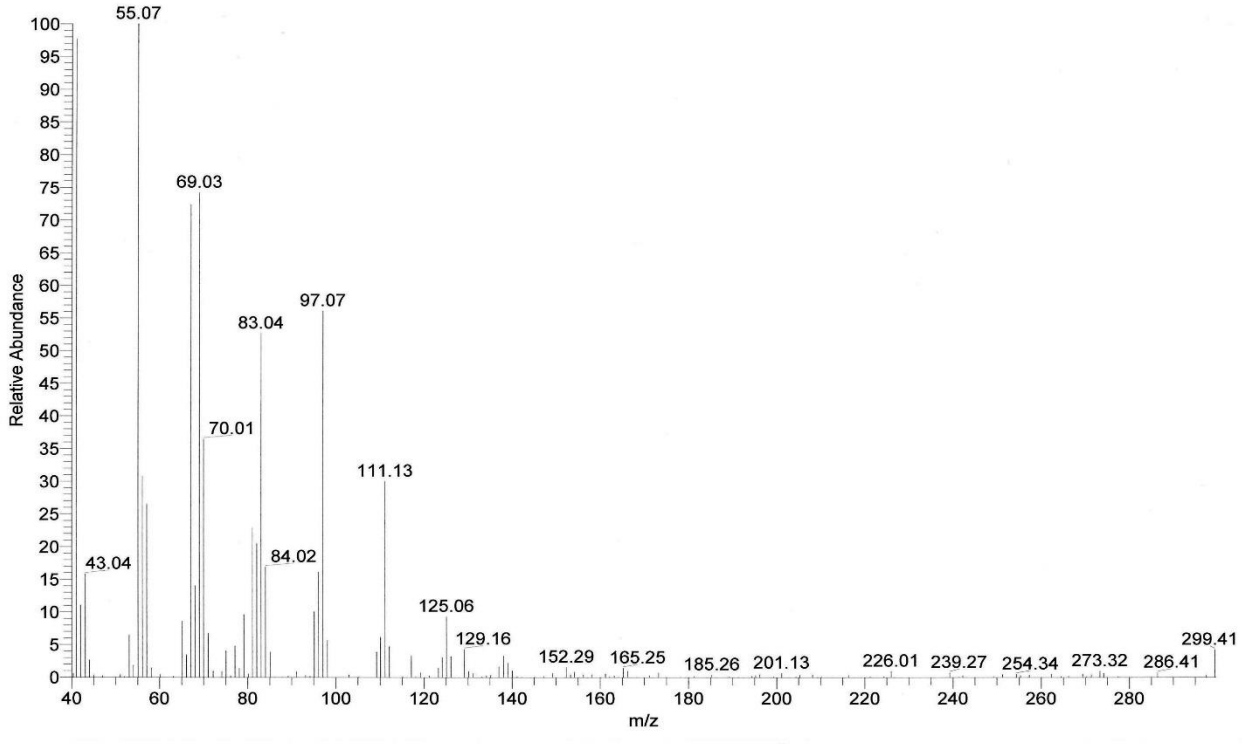


Mass Spectra for compounds found in the Dufour gland (see Fig 6.6)

D3

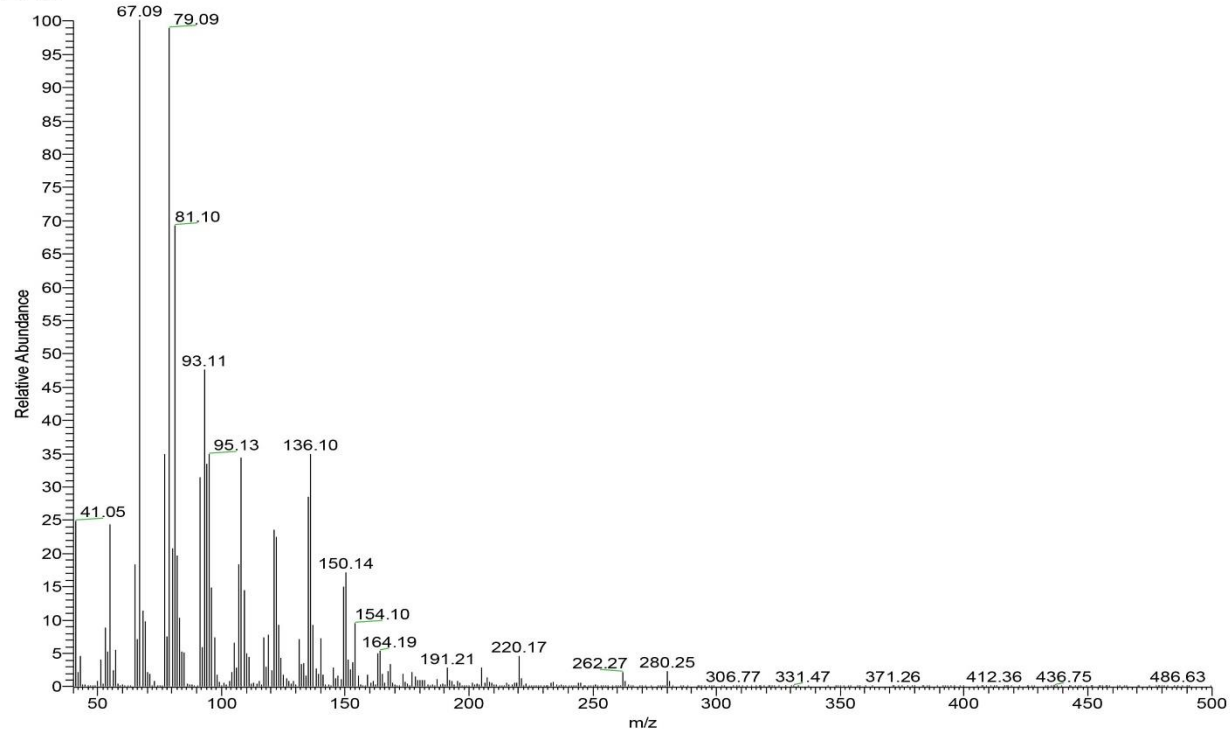


D4



Mass Spectra for compounds found in the Dufour gland (see Fig 6.6)

D5



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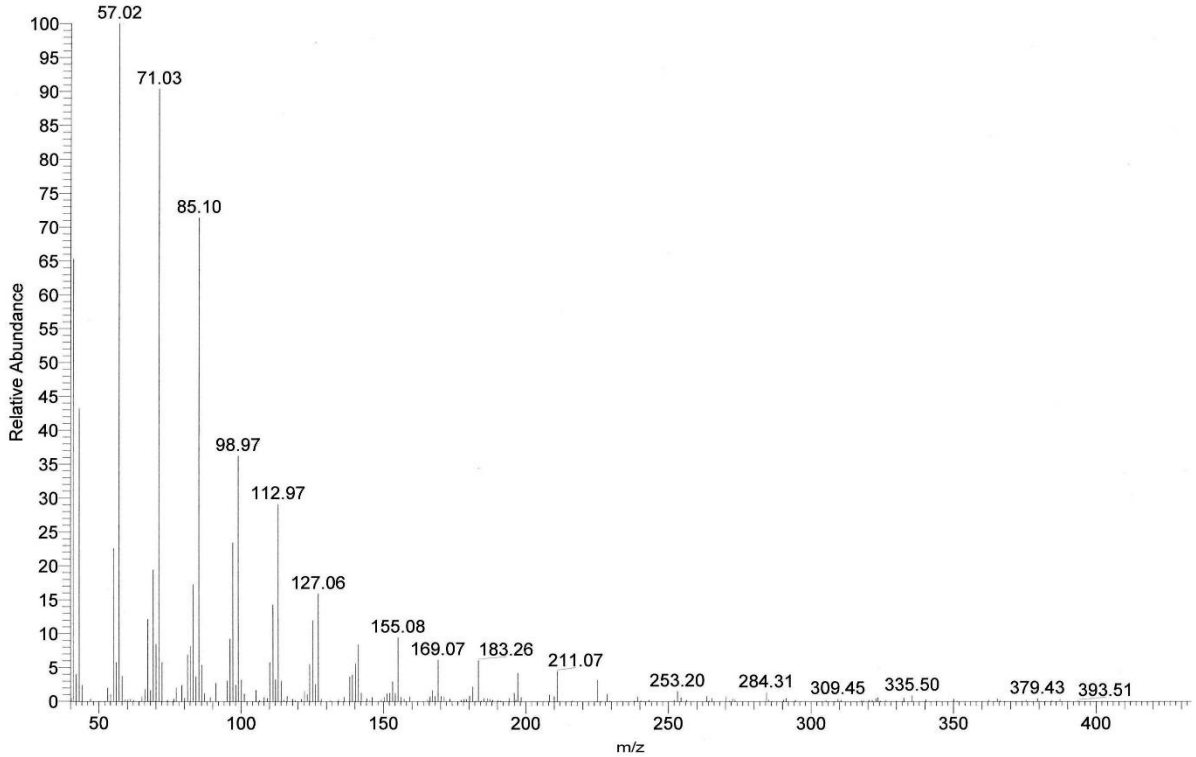
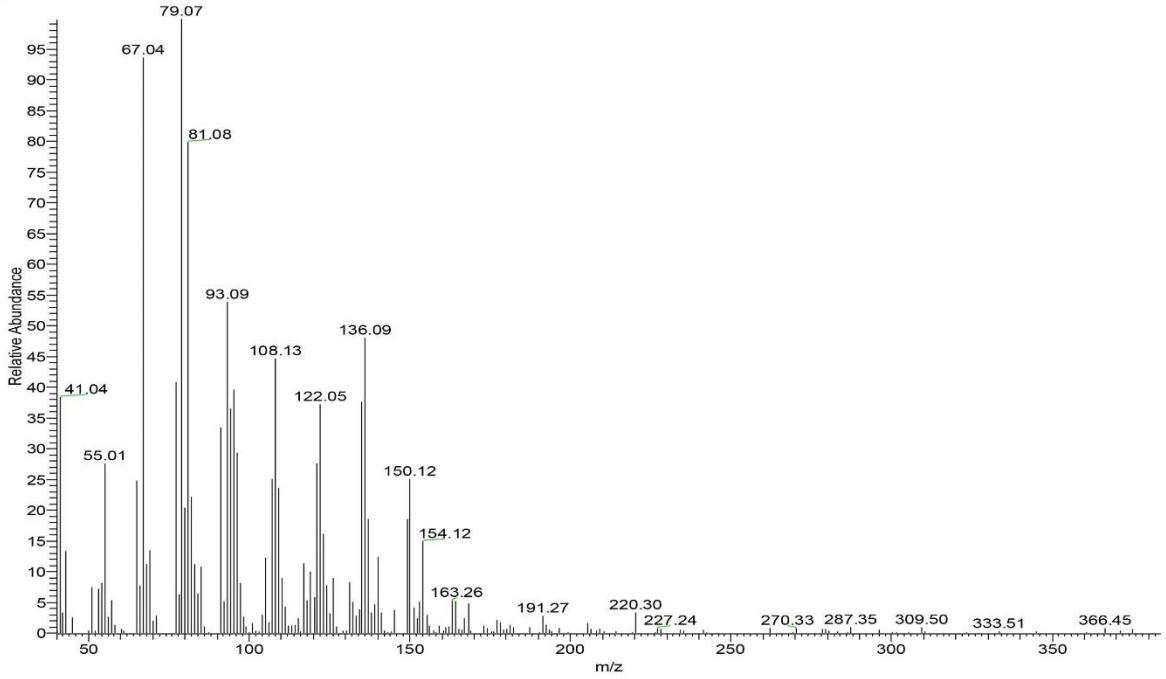
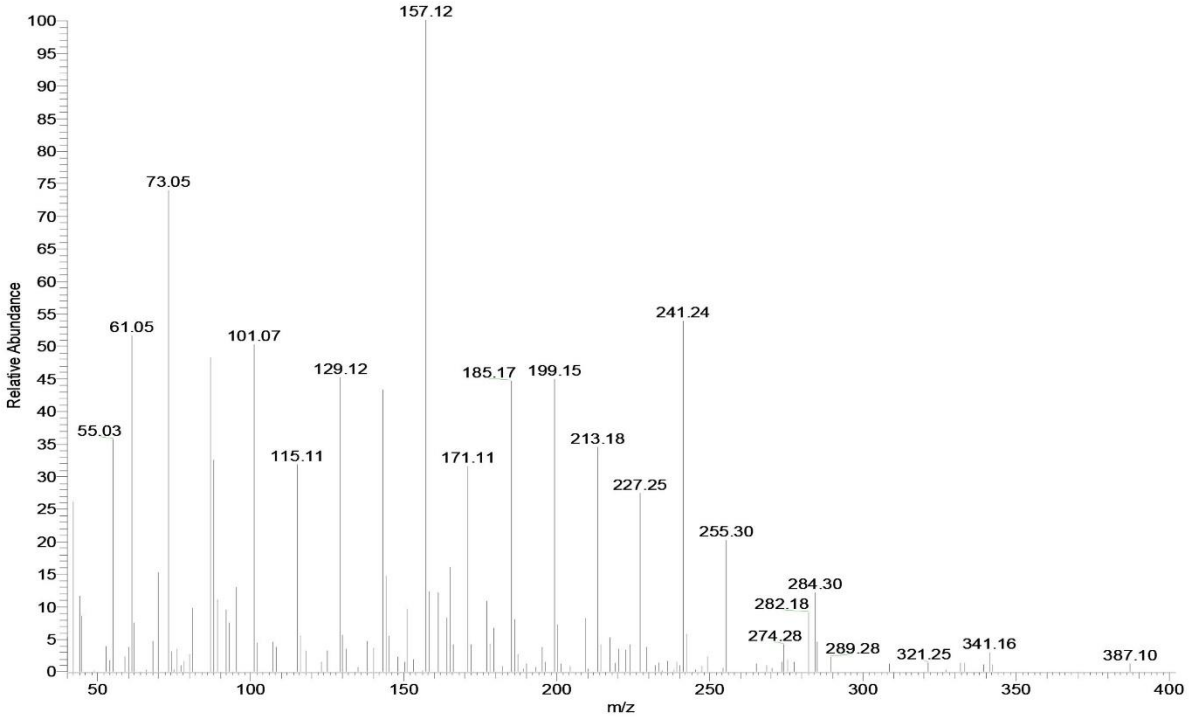


Mass Spectra for two compounds of interest found in the Hindgut (see Fig 6.8a)

H3

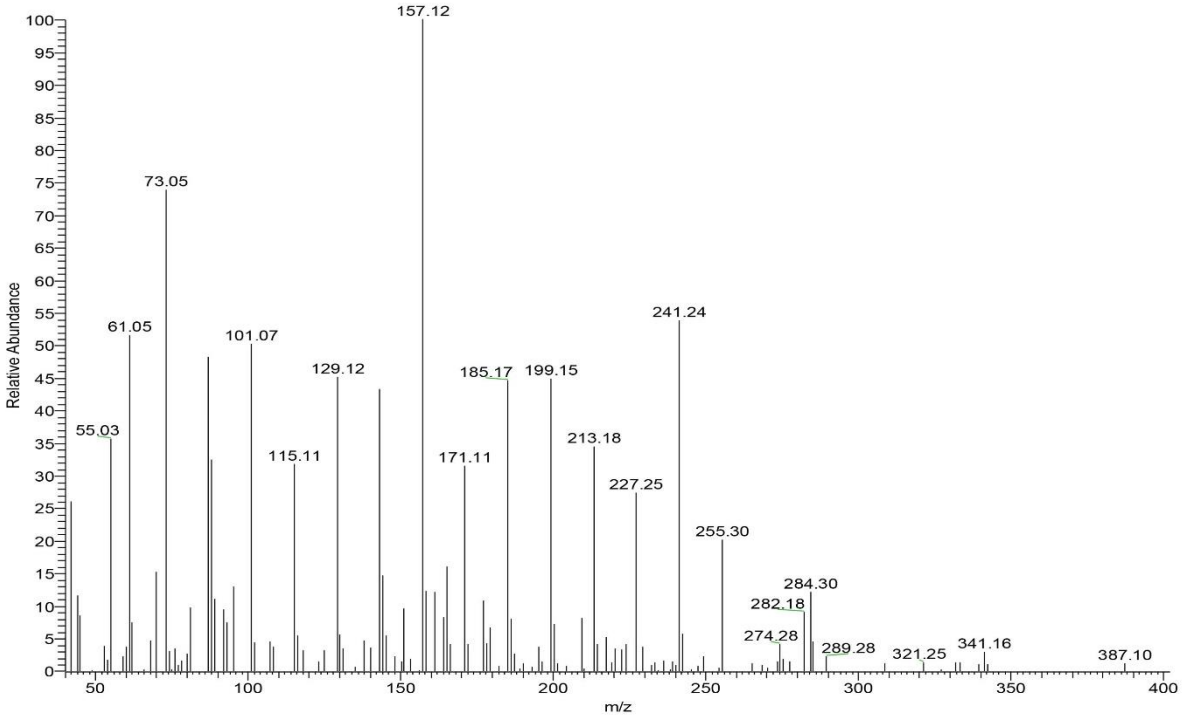


H6

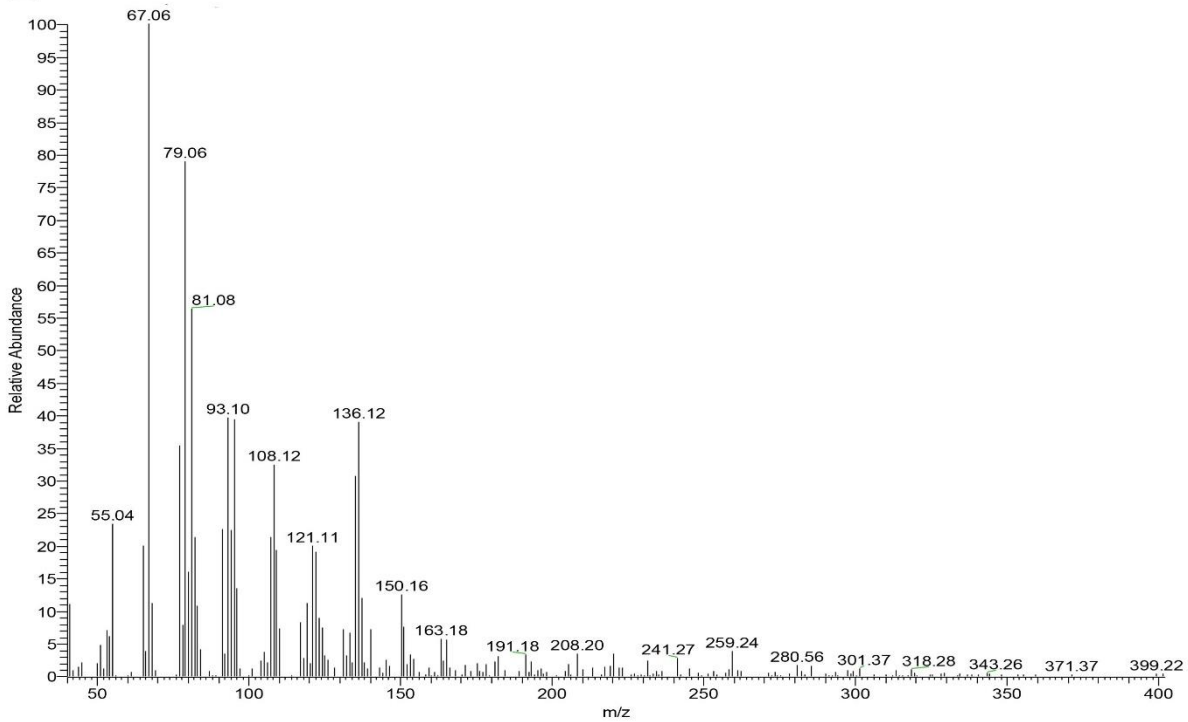


Mass Spectra for two compounds of interest found in the trail extract from filter paper (see Fig 6.8b)

P1



P2



Appendix 3 – Published Paper in Behavioral Ecology & Sociobiology

The role of wax and resin in the nestmate recognition system of a stingless bee, *Tetragonisca angustula*.

Behav Ecol Sociobiol
DOI 10.1007/s00265-011-1246-7

ORIGINAL PAPER

The role of wax and resin in the nestmate recognition system of a stingless bee, *Tetragonisca angustula*

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Abstract Recent research has shown that entrance guards of the stingless bee *Tetragonisca angustula* make less errors in distinguishing nestmates from non-nestmates than all other bee species studied to date, but how they achieve this is unknown. We performed four experiments to investigate nestmate recognition by entrance guards in *T. angustula*. We first investigated the effect of colony odours on acceptance. Nestmates that acquired odour from non-nestmate workers were 63% more likely to be rejected while the acceptance rate of non-nestmates treated with nestmate odour increased by only 7%. We further hypothesised that guards standing on the wax entrance tube might use the tube as an odour referent. However, our findings showed that there was no difference in the acceptance of non-nestmates by guards standing on their own colony's entrance tube versus the non-nestmate's entrance tube. Moreover, treatment of bees with nestmate and non-nestmate resin or wax had a negative effect on acceptance rates of up to 65%, regardless of the origin of the wax or resin. The role of resin as a source of recognition cues was further investigated by unidirectionally transferring resin stores between colonies. Acceptance rates of nestmates declined by 37% for hives that donated resin, contrasting with resin donor hives where acceptance of non-nestmates increased by 21%. Overall, our results confirm the accuracy of nestmate recognition in *T. angustula* and reject the hypothesis that this high level of accuracy is due to the use of the wax entrance tubes as a referent for colony odour. Our findings also suggest that odours directly acquired from resin serve no primary function as nestmate recognition cues.

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The lack of consistency among colonies plus the complex results of the third and fourth experiments highlight the need for further research on the role of nest materials and cuticular profiles in understanding nestmate recognition in *T. angustula*.

Keywords Jataí · Meliponini · Stingless bees · Recognition template · Nestmate recognition

Introduction

Recognition of self versus non-self is ubiquitous among organisms, operating at several different levels and involving a variety of mechanisms (e.g. Beale 1990; Janeway and Medzhitov 2002; Nasrallah 2002; Glass and Kaneko 2003). Eusocial insects demonstrate self versus non-self recognition predominantly at the colony level (but see Tibbetts 2002; d'Ettorre and Heinze 2005). In many species, the nest entrance is defended by guards who deter both allospecific and conspecific intruders (Butler and Free 1952; Bell et al. 1974; Wittmann et al. 1990). Conspecific recognition requires the matching of a set of cues carried on the cuticle of an encountered individual (the label) with a previously acquired representation of colony odour (the template) of an evaluating individual (van Zweden and d'Ettorre 2010). Depending on the degree of similarity/dissimilarity, the encountered conspecific is accepted or rejected (Lacy and Sherman 1983; Vander Meer et al. 1998). Ideally, nestmate recognition should categorise all incoming individuals without error (Sherman et al. 1997), but mistakes are made: nestmates may be rejected (rejection errors) or non-nestmates admitted (acceptance errors). Which of these two errors is minimised can vary adaptively via adjustment of the acceptance threshold. For example, increased rejection errors may be traded off for decreased acceptance errors when the frequency of intruders or the cost of admitting them is higher (Reeve 1989; Downs and Ratnieks 2000; Couvillon et al. 2009).

In the honeybee, *Apis mellifera*, the number of entrance guards and the permissiveness of the acceptance threshold change adaptively, depending on nectar availability and robbing intensity (Downs and Ratnieks 2000; Couvillon et al. 2008). Overall, the recognition error rates are surprisingly high, with means of approximately 23% (range 8–48%) for rejection errors and 29% (range 21–62%) for conspecific acceptance errors (Breed 1983; Downs and Ratnieks 1999, 2000; Couvillon et al. 2007a, 2008, 2009, 2010). This gives a total error of approximately 52%, almost exactly midway between the two extremes of perfect (0%) and zero information (100%) (Ratnieks 1991). This is in stark contrast to recent results for the Neotropical stingless bee, *Tetragonisca angustula*. Guards of *T. angustula* made few errors in discriminating nestmate workers from non-nestmate conspecifics, accepting all nestmate workers (0% rejection errors) while rejecting 92% of conspecific non-nestmate workers, giving a total error of only 8% (Kärcher and Ratnieks 2009). This is also considerably lower than the error rates reported for five other Neotropical stingless bees (Table 1).

This raises the question on what the underlying mechanisms are that allow *T. angustula* to have lower recognition error rates than honeybees or other stingless bees. One obvious difference between *T. angustula* and the six other bee species is that *T. angustula* is the only one that constructs a wax entrance tubes for its nest. Nests of *Frieseomelitta varia* have a round entrance hole surrounded by resin, nests of *Trigona fulviventris* have wide, sometimes tubular, resin openings, while those of the three *Melipona* species, *Melipona quadrifasciata*, *Melipona rufiventris* and *Melipona scutellaris*, all possess a small entrance hole surrounded by dry mud (Roubik 2006; Couvillon et al. 2007b; M.J. Couvillon, personal communication; S.M. Jones, personal observation). Wax is important in honey bee recognition, functioning as the primary source of colony odour cues and a wax entrance tube might provide guards with a more direct template with which to compare incoming bees (Breed et al. 2004; Couvillon et al. 2007a). This might allow guards to update their template more frequently to allow peripheral sensory detection via desensitisation (c.f. Ozaki et al. 2005) or to simply enable a direct comparison.

A further difference between *T. angustula* and *A. mellifera* is the former's greater use of plant resins. Leonhardt et al. (2009) recently demonstrated that terpenoid profiles, derived from resin, extracted from the cuticles of seven Paleotropical stingless bee species varied quantitatively between colonies of the same species, leading them to suggest that this may potentially serve some communicative function in these stingless bee species. This is entirely feasible given that conspecific recognition may rely on quantitative differences within the same set of compounds (vander Meer et al. 1989; Espelie et al. 1990; Martin et al. 2008; van Zweden and d'Ettorre 2010). Nests of *T. angustula* contain substantial amounts of resin stored in numerous piles throughout the nest. Under a microscope (magnification × 240), resin can also be seen in a layer on the legs, head and thorax of foragers (J.S. van Zweden, unpublished data) and is also mixed with wax to form cerumen, which is used to construct the combs and surrounding involucrum (Nogueira-Neto 1997; S.M. Jones, personal observation; Wille 1983). Thus, the ubiquitous presence of resin within the nest, either in its pure form as piles or as cerumen, should be sufficient for acquisition of a colony-encompassing odour profile. Indeed this would in many ways be analogous to the ubiquitous presence of wax in the combs of honeybees, although wax is secreted by the

Table 1 Error rates for *T. angustula* and six other bee species

Bee species	Rejection error rate (%)	Acceptance error rate (%)	Total error rate (%)	Reference(s)
Apis mellifera	33	31	64	Breed 1983
	26	18–30	44–56	Couvillon et al. 2007a
	26–48	30–59	56–107	Couvillon et al. 2008
	19–24	57–62	76–86	Couvillon et al. 2009
	8	30	38	Couvillon et al. 2010
	18	21	39	Downs and Ratnieks 1999
	17	22	39	Downs and Ratnieks 2000
Frieseomelitta varia	11	27	38	Couvillon and Ratnieks 2008
Melipona quadrifasciata	0	26	26	Breed and Page 1991
Melipona rufiventris	0	86	86	Breed and Page 1991
Melipona scutellaris	0	40	40	Breed and Page 1991
Tetragonisca angustula	0	8	8	Kärcher and Ratnieks 2009
Trigona fulviventris	24	24	48	Buchwald and Breed 2005

bees while resin is collected (Breed et al. 1995; d'Ettorre et al. 2006).

The aim of this study was to investigate conspecific recognition in *T. angustula*, with emphasis on the effects of odours derived from wax entrance tubes, plant resins and worker bees. This was achieved by investigating whether the acceptance of introduced nestmates and non-nestmates by guards standing on the entrance tube was influenced by: (1) the acquisition of cuticular odours derived from nestmates and non-nestmates onto the cuticle, (2) swapping wax entrance tubes between colonies, (3) the acquisition of resin and wax derived from nestmates and non-nestmates onto the cuticle and (4) the unidirectional swap of entire resin stores between hives.

Methods

Study site and organism

Data were collected in February 2009 (experiments 1 and 2) and 2010 (experiments 3 and 4) at Fazenda Aretuzina, São Simão, São Paulo State, Brazil. During both study periods, the weather was hot, with daytime high temperatures of ca. 24–32°C and periodic heavy rain. Data were only collected on non-rainy days during active foraging (between 9.00 and 17.00 hours).

T. angustula, local name Jataí, is unique among the stingless bees in possessing two types of guards: both hovering and standing (van Zweden et al. 2011). Hovering guards flank the flight path leading to the nest and readily attack allospecific bees approaching the nest vicinity (Wittmann 1985; Grüter et al. 2011), thus increasing the defensive perimeter of the nest (van Zweden et al. 2011). While hovering guards are efficient at detecting individuals visually dissimilar to themselves, it is the role of the standing guards, which stand around the opening on the tip of the entrance tube, to distinguish non-nestmate conspecifics from nestmates, which they do by contact chemoreception (Kärcher and Ratnieks 2009). The two types of guards complement one another and increase the defensive efficiency of the colony.

We studied five colonies of *T. angustula* in 2009 (experiments 1 and 2) and six in 2010 (experiments 3 and 4). Each colony was housed in a wooden hive box (ca. 50-cm high× 20 cm×30 cm), with a circular entrance hole of 1.8 cm in diameter (for more details, see Nogueira-Neto (1997)). Each colony had built a wax entrance tube from this hole. Entrance tubes were ca. 1–3 cm long and had a circular opening at the tip ca. 0.6 cm in diameter (see also figures in Wittmann (1985); Couvillon et al. 2007b; for more detail, see Grüter et al. (2011)). The entrance tubes on the study colonies appeared identical to those of unmanaged colonies nesting in walls. Hives were raised ca. 1 m aboveground on hive stands or attached to the walls of buildings. The study colonies were queenright and thriving, with the hive nearly full of combs, covered by involucrum and numerous honey pots with a population of many thousands of workers. Mature colonies of *T. angustula* in Costa Rica were estimated to have approximately 10,000 workers (van Veen and Sommeijer 2000).

Introduction of worker bees to guards and their acceptance or rejection

The acceptance or rejection of conspecific workers by guards standing on the entrance tube was determined using a standard bioassay (Downs and Ratnieks 2000) developed for studying honey bees, *A. mellifera*, and modified for use with *T. angustula* (Kärcher and Ratnieks 2009). Returning

foragers were collected at the hive entrance, placed in a tube and immediately chilled in an ice chest for 10–20 min, then removed one at a time and allowed to warm to ambient temperature. Once warmed, these workers walked actively but were less likely to fly than previously unchilled workers. A worker was taken from the ice chest and, once warmed up, allowed to grasp a clean wooden toothpick and walk onto the outer surface of the tip of the entrance tube of a discriminator hive. On contact with the guards standing on the entrance tube behaviour was observed for up to 2 min. The introduced worker was considered "rejected" if it was bitten and tugged for the duration of the observation period or fell off the tube while grappling with a guard (Kärcher and Ratnieks 2009). The worker was considered "accepted'" if she was subjected only to licking and antennal contact or bitten and tugged for a few seconds and then left alone. Each time four bees were introduced in a row to a particular discriminator hive pseudorandomly with approximately 5 min between each introduction. Once the four bees had been introduced, the same protocol was repeated at the next discriminator hive, ensuring that a minimum of 45 min had passed before returning to a particular discriminator hive. The observer was unaware of the treatment group of the introduced workers. The number of standing guards present on the entrance tube was recorded before introductions commenced (mean ± SD=15.43±4.38).

Experiment 1: the effect of bee-derived odours on acceptance rates of worker bees

The aim of this experiment was to determine how the transfer of nestmate and non-nestmate odours onto worker bees affected the acceptance of both nestmates and non-nestmates.

Four hives (A–D) were used both as discriminator and donor colonies. These were grouped into two pairs (A and B, C and D) to serve as donors and discriminators to each other (Fig. 1a). A fifth hive (E) was used as an additional source of non-nestmates. Worker bees (n=20±3) were collected at hive entrances and placed in a 6-ml clear plastic vial for 60 min to transfer odours to the vial at two vials per hive per study day. The bees were then released. Odours deposited on the inside of the tubes by these bees were then indirectly transferred to returning foragers by placing 12 individuals into a prepared vial for 15 min. Each vial was used only once. Fresh vials were prepared on each study day and used within 4 h.

The acceptance rate of the following seven treatments of workers were compared (Fig. 1a): (1) nestmates, untreated; (2) nestmates, treated with nestmate odour using the vial; (3) nestmates, treated with non-nestmate odour from the paired hive using the vial; (4) non-nestmates from the paired hive, untreated; (5) non-nestmates from hive E, untreated; (6) non-nestmates, treated with non-nestmate odour from the paired hive; and (7) non-nestmates, treated with nestmate odour.

Combined sample sizes for each of the seven treatments ranged between 99 and 111, with similar numbers introduced to each of the four discriminator hives.

Experiment 2: is the wax entrance tube used as a referent?

The aim of this experiment was to determine whether *T. angustula* guards use the wax entrance tube as a template or referent for colony odour. To achieve this, we swapped entrance tubes between paired colonies using the same pairings as in experiment 1. The entrance tube was gently cut away from the hive entrance hole using a penknife. By using the natural stickiness of the wax, the entrance tube could be attached to the end of a 1.5-cm-long plastic tube that exactly fitted into the hive entrance hole. The plastic tube was then placed into the hive's entrance hole and the colony was given 1–3 days for the entrance tube to attach firmly to the plastic tube using additional wax. Entrance tubes could then be swapped between hives in minutes, without physical damage and with minimal disturbance. Following tube swapping, guards appeared to behave normally on the new entrance tube.

The experimental design was the same as that used for experiment 1, with the exception that acceptance rates were determined only for the following treatments: (1) nestmates, (2) paired hive non-nestmates and (3) hive E non-nestmates. These were compared before and 1, 5 and 24 h after the swap (Fig. 1a).

Combined sample sizes ranged between 101 and 120 introduced bees for each of the three treatments both before and after the tube swap. Similar numbers were introduced to each of the four discriminator hives for each treatment.

Experiment 3: transfer of wax and resin odours to workers

The aim of this experiment was to determine if resin- or wax-derived recognition cues were utilised by *T. angustula* for the purpose of nestmate recognition. Six hives (F–K) were used: three discriminator colonies and three donor colonies for wax, resin and non-nestmates (Fig. 1b). Each discriminator colony thus received workers from all of the donor colonies in addition to their own nestmates. This change to the design from that used in experiments 1 and 2 diminishes any possible colony-specific effects as these can be dealt with statistically. Each discriminator hive received bees from its own hive and each of the three donor hives (i.e. a full factorial design) were treated as follows: (1) nestmates untreated, (2) nestmates treated with nestmate resin/wax, (3) nestmates treated with non-nestmate resin/wax, (4) non-

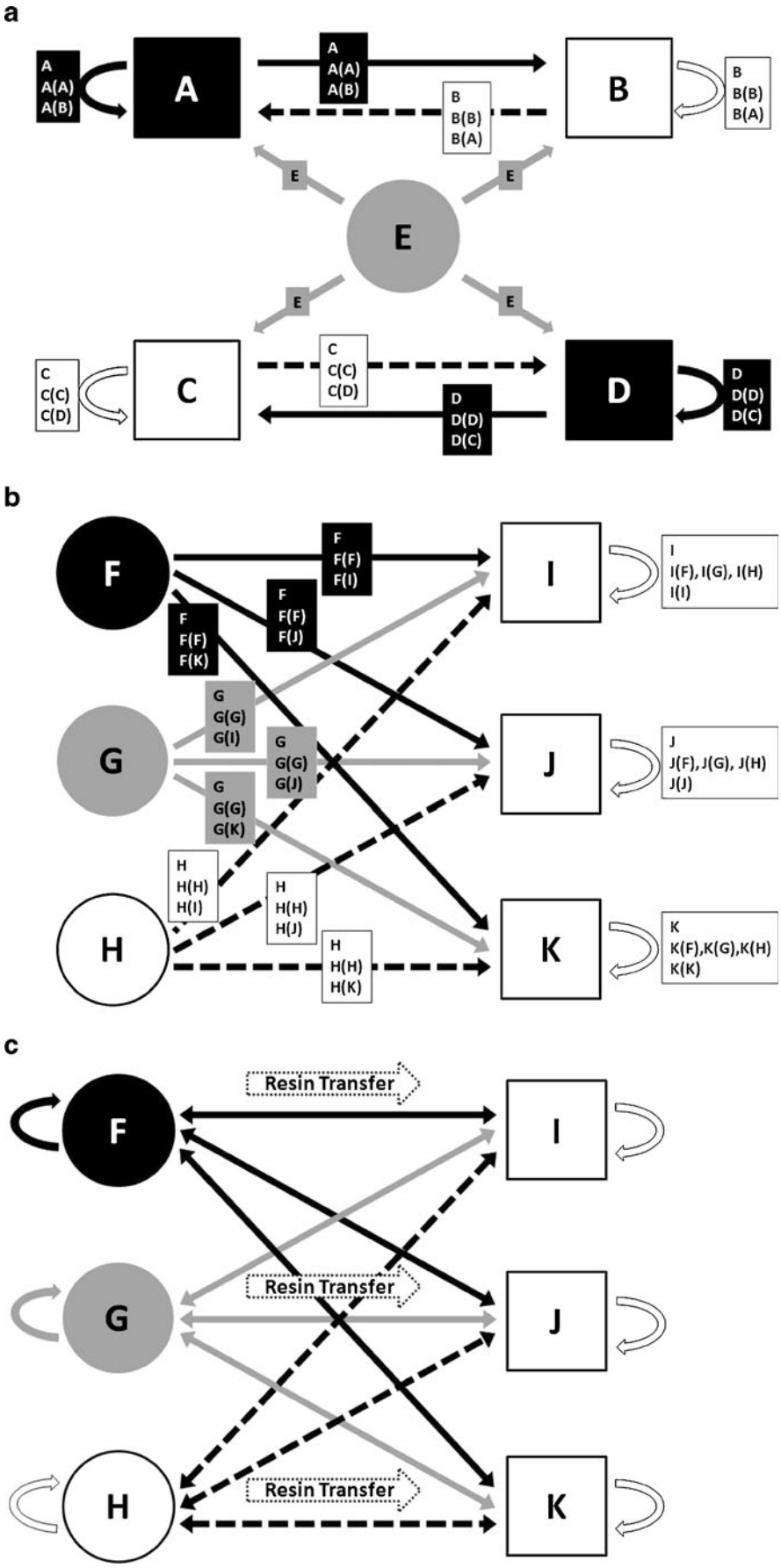

Fig. 1 **a** Experimental design of experiments 1 and 2. Treated and untreated worker bees were introduced to the entrance tubes of each discriminator hive. There were two pairs of discriminator hives (one black and one white). Hive E served as an additional source of non-nestmates, common to all four colonies. In the *boxes on the arrow*, *unbracketed capital letters* refer to the colony the introduced bee is from and the *bracketed letters* refer to the colony that the odour treatment, if any, originates from. **b** Experimental design of experiment 3. Treated and untreated worker bees were introduced to the three discriminator hives, *I*, *J* and *K*, from the donor hives, *F*, *G* and *H*. **c** Experimental design of experiment 4. Untreated worker bees were introduced to the entrance tubes of six discriminator hives *F–K*. Hives *F–H* acted as "resin donors" and hives *I–K* as "resin recipients". Resin was swapped unidirectionally from the donor hives to the recipient hives. Worker bees were subsequently introduced to all hives 24 h afterwards for a period of 7 days

nestmates untreated, (5) Non-nestmates treated with nestmate resin/wax and (6) non-nestmates treated with non-nestmate resin/wax. Combined sample sizes per treatment ranged between 35 and 102 introduced bees with similar numbers introduced to the three discriminator hives.

Resin was collected from resin piles within each colony and white wax was collected from newly constructed entrance tubes. We are confident that the resin we collected from the piles contained little or no wax because its dark colour and viscous consistency was identical to the resin carried in the corbiculae of returning foragers. Moreover, Gastauer et al. (2011) observed no mixing of wax or other substances with the resin collected by worker bees of seven Neotropical stingless bee species, including *T. angustula*. Each 4-ml glass vial was treated with 0.5 ml of hexane containing 2.5 mg of either wax or resin. Evaporation left a thin, barely visible coating within each vial. Up to four workers were transferred to a treated vial and left for at least 15 min to allow indirect transfer. The bees were then chilled and introduced individually to the entrance tube of a discriminator colony as in experiment 1. Each vial was used up to three times to treat a maximum of ten bees.

Experiment 4: one-way transfer of resin between hives

The aim of this experiment was to investigate the effect on the nestmate recognition label and/or template of unidirectional transfer of resin between *T. angustula* hives. Hive inspections showed that all the *T. angustula* nests had resin piles of varying sizes, all dark brown in colour, which were distributed throughout the nest. The mean weight of the entire resin reserves for the six colonies was 7.79±2.01 g (mean±1 s.e., range=2.04–16.05 g).

Entire resin stores were removed from a donor hive, weighed and distributed as new piles within a receiving hive that had been cleared of existing resin piles the day before. Six colonies were used (F–K), paired up as three groups containing a ‘resin donor’ and ‘resin acceptor’ (F and I, G and J, H and K; Fig. 1c). Bees were introduced to all hives prior to and following the swap. Each donor hive received nestmates and non-nestmates from each of the three resin acceptor hives and vice versa for the receiving hives. Depending on the treatment, combined sample sizes ranged between 17 and 126 introduced bees. Introductions were undertaken at four different time periods: between 12 and 96 h before the resin transfer (control) and then at 12, 60 and 84 h after.

Statistical analyses

For data analysis, we used generalised linear mixed-effect models (GLMM) with binomial errors in R 2.9 (R Development Core Team 2009). We fitted the models using the lmer function (Bates 2007). Colony was included as a random effect throughout to control for the non-independence of data points from the same colony (Bolker et al. 2009; Zuur et al. 2009). For model selection, we used the protocol proposed by Zuur et al. (2009). We first explored the optimal structure of the random components by comparing random intercept models with random intercept and slope models. Then, we explored the optimal fixed component structure. Wald tests were used to determine the significance of the fixed effects (Bolker et al. 2009).

For all cases, the dependent variable was the response of the guards (accept or reject). The random variable was “discriminator colony” in all experiments. Fixed variables were “treatment” in experiment 1, “time (time following entrance tube swap)” in experiment 2, “treatment” and “origin” of bee (nestmate or non-nestmate) for experiment 3 and “treatment”, “time” (before or after swap) and “origin” of bee for experiment 4.

Results

Experiment 1: the effect of odour transfer on acceptance rates of worker bees

Guards standing on the entrance tubes made few recognition errors with untreated introduced bees, accepting significantly more nestmates than non-nestmates as expected (Fig. 2; 94.6% vs. 4.5%, GLMM, Wald’s $z=-14.15$, $p=<0.001$). The strongest effect of odour treatment came from treating nestmates with non-nestmate odour, which resulted in an acceptance 54.3% lower than for nestmates treated with nestmate odour (31.3% vs. 85.6%, $z=-7.41$, $p=<0.001$). Conversely, the acceptance rate of non-nestmates was not significantly affected by treatment; only 6.8% more non-nestmates were accepted when treated with nestmate odour than when treated with non-nestmate odour (9.6% vs. 2.8%, $z=1.92$, $p=0.054$). A small but significant effect of the vial treatment itself could be seen on acceptance rates of bees treated with nestmate odour (94.6% vs. 85.6%, $z=-2.03$, $p=0.04$).

Experiment 2: is the wax entrance tube used as a referent?

Swapping entrance tubes did not affect the acceptance of either nestmates or non-nestmates (Fig. 2). There was no significant difference between the acceptance rates of nestmates for the four different time periods individually (0 h vs. 1 h: $z=-0.46$, $p=0.65$; 0 h vs. 5 h: $z=-1.83$, $p=0.067$; 0 h vs. 24 h: $z=0.14$, $p=0.91$) and combined (0 h vs. 1/5/24 h: 94.6% vs. 90.8%, $z=-1.05$, $p=0.29$). Similarly, there was no significant difference among the acceptance

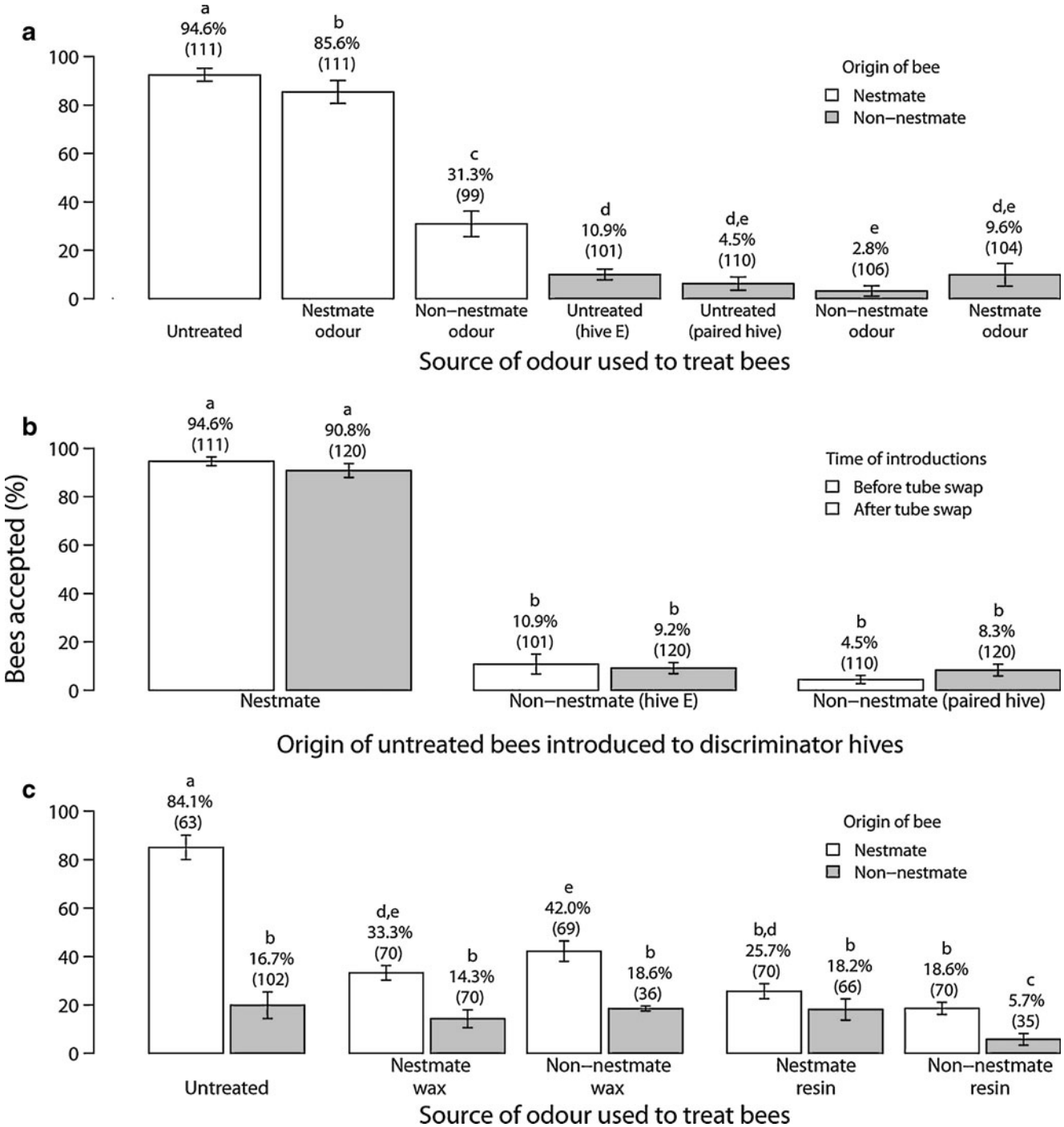

Fig. 2 Acceptance rates of introduced bees in experiments 1, 2 and 3. **a** Experiment 1: Treated and untreated worker bees were introduced to four discriminator hives, A, B, C and D. Non-nestmates introduced to discriminator hives originated from the paired hive and a fifth colony, colony E (untreated only), which served as a control. **b** Experiment 2: Nestmate workers and non-nestmate workers originating from both the paired hive and hive E (control) were introduced to four discriminator hives, both before and after swapping the wax entrance tubes. **c** Experiment 3: Nestmates and non-nestmate workers, either untreated or treated, were introduced to three discriminator hives. Treated bees bore wax- or resin-derived odours from either their own hive or a foreign hive. *Different letters* denote significant differences. Exact percentage acceptance rates and sample sizes are given above the *bars*

rates of paired hive non-nestmates for the different time periods, both individually (0 h vs. 1 h: z=0.79, p=0.43; 0 h vs. 5 h: z=0.79, p=0.43; 0 h vs. 24 h: z=1.32, p=0.19) and combined (0 h vs. 1/5/24 h: 4.5% vs. 8.3%, z=1.27, p=0.21). As expected, there was also no change in the acceptance of non-nestmates from hive E post-swap (0 h vs. 1/5/24 h: 10.9% vs. 9.2%, z=1.26, p=0.21). In addition, there is no indication that tube swapping affected the

acceptance of non-nestmates from the paired colony any differently than non-nestmates from the control, hive E, with no significant interaction between treatment and tube swapping (pre-swap vs. post-swap; z=1.27, p=0.21).

Experiment 3: transfer of wax and resin odours to workers

Treatment of nestmate bees with either wax or resin lowered their acceptance rates significantly to that of non-nestmates, irrespective of whether the wax/resin originated from a nestmate or non-nestmate hive (Fig. 2; 84.1% vs. 33.3%, z=4.78, p=<0.001; 84.1% vs. 42.0%, z=4.71, p=<0.001; 84.1% vs. 25.7%, z=5.27, p=<0.001; 84.1% vs. 18.6%, z=6.81, p=<0.001). Acceptance of non-nestmates remained low regardless of treatment (16.7% vs. 14.3%, z=0.41, p=0.67; 16.7% vs. 18.6%, z=−0.33, p=0.73; 16.7% vs. 18.2%, z=−0.26, p=0.79; 16.7% vs. 5.7%, z=2.05, p=0.040). Interestingly, there were no pronounced differences between resin and wax sourced from nestmate and non-nestmate hives. The acceptance rates of nestmates treated with nestmate or non-nestmate wax did not differ significantly (33.3% vs. 42.0%, z=−0.86, p=0.39), reflecting what we found for non-nestmates with the same treatments (14.3% vs. 18.6%, z=−0.68, p=0.49). Similarly, the acceptance rates of nestmates treated with nestmate or non-nestmate resin did not differ significantly (18.6% vs. 25.7%, z=0.84, p=0.39). However, non-nestmates treated with non-nestmate resin were rejected to a greater extent than nestmates treated with non-nestmate resin (5.7% vs. 18.2%, z=2.14, p=0.03).

Experiment 4: one-way transfer of resin between hives

After the unidirectional transfer of resin, the acceptance rate of nestmates dropped by 37.5% for resin donor hives (from 81.9% to 44.4%, z=−3.51, p=<0.001; Fig. 3), while only a decline of 4.5% was seen for resin recipient hives (from 82.3% to 77.8%, z=−0.36, p=0.71). Conversely, for non-nestmates, a non-significant increase of 1.6% in acceptance rates was seen in donor hives (from 14.3% to 15.9%, z=0.35, p=0.72), while a significant rise of 21.6% was observed for recipient hives (from 6.2% to 27.8%, z=2.75, p=0.005). This effect was independent of the resin source, that is, acceptance rates did not differ between non-nestmates from the paired hive and non-nestmates from other hives (z=−1.03, p=0.30). The overall trends were somewhat inconsistent amongst the hives with notable variation apparent (see "Electronic supplementary material").

Discussion

The results of the first experiment involving transferral of bee-derived odours confirm the exceptional recognition abilities of *T. angustula* compared to other studied species, with combined recognition errors of 10%. Acceptance rates of nestmates treated with non-nestmate odour were greatly and significantly reduced, while the small positive effect on acceptance from treating non-nestmates with nestmate odour was non-significant. Together with recent studies on the honeybee (Ratnieks et al. 2011), the stingless bee *F. varia* (Couvillon and Ratnieks 2008) and *Camponotus* ants (Guerrieri et al. 2009), these results support the idea that the odour space of a particular colony odour utilised in nestmate recognition is complex and multidimensional. In particular, a multidimensional odour space helps explain why it is far easier for a nestmate odour to diverge from the colony odour rather than a non-nestmate odour to converge on the colony odour of the guards (Ratnieks et al. 2011). In this framework, the average chemical distance that an individual is moved following odour transfer is enough to make a nestmate unacceptable but not enough to make a non-nestmate acceptable even though both changes are equal in chemical distance. Nestmate odours are chemically similar and therefore fall within the overall colony odour profile. Any deviation therefore is easily recognised; however, non-nestmate odours are sufficiently distant in chemical space that the same deviation, even towards the nestmate odour profile, is not enough to fall within the colony odour profile (for a visual representation, see Fig. 3 in Ratnieks et al. 2011).

Swapping of entrance tubes had no effect on acceptance and allows us to reject the hypothesis that accurate nestmate recognition in *T. angustula* is due to the wax entrance tube serving as a convenient or immediate template for colony odour. Our data show a small but non-significant 3% increase in the acceptance rates of non-nestmates introduced after tube swapping, which is only 1/30th of the 90% difference seen between the acceptance of untreated nestmates (94.6%) and untreated non-nestmates (4.5%) observed in our first experiment. Our results also show no significant change in acceptance rates with time, from 1 to 24 h post-tube swap. We would expect to see an effect in this time frame as swapping of wax combs in honeybees leads to a change in behaviour within hours (Couvillon et al. 2007a).

While the first two experiments both provided very clear findings, the results of the subsequent two were less clear. Following treatment of worker bees with wax and resin odours, we found no difference between acceptance rates of bees treated with nestmate wax or resin versus non-nestmate wax or resin. If wax or resin serves as a source of colony odour, we would have expected to see a disparity between nestmates treated with their own wax or resin versus non-nestmate wax or resin, as seen in the first experiment where the odours in the vial were derived from live bees. Acceptance rates of nestmates dramatically

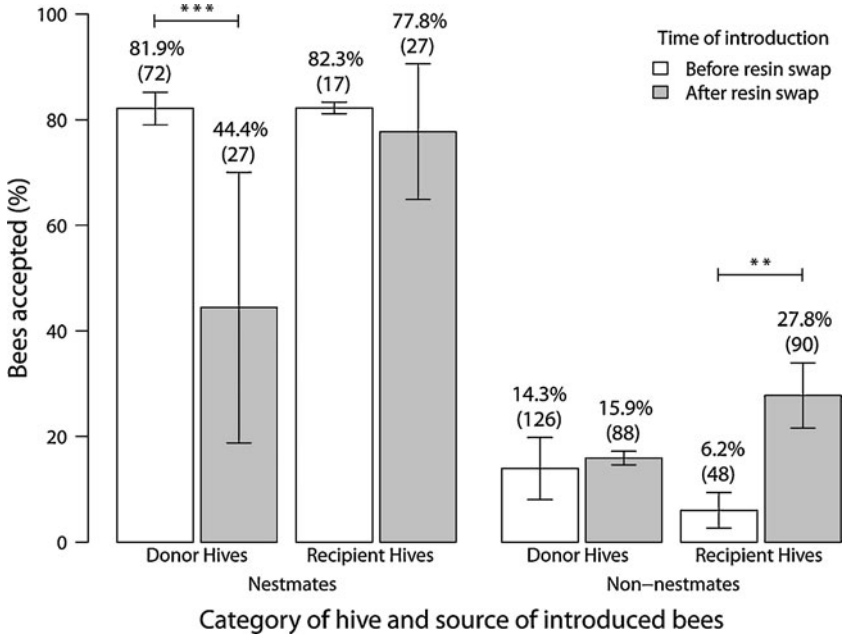

Fig. 3 Experiment 4: Nestmate and non-nestmate workers were introduced to six discriminator hives, both before and after a unidirectional resin transfer. Three discriminator hives were resin donors and three were resin recipients, forming three pairs. Exact percentage acceptance rates and sample sizes are given above the *bars*. Statistically significant differences are indicated (**$p<0.01$, ***$p<0.001$)

dropped instead (by 50.8% and 42.1% for wax and by 58.4% and 65.5% for resin) regardless of whether the wax or resin originated from a nestmate or a non-nestmate hive, respectively.

Transfer of resin stores between hives was our second approach to investigate the possible role of resin in nestmate recognition. In hives that had donated resin, acceptance rates of non-nestmates remained the same following resin transfer while acceptance rates of nestmates declined by over 37% (Fig. 3). Conversely, hives that had received resin accepted a significantly greater number of non-nestmates (an increase of 21%), while the acceptance rate of nestmates remained the same. We had expected to see a trade-off in which an increase in acceptance errors and a simultaneous decrease in rejection errors both occurred (Reeve 1989; Couvillon et al. 2009), and vice versa, but this negative correlation was not observed. If the template of the guards had been updated following the introduction of resin, then we would have expected to see a rise in the acceptance rate of non-nestmates from the partnered donor hive (c.f. Couvillon et al. 2007a). Although this effect is apparent, the acceptance rate of non-nestmates from non-partnered hives also increases to the same degree. At face value, it appears that guards were unable to distinguish between non-nestmates introduced from their partnered hive versus other non-nestmate hives. However, the interpretation of these trends is complicated by the fact that there was great variation in acceptance rates between the six discriminator hives (see "Electronic supplementary material"). For example, acceptance rates of nestmates by resin donor hives varied from 0% to 89% following the resin transfer. This marked variation suggests that something else may be occurring which our experiment was unable to reveal and therefore warrants further investigation.

The behaviour shown by the guards of resin donor hives is also puzzling. The post-transfer decline in acceptance of nestmates may be a response to the loss of the colony's entire resin store, but if this were the case we would predict a simultaneous increase in rejection rates of non-nestmates, which was not seen. The high variation in acceptance rates evident within both the donor and recipient colonies is perhaps indicative of guard confusion. Indeed this was conspicuous with guards exhibiting frequent and intense antennation with greater periods of time preceding rejection (S.M. Jones, personal observation). This lack of consistency in changes in acceptance rates among the discriminator colonies was also apparent in the wax and resin odour transfer experiment and is notably different from the consistent changes seen in the first two experiments. Our findings appear to show that *T. angustula* do not use pure resin as a source of cues for nestmate recognition. Several studies have failed to identify the presence of terpenoids on the wings of various Neotropical stingless bees (Abdalla et al. 2003; Jungnickel et al. 2004; Kerr et al. 2004; Nunes et al. 2008). To our knowledge, no study has yet analysed the cuticular chemical profiles of *T. angustula* but it would be surprising if terpenoids were absent when we know that resin, which is a rich source of terpenoids (Velikova et al. 2000; Sawaya et al. 2006), is found on the thorax of many foragers (J.S. van Zweden, unpublished data) and is universally present within the hive both in resin piles or mixed with wax as cerumen (S.M. Jones, personal observation; Michener 1974).

It is possible that the inconsistent results seen for the resin transfer experiment may have arisen because the

terpenoid composition of the pure resin we collected from the resin piles does not reflect the terpenoid profile present on the cuticles of the bees. Leonhardt et al. (2011) showed that terpenoids present on the cuticles of six different Paleotropical stingless bees differed from those found on nest material. Leonhardt et al. (2011) were also able to show that, from a total of 1,117 terpenoids available in stored resin, only 10% (105) were actually present on the cuticle of the Paleotropical stingless bee, *Tetragonilla collina*. To explain this, Leonhardt et al. (2011) proposed a hypothesis whereby stingless bees are able to perform some form of post-collection manipulation of resin terpenoids to ensure odour constancy. Resin stored by colonies of *T. angustula* from across Brazil was found to have a remarkably consistent composition, regardless of location (Sawaya et al. 2006). Therefore, for terpenoids to function as cues for nestmate recognition, quantitative differences between a discrete set of these compounds must be apparent among colonies and this would have to be achieved by some form of post-collection manipulation. Confirmation of whether terpenoids can be manipulated in this manner or indeed function as suitable recognition cues will require further behavioural and analytical study. A more parsimonious explanation may be that resin simply does not function as a primary source of recognition cues in *T. angustula*. An inherent problem with using collected materials, such as resin or food, as odour cues is the likelihood that the availability of the sources will change with time (Downs et al. 2000, 2001). Once a bee collects new material which is not consistent with its colony odour, there is a strong possibility that it will be rejected. For example, floral odours, most of which are terpenoids, were found to have no function in honey bee nestmate recognition (Downs et al. 2000).

Overall, our results confirm the accuracy of the nestmate recognition system in *T. angustula*. When results of the controls from the first three experiments were combined, a typical average of 10% was observed for both acceptance and rejection errors, giving a total error rate of 20%. Despite the variation that exists between colonies and studies, the error rate remains considerably lower than those reported for honey bees (Downs and Ratnieks 1999, 2000; Couvillon et al. 2007a, 2008, 2009, 2010) and lower than all stingless bee species studied to date (Breed and Page 1991; Buchwald and Breed 2005; Couvillon and Ratnieks 2008). Although our results do not show how *T. angustula* achieves this accuracy, we have ruled out one strong contender: the wax entrance tubes of *T. angustula* nests appear to play no role in nestmate recognition. Our results from the resin odour treatment and resin transfer experiments suggest that odours acquired directly from resin also serve no function as nestmate recognition cues, although the observed shifts in the acceptance threshold seen for the resin transfer suggest a possible secondary role. However, the variation and inconsistency of our results in the last two experiments together highlight the need for future chemical analysis of resin stores, cerumen and the cuticular profiles of worker bees. Indeed the results of a recent study by Nunes et al. (2011) suggest that cerumen may be a source of recognition cues, used by colony members of the stingless bee *F. varia*. It also remains to be seen whether this proficient recognition system has evolved as a result of low genetic variability or high parasite pressure.

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Appendix 4 – Published Paper in Behavioral Ecology & Sociobiology

Hovering guards of the stingless bee *Tetragonisca angustula* increase the colony's defensive perimeter as shown by intra- and inter-specific comparisons.

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ORIGINAL PAPER

Hovering guards of the stingless bee *Tetragonisca angustula* increase colony defensive perimeter as shown by intra- and inter-specific comparisons

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Abstract Social insects need to defend their nest against robbery, parasitism and predation. The stingless bee *Tetragonisca angustula* is unique in that it has guards that hover near the nest entrance in addition to guards that stand at the entrance. We tested both the general hypothesis that hovering guards increase the effectiveness with which flying intruders are detected and the specific hypothesis that hovering guards improve the detection of workers of the obligate robber bee, *Lestrimellita limao*. In an intraspecific study comparing colonies, we found a strong positive relationship between the number of hovering guards and the distance at which a dummy robber bee or *L. limao* worker, experimentally moved towards the nest entrance, was detected. These results were mirrored in an interspecific study showing that four species of stingless bees with similar population colonies but which lacked hovering guards, detected *L. limao* only at the nest entrance, in contrast to *T. angustula*. In addition, we found that a greater number of attacks by guards occurred when dummies were impregnated with citral, a major component of *L. limao* mandibular gland odour. Our results support the hypothesis that *T. angustula* hovering guards increase the detection perimeter for flying intruders, especially *L. limao*.

Keywords Nest defence · Nestmate recognition · Defensive perimeter · Robber bees · *Lestrimelitta limao*

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Introduction

Many animals build nests and protect these against predation (e.g. Armstrong 1949) and parasitism (e.g. Lotem et al. 1995), which is key to their reproductive success. Nests of social insects (e.g. bees, wasps, ants, and termites) contain resources such as stored food and brood subject to robbery by a variety of animals, including conspecifics and closely related species (Wilson 1975). Eusocial bees variously defend their nest by stinging, biting, the use of alarm pheromones, releasing caustic secretions, choosing a good defence site, and closing the entrance (Butler and Free 1952; Michener 1974; Wille 1983; Johnson et al. 1985; Seeley 1985), often displaying a combination of these defensive elements. For example, honey bees, *Apis mellifera*, usually nest inside a defensible cavity with a small entrance (Seeley 1985). Guards standing at the nest entrance check incomers and reject conspecific and allospecific intruders (e.g. Downs and Ratnieks 1999). Guards also sting and release alarm pheromones that recruit nestmate workers to defence. Remarkably, the detached sting continues to pump venom and release alarm pheromone, guiding additional workers to the intruder (Free 1987).

Stingless bees (Meliponini) are a diverse pantropical taxon of more than 350 described species (Michener 2000), in which nest defence is both diverse and sophisticated. Some species retreat into their nest when disturbed, relying on the cavity substrate itself to provide defence (Couvillon et al. 2008). Other species use unpleasant smells and sticky substances like plant resin to repel intruders and yet others rely on a constricted nest entrance or even fake entrances (Kerr and de Lello 1962; Michener 1974; Roubik 1989). There are some remarkable features, such as in *Partamona helleri*, which have an elaborate "toad mouth" double-

entrance structure whose wide outer entrance permits high-foraging traffic while also having a protective inner entrance (Pedro and Camargo 2003; Couvillon et al. 2008).

A unique defensive feature occurs in *Tetragonisca angustula* (Brazilian common name: Jataí), a relatively small (body length=4–5 mm) yellow/brown Neotropical stingless bee (Fig. 1). *T. angustula* is the only species currently known that has guards that hover near the nest entrance. It also has guards that stand at the entrance as in other stingless bees and cavity-nesting honey bees (Michener 1946; Wittmann 1985; Kärcher and Ratnieks 2009; Grüter et al. 2011). Hovering guards are present during daytime and often form a main group in front and two smaller groups, one on each side of the nest (Grüter et al. 2011). It has been hypothesised that they provide earlier detection of allospecific robber bees (Grüter et al. 2011). Hovering guards can only detect intruders that are differently coloured to *T. angustul*a (many robber bees are black) or which carry volatile odours (Bowden et al. 1994), whereas the standing guards discriminate against non-nestmate conspecifics using contact chemoreception (Kärcher and Ratnieks 2009). The two guard types, therefore, appear to complement each other in detecting intruders. An additional, and not mutually exclusive, hypothesis is that hovering guards play a particular role in defence against the recurring raids of *Lestrimelitta limao*, an obligate robber bee that raids nests of *T. angustula* and dozens of other stingless bees for honey, pollen and brood food (Wittmann 1985; Roubik 1989). Wittmann et al. (1990) found that hovering guards respond strongly to *L. limao*: upon presentation of *L. limao* mandibular gland odours (major component is citral, a mixture of the terpenoid isomers geranial and neral) there is a strong increase in the number of hovering guards. Mandibular gland odours of *L. limao* are normally thought to be used to disorient victim colonies (propaganda substances), but *T. angustula* guards have apparently adopted a strategy where they enhance their defences (Wittmann et al. 1990), by using the odour as a kairomone.

Fig. 1 The wax entrance tube of a natural nest of *T. angustula* (Brazilian common name: Jataí) built on a wall at Fazenda Aretuzina with standing and hovering guards. On the tube many standing guards can be seen and a hovering guard to the right. (Photo by F.L.W Ratnieks)

The aim of this paper was to test the hypothesis that hovering guards increase the defensive perimeter with which allospecific intruders are detected by measuring the distance at which dummies were attacked by *T. angustula* colonies with varying numbers of hovering guards. In addition, we compared the intruder detection ability of *T. angustula* with four other species of stingless bees without hovering guards. We also tested the hypothesis that hovering guards improve the detection of workers of the obligate robber bee *L. limao* by treating dummies with citral. We find that higher numbers of hovering guards do increase intruder detection distance in *T. angustula* and that the other four species all detect intruders at lesser distances, normally only when the intruder is at the entrance itself. Citral has a weak positive effect on detection distance, but it does significantly increase the number of attacks an intruder receives.

Methods

Study site and organism

Data were collected in February 2009 and 2010 at Fazenda Aretuzina, São Simão, São Paulo State, Brazil. Fazenda Aretuzina is a former coffee farm that has been converted into a centre for studying stingless bees and conserving Brazilian wildlife by its owner, Dr. Paulo Nogueira-Neto. Natural mature colonies of Jataí, *T. angustula*, can contain approximately 10,000 individuals (van Veen and Sommeijer 2000). Our study colonies were housed in wooden hives and had been allowed to build up for several years and so were similar in size and had normal foraging activity and appeared in good health (Paulo Nogueira-Neto, personal communication). Natural colonies in wall cavities were common at the farm and had similar entrance tubes and numbers of foragers and guards. During both study periods, the weather was hot with daytime high temperatures of approximately 24–32° C and periodic heavy rain. Data were only collected on non-rainy days during active foraging.

Experiment 1: attack distance

In 2009, we measured the distance at which hovering guards in 20 *T. angustula* colonies first attacked a black

modelling clay dummy (200.4±4.4 mg, width 3 mm, length 10 mm) of a *L. limao* worker (black, 13 mg, width 2 mm, length 8–10 mm). The dummy, either with or without citral (see: Odour application), was attached to a 15-cm piece of fishing line (diameter 0.14 mm), on the end of a thin wooden pole. The experimenter stood at the side of a test colony's nest entrance tube and moved the dummy slowly and with a consistent speed towards the entrance from an initial distance of approximately 40 cm and noted the distance between the entrance and dummy upon first attack by one of the guards. An attack was registered when a guard flew at the dummy and then tried to bite or grab it. In a natural situation with live robber bees, a single guard usually bites the intruder at the base of a wing, antenna, or leg, forcing it to the ground where it is immobilised for a few seconds to several hours, but usually is not killed (C. Grüter, personal observation).

In 2010, the procedure was replicated using freshly killed (by freezing at −20°C) *L. limao* workers. We used the same 20 *T. angustula* colonies as in 2009, and studied an additional ten colonies of *Frieseomelitta varia*, nine *Plebeia droryana*, nine *Nannotrigona testaceicornis*, and seven *Melipona quadrifasciata*, to compare *T. angustula* to species without hovering guards.

Experiment 2: attack preference

Experiment 1 indicated a weaker than expected effect of citral (see: Results). We, therefore, designed experiment 2 to determine whether guards have a higher tendency to attack citral-impregnated dummies (made of black modelling clay, as above) versus odourless or linalool-impregnated dummies. Like citral, linalool is a common odour component in floral odours (Knudsen et al. 1993), but is not used by *L. limao* and so was chosen as an additional control. We simultaneously attached three dummies to the fishing line, each separated vertically by 2.5 cm. One of these dummies was impregnated with citral ('C'), one with linalool ('L'), and one was left untreated ('U'). Six different permutations are possible with three different positions (C/L/U, C/U/L, L/C/U, L/U/C, U/L/C, and U/C/L). We tested three colonies, once with each permutation (N=18 trials in total). We held the three dummies at approximately 5 cm in front of the entrance and slowly moved them up and down for approximately 1 min. We recorded how many attacks occurred in the first 30 s.

Odour application

For both experiments 1 and 2, we impregnated a cotton swab with 10 μl pure odorant, and rubbed this against our dummy until it was covered with odour. The odour on the dummy was clearly perceivable by the human nose within 5 cm for at least 30 min. In order to ensure similar odour strength for all colonies we used newly prepared dummies after every four colonies (approximately every 5 min). Odours were obtained from Sigma Aldrich (Steinheim, Germany).

Statistics

Data were analysed in R 2.10.1 using the lme4 package, which allows the incorporation of general linear mixed models (GLMM). For experiment 1, we performed a GLMM with Poisson errors (goodness of fit, χ^2=3.54, df=2, p=0.17), distance of first attack as the dependent variable, the number of hovering guards ('guard number') as a continuous variable, the odour treatment ('odour') as a class variable, and the experimental colony as random variable. For experiment 2, the underlying error distribution of the GLMM was set to Poisson (goodness of fit, χ^2=8.29, df=4, p=0.08), the number of attacks was the dependent variable, the odour treatment ('odour') as a class variable, and the experimental colony as random variable.

Results

Experiment 1: attack distance

All intruders in our experiments were attacked at some stage. The distance from the nest entrance at which *T. angustula* hovering guards first attacked dummies ranged from 0 to 22 cm (mean ± S.D.=8.2±5.7 cm). Distance was significantly greater in colonies with more hovering guards (Fig. 2; effect of 'guard number', χ^2=26.31, df=1, p< 0.001). There was no significant effect of citral (effect of 'odour', χ^2=1.94, df=1, p=0.164), although there was a weak but significant 'guard number' × 'odour' interaction (χ^2=5.03, df=1, p=0.025). The attack distance of freshly killed *L. limao* workers also increased with the number of hovering guards (Fig. 2; effect of 'guard number', χ^2= 28.56, df=1, p<0.001), but since observations were made in different years, we did not statistically compare the data from dummies with freshly killed workers. In all trials, it was a hovering guard from the central group (Grüter et al. 2011) that initiated the attack rather than a hovering guard from a side group or a standing guard.

In non-*T. angustula* bees, almost all colonies attacked the freshly killed *L. limao* workers at the entrance (0 cm; Table 1). Across these four species, *L. limao* workers were only attacked before they reached the entrance in 4% of the trials (versus 90% for *T. angustula*). Only in *F. varia* did a guard attack the dummy before it reached the entrance, which occurred in 2 of the 20 trials at distances of 13 and 6 cm. All other attacks were at the entrance in this species, leading to a mean attack distance of 1.0±3.1 cm.

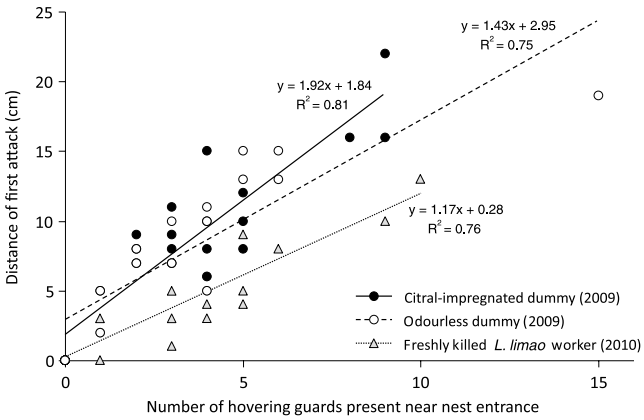

Fig. 2 Relationship between the number of *T. angustula* hovering guards and the distance of first attack. In 2009, the responses to odourless and citral-impregnated dummies, similar in size and colour to a *L. limao* worker, were studied, and in 2010 the responses to a freshly killed *L. limao* worker

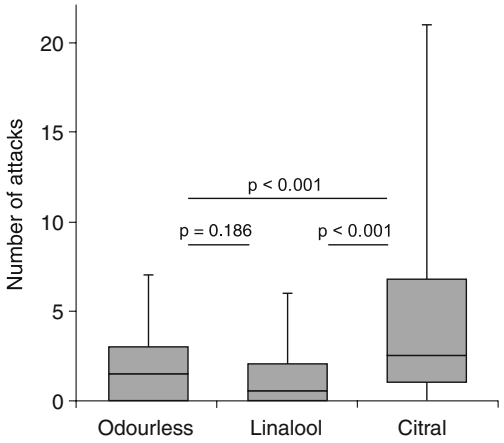

Fig. 3 Attack preference of *T. angustula* hovering guards when dummies carrying no additional odour or treated with linalool or citral were presented simultaneously. The Y-axis shows the number of attacks each of the dummies received in the first 30 s of presentation in front of the nest entrance. *Bars* show 75% quartile, median and 25% quartile, whiskers show maximum and minimum

Experiment 2: attack preference

Hovering guards of *T. angustula* attacked dummies impregnated with citral significantly more often within 30 s than those without odour (Fig. 3; GLMM; effect of 'odour', χ^2=46.90, *df*=2, p<0.001; odourless versus citral, Z=4.72, p<0.001). Linalool had no such effect, with the trend actually being slightly in the opposite direction although not significant (odourless versus linalool, Z=−1.32, p=0.186). In 9 out of 18 observations the dummy impregnated with citral was attacked first, versus 6 and 3 for odourless and linalool, respectively.

Discussion

Our results show that the presence of hovering guards in *T. angustula* improves the detection of both dummy intruders and real intruders (freshly killed *L. limao* workers) being moved towards the nest entrance. This is shown by both the intraspecific comparison, as the first attack by *T. angustula* guards occurred at greater distances from the nest entrance in colonies with more hovering guards, and the interspecific comparison, as all four species of stingless bees lacking hovering guards detected *L. limao* workers at lesser distances than *T. angustula*. In addition, we found a positive effect of citral in experiments 1 and 2. Our results, therefore, support both the general hypothesis that hovering guards enable earlier detection of allospecific robber bees and the specific hypothesis that they play a role in defence against the obligate robbing bee *L. limao*.

Because the number of hovering guards and the distance from the entrance at which they hover is correlated (J.S. van Zweden, personal observation), this may be the cause behind the greater distance detection seen in experiment 1

Table 1 Distance of first attack on a freshly killed *L. limao* worker by five species of stingless bees

Species	Nr. of colonies	Nr. of trials	Mean ± S.D. attack distance (cm)	Mean ± S.D. number of (hovering) guards[a]	Worker size (mm)[b]
Plebeia droryana	9	3	0.0±0.2	2.9±0.6	3.0–6.0
Frieseomelitta varia	10	2	1.0±3.1	2.5±1.4	4.0–6.5
Nannotrigona testaceicornis	8	2	0.0±0.0	5.6±2.3	3.0–5.0
Melipona quadrifasciata	7	2	0.0±0.0	1.3±0.6	8.0–15.0
Tetragonisca angustula	20	1	4.9±3.3	3.9±2.5	4.0–5.0

Nr number, *S.D.* standard deviation

[a] This column shows the number of hovering guards for *T. angustula* and the number of standing guards for the other four species, none of which have hovering guards. In addition to hovering guards, *T. angustula* has approximately twice as many standing guards on the nest entrance tube as hovering guards (Grüter et al. 2011). See also Couvillon et al. (2008)

[b] Sizes given here are based on those given for the genus by Michener (2000)

for colonies with more hovering guards. Alternatively, it may be because having more hovering guards gives greater vigilance, as can occur in the detection of predators by groups of birds (e.g. Kenward 1978). Although the underlying mechanism remains to be determined, one significance of hovering guards is that they allow earlier detection of intruders. Upon detection of an intruder, hovering guards of *T. angustula* will grab the intruder by the wings, legs or antennae and force it to the ground, where it is immobilised for up to half an hour (C. Grüter, personal observation). Because *T. angustula* hovering guards readily attack intruders, scouting robber bees may have significantly less success in recruiting nestmates to raid *T. angustula* nests compared to victim nests of other species. The other four species used in this study do not have hovering guards and detection of *L. limao* workers only occurred once the intruder was at the entrance. These species, however, have other means of defending against robber bees (Kerr and de Lello 1962). For instance, both *M. quadrifasciata* and *F. varia* have a narrow entrance hole that can be blocked by a single guard, and *M. quadrifasciata* also recruited workers from inside the hive and fiercely attacked the test *L. limao* worker. Nests of *Frieseomelitta* appear to be generally exempted from raids of *L. limao* (Bego et al. 1991), possibly due to their intensive use of sticky resin, a defensive substance (Patricio et al. 2002). *Plebeia* and *Nannotrigona*, on the other hand, seem to be the preferred genera for raids by *L. limao* (Sakagami et al. 1993) amongst dozens of species of potential social bee victims (Roubik 1989). Bees of the genus *Plebeia* are also known to use resin in their defence system (Patricio et al. 2002), whereas *Nannotrigona* seems to invest in many guards like *T. angustula* (Couvillon et al. 2008).

Hovering guards appear to respond mostly to visual cues, at least in the first instance, such as to bee-sized objects of a different colour to conspecifics moving towards the nest entrance (see also Kelber and Zeil 1990, 1997; Zeil and Wittmann 1993). Several Neotropical stingless bee species known to rob other species are black, including *L. limao* and *Scaptotrigona bipunctata*, which may therefore be easily detected. Nonetheless, hovering guards do respond to citral, as was shown by our attack preference experiment and the significant interaction between citral and the number of hovering guards. This specific recognition via a kairomone parallels the response of the honey bee *Apis cerana japonica* in detecting the aggregation pheromone used by scouts of the giant hornet *Vespa mandarinia japonica* to mark a victim nest. Preventing a scout hornet from recruiting nestmates can prevent a potentially lethal mass attack of hornets. On perceiving the pheromone, the worker bees kill the scout by forming a ball around her that heats up to lethal temperatures for the hornet (Ono et al. 1995). *T. angustula* is considered to be somewhat resistant to attacks of *L. limao* (Schwarz 1948; P. Nogueira-Neto, personal communication) and this may be due to the complementary role of hovering guards and standing guards (Kärcher and Ratnieks 2009). Hovering guards can take care of *L. limao* scouts using visual cues to detect them, probably aided by the kairomonal information from *L. limao*'s mandibular gland odour, thereby diminishing the chance of a successful raid and the loss of valuable resources.

Our results indicate that hovering guards can improve defence by extending the defensive perimeter of *T. angustula*, which raises the question as to why other species have not evolved this feature. The precise phylogenetic reletionships in the clade to which *T. angustula* belongs have been relatively well resolved (Rasmussen and Cameron 2010), but since the presence of hovering guards has thus far only been reported for *T. angustula*, the most parsimonious interpretation is that it is an autapomorphy and a comprehensive phylogenetic analyses is not possible. On the other hand, it may be possible to make comparisons of the presence or number of hovering guards within *T. angustula* between, for example, geographical areas with high and low densities of *L. limao*.

T. angustula has one of the most sophisticated guarding systems known amongst social bees, involving architectural, chemical and behavioural defences, e.g. a nest entrance tube made of wax and sticky resin that leads to a network of tunnels inside the nest cavity (J.S. van Zweden, personal observation), overnight closure of the nest entrance with wax (Grüter et al. 2011), the best discrimination abilities between nestmates and conspecific non-nestmates yet observed in bees (Kärcher and Ratnieks 2009), guards that remain as guards for a long duration (Grüter et al. 2011), and division of labour between standing and hovering guards (Kärcher and Ratnieks 2009). Is this sophistication possibly the result of a long evolutionary history of robbing by both conspecifics and allospecifics? Has *L. limao* been a specialist robber of *T. angustula* colonies, leading the latter to evolve a specialised system where guards seem to be in a constant state of alarm? Are hovering guards too costly to evolve for other species or simply not that useful compared to other defensive features? Future research may elucidate these questions, and provide us with novel and intriguing defensive features of stingless bees.

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