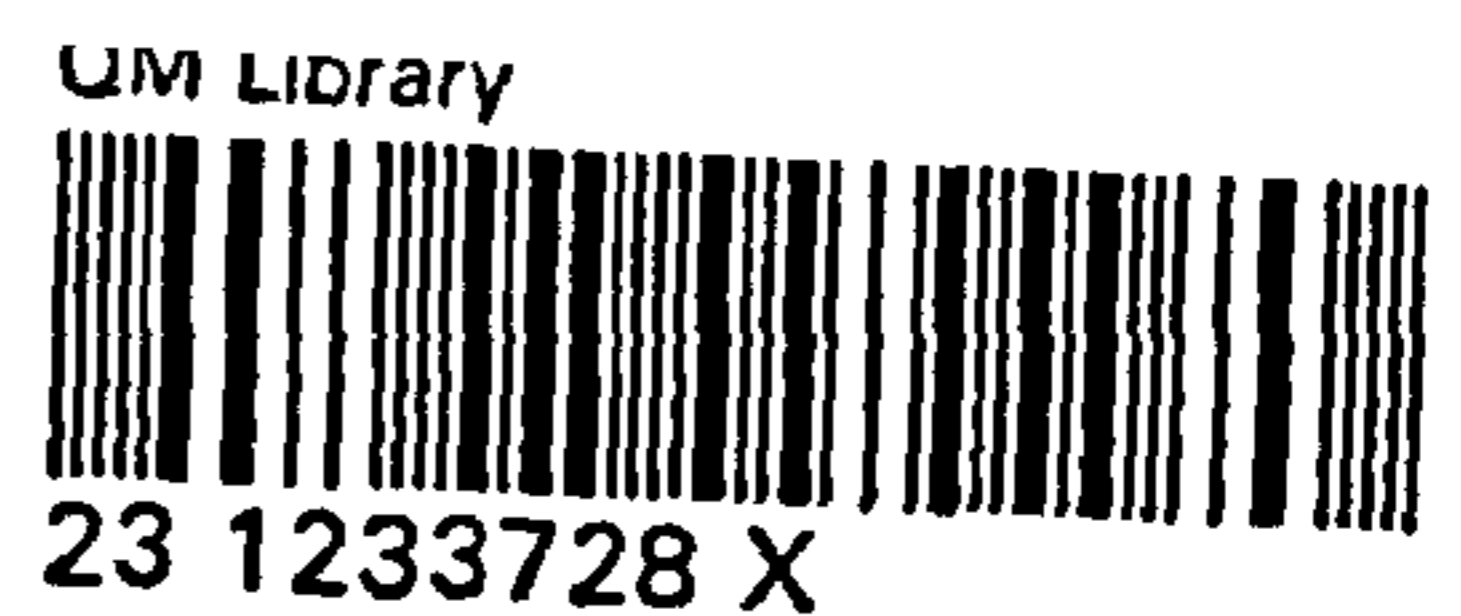


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Laboratory studies examining
aspects of scent marking, traplining
and remote detection of reward
in
the foraging bumblebee

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I declare that I have conducted the work in this thesis, and any collaborators have been acknowledged. I have composed this thesis, all quotations and sources of information have been acknowledged and this work has not previously been accepted for another degree.

A handwritten signature in black ink, consisting of several overlapping loops and a horizontal line at the base, positioned above a dotted line.

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ABSTRACT

Energy from food is essential for the survival of all animals. For decades, bumblebees have been used as model organisms for studying animal foraging strategies. Here, I use bumblebees to examine two foraging strategies: scent marking and traplining. I find that experience and long term memory play an important role in both of these strategies.

I show that bees interpret scent marks differently depending on context. They learn to rely on these scent marks to different degrees depending on flower handling time. Bees also learn to associate the same scent marks with high and low rewarding food, which means the same scent promotes and suppresses acceptance of flowers. Contrary to previous speculation, I find that these scent marks are not pheromonal signals specifically evolved to play a role in foraging. Rather they are incidental cues that bees learn to use to improve foraging performance and locate their nesting sites.

Experience is also important in developing repeatable stable routes between food sites i.e. traplines. I show that bees required long term spatial memory to gradually form traplines. They reduced their travel distance by linking near neighbour flowers, which did not result in using the shortest routes. Traplining bees were also less likely to revisit emptied flowers and spent less time searching for these flowers.

For decades, scientists have used water to control for remote effects of sucrose solution in experiments. I find that bees are able to detect the difference between these two liquids without contact chemoreception. The exact cue they use remains to be determined, but it is not humidity.

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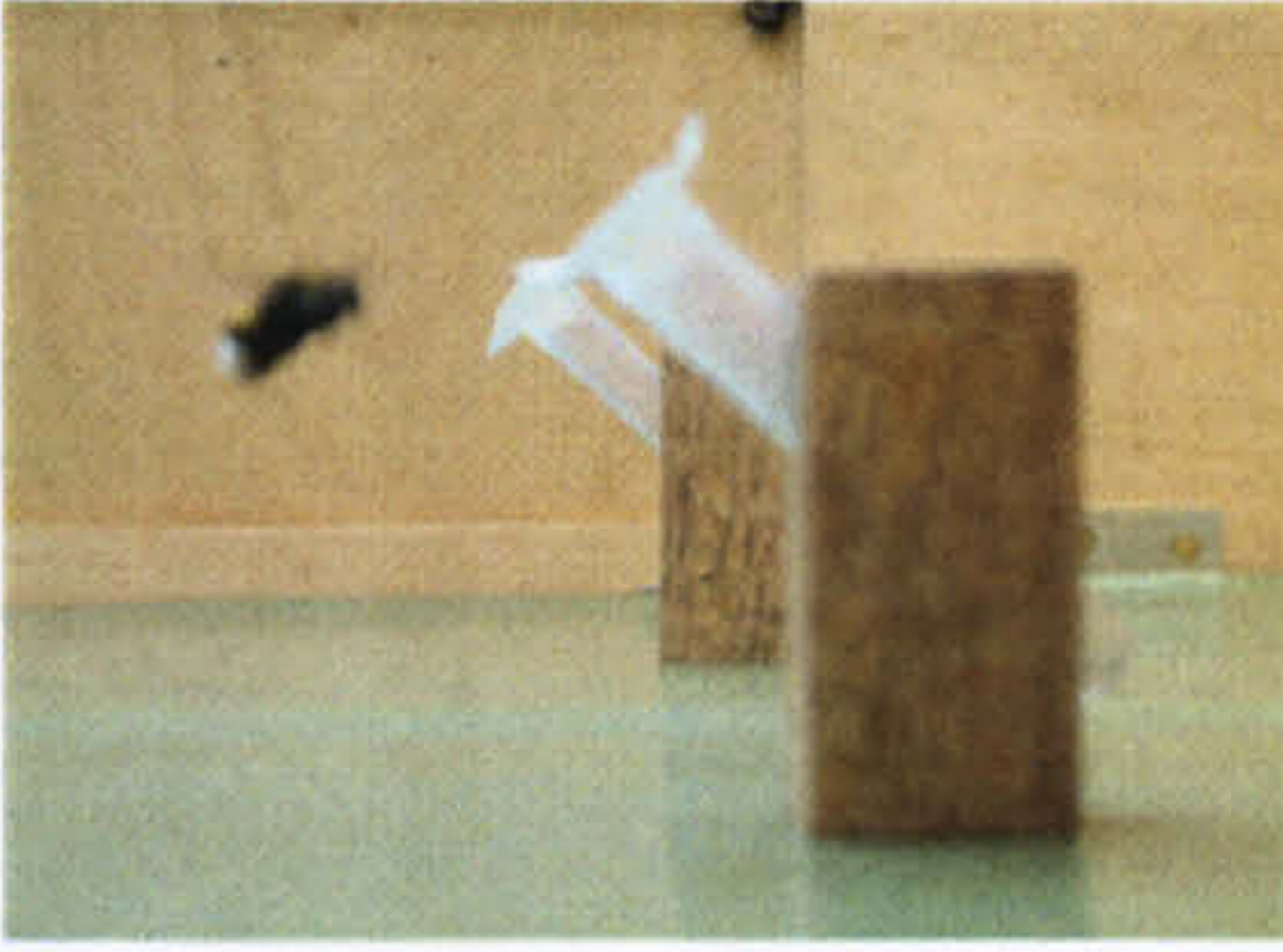


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LEGEND AND DEFINITIONS OF BEHAVIOURS ANALYSED

CXX- for example, C25, means a linear hydrocarbon molecule is composed of carbon and hydrogen atoms only and its formula is $C_{25}H_{52}$.

Bout- consists of the bee exiting the hive, feeding on flowers and returning to empty its honey crop.

	<p>Acceptance</p> <p>the bee entered to the bottom of the flower</p>	
Flower rejections	<p>Hovering</p> <p>bee hovered within 1cm of the filter paper of the flower for more than 1 second then flew away from the flower</p>	
	<p>Landing</p> <p>bee landed on the filter paper of the flower with all six legs then flew off the flower</p>	
	<p>Crawling-in</p> <p>bee entered halfway into the flower tube, crawled back out and flew off the flower</p>	

Introduction

Foraging strategies used by bumblebees

CHAPTER 1:

INTRODUCTION: Foraging strategies used by bumblebees

Foraging is a fundamental requirement for animal survival. Without the energy gained from food animals cannot avoid predators, find a mate or reproduce. For decades worker bumblebees have been popular model organisms for understanding foraging strategies. They are independent foragers (i.e. do not recruit to food sites, rather seek out and exploit floral resources individually). They are easily marked and observed in the field (Chittka & Thomson, 1997; Comba, 1999; Goulson, 2003). The ability to maintain them in the laboratory means we can easily test predictions from the field in laboratory studies. Bumblebees do not defend or maintain feeding territories, in fact aggressive interactions between bumblebees foraging on natural flowers are minimal (Comba, 1999; Goulson, 2003). Worker bumblebees do not search for nesting sites as they typically remain faithful to the parental nest throughout their life and, because they only reproduce without fertilisation, they do not search for mates. This means we can study movement patterns and their adaptive nature in a relatively pure foraging situation (Pyke, 1978). The knowledge we gain from the worker bumblebee's behaviour can then be compared and extended to other animals, such as birds, whose ecology involves locating mates and nesting sites, as well as resource defence and territoriality. Comparisons between animals with different foraging ecologies can give us insight into the evolution of foraging strategies and the role they play in animals with different life history traits and cognitive abilities. In this thesis I will examine two foraging strategies, scent marking and traplining. Both are believed to help bees avoid revisitation. I show that learning and long term memory are important factors in the use of both these foraging strategies.

1.1 FACTORS INFLUENCING FORAGING IN BEES

Because bumblebees do not defend territories to gain access to food, they have to compete indirectly with other nectar and pollen gatherers through scramble competition, where each individual attempts to remove reward before the next individual (Heinrich, 1979a). Their success depends on three major factors: the degree of competition from animals foraging on the same resources; the inherent variation in nectar/pollen production by the flowering plants; and the

strategies the bees use to locate and exploit the food. I will first give a brief overview of the first two factors, and then move to discuss the tactics used by bumblebees in more detail.

Bumblebees have to compete with other animals for access to floral rewards. In some areas this competition can be very high. For example, Thomson et al. (1982) found that 9 bumblebee species competed with at least 39 other species of insects (beetles, wasps, bees, and flies) for access to floral resources of one plant species. The behaviour of these other flower visiting insects impacts on resource availability resulting in patchy distributions of resources (Zimmerman, 1981). However, individual bumblebee workers have very little influence on the amount and impact of competition on resource distribution because they do not aggressively defend food sites (Comba, 1999). Therefore, they cannot exclude competitors from their foraging areas.

Worker bumblebees also have very little influence over the patchiness of resource distribution that occurs due to variation in pollen or nectar quality and production rates. A range of flowering plant species often co-occur in a habitat (e.g. Thomson et al., 1982; Gumbert et al., 1999). These flowering plant species differ in their nectar secretion rate (Lavery, 1980; Chittka & Schurkens, 2001; Biernaskie & Cartar, 2004), pollen production rate (Mazer & Hultgard, 1993) and nectar concentration (Lavery, 1980; Dupont et al., 2004; Chalcoff et al., 2006). In addition, individuals of the same plant species can differ drastically in these same characteristics (Cruden, 1976; Frankie & Vinson, 1977; Pleasants & Chaplin, 1983). This variation continues at the level of individual flowers within a plant (Mazer & Hultgard, 1993; Thakar et al., 2003; Biernaskie & Cartar, 2004) and can be influenced by many factors, for example the season (Pleasants, 1981; Pleasants & Chaplin, 1983; Emberlin & Norrishill, 1991; Herrera et al., 2006), and what parts of the plant are in the sun or shade (Corbet, 1978; Herrera, 1995). Such large, relatively unpredictable variation makes it difficult for bees to have complete knowledge of resource availability and, as with competition, worker bumblebees cannot directly influence intrinsic plant characteristics.

The only direct influence worker bumblebees have is on their own behaviour. They use behavioural strategies to improve their probability of gathering reward compared to random foraging (Levin et al., 1971; Dreisig, 1995). These strategies range from those that help naïve bees find floral

reward, to those that benefit bees with little information about resource distribution and finally those that can be used by bees with some knowledge of resource distribution. As most of the work dealing with bee foraging strategies focuses on nectar, rather than pollen, collection, I will restrict my analysis to this resource unless otherwise stated.

1.2 BUMBLEBEE FORAGING STRATEGIES

1.2.1 General

Naïve bumblebee foragers leave the hive for the first time armed with biases and tactics that increase their probability of encountering rewarding flowers. It is thought that naïve bees can identify flowers from a distance due to their innate preferences for blue and violet (Chittka & Briscoe, 2001; Chittka et al., 2004) as well as colour purity (Lunau, 1990). Violet is seen by the bees as a mix of blue and ultra-violet. In fact, it has been argued that these colour preferences are adaptive in some environments where blue and violet flowers contain high nectar rewards (Menzel & Shmida, 1993; Chittka et al., 2004). Bees also have an innate preference for symmetrical objects (Rodriguez et al., 2004), which presumably helps them confirm that the object is indeed a flower, because most flowers are symmetrical (Endress, 1999).

Visual cues play an important role in locating flowers from a distance (Lunau, 1992; Lunau, 1993; Gumbert, 2000). However, once a bee has located a flower, visual and olfactory cues act in combination to promote landing. Before landing on a flower for the first time, naïve bees will briefly touch the flower with their antennae (Lunau, 1992). On natural flowers with hidden anthers, the upper part of the stamen where the pollen is produced, these antennations are restricted to areas that mimic anther shape and colour. This area also has a different scent from the rest of the flower, and the presence of this scent results in more landings (Lunau, 1992). After attenuating this area, pollen odour further promotes landing (Lunau, 1992). Thus, at close range visual and chemical cues act together where each step of the process provides additional cues to confirm the object is a flower.

If these inexperienced bees' land on a flower with extremely simple morphology, for example *Asteraceae* (sunflower family) where nectar is often presented in open shallow cups (see Figure 1.1a), they have a good chance of locating nectar. All inexperienced bees tested on such simple flowers found the

nectar (Lavery, 1994a). However, not all flowers have a simple morphology. Nectar can be present at the bottom of a narrow corolla tube; in this case access to the nectar may be more difficult. The flower may have a long narrow open tube, the entrance of this tube may be hidden either fully or partially by overlapping petals, or the nectar may be presented in an unusual position (see Figure 1.1b-d). When landing on flowers with more complex morphology, inexperienced bees often land on flower areas that do not facilitate nectar extraction (Lavery, 1980). Yet, bumblebees have particular biases that improve their chances of finding the nectar. Specifically, they stereotypically target the central areas where the petals converge and the stamens, male part of the flower comprised of the anther and filament, are present (Lavery, 1980; Lavery, 1994a). These behaviours may be visually mediated by ultra-violet guides (Daumer, 1958; Waser & Price, 1983), high colour contrast (Lunau, 1990; Lunau et al., 1996) and/or the odour of the nectar (Lavery, 1994b). In the hive, bees sample honeypots filled with nectar from other foragers (Dornhaus & Chittka, 2004; Dornhaus & Chittka, 2005), therefore naïve bees may already be familiar with the nectar scent of flowering plant species.

Inexperienced bees can spend several minutes handling their first flower (Lavery, 1980; Lavery, 1994a; Chittka & Thomson, 1997). Their degree of success in extracting nectar will depend on the morphological complexity of the flower involved (Lavery, 1994a). If the bee is not successful at extracting nectar, it may visit a few more flowers before giving up for a short time (Lavery, 1994a). An inexperienced bee will make a small number of flower visits compared to experienced bees (Lavery, 1980). However, they quickly learn to forage effectively with some practice (Lavery, 1980; Lavery, 1994a; Keasar et al., 1996; Peat & Goulson, 2005).

Bees handle the encounter of unrewarding flowers by using two different strategies. 'Near-far search' has been described in both experienced (Pyke, 1978; Hodges & Miller, 1981; Thomson et al., 1982; Schmid-Hempel, 1984; Cartar & Real, 1997; Chittka et al., 1997; Cartar, 2004; Gegear & Thomson, 2004) and inexperienced bees (Keasar et al., 1996; Burns & Thomson, 2006). This strategy operates on small spatial scales (i.e. between flower movements) over short foraging periods. It involves reducing flight directionality and travel distance when encountering rewarding flowers while increasing these two factors when encountering unrewarding flowers (Pyke, 1978; Hodges & Miller,

1981; Thomson et al., 1982; Schmid-Hempel, 1984; Cartar & Real, 1997; Cartar, 2004; Gegear & Thomson, 2004). This behaviour causes the bee to stay on a plant/inflorescence (cluster of flowers on a plant) after encountering reward and to leave a plant/ inflorescence if no reward is encountered (Hodges, 1985). The other strategy that bees use when they encounter unrewarding flowers is to switch flower species. This has been shown in experienced bees (e.g. Chittka et al., 1997; Keasar et al., 2002; Gegear & Lavery, 2004), but has not been investigated in inexperienced bees. It is conceivable that inexperienced bees also switch species if they are unable to extract reward from a particular flower type.

Near-far search and switching flower species should promote sampling in both inexperienced and experienced bees. Flowers with extremely simple morphology are accessible to most flower visiting insects, because nectar extraction does not require special skills or body type (Heinrich, 1976; Thomson et al., 1982; Heinrich, 1983; Lavery, 1994b). This means that competition for these flowers will be high and, as a result, they are less likely to contain reward. Bees, especially large ones such as bumblebees, have the strength to move petals, and therefore can forage on flowers with other morphologies (Heinrich, 1976; Lavery, 1994b; Goulson, 2003). These flowers may contain more reward than flowers that can be exploited by any flower visitor. Indeed, at least in some species, complex flowers can have 80-300 times the reward offered by simple flowers (Heinrich, 1979c). By sampling other plants or flower species bees are able to gather information on which flowering plant species offers the best rewards. The bumblebee's preference for nectar with a high concentration of sucrose means that they are likely to change their foraging preferences when they detect changes in this parameter (Corbet, 1978; Lavery, 1980; Willmer & Stone, 2004).

Sampling also increases the bees' knowledge of the spatial location of rewarding plants and flower species. We still do not know the exact extent of a bee's foraging range. Although foraging ranges of up to 1750m have been reported for some bumblebee species (Walther-Hellwig & Frankl, 2000), most bumblebees seem to forage within a few hundred meters of their hive (Osborne et al., 1999; Walther-Hellwig & Frankl, 2000). This small foraging range may be explained by the fact that bees repeatedly return to patches over several days (Heinrich, 1976; Comba, 1999; Walther-Hellwig & Frankl, 2000; Osborne &

Williams, 2001). Site fidelity should allow bees to gain information about rewarding plants within a patch. Indeed, more bees forage and repeatedly return to the most rewarding plants in a patch (Cartar, 2004), indicating that they can detect differences in reward level between plants and can learn their spatial positions (Manning, 1956; Thomson et al., 1982; Williams & Thomson, 1998). Repeatedly returning to the same patch should also reduce the possibility of getting lost as bees can rely on landmarks as cues to aid travel between the patch and hive (Chittka et al., 1995; Collett & Collett, 2002; Collett et al., 2003).

Familiarity with specific plants allows bees to learn the position of rewarding flowers and their temporal pattern of nectar and pollen release. Bees that repeatedly return to particular plants are more effective at extracting reward from flowers within the plant (Williams & Thomson, 1998), suggesting they may learn the locations of rewarding flowers. Once bumblebees have identified rewarding plants, they begin to consistently return to them and eventually, we suspect, link them in a circuit or trapline. 'Traplining' is defined as visiting the same food sources in a stable repeatable order (Manning, 1956; Thomson et al., 1982; Williams & Thomson, 1998; Comba, 1999; Makino & Sakai, 2004). We expect that this type of behaviour will only happen once a bee is familiar with the spatial locations of its favoured rewarding plants. We still do not know what rules bees may use to link their favoured plants in traplines or what advantages this behaviour gives to foraging bees.

We know very little about the innate foraging strategies that inexperienced bees use to forage *within* a plant. Thus, we cannot speculate about what these bees do once they have experienced rewarding visits on their first ever foraging trips. However, we do know how experienced bees behave when they forage within a plant. One flower inflorescence may contain a dozen or more open flowers (Frankie & Vinson, 1977; Thomson, 1982), and plants usually have more than one inflorescence. In this situation, one very important factor comes into play: revisitation of emptied flowers. When bees lose their directionality in near-far search behaviour (Pyke, 1978; Thomson et al., 1982; Schmid-Hempel, 1984; Cartar & Real, 1997; Cartar, 2004; Gegear & Thomson, 2004), they are more likely to revisit flowers within that plant (Pyke, 1978). Aggregated floral displays (Cresswell, 2000) and variable distance between flowers (Pleasants & Zimmerman, 1979; Hodges & Miller, 1981) also cause

increased revisitation. If a bee does not avoid revisits, it may leave the plant too soon, not because the plant is no longer rewarding but because the bee keeps revisiting flowers it has already emptied. Bees have three strategies to cope with this problem.

The first is 'scent marking'. Bumblebees leave behind scent marks on flowers they have visited, which they use to avoid revisiting emptied flowers (Williams, 1998; Stout & Goulson, 2001; Gawleta et al., 2005; Reader et al., 2005). This strategy allows the bees to track their movements within a plant. Indeed, studies on honeybees have found that they are more likely to reject flowers scent marked by themselves than by hive mates (Giurfa, 1993). However, we do not know if these scent marks are evolved signals for foraging or if bees are learning to use cues that increase their foraging efficiency. The second is, once again, traplining. This behaviour has been shown, for example, when bees forage on vertical inflorescences, where they will move either up or down the inflorescence (Corbet et al., 1981; Haynes & Mesler, 1984). The exact direction is thought to depend on the direction the bee needs to face to access the nectar, but the behaviour is expected to result in reduced revisits within the plant (Corbet et al., 1981). In plants where flowers are not arranged on inflorescences bees will repeatedly visit particular flowers (Manning, 1956), and traplining on flowers has been reported in other bee species (Schlindwein & Wittman, 1997).

The third is a widely cited but poorly understood foraging strategy, the 'near-neighbour rule'. This strategy involves movement to the flower that is closest to the one just visited. This, as with traplining, occurs when bees move between flowers (Zimmerman, 1981) and between plants (Hodges & Miller, 1981; Dreisig, 1995; Cresswell, 2000; Makino & Sakai, 2004). If bees are visiting near neighbour flowers while maintaining directionality, then we might expect this strategy to reduce revisitation. It is interesting to note that both traplining and near-neighbour moves are used at the floral and plant spatial scale, suggesting that bees may use the same foraging strategies at more than one scale.

It is not clear when bees start to specialise on certain flower species i.e. exhibit 'flower constancy'. Flower constancy involves experienced foragers predominantly foraging on one flower species while visiting another one less often. This behaviour was first called 'majoring' and 'minoring' (Heinrich,

1979b). Studies on this foraging strategy have generally investigated experienced foragers (Chittka et al., 1997; Keasar et al., 2002; Gegear & Lavery, 2004; Gegear & Lavery, 2005). This behaviour has caught the attention of investigators because bees ignore other potentially rewarding flower species, causing seemingly inefficient behaviours such as increased travel distance between plants (Heinrich, 1979b; Chittka et al., 1997; Keasar et al., 2002; Gegear & Lavery, 2004; Gegear & Lavery, 2005). Flower constancy has been considered to result from cognitive constraints. By specialising on one or two flower species the bee can recall how to manipulate them more effectively, thereby saving time and energy. However, this idea does not agree well with the empirical data. The time cost for an experienced bee switching between flowers is minimal (Lavery, 1994b). Interestingly, bees are more likely to switch flower species if the flowers are similar in colour and morphology (Chittka et al., 1997). This suggests that cognitive constraints on visual and motor pattern memory may dictate how many species bees can use at one time. Indeed, bees were more constant when plants differed in more than one trait (e.g. colour, size and motor pattern) (Gegear & Lavery, 2005). When bees sample alternatives in the laboratory, they are less likely to switch from a rewarding plant species when its relative reward is significantly higher than the alternatives (Keasar et al., 2002; Gegear & Thomson, 2004), and when artificial flowers are farther apart (Gegear & Thomson, 2004).

There are other potential advantages, not often considered by researchers, which may cause a bee to restrict its visits to one or two flower species. Flowering plants differ in their temporal release of nectar (e.g. morning or afternoon) (Kakutani et al., 1989), and nectar production rate can also differ with flower age (Manning, 1956; Cruzan et al., 1988). Flowers can differ in nectar production rate and location of nectar in male and female flowers (Devlin et al., 1987; Willson & Agren, 1989). Many flowering plants undergo visual or chemical changes when they are pollinated and no longer produce nectar (Weiss, 1991; Schiestl et al., 1997; Negre et al., 2003). A bee acquainted with these intricate cues can adjust its foraging pattern to the particular plant species, and learn to identify the most rewarding flowers. In summary, it would be very difficult for bees to keep track of all these factors for more than a handful of species.

The innate biases and tactics of inexperienced bees allow them to locate rewarding flowers. However, the exact patches, plants and flowers these bees ultimately specialise on are a result of where each of them finds floral rewards. This explains why there are differences between individuals in where they apply their foraging strategies and to what degree they use them (Brian, 1952; Heinrich, 1976; Keasar et al., 1996; Thomson, 1996; Gegear & Lavery, 2004; Makino & Sakai, 2004).

This thesis will focus on two of the foraging strategies described above: scent marking and traplining. I will be investigating the role experience plays in the use of these foraging strategies. I will now move on to a brief description of our state of knowledge for these two strategies.

1.2.2 Scent Marking

Cameron (1981) was the first to report that bumblebees leave behind and use scent marks on flowers. She found that *Bombus vosnesenskii*, a North American bumblebee, left chemicals on rewarding flowers. These chemicals dissolved best in pentane and hexane, suggesting that the compounds involved were non-polar substances. A second report, nine years later, by Schmitt & Bertsch (1990) confirmed similar findings in the European bumblebee species, *Bombus terrestris*. Their findings showed that these bumblebees mark rewarding flowers with a non-volatile substance whose effects can last up to 20 hours. In a later study, Schmitt et al. (1991) showed similarities between the chemical compounds left on the rewarding flowers and those found inside the tarsal gland. (The tarsal gland is located on the fifth tarsomere on each of the bee's legs). They proposed that bees scent mark flowers with secretions from this gland. As these 'attractive' scent marks have only been described in the laboratory, we do not know how bees would use them in the field.

Another group of scent marks were suspected by other authors conducting field experiments in the early 1980s (Corbet et al., 1984; Wetherwax, 1986; Kato, 1988). These scent marks caused bees to reject flowers, and were eventually referred to as 'repellent' scent marks. It was not until the late 1990s that experimental evidence emerged to confirm this observation. Stout et al. (1998) and Goulson et al. (1998) were able to show the existence of scent marks on previously emptied flowers. They also showed that these scent marks were used by conspecific and heterospecific bumblebees

foraging on the same flowers. A parallel study by Williams (1998) found similar scent marks used by honeybees and bumblebees. There is contradicting evidence on the extent to which honeybees and bumblebees rely on each others' scent marks (Williams, 1998; Stout & Goulson, 2001). Recently, evidence has emerged that bumblebees use scent marks left by hoverflies (Reader et al., 2005) and the solitary bee species *Anthidium manicatum* (Gawleta et al., 2005). Thus, the use of the repellent scent marks by bumblebees is widespread in the genus *Bombus* and they are able to use scent marks left by insects in different orders. Investigations into the glandular source of this repellent scent mark showed that chemicals found in the tarsal gland elicited rejection behaviour comparable to flowers naturally scent marked by a visiting bee (Goulson et al., 2000). This means that the same chemicals are involved in eliciting the attractive and repellent effects of scent marks. It is not known what mechanism causes these opposite behaviours. I will discuss this in more detail in Chapter 3.

Once flowers have been emptied by an insect, they re-fill with nectar if unvisited for a period of time. The exact rate at which they re-fill depends on the nectar secretion rate, which differs between plants species (Lavery, 1980; Stout & Goulson, 2002). It, therefore, makes sense for the bees to either ignore the scent marks, or for the activity of the scent marks to cease, once the flower has refilled with sufficient reward. Investigating the time it takes for a flower to be accepted after it is scent marked should give us an indication of the longevity of the scent marks. Williams (1998) proposes a longevity of 37 seconds. This value differs drastically from that reported by Stout et al. (1998) who found that the scent marks' longevity was 20 minutes. In a later study, Stout & Goulson (2002) claimed that bumblebees are able to learn to adjust their reliance on the scent mark depending on the nectar replenishment rate of the flower species. They also demonstrate that the repellent effect can last up to 24 hours.

The fact that the repellent scent mark can still be active at 20 minutes or even 24 hours suggests that the bees are relying on relatively non-volatile chemicals to make their decisions. A similar argument can be made with the attractive scent marks, where an activity of 20 hours has been reported (Schmitt & Bertsch, 1990). The smallest molecular weight substance found in the tarsal gland and scent marks deposited on flowers so far is C₁₉, which has a boiling point of 330 °C and a vapour pressure of 1mmHg at 133 °C. As a comparison,

water has a boiling point of 100 °C and a vapour pressure of 17.5 mmHg at 20 °C. (Values obtained from Material Safety Data Sheets). Therefore, it seems that the molecules that make up the scent marks are relatively non-volatile and exist in both the attractive and repellent scent marks.

Schmitt et al. (1991) found that, in *B. terrestris*, the compounds left on rewarding flowers and the compounds in the tarsal gland were straight chain hydrocarbons ranging from C19-C31. A large number of these compounds were alkenes (one double bond in molecule), followed by alkanes and alkadienes (two double bonds in the molecule). However, the alkenes, although numerous in number, were each present in small amounts. It is the alkanes that were present in the biggest amounts. This result is confirmed to an extent by Goulson et al. (2000), who found that the tarsal gland of *B. terrestris* contained alkanes and alkenes ranging from C21-C29. These authors do not determine the exact locations of the double bonds for alkenes with the same chain length, and they do not report the presence of alkadienes.

Experiments with synthetic compounds, to determine what chemicals or chemical classes (i.e. alkanes, alkenes or alkadienes) are behaviourally active, found that high concentrations (i.e. 100 µg) of alkane and alkene mixtures induced rejection behaviours in bees that previously accepted flowers in response to the scent marks (Schmitt et al., 1991). Experiments on the repellent scent mark found that C23 > C21 > C25 > C27 elicited a rejection response in decreasing order compared to a pentane control (Goulson et al., 2000). The attractive and repellent effects are not caused by a difference in concentration, where, for example, high concentrations cause rejection while low concentrations cause acceptance. Bees foraging on natural flowers found the scent mark repellent whether in high or low concentrations (Goulson et al., 2000). It also seems that bees respond to most of the chemicals in the scent marks if not individually (e.g. 9-tricosene), then when present in a mixture of similar compounds (e.g. 9-alkenes) (Schmitt et al., 1991; Goulson et al., 2000). How the same chemicals can cause two opposite behaviours remains to be determined. The chemical compositions of the compounds in the tarsal glands of several bumblebee species have been characterized. The identities of the chemicals are very similar in *B. pascuorum*, *B. hortorum*, *B. terrestris* and *B. lapidarius*. There are, however, species specific differences in the relative amounts of these compounds (Schmitt et al., 1991; Goulson et al., 2000; Eltz,

2006). Recently, it has been shown that a single bee visit by *B. pascuorum* leaves behind chemicals that are distinguishable from the chemical profile of some flower species. These chemicals are alkenes with chain lengths of C25, C27, C29 and C31 (Eltz, 2006).

Numerous speculations have been communicated regarding the adaptive role attractive and repellent scent marks play to foraging bumblebees. Most researchers agree that the benefit of using the scent marks on natural flowers is to reduce the time and energy spent handling empty flowers. A bee can handle a flower every few seconds (Lavery, 1994b; Chittka et al., 1997), and will forage on hundreds of flowers per bout (Ribbands, 1949), therefore the reliance on scent marks to avoid handling empty flowers can greatly improve a bee's foraging efficiency; especially if the scent marks allow it to track the movement of other flower visiting insects as well as its own movements. Indeed, preliminary evidence does support the idea that scent marks can improve a bee's foraging efficiency (Schmitt & Bertsch, 1990; Giurfa & Núñez, 1992; Stout et al., 1998). However, what exact role these chemicals play and how or why they evolved is still uncertain (Schmitt et al., 1991; Stout & Goulson, 2002; Gawleta et al., 2005; Reader et al., 2005; Eltz, 2006).

The main areas of debate within this topic are: 1) how the same group of chemicals can cause both attractive and repellent effects on foraging bumblebees; 2) whether these scent marks evolved to signal food profitability or are by-products of another signalling system or body function; 3) can bees adjust their reliance on the scent mark through learning. The first part of my PhD thesis (Chapters 2-4) will attempt to answer these three questions.

1.2.3 Traplining

Anecdotal reports of bumblebees traplining between different plants have been around for decades (e.g. Manning, 1956; Heinrich, 1976; Heinrich, 1979c; Thomson et al., 1982). However, it was not until the late 1990s that researchers began to study it empirically. We still know very little about this foraging strategy.

Traplines are characterised by repeated visits to the same plants in a predictable order. These plants may belong to different species (e.g. Thomson et al., 1982). The traplines are usually unidirectional circuits that are repeated several times per foraging trip (Thomson et al., 1997; Comba, 1999). These

rounds are not perfect repetitions of each other, but traplines are more similar within a foraging trip than between foraging trips (Thomson et al., 1997; Makino & Sakai, 2004). Monitoring these gradual changes in traplines over successive trips suggests that they result from changes in plant reward status. For example, removing competitors from adjacent patches caused bumblebees to change their traplines within a few hours (Thomson et al., 1987), and traplining bees tolerated a reduction of 10-20 % of open flowers on their favoured plants but reductions of 40-70 % caused these bees to forage on new plants (Comba, 1999).

We do not know how traplines are formed. However, once formed, they can last several weeks and can cover an area of at least 312 m² (Heinrich, 1976; Comba, 1999). The size of the traplines seems to depend on the flower density of the foraging patch. Larger traplines (i.e. traplines that cover a greater area) are found in less dense areas and vice versa (Comba, 1999). If certain plants within a trapline are bagged, thereby rendering them unrewarding, bees will continue returning to these plants (Comba, 1999). This behaviour suggests that it takes time for bees to remove a plant entirely from its 'trapline memory'. This effect has been named trapline holdover (Thomson, 1996).

Traplining bees return to their favoured plants at regular intervals. These returns do not coincide with the presence of high reward. In fact, traplining bees were just as likely as 'non-resident' bees to return when the plant was not very rewarding (Williams & Thomson, 1998). Nonetheless, trapliners are believed to extract more reward because they visit more flowers on a plant and, more importantly, can locate the rewarding flowers on a plant (Williams & Thomson, 1998). It is not clear how traplining bees identify the rewarding flowers, because non-resident bees are also capable of using scent marks on unrewarding flowers (Williams & Thomson, 1998). It is possible that they learn the position of the bonanza or frequently overlooked flowers.

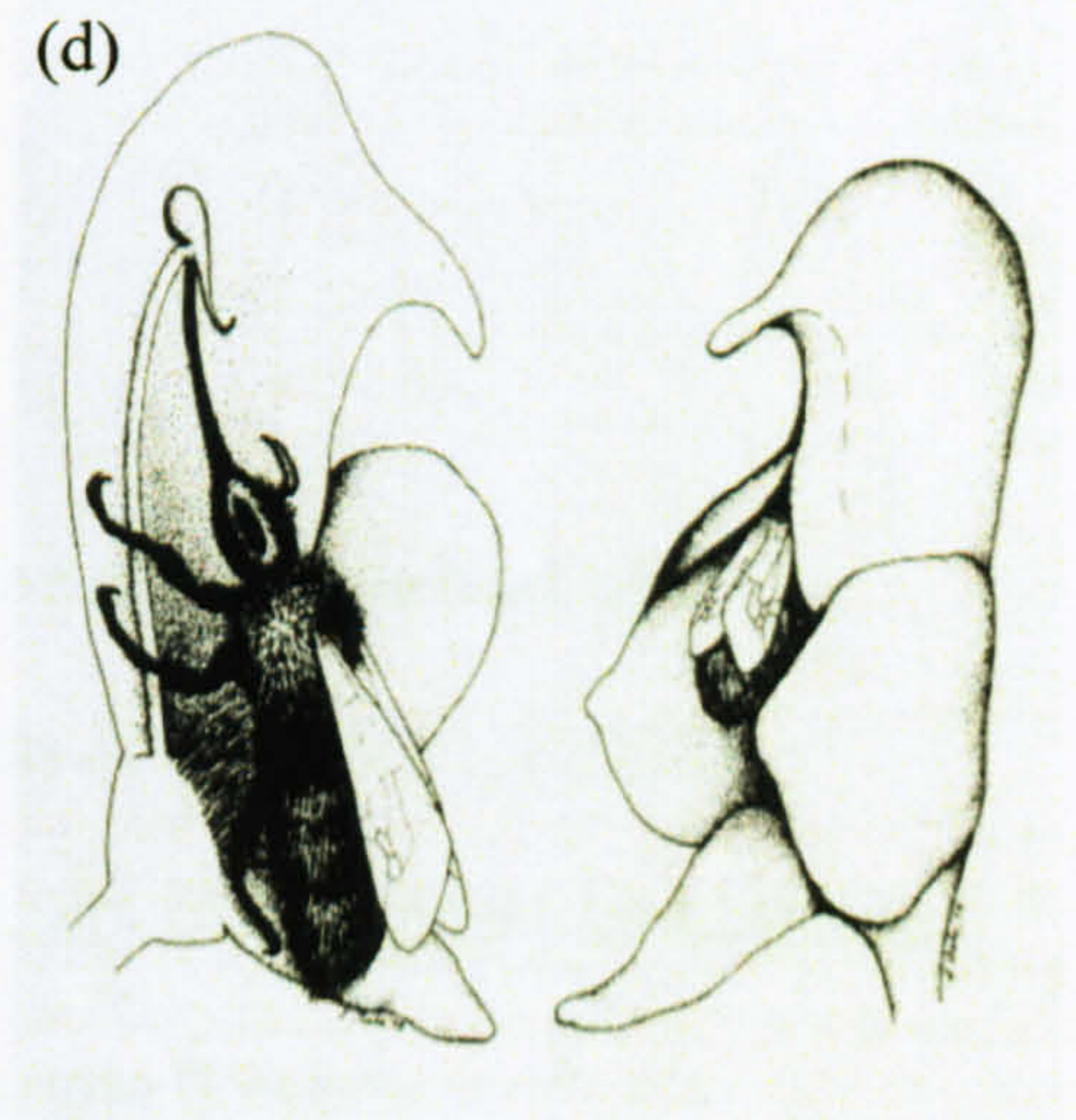
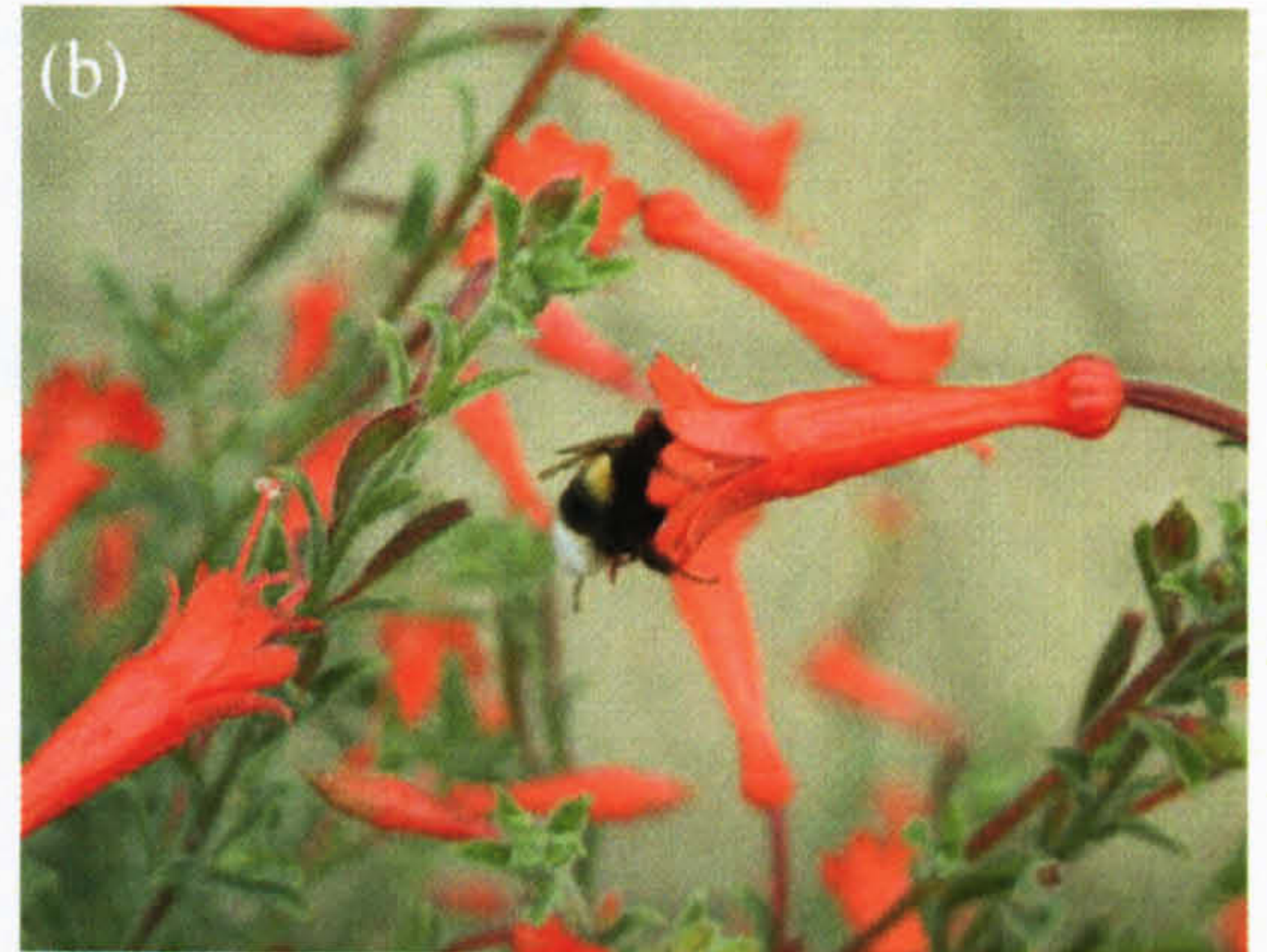
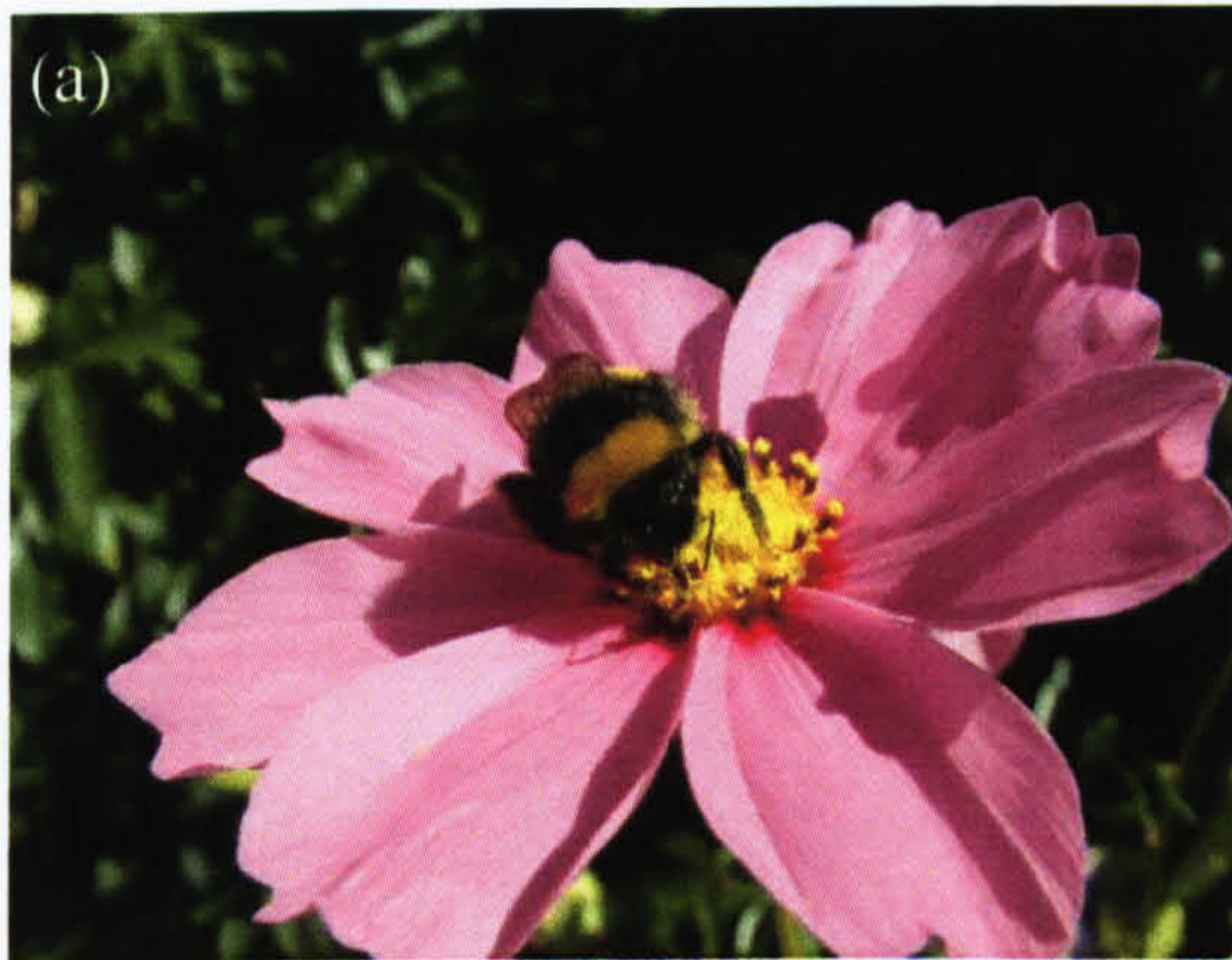
Regular visits also result in regular depletion of flowers, thereby making the plant less attractive to other foragers. Indeed, in one study, 57% of visits to one plant were made by 4 regularly returning bees (Williams & Thomson, 1998). Even when several bees trapline on the same plant, they differ in the degree with which they rely on it (Thomson et al., 1987; Makino & Sakai, 2004). Therefore, the bees' behaviour may in effect result in niche partitioning among

foragers within a patch. This remains to be shown empirically. We still have not clearly demonstrated the advantages this strategy gives a foraging bee.

In summary, we know that traplining between plants exists, changes in a plant's reward status probably cause gradual changes in traplines and different bees use different plants to different degrees. However, there are still many questions that remain unanswered. For example, how do bees develop their traplines and, more importantly, what advantages does this behaviour give a foraging bee. I will address these two questions in Chapter 4.

During the course of my PhD I discovered that bees are able to distinguish between water and sucrose solutions without direct antennal or proboscis contact. This result contradicts a widespread assumption among researchers that bees can only perform this feat through contact chemoreception. As a result, water is used to control for remote effects of sucrose. These findings will not only provide us with information on the sensory capabilities of these animals, they will have a large consequence on our current experimental practices. I present my preliminary discoveries in Chapter 5. I intend to pursue further research on this finding in the future.

FIGURE 1.1 (a) Mexican Aster (*Cosmos bipinnatus*): simple, composite flower. (b) *Penstemon* spp.: complex flower, the nectar is presented at the bottom of a narrow corolla tube. (c) Snapdragon (*Antirrhinumcorolla* spp.): complex flower, the corolla tube is hidden by petals. (d) European monkshood (*Aconitum napellus*): complex flower, the nectar is present in an unusual location. The bee needs to insert its proboscis in the top (hood) of the flower to locate the nectar [copied from Laverly (1980)]. Figures used with permission from the owner and publisher.



FACULTATIVE USE OF SCENT MARKS I:

Flower handling time

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Facultative use of repellent scent mark in foraging bumblebees:

complex versus simple flowers.

Animal Behaviour 71:847-854

2.1 ABSTRACT

Bumblebees leave scent marks on flowers to avoid recently depleted resources. Complex flowers require longer handling times and, when foraging on these flowers, bees show spatial foraging patterns that make revisitation more likely. We investigated if the bees' response to these scent marks is fixed or flexible, by using two artificial flowers that differed in handling time. Bees preferred foraging on short handling time flowers, but accepted both types. Bees were twice as likely to reject long handling time flowers that were marked, and the effect of scent marks lingered 60% longer in these flowers. We also determined how bees decide on rejecting a flower. Bees were able to reject flowers in flight. Therefore, they are able to make their decisions by relying on visual and olfactory memory, not motor pattern.

2.2 INTRODUCTION

Scent marking of depleted renewable food sources can be advantageous to animals that repeatedly return to the same food sites (Kruuk, 1992). By placing chemical markers on these temporarily unrewarding resources, animals can ease demands on memory. The two animal groups best represented in the literature are bees (Williams, 1998; Goulson et al., 2000; Gawleta et al., 2005; Reader et al., 2005) and canines (Henry, 1977; Harrington, 1981; Harrington, 1982), although many other animals probably share this behaviour. Here, we ask if the bees' response to these scent marks is hard wired by investigating if bees use them facultatively when foraging on flowers with different handling times.

Bees scent mark depleted natural and artificial flowers with a scent. This scent helps them avoid revisiting flowers emptied by themselves (Giurfa, 1993; Williams & Poppy, 1997) and other insects (Stout et al., 1998; Williams, 1998; Gawleta et al., 2005; Reader et al., 2005). In theory, we expect bees to avoid all scent-marked flowers. However, there are conditions when a strict rejection of all scent-marked flowers may be not be necessary. Firstly, bees sometimes leave nectar behind in flowers (Hodges & Wolf, 1981; Wetherwax, 1986). Most individuals may find extracting this nectar more costly than moving to the next flower. However, as flower visiting insects differ in body size, handling ability, and metabolic needs, some individuals/species, may find this nectar valuable. Secondly, grooming bees or bees collecting pollen may accidentally mark flowers containing nectar (Stout et al., 1998). A nectar forager relying on all scent marks would reject these rewarding flowers. Handling costs can differ by a factor of 10 between simple and complex flowers (Lavery, 1994b; Ohashi, 2002). Therefore, we expect bees to inspect scent-marked flowers when visits to these flowers do not cost more energy than that to be gained from the nectar. This may be the case with quickly handled flowers, such as buttercups, but is less likely to be true for flowers with longer handling times, such as snapdragons.

Another factor that may influence the facultative reliance on scent marks is the risk of revisitation. When bees encounter rewarding flowers, they lose their directionality (Pyke, 1978; Thomson et al., 1982; Schmid-Hempel, 1984; Cartar & Real, 1997; Cartar, 2004; Gegear & Thomson, 2004). Although this loss of directionality increases the probability of remaining in a rewarding patch, it also

increases the risk of revisitation (Pyke, 1978). This increased revisitation may be more costly, in terms of time and energy, when bees forage from long handling time flowers. Therefore, we expect bees to respond more strongly to scent marks on these flowers.

There is suggestive evidence that bees can facultatively rely on scent marks in the field (Goulson et al., 2001). However, given how difficult it is to manipulate handling time while keeping all other factors equal in the field, a controlled laboratory experiment is needed to confirm these observations.

2.3 MATERIALS AND METHODS

2.3.1 Test animals and flight arenas

Colonies of *Bombus impatiens* were obtained from Biobest Canada Ltd. (Leamington, Ontario, Canada). Each colony was connected to a flight arena by means of a transparent Plexiglas tube. This tube contained moveable plastic flaps (henceforth “doors”) to allow only selected individual foragers into the flight arena. Approximately 8g of pollen were placed directly into the nest everyday. Tests were conducted at a temperature of $\approx 20^{\circ}$ C and a light dark regime of 10:14 light-dark cycle.

Two flight arenas were used in parallel for all experiments. In one arena (henceforth, the scent-marking arena), bees foraged from artificial flowers freely, and in the process left scent marks. Flowers with these scent marks were then offered to test foragers in another arena (henceforth, the test arena), and their responses monitored. In the course of the experiments, a variety of flight arenas were used, with sizes of 100 (L) x 40 (S) x 70 (H) cm and 75 x 75 x 75 cm, 105 x 72 x 30 cm and 103 x 71x 30 cm. The flower array presented to bees, however, was identical in all cases (see below). A green Bristol board was taped to the entire floor of each arena to mimic natural foliage.

2.3.2 Artificial flowers

Two flowers types were designed from 5-mL Polypropylene Round-Bottom Tubes (12 x 75 mm style; Falcon, Becton Dickinson Labware, New Jersey, USA). The length of the flowers was adjusted so they differed in handling time. The short handling time flowers were 2 cm long (henceforth called short flowers). The ‘long’ handling time flowers were 7.5 cm long (henceforth called

long flowers). Each flower was inserted into a light blue extruded Styrofoam block (2.5 x 2.5 x 2 cm), which allowed them to stand upright. The long flower penetrated the Styrofoam block at a 45° angle to facilitate access for the bees. Each flower had a white filter paper collar of 2.4 cm diameter (3MM Qualitative, Whatman, W & R Balston Ltd., England) (see Figure 2.1). Bees foraged on 3 short and 3 long flowers spaced 15 cm apart (see Figure 2.1) and held in place with Velcro. The flower array was placed on a piece of green cardboard (20 x 32 cm), henceforth called a tray.

In preliminary trials, we determined the mean handling time of both flower types when empty. The long flowers took ca. 4 times longer to handle [1.66 ± 0.014 s (mean \pm standard error) and 6.50 ± 0.29 s for short and long flowers; $n = 17$ bees in both cases]. Thus, our artificial flower types were well suited to explore the question of whether bees respond flexibly to scent marks on flowers that differ in handling time. All experiments were videotaped and all bees foraged from both flower types.

2.3.3 Experimental procedures

2.3.3.1 Training: Familiarisation with setup

It is unknown whether the response of bees to scent marks on flowers is innate or learned. Therefore, we introduced an experimental phase to familiarise bees with the experimental setup and, if necessary, make the appropriate associations between empty flowers and scent marks. A forager in the test arena foraged on 3 short and 3 long flowers that were refilled between, but not within, bouts. Each flower initially contained 13 μ l of 30 % (w/w) sucrose solution and its position randomly changed between bouts, to exclude the possibility of spatial learning. The test bee foraged on this setup for 15 bouts then her association was tested.

2.3.3.2 Experiment 1: Effect of handling time on interpretation of scent marks

In experiment 1, one flower was scent marked and randomly compared to an unmarked flower of the same type within the array (see Figure. 2.1b). These 'test' flowers were either both long or both short. All flowers were brand new, touched only by powder-free latex gloved hands (SafeSkin PFE, Kimberly-Clark

Worldwide Inc., Roswell, USA), and contained 13 μ l of 30% (w/w) sucrose solution.

The test forager was kept outside the test arena until a scent-marked flower was ready. Meanwhile, live bees were used to scent mark the test flower. During the test bee's training, these bees were foraging *ad libitum* in the scent marking arena from one long and one short flower. To mark a test flower, we filled it with 13 μ l sucrose solution and placed it in the scent marking arena. The scent marking bees marked the flower as they fed on it. Once the flower was scent marked, we immediately added 13 μ l of sucrose solution, placed it on the tray, and put the tray into the test arena. The test forager was then allowed to enter the experimental arena. We repeated this procedure six times for each bee, 3 times with both flower types. We discarded flowers where the dispenser tip touched the inside of the flower to prevent bees from relying on residual sucrose to make their decisions. Twenty bees were each tested once.

2.3.3.3. Experiment 2: Do bees remember handling cost of flower type?

If bees facultatively use scent marks on flowers, then there are two possibilities we need to consider. A larger amount of scent marks may have been deposited on the long flowers, so bees may have been using the difference in the amount of scent marks. Alternatively, it is possible they remember the handling costs of the flowers. To tease these possibilities apart, only the filter paper collars were scent marked. With this procedure, bees were confronted with flowers of unequal handling time, but paired with (on average) equal amounts of scent marks.

After the test foragers 15th bout she was held between the two doors while the filter paper was collected. Bees of the scent-marking colony foraged continuously from six artificial flowers. These flowers were composed of 2cm test tubes (same design as short flowers). A filter paper collar was placed around the top of these test tubes. The flowers dispensed sucrose at 1.2 μ l/minute through a syringe needle by means of a motor. After one or more bees landed and probed the flowers in the scent-marking arena, the filter paper was removed using forceps. Bees were never forced off the flowers to ensure that they did not leave an alarm pheromone or distress signal that may have disrupted the experiment. When filter paper collars were placed on all test

flowers the tray was moved to the test arena and the experimental bee released. While she was foraging, a new set of filter paper was placed on the flowers in the scent-marking arena. Experimenters wore gloves throughout the test phase. Flowers were randomised with respect to position.

In the test colony, bees underwent the same training as described above, and then were tested with four unrewarding marked flowers (two long and two short). Each test trial lasted 5 minutes, but was terminated prematurely if the experimental bee did not interact with the flowers more than once in one minute. This was to avoid loss of foraging motivation or the development of a different association with the scent marks. In between test bouts, bees were allowed two non-test bouts. These bouts consisted of four rewarded unmarked flowers (two of each type). Five test sessions were conducted for each bee. Ten bees were each tested once.

2.3.4 Data analysis

To ensure that bees did not rely on spatial memory *within* bouts, we only analysed the bees' *first approach* (i.e. interaction) to the test flowers in each bout (unless otherwise stated). We investigated two behaviours: a) Acceptance and b) Hovering (see Legend for definitions). We also measured the time to first acceptance to determine if bees took longer to accept long marked flowers. We only analysed data from test, not training, bouts.

As the data were not normally distributed, we used Wilcoxon two-sample tests to evaluate the bees' responses. In Experiment 1, we compared the number of acceptances towards marked and unmarked flowers regardless of flower type to decide if bees were indeed relying on scent marks in our setup. Then, we investigated the acceptance of marked and unmarked long and short flowers. This was done for both Experiment 1 and 2. Hovering behaviours were also investigated in Experiment 2 to determine if bees retrieve their memory of flower handling time in flight. This would tell us if bees relied on visual input or motor pattern (i.e. memory of body movements) to assess the handling time of the flower. We also investigated if bees preferred foraging on short flowers by comparing the total number of acceptance behaviours directed towards each flower type. We used data from Experiment 2 for this, but similar trends were observed in Experiment 1.

For Experiment 1, the time to first acceptance was calculated from the time the test flowers were placed in the test arena until the bee landed on them. For Experiment 2, the time to first acceptance was calculated from the moment the filter paper collar was placed on the test flowers. The manner of measuring time to acceptance in the Experiment 2 allowed greater accuracy because it measured the time elapsed since the last scent mark was deposited to when the bees accepted the flower. If bees did not accept a flower, then the time to acceptance was recorded from the beginning of the trial to the end of the bout. Note that this is conservative, since bees might have taken even longer to accept such flowers.

Time to acceptance for the two flower types was compared by taking the median time to acceptance for each flower type in the test bouts and using this median for Wilcoxon two-sample tests with bees as the unit of replication. In the results, values for behavioural and time data are given as median \pm standard error. The symbol n is the total number of bees used and s is seconds.

2.4 RESULTS

2.4.1 Experiment 1: Effect of handling time on interpretation of scent marks

Bees were more likely to accept unmarked flowers than scent-marked flowers (Wilcoxon two-sample test: $W= 1034.5$; $p < 0.00001$; $n=40$; Figure 2.2a) (2.0 ± 0.15 versus 3.0 ± 0.067 acceptances for marked and unmarked flowers respectively). There was no significant difference in acceptance between short and long unmarked flowers (Wilcoxon two-sample test: $W= 400$; $p < 0.72$; $n=20$; Figure 2.2a) (3.0 ± 0.10 versus 3.0 ± 0.09 acceptances for long and short flowers respectively), but there was a significant difference in acceptance of marked flowers depending on flower type. The short marked flowers were more likely to be accepted at first approach than the long marked flowers (Wilcoxon two-sample test: $W= 334.5$; $p < 0.034$; $n=20$; Figure 2.2a) (1.0 ± 0.20 versus 2.0 ± 0.21 acceptances for long and short flowers respectively).

Bees also took longer to accept marked flowers than unmarked flowers (139.4 ± 13.3 s and 58.7 ± 5.05 s for marked and unmarked flowers respectively; Figure 2.2b) (Wilcoxon two-sample test: $W= 2221.5$; $p < 0.00001$; $n=20$). There was no significant difference in time to acceptance between unmarked long and short flowers (61.7 ± 5.90 s and 55.9 ± 3.93 s for short and long flowers respectively) (Wilcoxon two-sample test: $W= 445.5$; $p < 0.34$; $n=20$).

However, there was a significant difference between time to acceptance of marked long and short flowers (Wilcoxon two-sample test: $W= 315$; $p < 0.01$; $n=20$). Short marked flowers were more likely to be accepted before long marked flowers (106.9 ± 10.86 s and 173.9 ± 11.14 s for short and long flowers respectively; Figure 2.2b).

2.4.2 Experiment 2: Do bees remember handling cost of flower type?

Here, we investigated if the facultative use of scent marks found in Experiment 1 was due to the presence of more scent marks on long flowers, or the memory of handling costs. Bees were still more likely to accept short flowers at first approach, when both flower types had equal amounts of scent marks (Wilcoxon two-sample test: $W= 70.5$; $p < 0.009$; $n=10$; Figure 2.3a) (1.0 ± 0.45 versus 3.5 ± 0.69 acceptances for long and short flowers respectively). Time to acceptance was also significantly higher for long flowers than for short flowers (Wilcoxon two-sample test: $W= 77.0$; $p < 0.037$, $n=10$) (267.1 ± 32.41 s for long and 191.8 ± 38.04 s for short flowers; Figure 2.3b).

Overall, short flowers received more approaches than long flowers (70.8 ± 7.75 short and 44.0 ± 7.00 visits for short and long respectively; Wilcoxon two-sample test: $W= 135.5$; $p < 0.023$, $n=10$).

2.4.3 Experiment 2: How bees remember handling cost of flower type?

If bees reject flowers in flight, then they remember the handling cost from the visual image and not the motor pattern. Bees were able to reject flowers while hovering. Long marked flowers were more likely to be rejected in flight before landing (Wilcoxon two-sample test: $W= 148.0$; $p < 0.0009$; $n= 10$; Figure 2.4) (2.0 ± 0.13 versus 0.0 ± 0.057 hoverings for marked and unmarked flowers respectively).

2.5 DISCUSSION

At first approach, bumblebees were more likely to rely on scent marks when flowers took longer to handle. They accepted fewer long marked flowers, and this acceptance took longer. This result is further confirmed by the fact that bees performed more hovering rejections towards long marked flowers. The ability to perform hovering rejection means bees did not need to perform a motor pattern to remember the handling costs of the flower types. Bees also

approached short flowers more frequently than long flowers. Thus, bees appear to save time and energy by selectively approaching short handling time flowers and selectively rejecting marked long handling time flowers.

In Experiment 1, test flowers were marked directly by foraging bees. As a result, short flowers, most likely, contained less scent. Therefore, the bees' facultative use of scent marks may have been due to the difference in the amount of scent marks on the two flower types. However, an alternative explanation is that the bees relied on memory of the flower handling time to make their distinction. Distinguishing between these two modes of memory retrieval will help give us insight into how sensory cues are stored in the bee's brain.

We find bees continued to reject more long flowers when both flower types contained similar amounts of scent. Therefore, bees relied on previously stored information about floral complexity, not the amount of scent marks on flowers. Investigating the mechanism by which bees do this reveals that they can perform these rejections in flight. Thus, the bees identify the flower type ("species") from visual cues, and this helps them retrieve the memory of its handling costs. With this, they combine the chemosensory information of whether scent marks are present. Such facultative use of scent marks would place no small demands on cognitive ability.

There may be many scenarios in which facultative reliance on scent marks supports adaptive foraging. Consider the motivational state associated with hunger or starvation. For example, hungry wasps maintain significantly higher proboscis extension responses to a conditioned odour without reward than well-fed wasps (Tertuliano et al., 2004), suggesting that hunger state can influence reliance on sensory cues. Bees foraging in a resource-poor, highly competitive environment (or from a starving colony) may want to probe most flowers they encounter. We may find, in these conditions of low food availability, hungry bees rely less on scent marks when foraging from both complex and simple flowers.

In summary, bees have versatile abilities to make associative memories depending on context (Giurfa, 2003). Our study has shown that bees rely on scent marks differently when they are found on flowers that differ in handling time. They are more likely to reject recently visited flowers with a long handling time. Bees foraging on simple flowers in the wild are expected either to revisit

more, or rely on other cues or behavioural tactics to avoid revisitation. Bees were also able to reject flowers without landing on them, indicating that they rely on a visual memory of the flower type to retrieve information of the handling time associated with flowers. They then use this information in conjunction with the presence of scent marks to decide whether or not to visit flowers. This complex use of information further underlines the complex behavioural abilities of bees.

2.6 ACKNOWLEDGEMENTS

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FIGURE 2.1 (a) Long flowers penetrated the Styrofoam at a 45° angle to facilitate access for the bees; short flowers were placed in a groove on top of a Styrofoam base to allow them to stand upright. (b) Tray used for training and testing bees. Underlined flowers indicate an example of flower array used in Experiment 1. In Experiment 2 the last row of flowers was not presented. L= long flower; S= short flower. The two lines at the top indicate the arena entrance. All flowers were randomly moved between bouts.

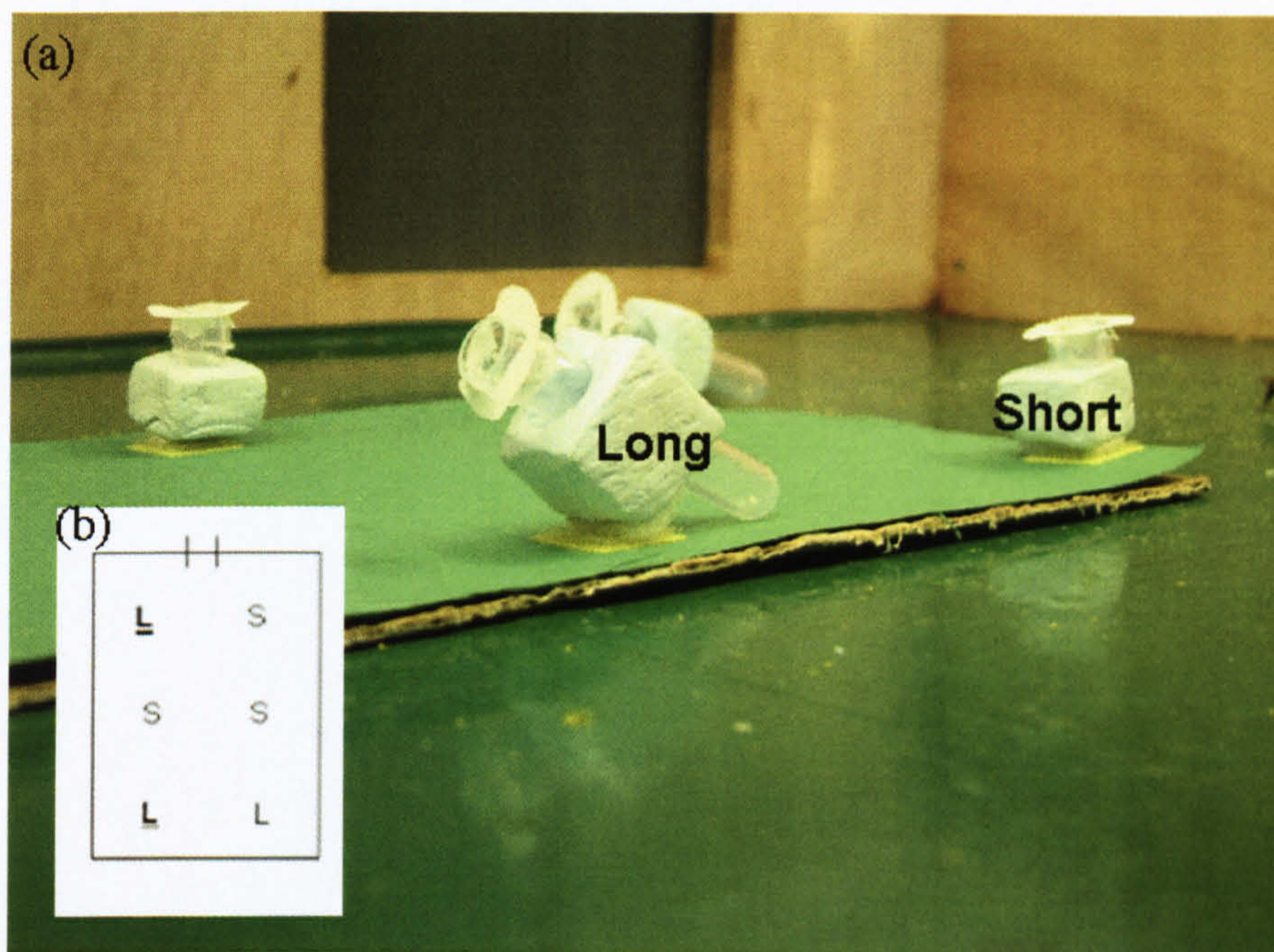


FIGURE 2.2 (a) Percentage of test flowers accepted by bees in Experiment 1. Marked long flowers were less likely to be accepted than marked short flowers. This difference was not present for unmarked flowers. (b) Time to first acceptance of flowers in Experiment 1. Long marked flowers took longer to accept than short marked flowers. This difference was not seen in unmarked flowers. Medians are the horizontal lines inside the boxes; the boxes indicate the interquartile range and the whiskers indicate the range of the data; n=20 for each bar.

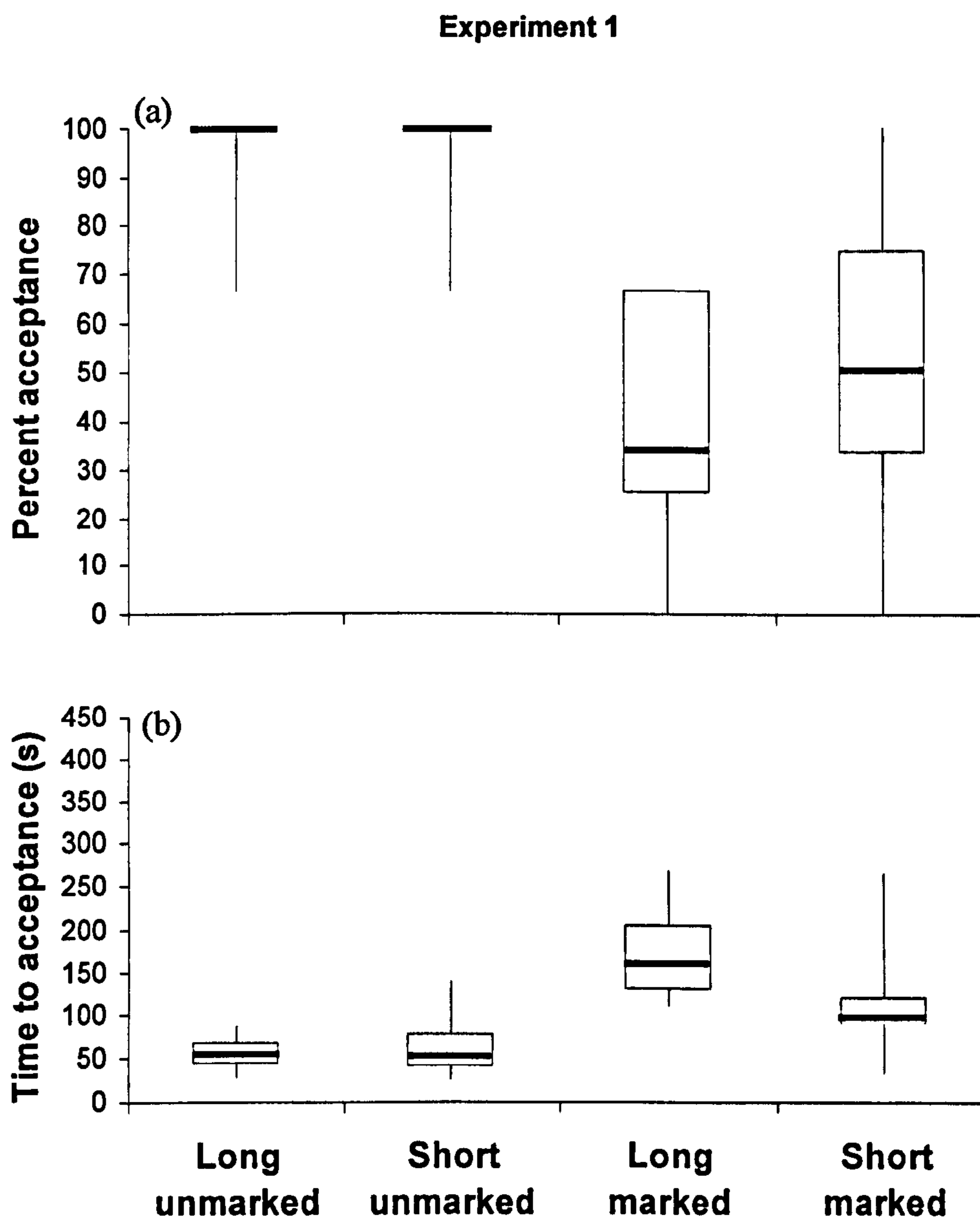


FIGURE 2.3 (a) Percentage of test flowers accepted by bees in Experiment 2. When flowers had similar amounts of scent mark, fewer marked long flowers continued to be accepted than marked short flowers. (b) Time to first acceptance of Experiment 2 test flowers. When flowers had similar amounts of scent mark, long marked flowers took longer to accept than short marked flowers. Medians are the horizontal lines inside the boxes; the boxes indicate the interquartile range and the whiskers indicate the range of the data; n=10 for each box.

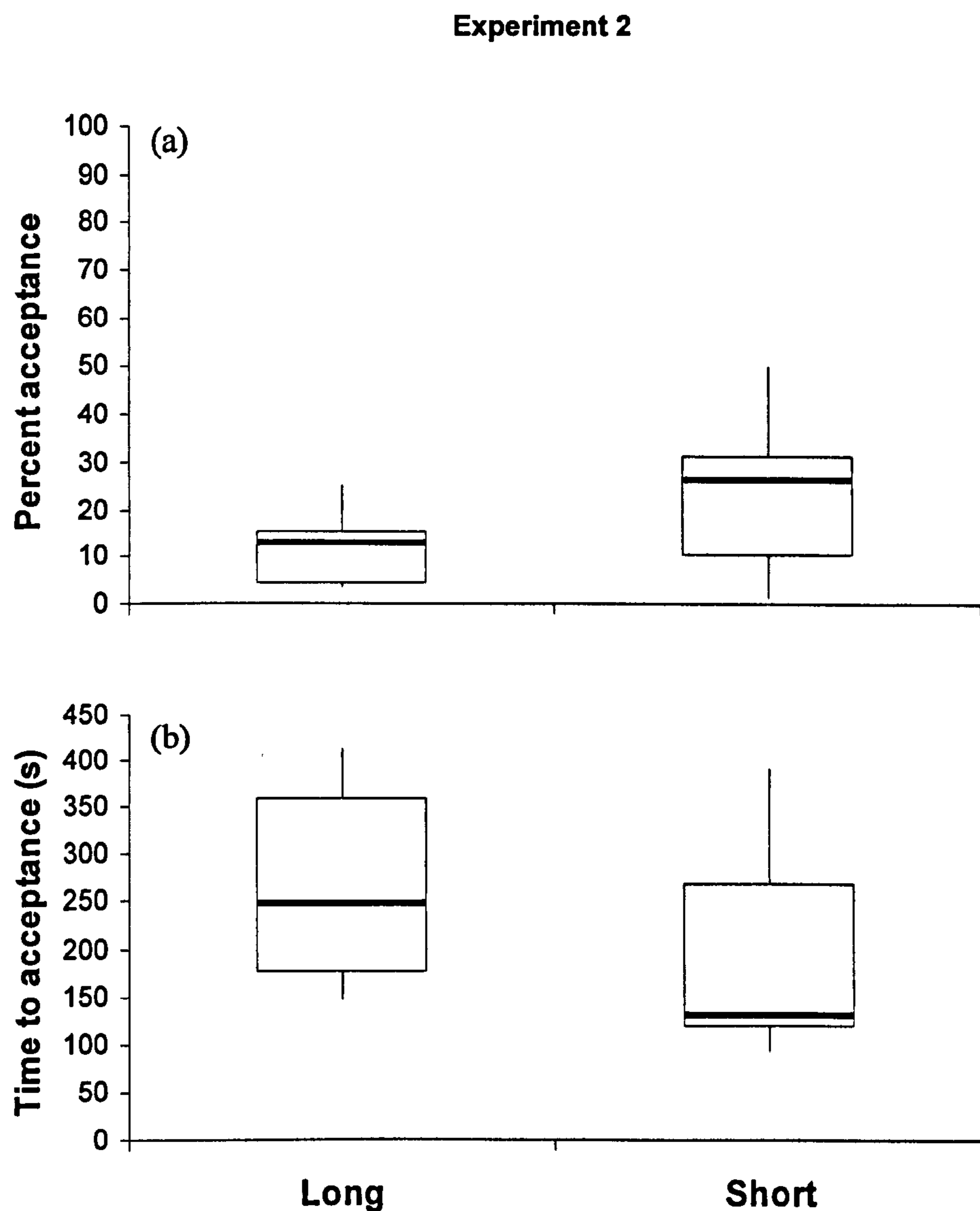
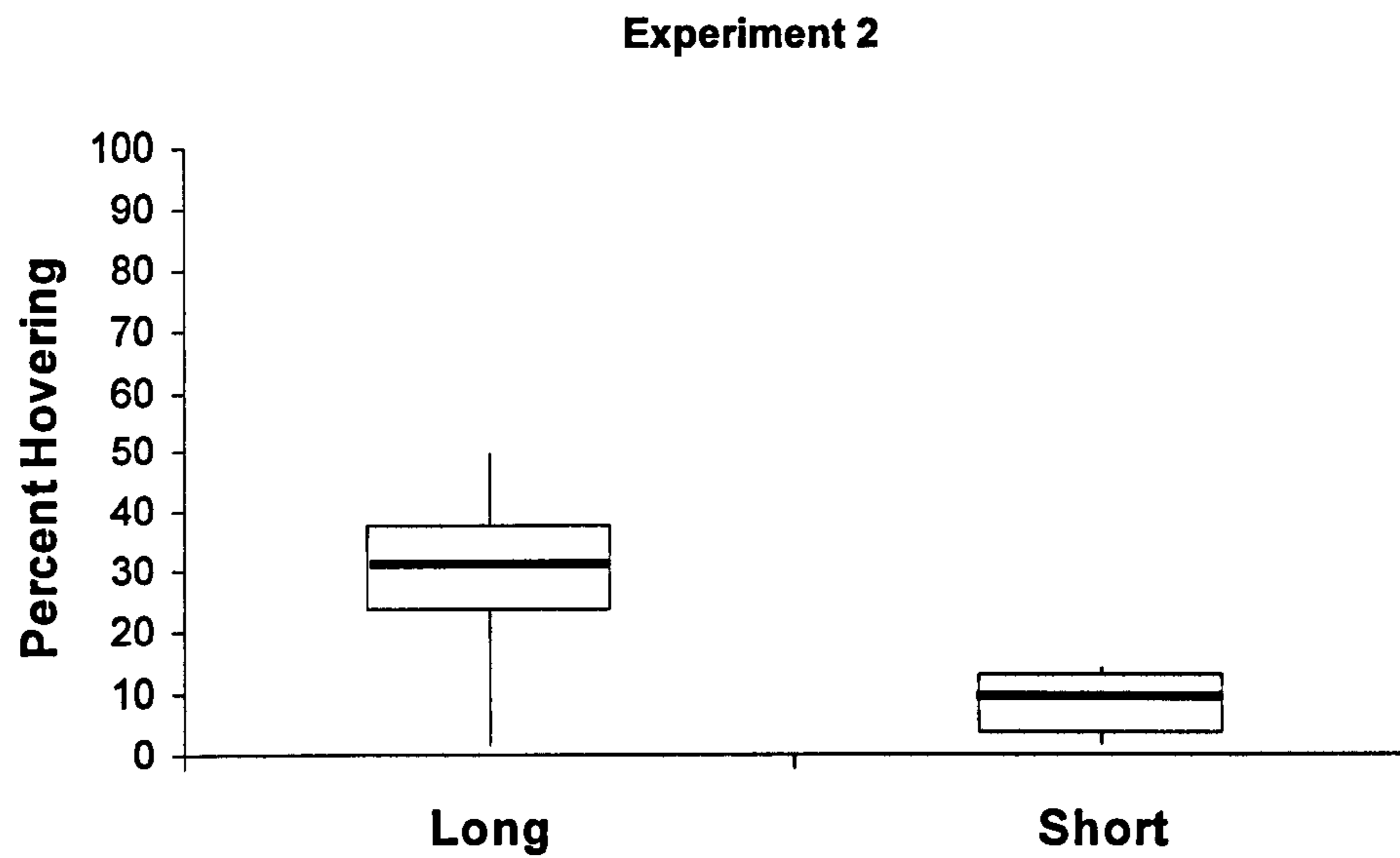


FIGURE 2.4 Percentage of hovering behaviours. Bees are able to reject flowers in-flight and were more likely to reject long flowers. Medians are the horizontal lines inside the boxes; the boxes indicate the interquartile range and the whiskers indicate the range of the data; n=10 for each box.



FACULTATIVE USE OF SCENT MARKS II:
Flower reward level

The results in this chapter are published as:

Saleh, N, & Chittka, L (2006)

The importance of experience in the interpretation of conspecific chemical signals.

Behavioral Ecology and Sociobiology 61: 215-220

3.1 ABSTRACT

Foraging bumblebees scent mark flowers with hydrocarbon secretions. Several studies have found these scent marks cause foraging bees to reject flowers. This is thought to minimise the risk of visiting recently depleted flowers. Other studies, however, have found a reverse, 'attractive' effect of scent marks left on flowers. Do bees mark flowers with different scents, or could the same scent be interpreted differently depending on the bees' previous experience with reward levels in flowers? We use a simple experimental design to investigate if the scent marks become attractive when bees forage on artificial flowers that remain rewarding upon the bees return after initial feeding. We contrast this with bees trained in the more natural scenario where revisits to recently emptied flowers are unrewarding. The bees' association between scent marks and reward value was tested with flowers scent marked from the same source. We find that the bees' experience with the flowers' reward level determines how the scent marks are interpreted: the same scent can promote and suppress landing and probing of flowers. How learning influences the interpretation of conspecific chemical marks has rarely been investigated.

3.2 INTRODUCTION

Chemical signals used in insect communication were once thought to trigger a mainly hard-wired, genetically predetermined response (Karlson & Luscher, 1959). We now know that the same chemical signals can elicit different behaviours in different contexts (Hölldobler & Wilson, 1990; Keeling et al., 2004). However, very few studies have differentiated between substances which have fixed meanings (e.g. through genetically hardwired responses) and those that can develop or change meanings through learning and experience. This distinction seems crucial if we are to understand what role chemical signals play in communication systems and how they evolved.

One behavioural context where the response to an insect chemical cue has been considered hard-wired is the scent marking of flowers by pollinating insects (Giurfa & Núñez, 1992; Stout et al., 1998; but see Stout & Goulson, 2002). Using scent marks to indicate a renewable resource's reward status is expected to reduce time and energy spent locating the food source as well as investigating emptied food sites (Giurfa & Núñez, 1992). Although this topic has mainly been explored in insects, such as bees and ants, it is probably widespread throughout the animal kingdom (Henry, 1977; Harrington, 1981).

Flowers generally offer small rewards, and bees need to forage on hundreds of flowers per foraging bout to fill up their honeycrop (Ribbands, 1949). Because nectar is a slowly replenishing resource, an obvious problem bees encounter is how to avoid revisiting flowers they already visited. Using spatial memory alone may not be sufficient given the enormous number of flowers they would need to remember, so instead bees use scent marks left by themselves and other visiting foragers to avoid visiting recently emptied flowers (Williams, 1998; Stout & Goulson, 2001; Gawleta et al., 2005; Reader et al., 2005). Relying on such marks has been shown to reduce time spent probing unprofitable artificial flowers (Giurfa & Núñez, 1992). Bees can adjust their reliance on scent marks depending on the flower handling time (Saleh et al., 2006), and nectar secretion rates of the flower species, suggesting that the meaning of the scent marks can be learned (Stout & Goulson, 2002).

In contrast to the 'repellent' effect found in several publications, some studies have found that the scent marks serve to promote landing and probing of flowers (Cameron, 1981; Schmitt & Bertsch, 1990). Several hypotheses have been suggested to explain these divergent observations. For example, Stout et

al. (1998) postulate that two types of marks with different chemistry may exist, which may come from different glands where at least one is actively secreted and controlled. This is highly unlikely to be the only possible explanation, because studies on bumblebees have shown that chemicals similar to tarsal gland secretions elicit both attractive and repellent properties (Schmitt et al., 1991; Goulson et al., 2000). Another conjecture made by both Giurfa and Núñez (1992) as well as Stout et al. (1998) is that for bumblebees and honeybees fresh scent marks may be repellent, but once the volatile compounds evaporate, the remaining non-volatile components become attractive. In bumblebees, this is unlikely to be applicable because Stout and Goulson (2002) found the repellent effects can last up to 24 hours. We also find that the bees rely differently on the scent marks depending on the handling time of the flower indicating that flowers carrying similar amounts of scent can elicit different degrees of repellent responses in bees (Saleh et al., 2006). A third explanation is that bees are simply interpreting the scent as attractive or repellent depending on their experience with the reward levels of the food source.

In bumblebees, studies that have found the scent marks to be attractive have been conducted in the laboratory where the reward levels were 1µl of 50 % sucrose per visit (Cameron, 1981; Schmitt & Bertsch, 1990). The bees received a reward each time they visited a scent marked flower. Studies that have found the scent marks to be repellent have been conducted in both the laboratory (Saleh et al., 2006) and the field (Stout & Goulson, 2001). The flowers in these studies were not rewarding upon immediate revisitation. This correlation is also found in honeybees. Studies that have found an attractive effect were carried out in the laboratory with either 50ml feeders (Free & Williams, 1983) or 200 µl feeders (Williams & Poppy, 1997). The repellent effect was found when bees foraged on natural flowers (Williams, 1998; Stout & Goulson, 2001; Reader et al., 2005) or on artificial flowers where immediate revisits were not rewarding due to low rates of sucrose solution secretion (Giurfa & Núñez, 1992). Therefore bees may interpret the scent marks as attractive when revisits yield rewards and as repellent when revisits do not yield rewards. We use bumblebees to investigate how the meaning of scent marks left on food sources can change depending on the reward levels at the food

source. We show that the same chemical mark can have opposite meanings and that this is directly attributed to learning.

3.3 MATERIALS AND METHODS

3.3.1 Test animals and flight arenas

Colonies of *Bombus terrestris dalmatinus* were obtained from Koppert Ltd. (The Netherlands). Each colony was connected to a flight arena [100 (L) x 40 (S) x 70 (H) cm] by a transparent Plexiglas tube. Moveable cardboard flaps within the tube allowed only selected individual foragers into the flight arena. Approximately 8g of pollen were placed directly into the nest every other day. Tests were conducted at a temperature of $\approx 20^{\circ}$ C and a light dark regime of 10:14 light-dark cycle. Green cardboard was taped onto the arena floor to mimic the green foliage background found in most natural situations of bees foraging from flowers.

3.3.2 Artificial flowers

Flowers were designed from 5-mL Polypropylene Round-Bottom Tubes (13 x 75 mm style; Plastiques Gosselin, Hazebrouck Cedex, France), which were 7.5 cm in length and 1.3 cm in diameter. Each flower was inserted into a brown wooden block (2.8 cm x 2.7 cm x 6 cm) at a 45° angle to facilitate access. A white filter paper collar of 3.0 cm diameter (3MM Qualitative, Whatman, W & R Balston Ltd., England) was placed around the top. (See Figure 3.1)

3.3.3 Experimental procedures

For the high reward treatment, the training phase consisted of bees foraging on 2 flowers for 15 bouts. One of these flowers contained 1mL of 50 % (w/w) unscented sucrose solution and the other 1mL of water (*B. terrestris dalmatinus* needed higher sucrose concentration for motivated foraging than *B. impatiens*, hence the higher concentrations used in Chapters 3,4 and 6). These flowers were placed side by side with wooden blocks touching and their position was alternated between bouts. Their distance away from the hive entrance was also randomly changed between bouts (see Figure 3.1a). Thus bees could not use spatial memory between bouts to locate the rewarding flower. Upon exiting the hive, the bees encountered two identical flowers, one of which was rewarding upon revisits. We expect any scent marks to indicate the position of the

rewarding flower. These flowers were not changed between bouts, to allow accumulation of scent marks and give the bees the opportunity to make the positive association between reward level and scent mark. Two flowers were used in this treatment because the bees generally filled up their crop from the first rewarding flower they encountered. This also ensured that the rewarding flower contained sufficient amounts of scent.

For the low reward treatment, the bees were allowed to forage on 6 flowers with 30 μ l of 50% (w/w) unscented sucrose solution, where revisits did not yield rewards, for 15 training bouts. The flowers were changed after each bout, so only unmarked flowers were available for each new foraging bout. In this situation, we expect any scent marks to indicate that the flower had already been visited and was, therefore, empty. The flowers were placed side by side as in the high reward treatment, forming three rows of two flowers each (see Figure 3.1b).

The bees in the two treatments were subsequently tested in an identical situation where scent marks were collected from the same source and presented to the test bees. In test trials the test bees would encounter two identical flowers where one was scent marked and the other was unmarked. To mark the test flowers, a clean, previously unused flower with 30 μ l of 50% (w/w) unscented sucrose solution was placed inside a separate marking arena where non-test foragers were foraging. These foragers were allowed to feed on the flower, thereby scent marking it. The number of foragers marking the flower was recorded and when all bees had left the flower, it was quickly removed and 30 μ l of water were placed at the bottom. This flower was presented to the test bee along with an identical unmarked flower also filled with 30 μ l of water. We conducted 3 test bouts for each bee where each test bout was followed by two non-test bouts similar to the bee's training bouts. We have previously shown that bees will deposit and detect scent marks left by other foragers in this setup (Saleh et al., 2006). All flowers were handled with non-powdered gloves and there were 12 bees in each treatment. Only bees with inter-bout times of less than 5 minutes were used to ensure motivation to forage (very few bees failed this criterion) and each bee was only used once. Flowers were only refilled between bouts. A flower was discarded if the dispenser touched the inner walls of the tube when injecting the sucrose reward; this was to ensure that bees

were not detecting the presence or absence of sucrose through residue on the inner walls.

3.3.4 Data analysis

We investigated four different behaviours: a) Acceptance b) Crawling-in c) Landing: and d) Hovering (see Legend for definitions). We added up the number of times each of these behaviours were used by each bee for the three test trials, and used this sum for statistical analysis, with bee as the unit of replication. We conducted a two sample t-test with context (high/low reward level) as the factor and the behaviour index of each bee as the response. The behaviour index was calculated by subtracting the number of behaviours performed towards the unmarked flowers from those performed toward the marked flowers. Negative values indicate a preference for unmarked flowers, positive values indicate a preference for marked flowers and a value of zero indicates no preference. We report averages of the behavioural indices as means \pm SE.

3.4 RESULTS

Bees in the high reward treatment performed an average of 7.91 ± 1.13 (SE) visits to unmarked flowers and 7.16 ± 1.02 to marked flowers for the three test bouts. Those in the low reward treatment did, on average, 10.23 ± 1.12 and 9.38 ± 0.94 visits to unmarked and marked flowers respectively. There was no significant difference in the number of bees marking the test flowers between the two treatments (two-sample t-test: $df= 14$; $t= -0.67$, $p= 0.51$). Therefore, any observed differences between the two bee treatments should not be due to differences in amount of scent mark deposited.

Bees in the high reward treatment accepted more marked flowers than those in the low reward treatment, in which bees accepted more unmarked flowers (two sample t-test: $df= 22$; $t= 5.39$, $p < 0.001$; see Figure 3.2)(high reward treatment= 2.75 ± 0.62 , low reward treatment= -2.42 ± 0.73). Bees in the high reward treatment were more likely to hover over, and subsequently reject, unmarked flowers than those in the low reward treatment, which were more likely to hover over the marked flowers (two sample t-test: $df= 22$; $t= -4.02$, $p < 0.001$; see Figure 3.2)(high reward treatment= -1.17 ± 0.60 , low reward treatment= 2.33 ± 0.63). There was no significant difference in the number of

crawling-ins and landings performed by bees in the two treatments (two sample t-test: $df= 22$; crawling-in: $t= -0.17$, $p< 0.87$; landing: $t= -1.23$, $p< 0.23$; see Figure 3.2) (crawling-in: -0.92 ± 0.90 and -0.75 ± 0.39 ; landing: -1.42 ± 0.70 and -0.17 ± 0.74 for high and low reward treatments respectively).

3.5 DISCUSSION

The bees in our study were tested in the same manner and with the same source and relative amounts of scent marks. The only difference between the two groups was in their experience with high or low rewarding flowers.

Bees in the high reward level treatment, where revisits were rewarding, accepted more scent marked flowers than those in the low reward treatment. Thus scent marks, in this case, were perceived as attractive. The opposite is true for bees trained in the low reward treatment; these bees were more likely to accept unmarked flowers compared to marked flowers, suggesting that the scent mark in this context served as a repellent. Indeed analysis of hovering/rejection behaviours indicates that bees trained in the low reward treatment were more likely to hover over marked flowers. The opposite is true for those trained in the high reward treatment, these bees were more likely to hover, or reject, unmarked flowers. Bees in both treatments hovered over flowers that did not offer reward. This may be an attempt to ensure that they have correctly detected the presence or absence of scent mark in order to minimize erroneously entering into emptied flowers.

Although the attractive effect of foraging scent marks in bumblebees, honeybees and stingless bees has only been found on artificial feeders where revisits are rewarding, we do expect bees to have a use for an attractive scent in nature. For example, bees may want to scent mark bonanza food sites such as flowering trees (Seeley, 1995), other bees' nests that are sometimes raided (Sakagami et al., 1993), or rotting fruit (used as food sources in foraging honeybees and bumblebees; Chittka, personal observations) in order to return to them later. To understand the true function of any of these chemical cues we need to test them in experimental setups that mimic natural foraging conditions. This will allow us to identify exactly what role they play to foraging bees and what their evolutionary significance may be. We have some clues on some of the meanings the scent marks can have to foraging bumblebees. In addition to attractant and repellent effects, bees will rely on the scent marks to different

degrees depending on the handling time of the flowers (Saleh et al., 2006). They also seem to adjust their reliance on scent marks depending on the nectar secretion rate of the flower species (Stout & Goulson, 2002).

As bumblebees do not recruit to food sites, and forage in areas where they mix at random with non-colony members (Thomson & Chittka, 2001; Chapman et al., 2003), it would seem that the attractive effect of the foraging scent mark could be used by a bee to signal a rewarding food site to itself. This can be advantageous because, although detectable to other bees and insects (Williams, 1998; Stout & Goulson, 2001; Gawleta et al., 2005; Reader et al., 2005), they would not be able to interpret its meaning unless they were also aware of the value of the food site. This is especially true if their interpretation of the scent is as a repellent due to the fact that pollinating insects mainly forage on low rewarding flowers that replenish too slowly for immediate revisits to be rewarding (Seeley, 1995). This may act to reduce competition for the food source allowing the bee to exploit it more thoroughly.

We do not know if the foraging scent marks have an innate meaning that can change with experience or if they acquire their meaning independently. Distinguishing between these two modes of learning is important. Nonetheless, both scenarios may involve simple associative learning. Yet simple associative learning does not exclude the ability of the association to influence biologically important behaviour. The foraging scent marks can increase the bees' foraging efficiency (Giurfa & Núñez, 1992), therefore they are able to spend more time investigating rewarding flowers. More food can directly impact the reproductive output of the colony (Schmid-Hempel & Schmid-Hempel, 1998; Pelletier & McNeil, 2003). Thus it is important to identify the versatile and learned cues used in communication.

Bees are very versatile learners. They can associate an odour with reward after only one rewarded experience (Menzel, 1985), and they are capable of contextual learning (Chittka et al., 1995; Chittka & Thomson, 1997; Chittka, 1998). Although we know that the same pheromonal cue can elicit different behaviours in a variety of different contexts (Keeling et al., 2004), we know very little about the role learning plays in the interpretation of marks left behind by the bee itself. Several studies using proboscis extension response (PER), where honeybees and wasps were harnessed and tested for their response to a conditioned stimulus, have shown that these insects can learn to

associate alarm (Sandoz et al., 2001; Guerrieri et al., 2005) and sex pheromone chemicals (Hartlieb et al., 1999) with food. This indicates that even substances that are innate or have a tendency to produce very specific behaviours can be associated with a different meaning.

This study has highlighted the importance of learning and experience in determining the meaning of the scent marks left on food sources by bumblebees. It has shown that these bees will interpret foraging scent marks differently depending on the reward status of the food source where revisits do or do not yield reward. The evidence so far suggests that the use of foraging scent marks in bumblebees is flexible, ultimately depending on the bee's personal experience with the marks. Flexible cues can play important roles in influencing an animal's behaviour and ultimately its fitness. Distinguishing between scents whose effects are highly flexible and those that are less flexible will give us a better understanding of the role chemical signals play in communication. We expect that learning and experience actually have a large role to play in the interpretation of chemical signals and may explain conflicting reports in the literature.

3.6 ACKNOWLEDGEMENTS

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FIGURE 3.1 Arrangement of flowers during training phase. (a) In the high reward treatment only 2 flowers were used; these were alternated in position relative to each other and distance from the hive entrance at each bout. (b) For the low reward treatment six flowers were used with their distribution as shown.

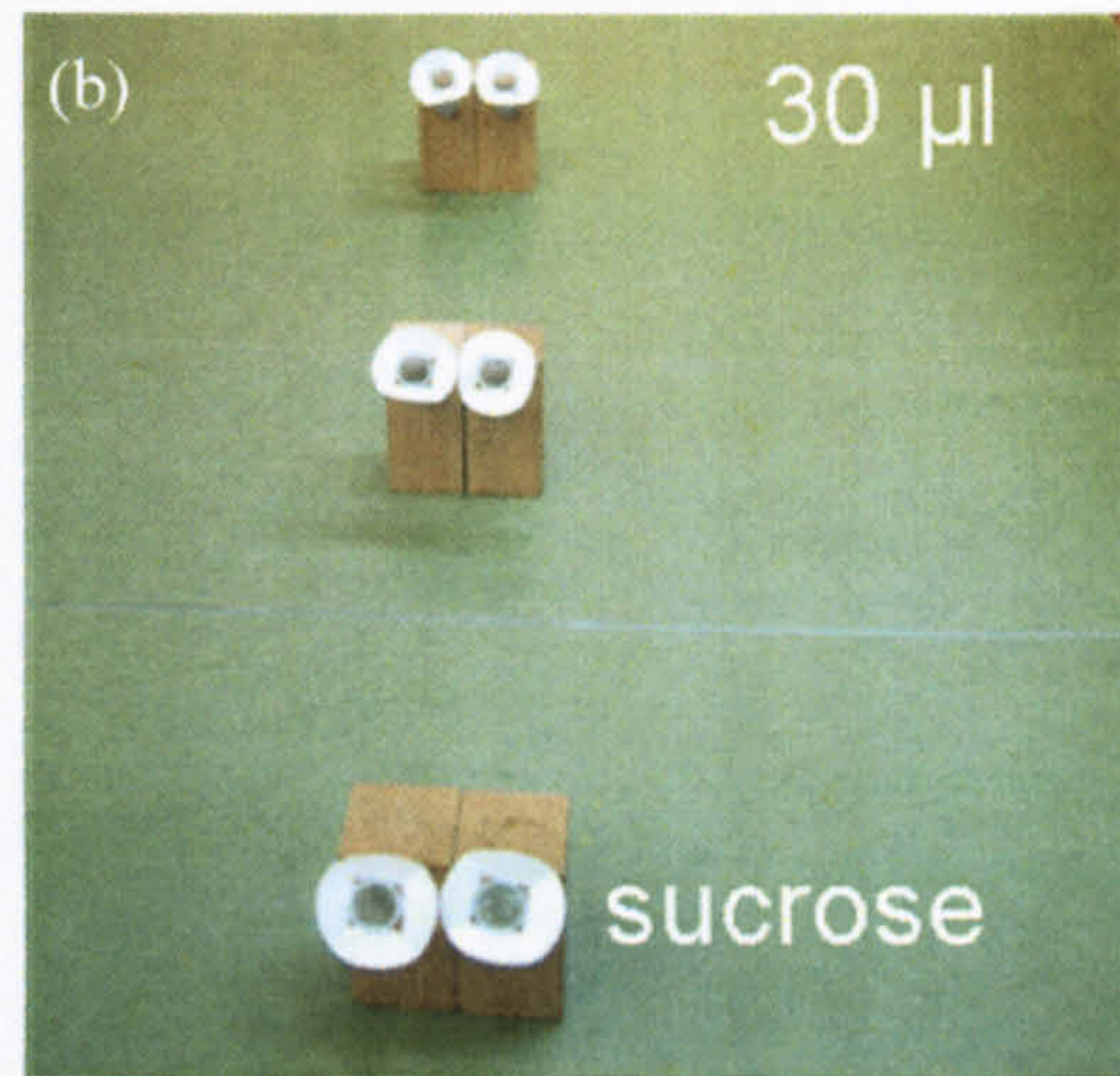
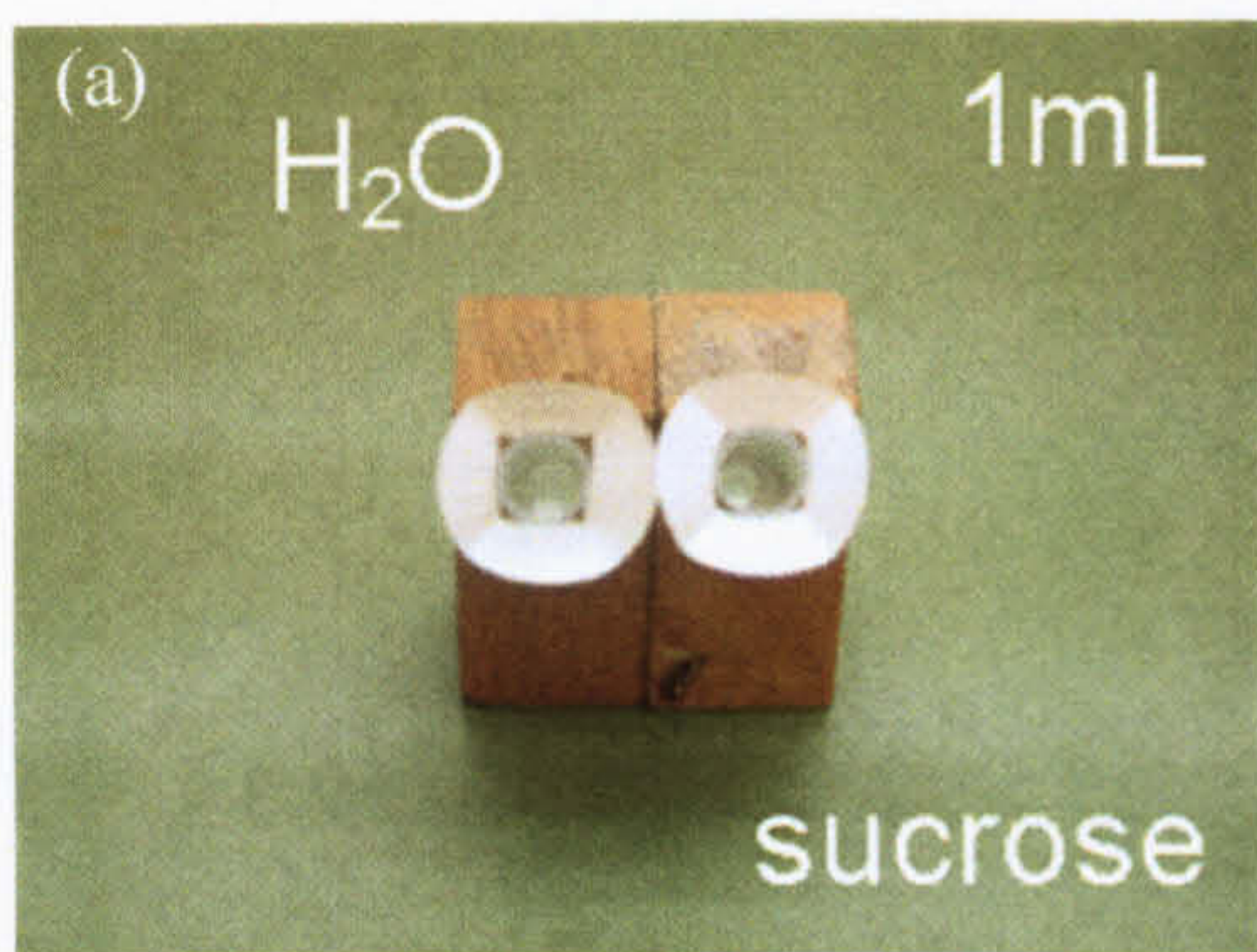
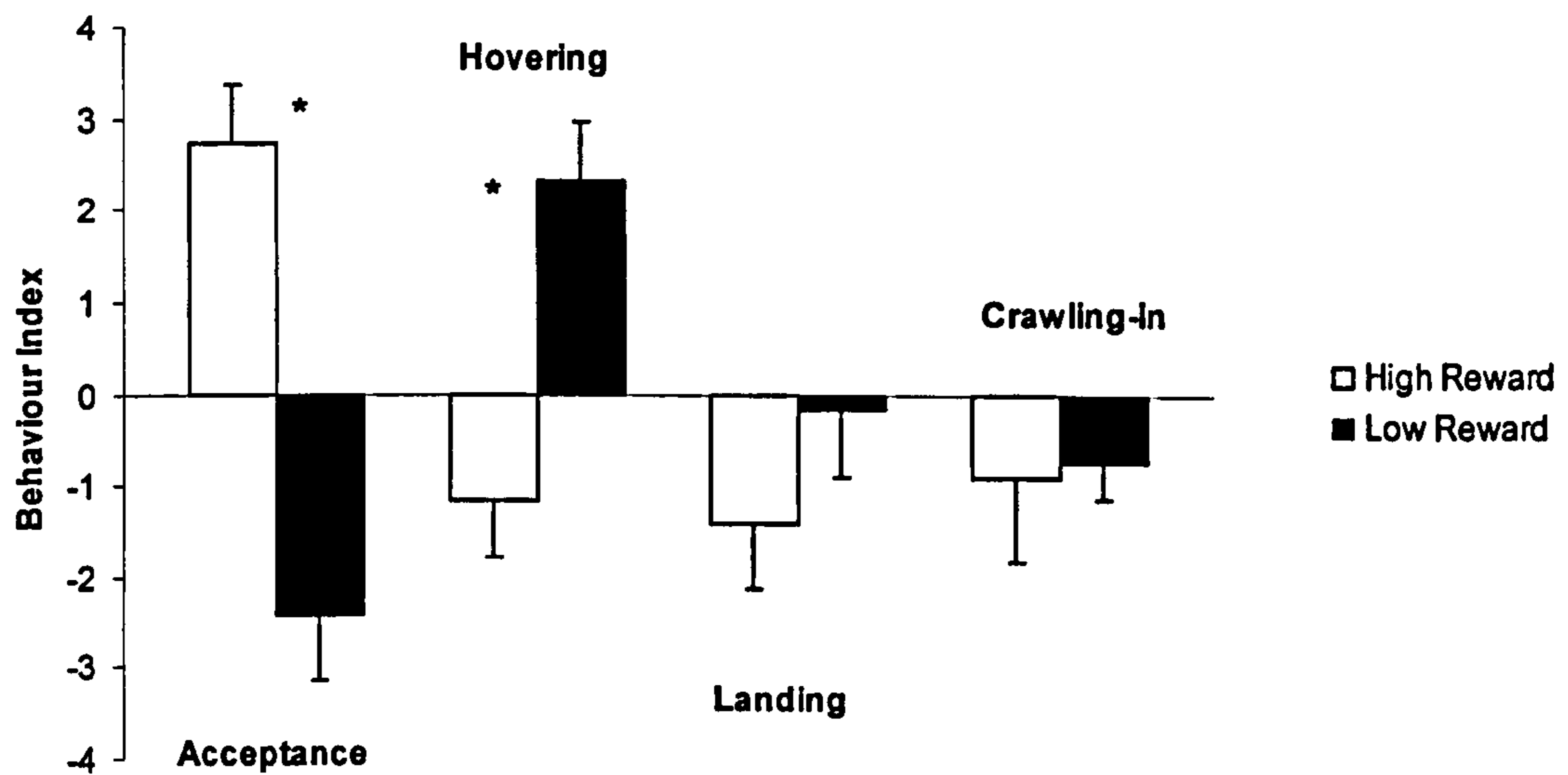
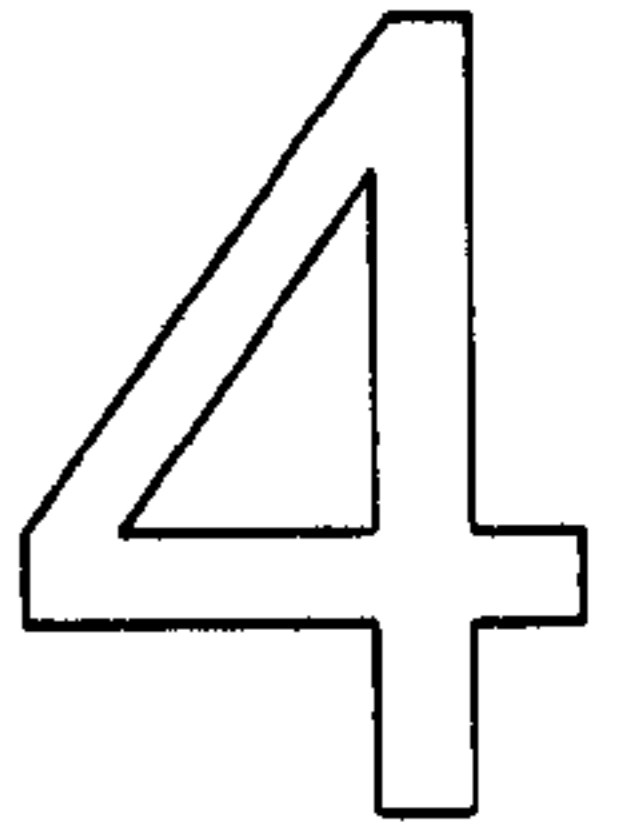


FIGURE 3.2 Bees showed a difference in acceptance and hovering rates towards marked and unmarked flowers depending on whether flowers they experienced prior to testing had high or low reward. There was no significant effect of treatment on crawling-in and landing behaviours. Mean behavioural indices are shown with standard error on mean. Negative values indicated a preference for unmarked flowers and positive values indicate a preference for marked flowers. Asterisk indicates significant a difference ($p < 0.05$) between the two bee treatments for that behaviour.





Are scent marks pheromones?

The results in this chapter are submitted as:

Saleh, N., Scott, A.G., Bryning, G.P., & Chittka, L. (submitted)

**Bumblebees use incidental footprints to generate adaptive behaviour
at flowers and nest**

Journal of Experimental Biology

4.1 ABSTRACT

Chemicals used in communication are divided into signals and cues. Cues are incidentally deposited by an animal and the information they carry is not moulded by natural selection. Distinguishing between these two modes of information transfer is difficult when animals do not perform obvious secretion behaviours. Although a number of insects have been suspected of using cues to mark food sites and nest entrances, studies have not attempted to experimentally distinguish between cues and signals. Here, we examine the chemical composition of the scent marks left by the bumblebee *Bombus terrestris* at food sites and compare it to those found at a neutral location. If bees are depositing a cue, we expect the same chemicals to be found at both sites, but if they deposit a signal we only expect to find the scent marks at the food site. We were also interested in identifying the chemicals left at the nest entrance to determine if they differed from those used to mark food sites. We find that bees deposit the same chemicals at food, nest and neutral sites. Therefore bumblebees leave behind chemical footprints everywhere they walk and we propose that they learn to use these footprints in a manner that ultimately enhances their fitness, for example, to improve their foraging efficiency and locate their nest. Experimentally distinguishing between cues and signals is crucial for understanding how they interact to shape animal behaviour and what chemical bouquets are under natural selection.

4.2 INTRODUCTION

Sources of information used by animals are generally placed into two broad categories: signals and cues. Signals are defined as traits that evolved for a specific role in communication (Karlson & Luscher, 1959) and are often believed to elicit hard-wired responses (Beauchamp et al., 1976). Chemical signals are often referred to as pheromones. Cues, on the other hand, are defined as incidental features present in the environment (Seeley, 1995). They have not been moulded by natural selection to carry a specific meaning for that particular animal species. Animals should rely on both cues and signals to generate adaptive behaviour (Seeley, 1998). Distinguishing between signals and cues is essential for identifying which chemical bouquets are shaped and maintained through natural selection. Several authors have proposed that scent marks deposited by bumblebees to improve their foraging efficiency and mark their nests may be signals, moulded by natural selection to carry specific information (Giurfa & Núñez, 1992; Pouvreau, 1996; Stout et al., 1998), while others have suggested they are incidental cues, left everywhere bees walk (Butler et al., 1969; Stout & Goulson, 2002; Eltz, 2006). Here we show that these scent marks are chemical footprints left everywhere bees walk. We do this by identifying the chemicals left behind at these sites and comparing them to those left at a neutral site.

In bumblebees, the tarsal gland has been suggested as the source of the scent marks (Schmitt et al., 1991). However, the tarsal gland has no openings to the exterior of the bee (Pouvreau, 1991). In addition its contents, linear hydrocarbons, resemble those found on the cuticle (Oldham et al., 1994), whose secretion most likely comes from cuticular tissue (Schal et al., 1998). These hydrocarbons are liquids that can easily be leaked passively onto a substrate (Oldham et al., 1994). Therefore it is not clear if these chemical marks are left behind on flowers through active secretion from the glands, or if the cuticular hydrocarbons on the bees' feet are passively left behind generating chemical footprints.

Bumblebees also leave a chemical trail to their nest, which they follow to locate their nest entrance (Cederberg, 1977; Foster & Gamboa, 1989; Pouvreau, 1996). Such trails can also be laid to feeders when bees are forced to forage in darkness (Chittka et al., 1999). Bumblebees nest in underground cavities, which can become covered with grass and shrubs obscuring the

entrance of the colony. Reliance on these chemical marks is expected to allow bees to locate their nest faster, and distinguish it from other nests nearby (Foster & Gamboa, 1989). In addition, the vegetation surrounding the nest can change, rendering visual cues less reliable. Therefore olfactory cues may provide a reliable backup system (Jandt et al., 2005). Bees can discriminate between chemical trails laid by their nestmate, conspecific and heterospecific bumblebee workers, and are more likely to choose entrances marked by their nestmates (Pouvreau, 1996). However, we do not know the chemical composition of these chemical trails.

The purpose of this study was to 1) compare the chemicals deposited at a neutral site to those deposited at the food site to resolve if the scent marks are footprints or pheromones 2) determine the chemical composition of the scent marks left at the nest entrance.

4.3 MATERIALS AND METHODS

If the scent marks are pheromones (signals) we expect to find them only in areas that hold a resource value, such as the food and nest sites, not in a neutral site. However, if they are cues, left everywhere bees walk then we should also find them at neutral sites. In order to test this we needed bees to walk in neutral areas that held no resource value. We did this by having the bees exit the hive into an unrewarding arena connected via a large tunnel to another empty unrewarding arena (see Figure 4.1). This section of the setup should hold no resource value to the bees and therefore should have no scent marks, of the kind left at food sites, if the secretion on flowers is actively controlled.

4.3.1 Test animals and experimental setup

Colonies of *Bombus terrestris dalmatinus* were obtained from Koppert Ltd. (Netherlands). They were housed in a wooden nestbox [16 (w) x 28 (l) x 11cm (h)] and fed approximately 4g of pollen into the nest every day. Tests were conducted at a temperature of $\approx 20^{\circ}$ C and a light dark regime of 10:14 light-dark cycle. The bees foraged on 50% sucrose solution from gravity feeders described below.

The experimental apparatus consisted of three arenas connected to each other via tunnels. Nest samples were collected from a clear plastic tunnel (3.5 x

30 x 3.3 cm) connected to the colony's nest box. Plastic shutters at the entrance and exit of the tunnel could be used to isolate it from incoming bee traffic and a removable top allowed the experimenter access to the tunnel. This tunnel connected the nest to Unrewarding arena 1. Unrewarding arenas (40 x 60 x 30 cm) never contained food and were empty throughout the experiment. Unrewarding arena 1 was connected to Unrewarding arena 2 via a large black plastic tunnel (10 x 5 x 195 cm) where the neutral samples were collected. The top of this tunnel was covered with wire mesh except for the sample collection area. This area was covered with a clear plastic sheet taped to the top of the tunnel, thereby allowing the experimenter easy access to the tunnel. Cardboard shutters were used to control bee traffic. These were inserted before and after the plastic sheet when needed. To encourage bees to walk in this area, we reduced the light level by placing pieces of wood over it. However, bees were still able to rely on visual cues to move around in the experimental setup. Unrewarding arena 2 was connected to the Food arena (72 x 104 x 30 cm) via a second large tunnel (10 x 5 x 105 cm) which was covered entirely with wire mesh. We collected food samples from the Food arena. This was the only arena where food was presented to the bees. Bees were fed 50% (w/w) reagent grade sucrose solution from a gravity feeder (Frisch, 1967). It was composed of a glass dish ($\varnothing = 5$, $h = 3$ cm) inverted onto a circular Plexiglas plate ($\varnothing = 6$, $h = 0.5$ cm). Eighteen equidistant grooves were cut in a radial arrangement on the top surface of the Plexiglas plate. This feeder was elevated from the ground using a platform (8 x 8 x 3.5 cm). Green cardboard was taped onto the arena floors to mimic the green foliage background found in most natural situations of bees foraging.

4.3.2 Data collection

Teflon[®] disks ($\varnothing = 2$ cm; Supelco, Bellefonte, USA), vials and low-volume inserts (QMX, Thaxted, UK) used to collect and treat the samples were sterilised by rinsing them in ethanol, acetone and pentane solutions (HPLC grade, Sigma-Aldrich Co. Ltd, Gillingham, UK) then placing them in the oven for 3 hours at 230 °C. They were rinsed again with pentane before use. Flame sterilised tweezers were used to handle the vials and Teflon[®] disks. Glass pipettes, used to transfer the solvents, were new and rinsed at least three times in pentane before use.

We placed aluminium foil along the floor of the nest and neutral tunnels. Aluminium foil also covered the bottom of the gravity feeder and the top of the feeder platform. This minimized contamination from the plastic. We changed the aluminium foil, with tweezers, after each collection. All our samples were compared to an Arena control. To collect the Arena control 12 Teflon[®] disks were placed onto a sheet of aluminium foil in an 800 ml beaker ($\varnothing = 9.2$, $h = 13.4$ cm). The beaker was covered with wire mesh, to prevent bees from walking on the disks, and placed inside one of the arenas.

The nest, neutral and arena control samples were collected together; food samples were collected separately. This prevented contamination by the food foraging pheromone which alerts nestmates to the presence of food (Granero et al., 2005). Twelve Teflon[®] disks were placed on the floor of the tunnels to collect the nest and neutral context samples. For the food context, a small part of each of the 12 disks was slipped between the bottom of the feeder and the platform to prevent it from falling on the floor. All Teflon[®] disks were left for 3 hours; meanwhile the bees were allowed to walk on them. The disks were then removed using tweezers and placed into a 4 ml vial containing 1.5 ml of pentane (Schmitt et al., 1991; Jarau et al., 2004). The liquid was swirled for 1.5 minutes and then transferred, via a glass pipette, to another clean 4 ml vial. Samples not immediately analysed were stored in a -20 °C freezer until analysis. When the samples were analysed, they were concentrated using a gentle stream of dry nitrogen to 200 μ l. Two colonies (A and B) were used and at least three sets (set = one nest, one neutral and one food context) of samples were collected from each colony.

4.3.3 Data analysis

Samples were analysed using a Gas Chromatograph-Mass Spectrometer (henceforth GC- MS) (Agilent Technologies: GC 6890N/MS 5973N) with helium as carrier gas on pulsed splitless mode. An HP-1MS column was used (Hewlett Packard: 25.0 m length, 320 μ m internal diameter and 0.52 μ m film thickness). The temperature program was initially held at 60°C for 1 minute, then increased to 300°C at 10°C/minute and kept at this temperature for an additional 30 minutes. The alkanes were identified through retention time comparisons with synthetic compounds (Sigma-Aldrich Biotechnology). A set of samples from one of the colonies was treated with dimethyl disulphide (DMDS) as described in

Carlson (1989) to identify the alkenes. Percent peak areas (i.e. relative amounts) of the compounds in each sample were compared via principle components analysis (henceforth PCA) using Brodgar version 2.4.6 (Highland Statistics Ltd).

4.4 RESULTS

Samples from all three contexts have very similar chromatograms (see Figure 4.2). Detailed identification of the compounds left in each context revealed that 76 out of 77 compounds were present in all three contexts and in similar relative amounts (see Table 1). This similarity was confirmed by the PCA where Axis 1 explained 95.19% of the variation. There was no clustering of samples based on the collection context (see Figure 4.3), nor were any differences detected between contexts in relative amounts of any compound. Therefore we conclude that samples collected in the food, nest and neutral contexts are composed of the same chemicals present in similar proportions. The extracts were a mixture of 13 alkanes, 55 alkenes, 4 alkadienes and 5 aldehydes. The alkanes were the most abundant compounds, while the majority of the alkenes were present in small amounts (i.e. <1%) (see Table 1).

4.5 DISCUSSION

This study provides a simple means for distinguishing cues and signals, which can be used to categorize chemical marks when the animals do not perform obvious marking behaviour to a human observer. We did this by identifying the chemicals left at the food and nest sites and comparing them to chemicals found at a neutral site. Other studies generally either draw conclusions based on the presence of correlations between the compounds left on the substrate and a gland or cuticle (e.g. Schmitt et al., 1991; Goulson et al., 2000; Steinmetz et al., 2003; Jandt et al., 2005), or do not control for the possibility that the same marks may be actively left behind in places that hold a resource value (Schmidt et al., 2005). Our results indicate that the same compounds were present in the feeding site, neutral site and nest entrance contexts. Thus bees are leaving behind a footprint, which they learn to associate with different meanings depending upon context and experience.

It is extremely difficult to experimentally show that any area is absolutely neutral. However, we can show that the neutral area in our experimental setup was relatively neutral to the food and nest sites. Firstly, the neutral area did not hold any obvious resource value, because the bees neither fed nor nested in it. Secondly, we collected the food samples separately to the neutral samples; therefore it is unlikely that the bees were marking the neutral area with a trail to the food source. Thirdly, it is unlikely we neglected to detect chemicals of high volatility that may be used to distinguish between the different areas because the effect of the chemicals left at the food and nest sites have been reported to last over 20 hours (Cederberg, 1977; Schmitt & Bertsch, 1990; Stout & Goulson, 2002), suggesting that the compounds used by the bees are relatively non-volatile. Therefore, we feel that our comparisons of food and nest sites to the neutral site should have revealed differences in deposited chemicals if there were any.

Consistent with previous studies on the scent marks left by bumblebees at food sources, we have found that the footprint is a mixture of alkanes and alkenes with a minor occurrence of alkadienes and aldehydes, which resemble those found in the tarsal gland and cuticle extracts (Schmitt et al., 1991; Oldham et al., 1994). There are some variations in the compounds present and their quantities in our study compared to those of Schmitt et al. (1991) and Goulson et al. (2000). These differences are probably due to natural variation within the species, as similar differences were observed in honeybee cuticular extracts (Dani et al., 2005). Although all three studies used *B. terrestris*, it is possible that the subspecies used may influence the ratio of hydrocarbons present. There is pronounced variation in the behaviour and sensory systems between the subspecies of *B. terrestris* (Chittka et al., 2004), hence it is possible that there might also be variation in chemical signatures. We used the South-Eastern European variety *dalmatinus*, but subspecies information was not provided in the previous studies.

It is unlikely that we overlooked differences in minor compounds in the samples. This is because each sample was compared to the arena control with which it was collected and only compounds that were not present in the control or present in quantities above those of the control were included in the analysis. Therefore, any consistent differences, even if minor, would have been noticed by the experimenter and should have been detected by the PCA analysis. In

addition, it is unlikely that bees are responding to only one compound, rather a mixture of different compounds (Schiestl & Ayasse, 2000).

Although we do not know the innate meaning, if any, of this chemical footprint, it is known from previous studies that learning greatly influences a bee's reliance on it. We know that during foraging these scent marks can be attractive or repellent (Saleh & Chittka, 2006), and they are relied on to different degrees depending on the handling time of flowers (Saleh et al., 2006) and its replenishment rate (Stout & Goulson, 2002). Now we know that the same scent marks are used to locate the bees' nest entrance. Therefore bees might be able to associate these scent marks with multiple meanings that may, at times, be unique to each individual.

There is strong suggestive evidence that honeybees also evaluate footprints left behind at their nest entrance and food sites, in addition to the active (signalling) use of Nasonov glands (Frisch, 1967). Butler et al. (1969) have shown that scent marks collected at the nest entrance increase the frequency of honeybees landing on rewarding food sources. This behaviour was also elicited from the scent marks left on the hive floor, suggesting that the hive floor, nest entrance and food source are marked by the same chemicals. The scent marks are most likely a footprint because non-foraging bees left scent marks at unrewarding feeders (Ferguson & Free, 1979) and the attractiveness of an entrance tube was a factor of the number of bees walking on it (Butler et al., 1969). However, empirical tests need to eliminate the possibility that the two sites are actively marked with the same signal by comparing compounds left at a site that does not hold a resource value to those at food and nest sites.

Although many stingless bee species perform behaviours that indicate active marking of highly rewarding food sources (Nieh, 2004), there are two reports that suggest a footprint mechanism may be acting in some stingless bees. *Melipona seminigra* leaves compounds on food sites very similar to those left behind by bumblebees (Jarau et al., 2004). The claw retractor tendon glands are believed to be the source of these chemical marks, but the secretion mechanism is thought to be passive. Schmidt et al. (2005) found that *Nannotrigona testaceicornis* leaves the same scent marks at the nest entrance and food sites. Although the authors suggest that the scent marks are footprints, they do not control for the possibility that both sites are actively marked by the same substance. This can be easily achieved through

comparison with a neutral site. There are also reports on *Vespula vulgaris* (Steinmetz et al., 2003) and *V. germanica* (Jandt et al., 2005) that suggest a footprint mechanism may operate in some wasps. It is, therefore, possible that the use of conspecific chemical cues for adaptive behaviour is common among social insects. This remains to be shown.

When active marking cannot be demonstrated through behavioural observations, very few studies, investigating the mechanisms involved in conspecific communication of resources, have shown that the compounds left in a specific context are indeed signals, and not passive cues also left in a neutral context. Passive conspecific cues left and used by insects have received little attention in the context of generating adaptive behaviour. However, these types of cues can contain biologically important information such as reproductive status (Ayasse et al., 1995), and can be used to influence biologically important behaviour such as foraging (Giurfa & Núñez, 1992), locating nesting sites (Cederberg, 1977; Foster & Gamboa, 1989; Pouvreau, 1996) and the detection of intruders (Dronnet et al., 2005). In order for us to understand the roles certain chemicals play in communication, more studies will need to experimentally determine if the chemical bouquet is a cue or signal. This will increase our understanding of animal communication and provide us with greater insight on how signals and cues interact to shape animal behaviour.

4.6 ACKNOWLEDGEMENTS

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FIGURE 4.1 Experimental setup. Nest samples were collected in tunnel connecting nest box to first unrewarding arena. Neutral samples were collected in large tunnel connecting unrewarding arena 1 and 2. Food samples were collected at a feeder, indicated by the circular symbol surrounded by a rectangle.

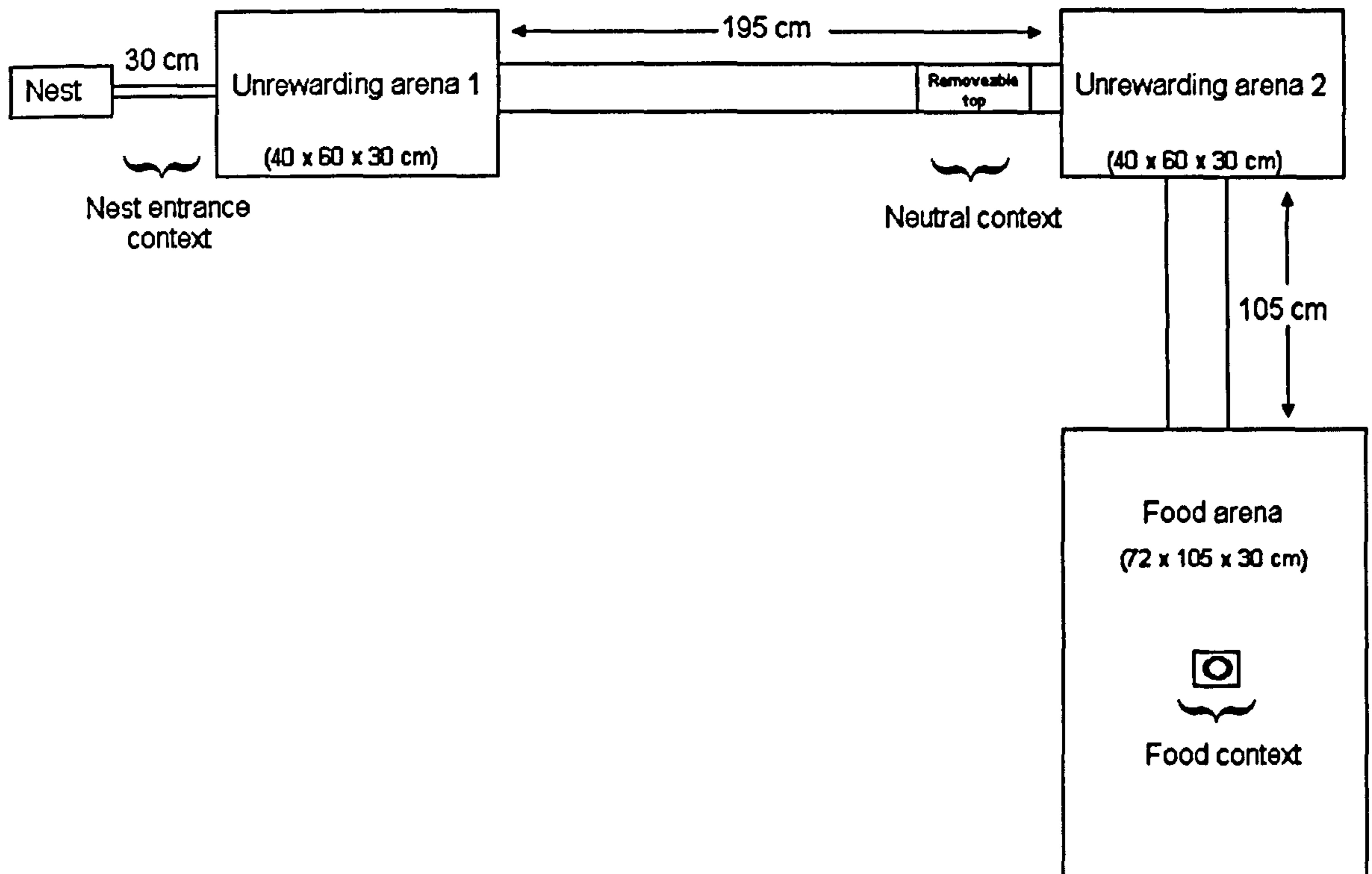


FIGURE 4.2 Chromatograms of samples collected in food, nest and neutral contexts. The three chromatograms are very similar indicating the presence of similar compounds in each context. Results shown here are for samples collected from colony A. Numbers correspond to compounds identified in Table 1.

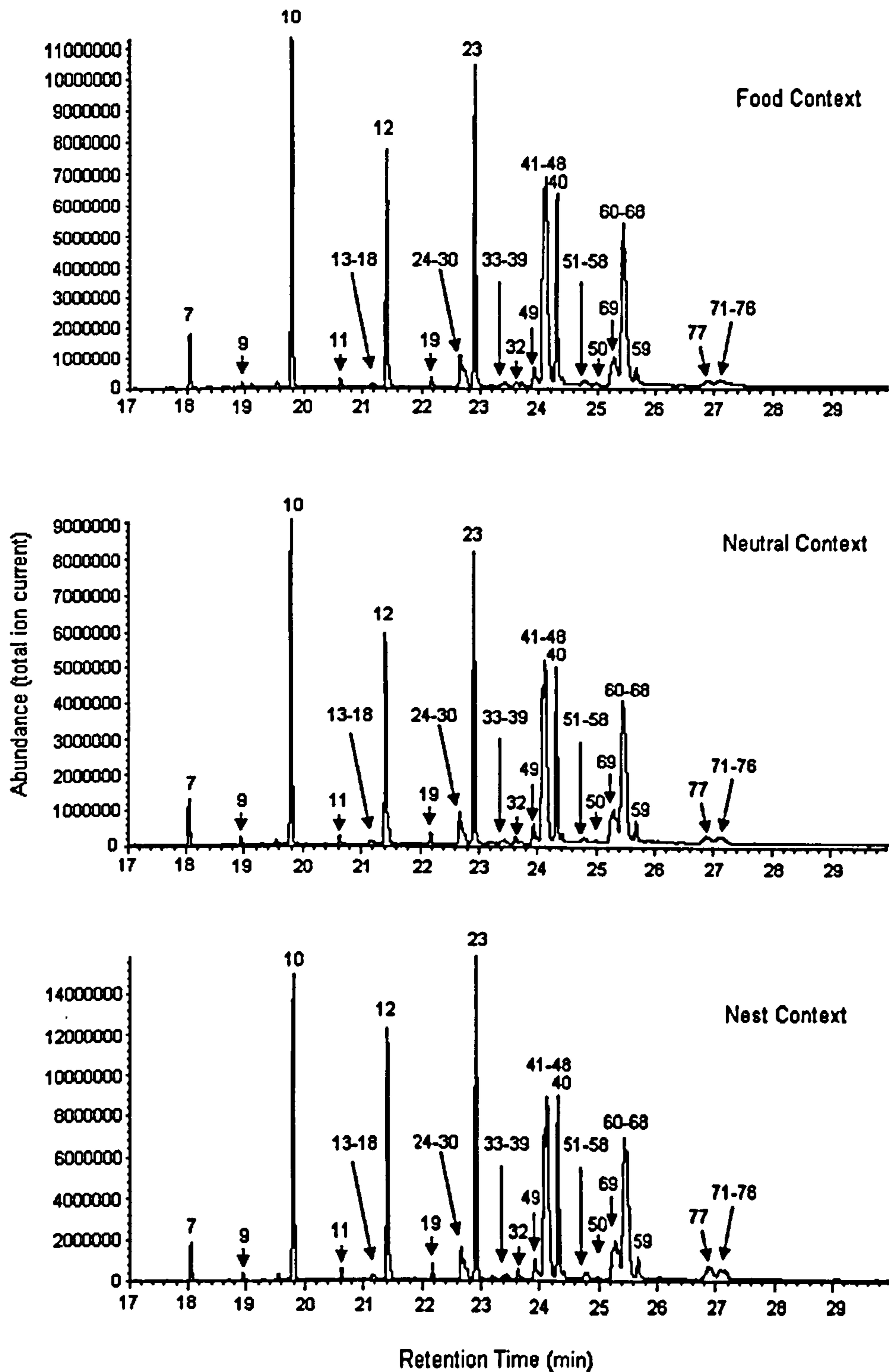


FIGURE 4.3 Results from PCA analysis. (a) Samples were highly correlated and grouped together. Axis 1 explains 95.19% of the variation. (b) Magnified view of x-axis: there was no clustering of samples depending on context, indicating that the scent mark is a footprint. F= sample from food context; N= sample from nest context; T= sample from neutral context; A= sample from colony A; B= sample from colony B.

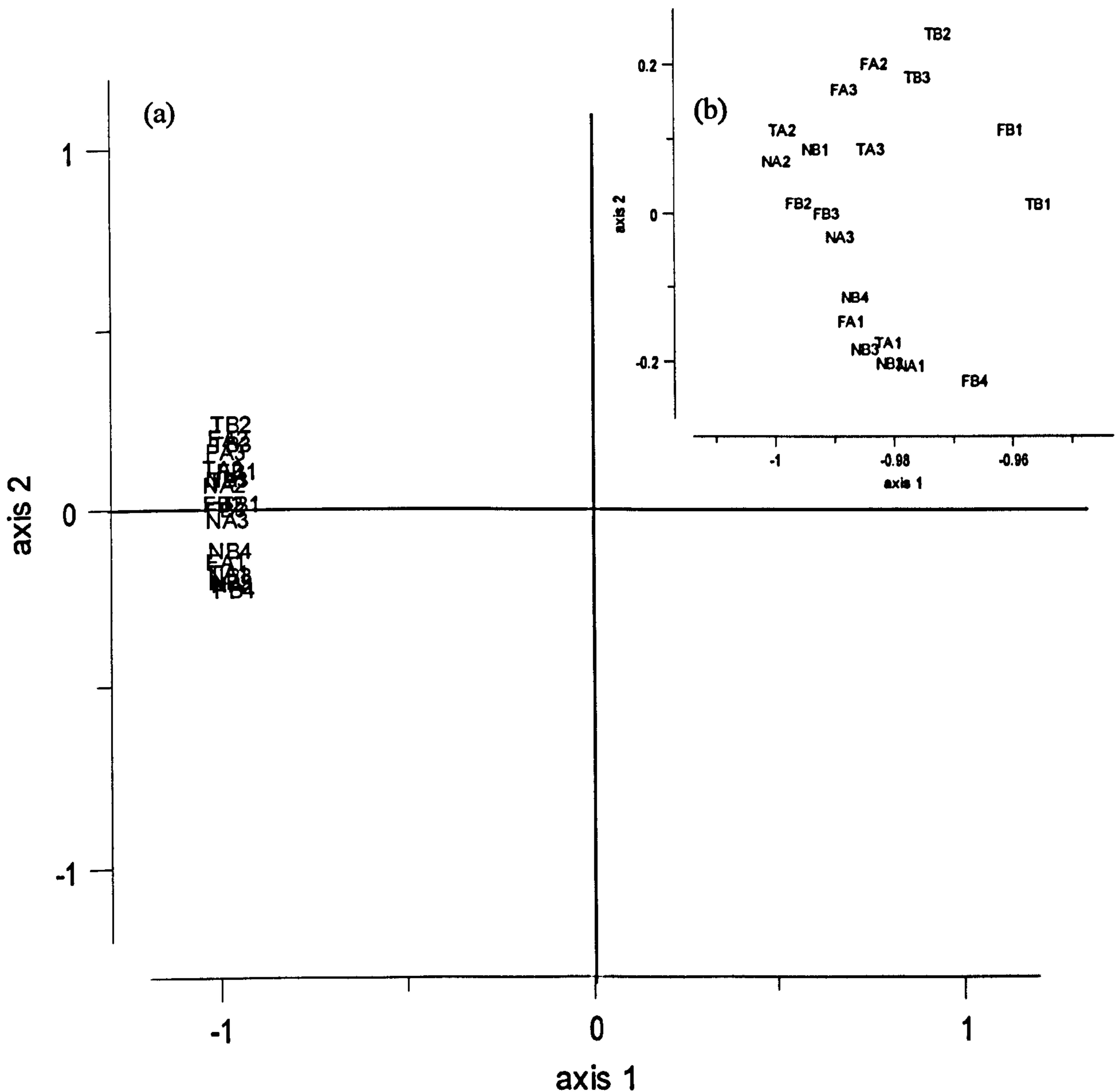


TABLE 4.1 Compounds identified in nest, neutral and food contexts. 76/77 compounds are present in all three contexts, indicating the scent mark is a footprint. Identification of compounds was done for one set of samples from Colony B. Table continues on next page. += <1 %, ++= 1-10%, +++= over 10% abundance; - = compound not present. Alkenes were identified using DMDS treatment.

<i>Number</i>	<i>Compound</i>	<i>Mol wt</i>	<i>Diagnostic El ions</i>	<i>Food</i>	<i>Nest</i>	<i>Neutral</i>
1	Nonanal	142		+	+	+
2	Decanal	156		+	+	+
3	undecanal	170		+	+	+
4	dodecanal	184		+	+	+
5	Tridecanal	198		+	+	+
6	n-Nonadecane (C ₁₉ H ₄₀)	268		+	+	+
7	n-Heneicosane (C ₂₁ H ₄₄)	296		++	++	++
8	9-heneicosene	388	173/215	+	+	+
9	n-Docosane (C ₂₂ H ₄₆)	310		+	+	+
10	n-Tricosane (C ₂₃ H ₄₈)	324		+++	+++	+++
11	n-Tetracosane (C ₂₄ H ₅₀)	338		+	+	+
12	n-Pentacosane (C ₂₅ H ₅₂)	352		++	++	++
13	7-pentacosene	444	145/299	+	+	+
14	8-pentacosene	444	159/285	+	+	+
15	9-pentacosene	444	173/271	+	+	+
16	10-pentacosene	444	187/257	+	+	+
17	11-pentacosene	444	201/243	+	+	+
18	12-pentacosene	444	215/229	+	+	+
19	n-Hexacosane (C ₂₆ H ₅₄)	366		+	+	+
20	9-hexacosene	458	173/285	+	+	+
21	11-hexacosene	458	201/257	+	+	+
22	13-hexacosene	458	229/229	+	+	+
23	n-Heptacosane (C ₂₇ H ₅₆)	380		+++	++	++
24	7-heptacosene	472	145/327	+	+	+
25	8-heptacosene	472	159/313	+	+	+
26	9-heptacosene	472	173/299	+	+	+
27	10-heptacosene	472	187/285	+	+	+
28	11-heptacosene	472	201/271	+	+	+
29	12-heptacosene	472	215/257	+	+	+
30	13-heptacosene	472	229/243	+	+	+
31	Heptacosadiene	376	376	-	+	+
32	n-Octacosane (C ₂₈ H ₅₈)	394		+	+	+
33	8-octacosene	486	159/327	+	+	+
34	9-octacosene	486	173/313	+	+	+
35	10-octacosene	486	187/299	+	+	+
36	11-octacosene	486	201/285	+	+	+
37	12-octacosene	486	215/271	+	+	+
38	13-octacosene	486	229/257	+	+	+
39	14-octacosene	486	243/243	+	+	+
40	n-Nonacosane (C ₂₉ H ₆₀)	408		++	++	++
41	7-nonacosene	500	145/355	+	+	+
42	8-nonacosene	500	159/341	+	+	+
43	9-nonacosene	500	173/327	++	++	++
44	10-nonacosene	500	187/313	++	++	++
45	11-nonacosene	500	201/299	++	++	+
46	12-nonacosene	500	215/285	+	+	+
47	13-nonacosene	500	229/271	++	++	++
48	14-nonacosene	500	243/257	+	+	+

Number	Compound	Mol wt	Diagnostic EI ions	Food	Nest	Neutral
49	Nonacosadiene	404	404	+	+	++
50	n-Triacontane (C ₃₀ H ₆₂)	422		+	+	+
51	8-triacontene	514	159/355	+	+	+
52	9-triacontene	514	173/341	+	+	+
53	10-triacontene	514	187/327	+	+	+
54	11-triacontene	514	201/313	+	+	+
55	12-triacontene	514	215/299	+	+	+
56	13-triacontene	514	229/285	+	+	+
57	14-triacontene	514	243/271	+	+	+
58	15-triacontene	514	257/257	+	+	+
59	n-Hentriacontane (C ₃₁ H ₆₄)	436		++	++	++
60	7- Hentriacontene	528	145/383	+	+	-
61	8-Hentriacontene	528	159/369	+	+	+
62	9-Hentriacontene	528	173/355	++	++	++
63	10-Hentriacontene	528	187/341	+	+	+
64	11-Hentriacontene	528	201/327	++	++	++
65	12-Hentriacontene	528	215/313	+	+	+
66	13-Hentriacontene	528	229/299	+	+	+
67	14-Hentriacontene	528	243/285	+	+	+
68	15-Hentriacontene	528	257/271	+	+	+
69	Hentriacontadienes	432	432	++	++	++
70	9-tritriacontene (C ₃₃ H ₆₆)	556	173/383	+	+	+
71	10-tritriacontene	556	187/369	+	+	+
72	11-tritriacontene	556	201/355	+	+	+
73	12-tritriacontene	556	215/341	+	+	+
74	13-tritriacontene	556	229/327	+	+	+
75	14-tritriacontene	556	243/313	+	+	+
76	15-tritriacontene	556	257/299	+	+	+
77	Tritriacontadiene(s)	460	460	+	+	+

Traplining in bumblebees:
Ontogeny and importance of
long term spatial memory

The results in this chapter are in press as:

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the importance of spatial reference memory in short range foraging

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5.1 ABSTRACT

To test the relative importance of long term and working spatial memories in short-range foraging in bumblebees, we compared the performance of two groups of bees. One group foraged in a stable array of 6 flowers for 40 foraging bouts, thereby enabling it to establish a long term memory of the array, and adjust its spatial movements accordingly. The other group was faced with an array that changed between (but not within) foraging bouts, and thus had only access to a working memory of the flowers that had been visited. Bees in the stable array started out sampling a variety of routes, but their tendency to visit flowers in a repeatable, stable order ("traplining") increased drastically with experience. These bees used shorter routes and converged on four popular paths. However, these routes were mainly formed through linking pairs of flowers by near-neighbour movements, rather than attempting to minimize overall travel distance. Individuals had variations to a primary sequence, where some bees used a major sequence most often, followed by a minor less used route, and others used two different routes with equal frequency. Even though bees foraging in the spatially randomized array had access to both spatial working memory and scent marks, this manipulation greatly disrupted foraging efficiency, mainly via an increase in revisitation to previously emptied flowers and substantially longer search times. Hence, a stable reference frame greatly improves foraging even for bees in relatively small arrays of flowers.

5.2 INTRODUCTION

Traplining is a foraging strategy that involves visiting food sources in a stable repeatable sequence. It has been reported in ten bumblebee species (Manning, 1956; Thomson et al., 1982; Williams & Thomson, 1998; Comba, 1999; Makino & Sakai, 2004), euglossine bees (Janzen, 1971; Ackerman et al., 1982), honeybees (Ribbands, 1949), hummingbirds (Gill, 1988; Garrison & Gass, 1999), tamarins (Garber, 1988), rats (Reid & Reid, 2005), pied wagtails (Davies & Houston, 1981), long-nosed bats (Lemke, 1984) and several species of *Heliconius* butterflies (Gilbert, 1980). Despite the widespread nature of this strategy, the question of how traplines are formed remains relatively unexplored.

Once established, traplines can remain stable for extended time periods (Thomson, 1996; Comba, 1999). However, very little is known on how animals decide on suitable routes to link a set of known locations, i.e. whether they try out multiple routes and ultimately settle on the optimal solution. Janzen (1971) suggested that bees link plants in the order they encounter them, but there is no empirical evidence for this. More importantly, as Janzen points out, such a strategy might produce suboptimal results, because it would not serve to minimize travel paths. Research on honeybees has shown that bees can travel novel shortcuts between familiar locations (Menzel et al., 1998; Menzel et al., 2005), indicating that they might be able to connect multiple locations in a different sequence from that in which they were encountered. One of the goals of this study was to quantify how searching among flowers of a stable array, gradually turns into a trapline. Bees may do this by experiencing multiple possible paths and finally settling onto a near-optimal solution.

Bees have several ways of improving their foraging efficiency in an unfamiliar environment. They can use spatial working memory (Brown et al., 1997), which can help to keep track of recently visited flowers or plants. They also have hard-wired strategies, such as near-far-search, where animals foraging in patchy environments make short movements when encountering high rewards (maximizing the probability of staying in a rich patch), but travel longer distances after receiving poor rewards (Pyke, 1984; Chittka et al., 1997). However, bees also have an impressive long term spatial memory, which they use when foraging from multiple food sources (Collett, 1993; Chittka et al., 1995; Menzel et al., 1998). We use a simple, yet efficient procedure to identify

the relative benefits of long term spatial memory: we randomize flower positions between subsequent foraging bouts, thus preventing bees from using long term spatial memory of flower position *between* bouts, but retaining the possibility to resort to classic foraging algorithms as well as working memory of visited flowers *within* bouts. The performance of these bees is compared to bees allowed access to long term spatial memory, and thereby the ability to form traplines.

There are obvious potential advantages to traplining. Trapliners might learn to link resources in a more direct path, cutting down on travel distance. This can be seen in rats repeatedly visiting food sources (Reid & Reid, 2005). Because traplining bees know the food source locations, they can spend less time searching for them, potentially even identifying individual, rewarding flowers with higher probability than naïve bees (Williams & Thomson, 1998). Thus search times should be lower than for animals that do not, or in our case cannot, use this information. A traplining animal should also make fewer revisits to recently depleted food sources because it can circuit through them, making it less likely to back track on its path. This is especially important as flowers take time to replenish so revisiting at an incorrect schedule would waste time and energy (Williams & Thomson, 1998).

This study uses bees as a model to determine: 1) the ontogeny and characteristics of stable traplining routes within arrays of multiple feeding sites; 2) whether access to long term spatial memory results in stable routes that reduce flight time in search of food, revisits to the food source and travel distance between food sources.

5.3 MATERIALS AND METHODS

5.3.1 Test animals and flight arenas

A bumblebee colony (*Bombus impatiens*) obtained from Biobest Ltd. (Leamington, Canada) was housed in a nest box and connected to a flight arena [105 (l) x 75 (w) x 30 (h) cm] via a clear Plexiglas tunnel. Shutters in the tunnel allowed single bees to be tested by restricting access of other bees. Approximately 8g of pollen were placed directly into the nest on a daily basis. Tests were conducted at temperatures of ≈ 20 °C and a light-dark cycle of 10:14.

5.3.2 Artificial flowers

Flowers were circular with a 1cm diameter, made of blue cardboard and attached to the arena floor. Each bee foraged on 6 rewarding flowers (30% (w/w) sucrose solution placed in the centre of the flower) for 40 continuous bouts. We adjusted the nectar rewards of each bee to its honey crop capacity by allowing it to forage on 15 flowers with 10 μ l of 30% sucrose solution for three pre-experimental bouts. The average amount of sucrose ingested in all three bouts was divided by six and placed on each test flower.

5.3.3 Experimental procedures

The flowers were replaced after each bout, so there were no scent marks from previous bouts available in any given foraging circuit. Individual bees were placed in one of two treatments. In the stable flower treatment flowers remained in fixed positions throughout the 40 bouts, allowing use of spatial memory (and the formation of traplines) within and between bouts (n= 7 bees)(see Figure 5.1a). In the random flower treatment, spatial positions were randomly allocated (using a computer algorithm) onto a 7 x 5 points square grid with 15cm between points, in each new foraging bout (see Figure 5.1b). This protocol allowed the use of spatial working memory within bouts but did not allow use of long term spatial memory between bouts (n= 5 bees). Bees in this treatment could not form traplines and their results were used only to test possible adaptive advantages to traplines. Trials were video taped and analyzed using Behavior Tracker software (Version 1.5). Each individual marked bee was tested once and bees with inter-bout times of less than five minutes were chosen to ensure that only highly motivated bees were used in the tests (very few bees failed this criterion).

Based on our knowledge of the visual system of a different bumblebee species, *Bombus terrestris* (Spaethe & Chittka, 2003), it is unlikely that bees in the stable flower array could see the nearest flower from any one of the flower positions. In that species, large workers can detect a target that subtends 3.5°, corresponding to a distance of 16cm in a target with a diameter of 1cm. Since *Bombus impatiens* workers are smaller, their visual spatial resolution is likely to be less fine-grained, so that data from *B. terrestris* provide a conservative estimate. The smallest inter-floral distance was 21cm for the stable array, therefore bees foraging on the stable array could not detect the next nearest

flower. However, bees foraging on the random arrays may have been able to detect at least one nearest flower in 58% of the spatial arrangements. Thus, if bees employed a simple visually mediated near-neighbour search strategy, the random floral arrays should, on average, have been the easier task of the two.

5.3.4 Data analysis

We terminated data analysis once the bee finished feeding on the sixth rewarding flower. We tested for normality of data where necessary and used appropriate tests utilizing Minitab Version 12. Individual distances between each flower and the other five in every bout were not significantly different amongst the two treatments (Mann Whitney test: $n= 870$; $W= 7122.0$; $p= 0.5415$; Median ± 1 standard deviation: stable= 47.5 ± 19.4 ; random= 46.0 ± 21.8 cm). Thus, flower distances between the groups were comparable and observed differences are not due to this factor.

5.3.4.1 Ontogeny of traplines

We wanted to explore the similarity of individual bees' spatial visitation sequences from one bout to the next, to see whether the tendency to repeat such sequences increased with experience. In comparing the sequences, the crucial question was how many substitutions, insertions and deletions of flowers are necessary to make two sequences identical. This is essentially the same as in DNA alignment, where DNA sequences are compared with one another to assess similarity between them (Waterman & Jones, 1990). We calculated similarity indices using a computer program (courtesy of K. Ohashi & J.D. Thomson) formulated on a technique similar to DNA alignment where the endpoints of the sequences were fixed (Thomson et al., 1997). This technique takes into account insertions, deletions and substitutions to any primary sequence. We defined the two fixed end points as the bee's nest entrance, where each foraging bout originates and eventually terminates. Similarity indices of 0 mean the visitation sequences are completely different and of 1 mean they are identical. We calculated similarity indices between each bout and its subsequent bout. We then averaged these values to give a mean value representing a moving average bin. The similarity indices were analyzed by using a moving average of 5 bouts. For example, we had an average for bouts 1-5, 2-6, 3-7 etc; this averaging removed the effect of variation between pairs of

bouts and revealed gradual changes in the bees' tendency to trapline (Kenney, 1967). For statistical analyses of the bees' similarity indices, we only compared independent moving average bins with no overlapping bouts, so for example bin 1-5 to bin 6-10. Henceforth, when discussing the bees' progress as a function of experience, we only refer to the midpoints of each bin.

To determine whether bees showed a higher tendency to trapline than expected by chance, we compared similarity indices based on observed sequences with those generated by a null model. We generated 5000 random sequences of 10 flower visits (the mean length of the bees' sequences). Then we took sequences 1-5, 6-10, until 4996-5000, and calculated similarity indices for each set of 5, so that we produced a distribution of 1000 randomly generated similarity indices in total. A frequency histogram of these indices is shown in Figure 5.3; 95% of the randomly determined indices fall below a threshold of 0.269. We defined an individual bee's similarity index as non-random (at the 5% level) if it exceeded this threshold. To examine the characteristics of the bees' traplines, we re-examined all visitation sequences this time excluding any revisits to determine trends in the basic visitation sequence. We also compared the distances of these basic visitation sequences to see if bees used shorter ones with experience. To do this, we divided the 40 bouts into early, middle and late bouts, which corresponded to bouts 1-13, 14-26 and 27-40 respectively.

5.3.4.2 Advantages of long term spatial memory for foraging

We investigated how experience affected the number of revisits, and flight time per bout. In this case we compared all the revisits performed per bout by the bees in each treatment. Flight time is a function of search time as well as travel time between flowers, given that the distances between flowers within the two treatments are comparable, we expect any differences in flight time to be due to differences in time spent searching for the flowers. The mean number of revisits or flight time in the first bout was compared to the mean of the last 20 bouts for each group to determine changes within the group with experience. We chose to analyze 20 bouts because by then bees had stabilized in their use of traplines, see Figure 5.3. To evaluate differences between groups, only the last 20 bouts were examined so that comparisons were made after performance saturated for the stable treatment bees. Next, we wanted to investigate if the stable treatment bees performed fewer revisits when using their two favoured

routes than the other less used routes. To this end, we compared the total revisits performed per bout when bees used their two favoured routes with the total revisits performed when using all other routes.

We were also interested in detailed investigation of the revisits bees performed after visiting a rewarding flower to determine if there was a difference between the two bee treatments. We divided the revisits into two types: same-flower (revisit to the same flower immediately after the bee finished feeding from it) and different-flower (revisit to a different previously visited flower). We compared the stable and random flower treatments to investigate how they may differ. We also compared the number of each type of revisit performed per bout when bees in the stable array treatment used their two favoured routes compared to all other routes.

Flight distances were determined through frame by frame playback (using a JVC DV Video Cassette Recorder, BR-DV3000E) of the first and last three bouts, when the bees had the least and the most experience with the setup. We arbitrarily chose to analyze three bouts before we began data analysis. A piece of clear cellophane was taped onto the television screen and the bee's path was marked. A string was used to determine the distance travelled by connecting these marks. We had complete video tapes for nine bees.

We needed to standardize the routes, because longer flight distances may be due to larger inter-floral distances. We divided the total travel distance performed by each bee in each bout by the summed value of individual inter-floral distances for that bout. We then averaged these standardized values to generate one value for the first three bouts and one for the last three bouts for each bee. The performance of the two groups of bees in the first and last three bouts was compared using a two sample t-test with bee as the unit of replication. We verified that inter-floral distances were comparable between the two bee treatments for the first and last three bouts. To do this, we performed two sample t-tests comparing the inter-floral distances of the stable arrangement with each of the random arrangements. Twenty three out of twenty six comparisons were not significant ($p > 0.05$). In the three cases where they were significant, the random array inter-floral distances were much smaller than the stable distances (mean \pm 1 standard deviation: 29.7 ± 15.9 , 28.5 ± 11.9 and 37.8 ± 16.9 cm for the random arrangements compared to 52.9 ± 19.6 cm for

the stable arrangement). Henceforth, average values are reported as mean \pm 1 standard deviation throughout and sample sizes (n) are number of bees per treatment.

5.4 RESULTS

5.4.1 Changes in sequence similarity with experience

Visitation sequences became more similar with experience until approximately bout 20, where repeatability saturated (see Figure 5.3). There was a significant difference in the route similarity indices of bees foraging in the stable spatial array of flowers from the start level to bout 10 (paired t-test: $t = 2.76$, $p < 0.033$; $n = 7$). This significance was consistent between the first and all subsequent bins. Comparing the randomly generated sequences to bees in the stable flower array, we find that bees, as a group, had a significantly higher similarity index by bout 5 (see Figure 5.3). Bees C and F reached significance by bout 3, bees B, E and G by bout 5 and bees A and D by bout 12 and 11 respectively.

When revisits were removed from the analyses, bees foraging on stable spatial arrays had one of two strategies for traplining. They either relied heavily on one visitation sequence and less on another (bees B, D, E and F), or had two sequences that they equally used (bees A, C and G) (see Table 5.1). None of these preferred visitation sequences were performed in the first three bouts. The earliest was at bout 4 (bee A, sequence 2-3-4-5-6-1) and the latest by bout 11 (bee C, sequence 2-4-5-6-3-1). Four main sequences were used as traplines by 5 out of 7 bees (henceforth called the four popular routes). Of the remaining two bees, bee E used none of the four popular routes and bee G used only one. These sequences are 1-2-4-5-6-3, 1-2-3-4-5-6, 2-4-5-6-3-1 and 2-3-4-5-6-1. These routes are similar in some components, for example they all contain the sequence 4-5-6. The main differences between these routes are the start flower position and whether the bees visited flower 3 or 4 first after feeding from flower 2.

Investigating these routes in detail suggests that bees are minimising distance between flowers generally, except in the case of flowers 3 and 4, which were located at very similar distances, 33.5 and 33.75 cm respectively, away from flower 2. In addition, when deciding on the start flower, bees did not always minimize distance travelled from the hive to the first flower. Flower 2 was about 7cm farther away from the hive entrance than flower 1 (32 and 39.25

cm beeline distance from the hive entrance for flowers 1 and 2 respectively). Bees started at flower 2 in half of the four most popular routes.

Bees generally used longer routes in the early bouts compared to the middle and late bouts (see Figure 5.4). There is also an obvious increase in the use of the four popular routes with experience, which is detectable from the early bouts. The number of routes used by the bees as a whole decreased with experience from 29 in the early bouts to 18 in the late bouts.

5.4.2 Revisits

Bees in both treatments reduced their revisitation from the first bout (stable array: 11.14 ± 7.63 first bout, 2.36 ± 0.48 last twenty bouts; random array: 13.40 ± 5.85 first bout, 4.9 ± 1.6 last twenty bouts) (paired t-test: stable array: $t = 3.04$, $p < 0.023$; $n = 7$; random array: $t = 3.22$, $p < 0.032$; $n = 5$; see Figure 5.5a). In the last 20 bouts, traplining bees that had access to long term spatial memory performed fewer revisits than those unable to use this memory (two sample t-test: $t = -3.45$, $p < 0.026$; $n = 12$). The bees foraging on a stable spatial array of flowers made fewer revisits in 34 out of 40 bouts (85%). Traplining bees performed, on average, fewer revisits when using their favoured routes compared to other routes (3.13 ± 0.66 and 4.07 ± 1.36 revisits per bout for favoured and all other routes respectively). However, this difference was not significant (Mann Whitney U test: $W = 44.0$; $p < 0.31$; $n = 7$).

Bees foraging on the stable array were more likely to revisit the same-flower in the first few bouts (see Figure 5.6). For example, they performed 3.42 ± 1.81 same-flower revisits compared to 0.86 ± 1.3 different flower revisits in the first bout. This quickly reversed to 0.71 ± 0.98 and 1.43 ± 1.27 by bout 4 for same- and different-flower revisits respectively. Interestingly, bees foraging on the random array, without access to long term spatial memory, do not seem to change their use of same- and different-flower revisits. These bees used fewer same-flower revisits in only 25 out of 40 (62.5 %) bouts. However, there appears to be more individual variation per bout in the number of revisits to different flowers compared to revisits to the same flower for these bees.

After traplining bees finished feeding on a flower, they made similar numbers of same (Mann Whitney test: $W = 50$; $p = 0.79$) and different (Mann Whitney test: $W = 49$; $p = 0.70$) flower revisits when using their favoured routes compared to other routes. Bees performed 0.90 ± 0.40 revisits to the same

flower and 1.22 ± 0.52 revisits to a different flower when using their favoured routes. They performed 1.03 ± 0.48 revisits to the same flower and 1.3 ± 0.37 revisits to a different flower when using the other routes.

5.4.3 Flight Time

Bees in both treatments reduced their flight time from the first bout, however, only the improvements for bees foraging on stable arrays are significant (stable array: 63.9 ± 33.1 s first bout, 22.5 ± 4.03 s last twenty bouts; random array: 71.0 ± 34.60 s first bout, 32.3 ± 5.29 s last twenty bouts) (paired t-test: stable array: $t= 5.04$, $p< 0.002$; $n=7$; random array: $t= 15.66$, $p< 0.072$; $n=5$; see Figure 5.5b). Overall, bees foraging from stable spatial arrays with access to long term spatial memory spent less time flying per bout (22.5 ± 4.03 s for stable, 32.3 ± 5.29 s for random spatial array of flowers; two sample t-test: $t= -3.48$, $p< 0.01$; $n= 12$). Bees foraging on stable spatial arrays flew less than those on random arrays in 30 out of 40 bouts (75%). There was no significant difference in inter-floral flight times per bout (2.55 ± 0.52 s for stable and 2.95 ± 0.18 s for random spatial arrays; two sample t-test: $t= -1.86$; $p< 0.11$; $n= 12$).

5.4.4 Flight distance

Bees in both treatments travelled similar distances in the first three bouts (919.9 ± 276.93 cm for stable and 1007.9 ± 282.42 cm for random spatial array) (two sample t-test: $t= - 0.87$, $p< 0.65$; $n= 9$). Although there is suggestive evidence that bees foraging on stable arrays travel less distance than those on random arrays in the last three bouts (526.3 ± 55.51 cm for stable, 795.4 ± 216.67 cm for random spatial array), these results are not significant (two sample t-test: $t= - 2.63$, $p< 0.062$; $n= 9$). Distance travelled was correlated with revisitations (Pearson Correlation: $r = 0.894$; $p< 0.001$; $n=9$), and bees foraging on random spatial arrays revisit more than those on the stable array. Thus, it is likely that with more bees, the difference in travel distance between the two treatments would become significant.

5.5 DISCUSSION

Previous reports on traplining in bees and other animals have observed this behaviour in field conditions, where either reward levels, plant visitation patterns and the bees' previous experience were not controlled, or in case of more

controlled studies, the visitation sequences were entrained by an experimenter (e.g. Thomson, 1996; Comba, 1999). This study investigates traplining behaviour in a controlled laboratory study where bees are able to freely optimize routes as they accumulate experience. It is the first to look at how spatial foraging strategies develop in naïve bees.

5.5.1 Bees form traplines with experience

To assess if the bees' visitation sequences became more similar with experience, we produced a null model based on similarity indices from randomly generated sequences and compared it to similarity indices generated by the bees' visitation sequences. While complete randomness of movements is perhaps a simplistic assumption of how bees might move in the absence of traplining, such a random null model has the virtue of being free of ad-hoc assumptions about alternative strategies that bees might use.

Traplining bees began stereotyping their routes between bouts 8 and 12 (see Figure 5.3), and continued to strengthen this repeatability until approximately bout 20, showing that bees develop their traplines after experiencing different routes. Indeed, the earliest one of the later preferred visitation sequences was performed at bout 4 (bee A) and the latest at bout 11 (bee C).

Thus, bees foraging on small spatial scales do not form traplines simply by following the visitation sequence in which they originally encountered the flowers (Janzen, 1971). In order for a trapline to form, bees first need to locate the rewarding plants, and experience several subsequent rewarding revisits before linking them in a repeatable sequence. They also need to learn the locations of these flowers relative to each other. We expect that naïve bees with little foraging experience or experienced bees naïve to the area may sample different plants a few times before deciding to return to specific plants on a regular basis. In doing this, they may experience different routes within these plants until they find preferred ones. Bees that have experience foraging in an area may also sample different routes as resources change.

5.5.2 Preferred visitation sequences and minor variations to primary sequence

The two most used visitation sequences of each bee were used for about half of the foraging bouts. Of these, bees either had one preferred sequence and a

second less used one, or they had two that they used with equal frequency. The differences between these two most used visitation sequences for each bee are very small, involving a change in one flower visit in the sequence. Thus, bees use variations to one primary visitation sequence when they trapline. The variations in the top two preferred visitation sequences where the first visited flower was moved to the last arise from starting at a different flower. For example, the two preferred visitation sequences used by bees A, B, and E started with flower 1 instead of 2 and vice versa. Thus, in order to complete the circuit, they needed to visit the flower they skipped at the start. The three cases where replacements within the sequence took place (bees C, D and F), the start flower remained the same. In these cases the bees reversed their visitation of flowers 3 and 4. Variation in traplining routes have been reported previously (Thomson et al., 1982; Thomson et al., 1987; Comba, 1999). Although changes between days have been attributed to changes in plant status (Thomson et al., 1982; Williams & Thomson, 1998), we show here that some variations can still occur, because traplines varied even though our flowers were entirely constant in position and reward status.

5.5.3 Optimal routes, and similarities in traplines used by individual bees

Most bees converged on two of the four most popular sequences as their favoured routes, indicating that they were using a common foraging strategy. A bee feeding from a flower in the stable array probably could not visually detect the next nearest flower, thus near-neighbour movements by direct detection of nearest flowers were not possible. Further evidence against such near neighbour movements is that bees developed their traplines with experience, suggesting that they needed to become familiar with the spatial layout.

Analysis of the four most popular sequences suggests that bees learn to minimize travel distance between flowers, rather than minimizing overall travel distance. The four popular routes mainly involve movements between nearest flowers with two main differences: the start flower and the order with which flowers 3 and 4 were visited after feeding from flower 2. Flowers 3 and 4 were located 33.5 and 33.75 cm away from flower 2. Thus the switch in the visitation order is most likely due to the inability of the bees to differentiate between such small differences in distance. It is interesting that they did not choose one of the two variants but rather used both even though one of the routes always

provided a longer overall travel path. Flower 2 was about 7cm farther away from the hive entrance than flower 1 thus it seems bees did not always minimize distance travelled from the hive to the first flower when deciding on the start flower. The bees often followed a straight trajectory from the hive entrance into the foraging arena, where they encountered flower 2 and probably choose to feed there rather than change their path to find flower 1. However, the fact that half of the favoured routes began by visiting flower 1 indicates that some bees were assessing distance of flowers from the hive entrance. It is interesting to note that bee E had her own unique routes, indicating that not all bees are minimizing flight paths by minimizing flight distance between flowers. Her routes resulted in longer flight distances (352.3 and 361.5 cm) than the four popular routes.

The bees as a group used 38 different visitation sequences throughout the 280 foraging bouts. In the early bouts they used 29 different routes. However, with experience, the number of routes used, especially those involving the longest visitation sequences, were reduced by about half, while usage of the subsequent four popular routes increased.

If the bees were trying to link the flowers optimally, we would expect their favoured visitation sequences to be very close to the shortest sequence. In addition to the optimal route, bees chose the 8th, 14th and 19th shortest routes. However, all these choices can be explained by attempts to reduce travel distance between pairs of flowers as opposed to minimizing total travel distance.

Thus, bees can reduce their travel distance with experience, but, at least for small foraging scales, they use simple rules that do not necessarily produce the shortest path. Rats (Reid & Reid, 2005) also reduced their distance travelled between point sources with experience. Although that study was not analyzing traplining behaviour, the rats' behaviour can be explained by attempts to minimize travel distance between food sources. These rats never reached the optimal distance. It would be interesting to see if bees foraging on larger spatial arrays also reduce their travel distance by minimizing flight time between plants.

5.5.4 Revisits

Both groups reduced their revisitations from the initial bout (see Figure 5.5a). Bees can use tactics such as working spatial memory and scent marks within

bouts to reduce their revisitation rate. However, bees that were allowed to use long term spatial memory, and thereby form traplines, reduced their revisits to a greater extent than bees that could not form traplines. The bees on the stable spatial array revisited less than those on random arrays in 85% of bouts. Therefore, the ability to use long term spatial memory to locate and circuit through food sources in a repeatable order reduces the likelihood of revisiting recently visited food sources. We expect this to be especially important when the food sources are spatially aggregated and therefore revisits more likely.

There is suggestive evidence that bees foraging on the stable array revisited less when using their preferred sequences compared to the other sequences. However, these results were not significant.

We investigated the types of revisits performed by bees in the two treatments. We find that when bees have access to long term spatial memory they reduce their same-flower revisits and use more different-flower revisits within the first few bouts. However, bees foraging on the random array did not change their performance of the two types of revisits. Both groups of bees had the opportunity to learn that flowers do not refill once emptied. Bees have been shown to reduce their flight angle upon encountering rewarding flowers, helping the bee stay in a rewarding patch (Pyke, 1984; Chittka et al., 1997). It is possible that the immediate revisits to the same flower are similar to area restricted searching. In our case the flowers were not spatially aggregated and often the bees could not see the next nearest flower, which may have resulted in the bees revisiting the same flower they just fed from in an attempt to perform area restricted searching. The bees in the stable array learned not to use this behaviour while those without access to long term spatial memory continued to employ it as a search strategy. The fact that bees foraging on the stable flower array increased their performance of different-flower revisits provides further evidence against the idea that bees are optimising their route by finding the shortest one, because if this was the case we would expect a reduction in any kind of revisitation not just same-flower revisits.

5.5.5 Flight Time and distance

We also find an advantage to traplining in terms of the time spent searching for flowers. Bees foraging on the stable spatial array had lower flight times and they reduced their flight time with experience, but those on random spatial arrays did

less so. Bees having access to long term spatial memory had lower flight times in 75 % of the 40 bouts. This is found in other animals as well. Rats also took less time to move between food sources as they gained experience with the location of these resources (Reid & Reid, 2005).

Both bee treatments had similar travel distances in their first three foraging bouts, and bees in both treatments reduced their travel distance with experience. There is suggestive evidence that traplining bees, with access to long term spatial memory, have shorter flight distances than bees unable to form traplines, but our results are not statistically significant.

5.5.6 Conclusion

After sampling multiple routes, bees with access to long term spatial memory arrive at traplining routes that help reduce revisitations and search times. Our study found large differences in performance between traplining bees, and those not allowed to trapline, despite its small spatial scale. Bees can forage in patches located several kilometres apart (Janzen, 1971; Osborne et al., 1999; Goulson, 2000; Walther-Hellwig & Frankl, 2000) and their traplines can cover an area of at least 312 m² (Comba, 1999). Therefore, we expect the advantages of traplining to be greatly magnified in field conditions, where bees often cannot easily detect one flower (or patch) from another, and therefore a continuous search or relying solely on short term memory may be a highly inefficient strategy. Thus, experiencing different routes and subsequently linking distant foraging locations by memorized vectors, while minimizing travel distance, should greatly enhance a bee's foraging efficiency. We used a small spatial scale, which means we were investigating within patch behaviour. It is important to identify if bees behave in a similar manner when foraging between patches and on food sources with varying levels of reward.

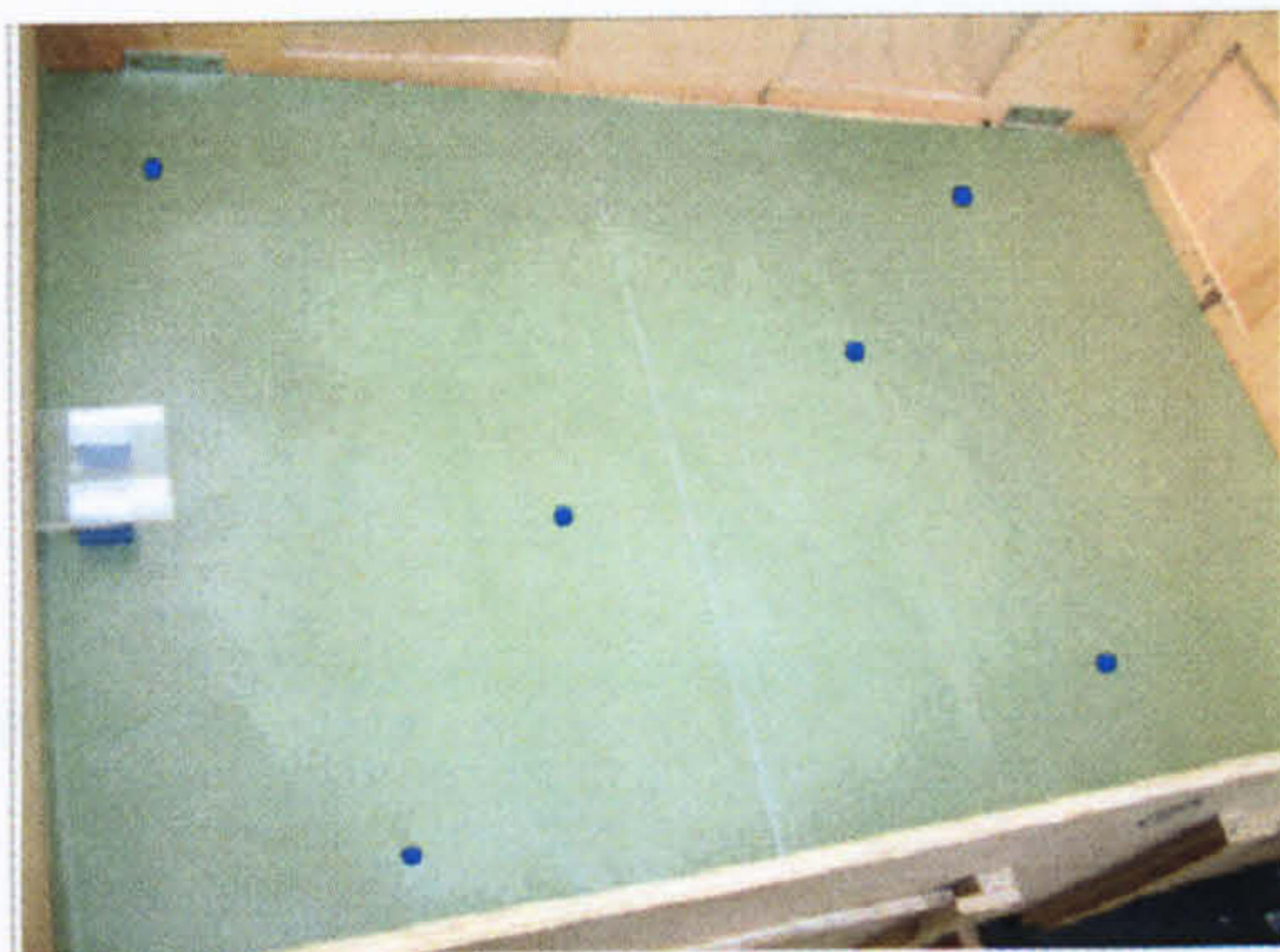
Traplining behaviour has been reported in over 20 animal species. This study provides clues to some of the advantages of this behaviour. Further research in this area should attempt to identify if the same advantages are present in other traplining animals. This will help clarify why this foraging strategy is so popular.

5.6 ACKNOWLEDGEMENTS

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FIGURE 5.1 Experimental setup. (a) Arrangement of flowers in the stable array
(b) Examples of flower arrangement in the random arrays.

(a) Stable array



(b) Examples of random array

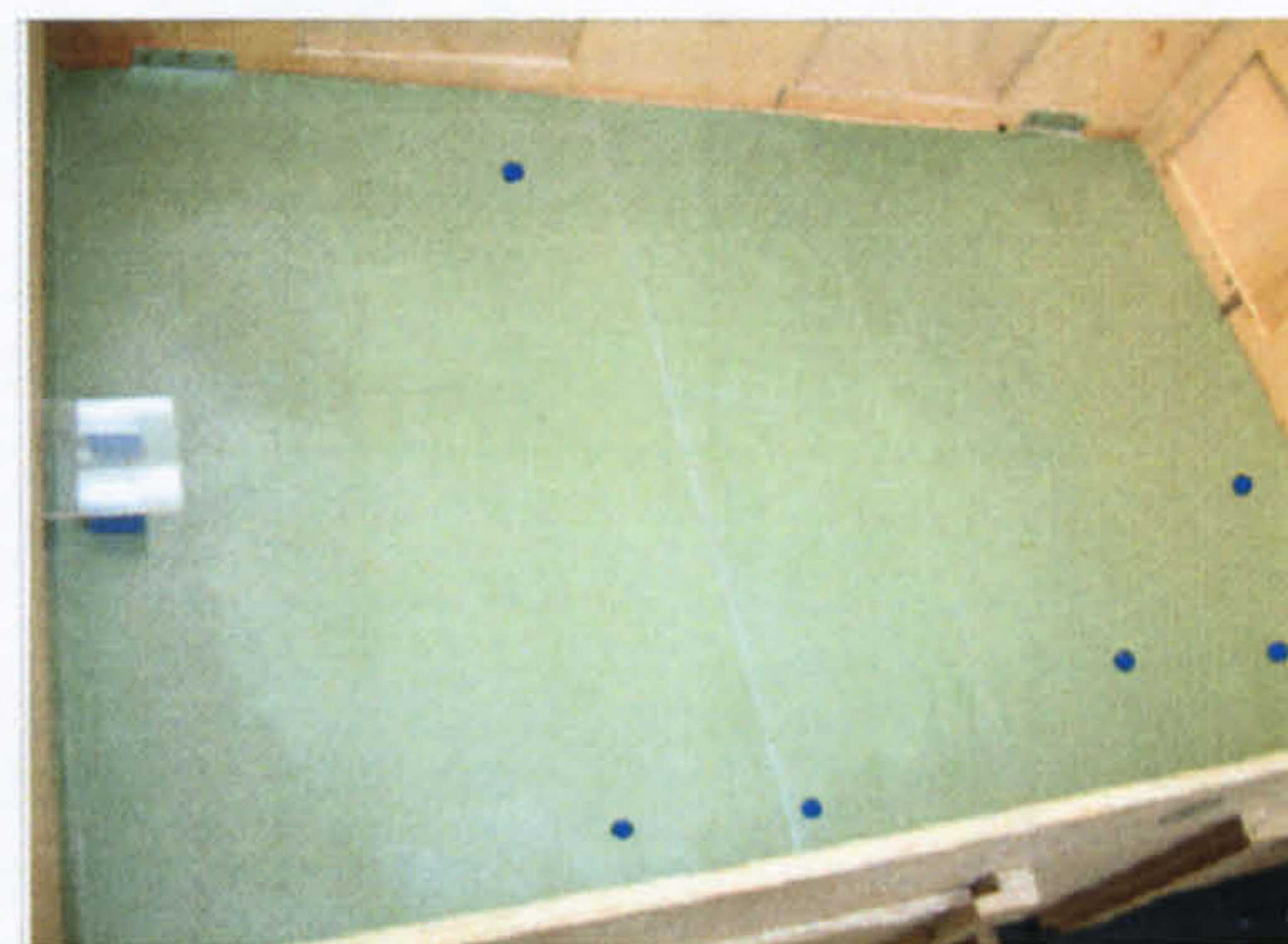
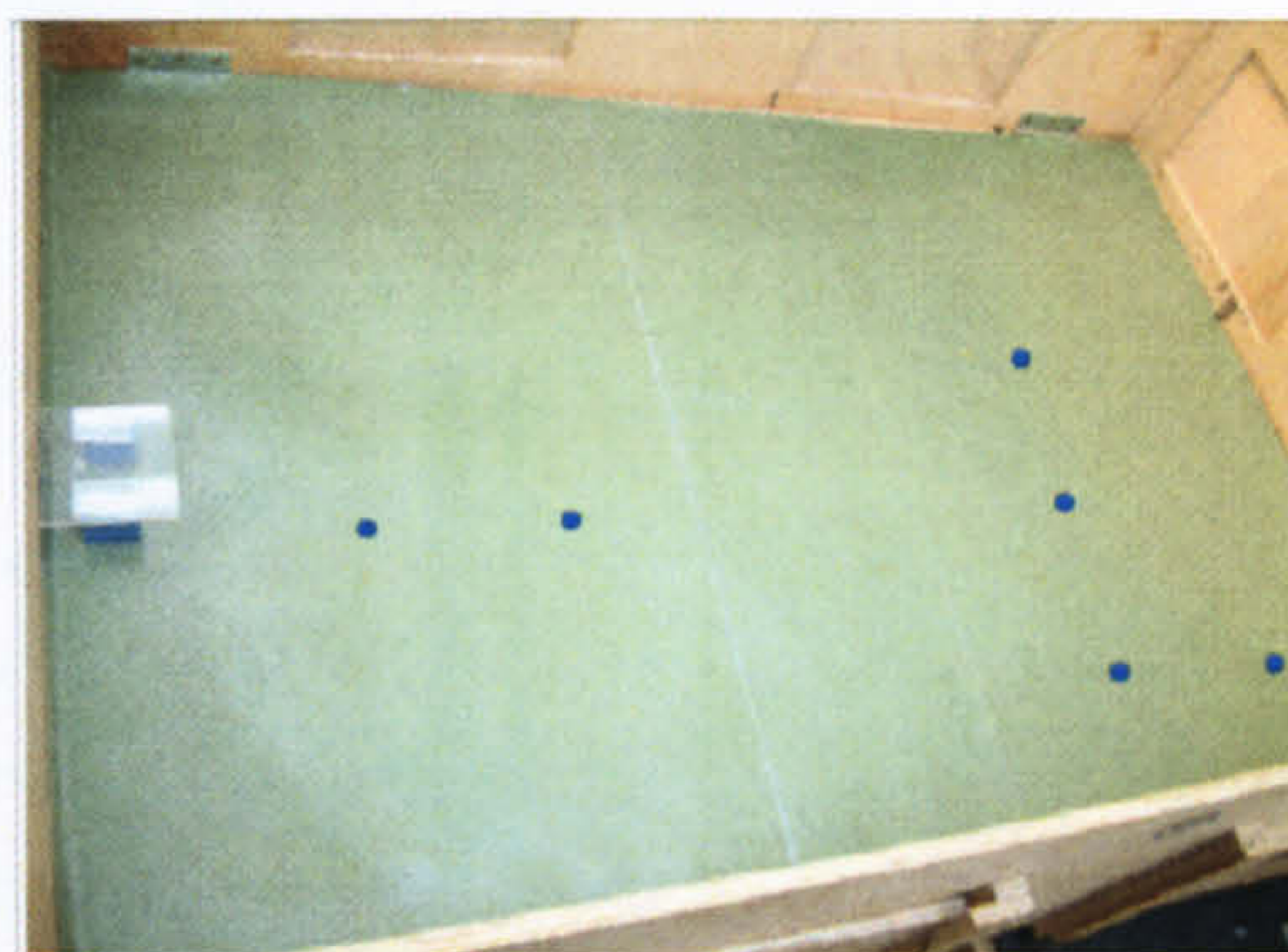
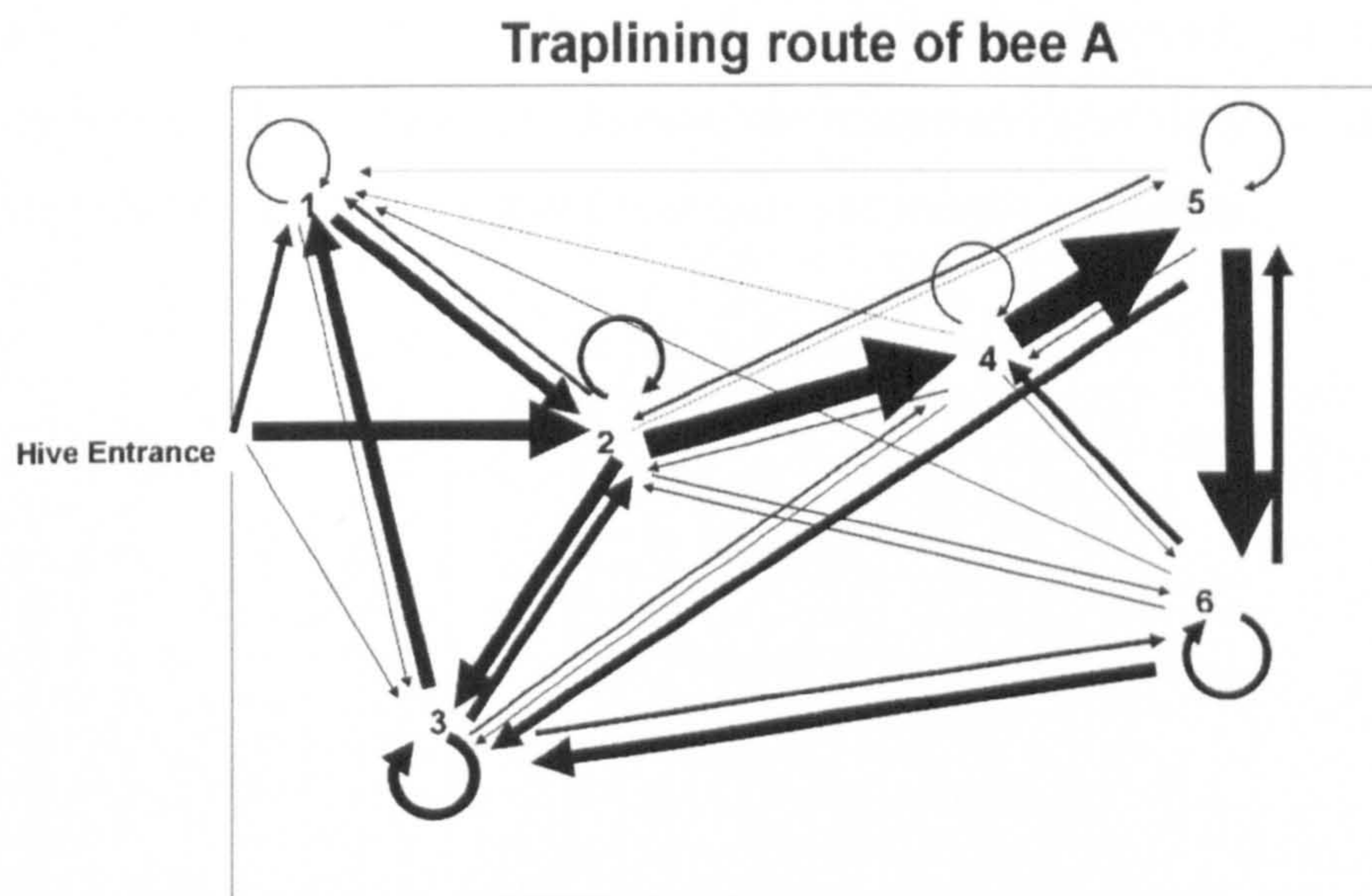


FIGURE 5.2 (a) and (b) Examples of routes taken by bees foraging on six fixed artificial flowers. Numbers represent flower positions. The width of the arrow corresponds to the frequency each trajectory was taken throughout the 40 bouts. The arena was enclosed by four walls [105 (l) x 75 (w) x 30 (h) cm], illustrated in the figure. Flowers were 1cm in diameter.

(a)



(b)

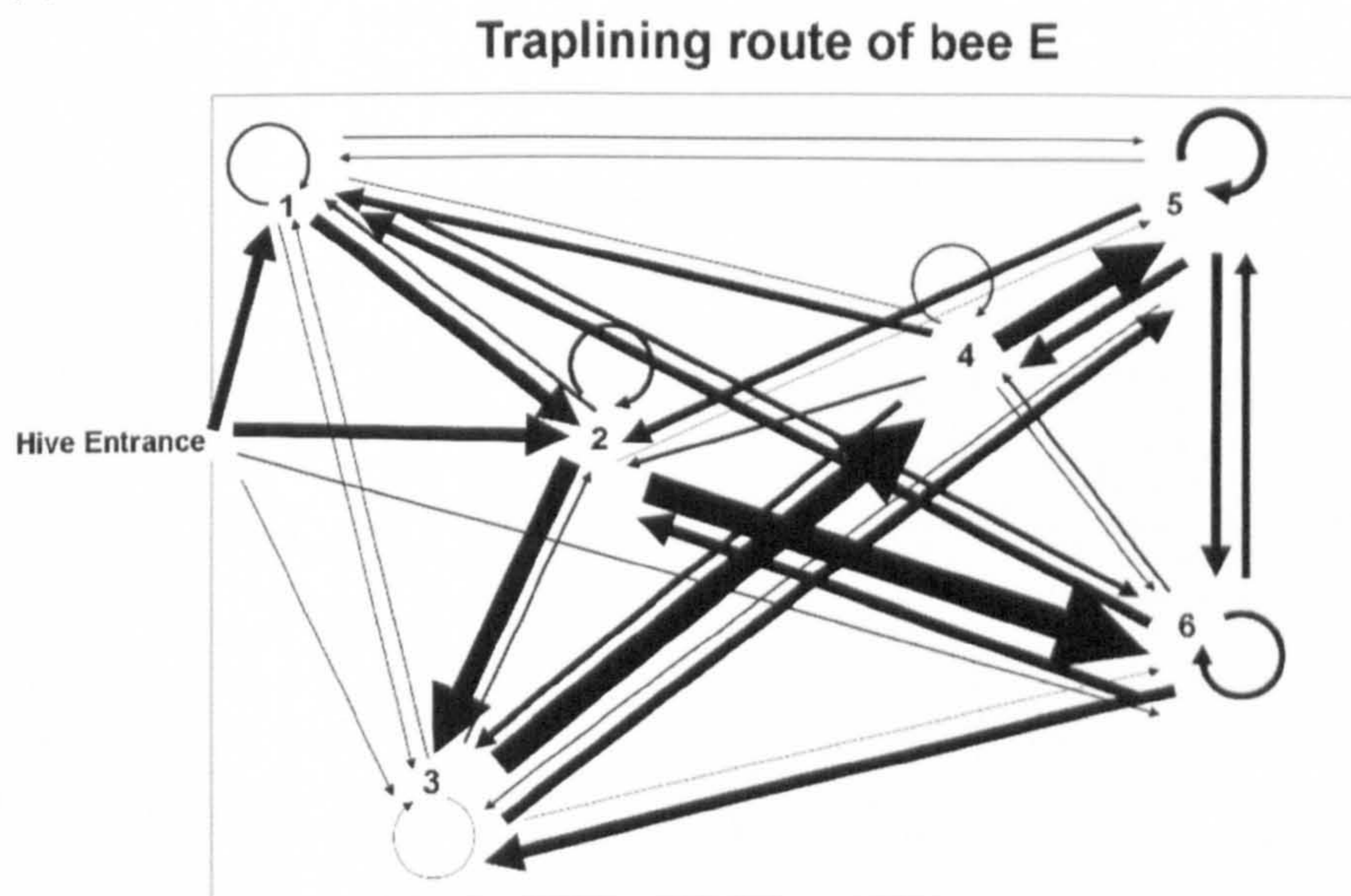


FIGURE 5.3 (a) Moving average for mean similarity indices of traplining (stable treatment) bees. The higher the value of the similarity index the greater the similarity between bouts (i.e. tendency to trapline). Similarity indices were calculated using a technique similar to DNA sequence alignment. (b) The bees' similarity indices were compared to 1000 indices produced from randomly generated sequences; 95 % of the indices from randomly generated sequences fall below the threshold of 0.269, indicated by the dashed line. The bees' similarity index was considered significantly different (at the 5% level) if it exceeded this threshold. Bees show increased similarity in visitation sequences with experience. Error bars represent standard deviation.

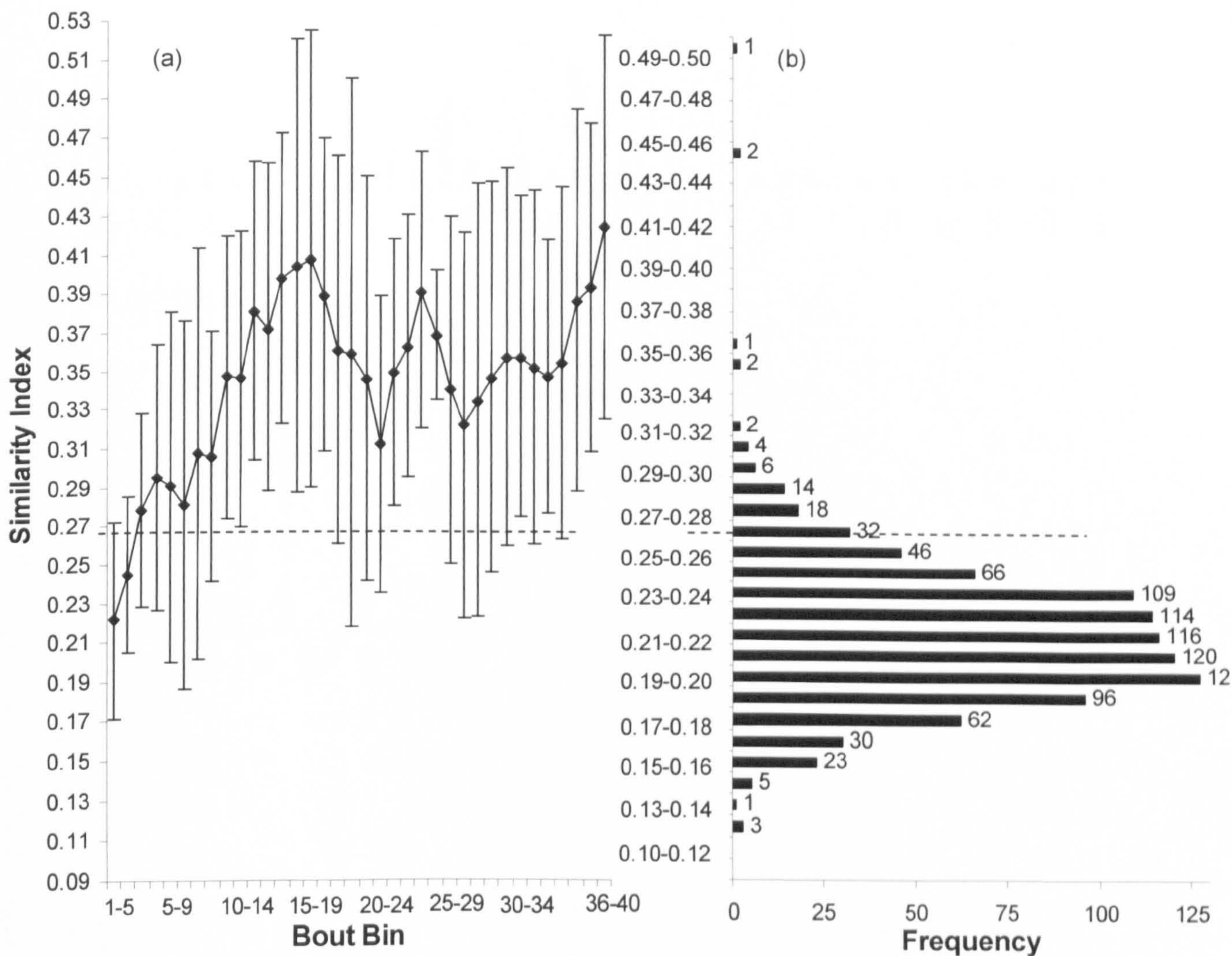


FIGURE 5.4 Percentage of times bees used each visitation sequence listed in Table 1 as a function of experience. These distances are for sequences that exclude revisits. The numbers on the x-axis represent the length of each route. Each sequence had its own unique distance and the length of every other sequence is marked on the x-axis. Bees followed longer routes in the early bouts, which were not used in the middle and late bouts. They also start to show a preference for the subsequent four popular routes in these early stages.

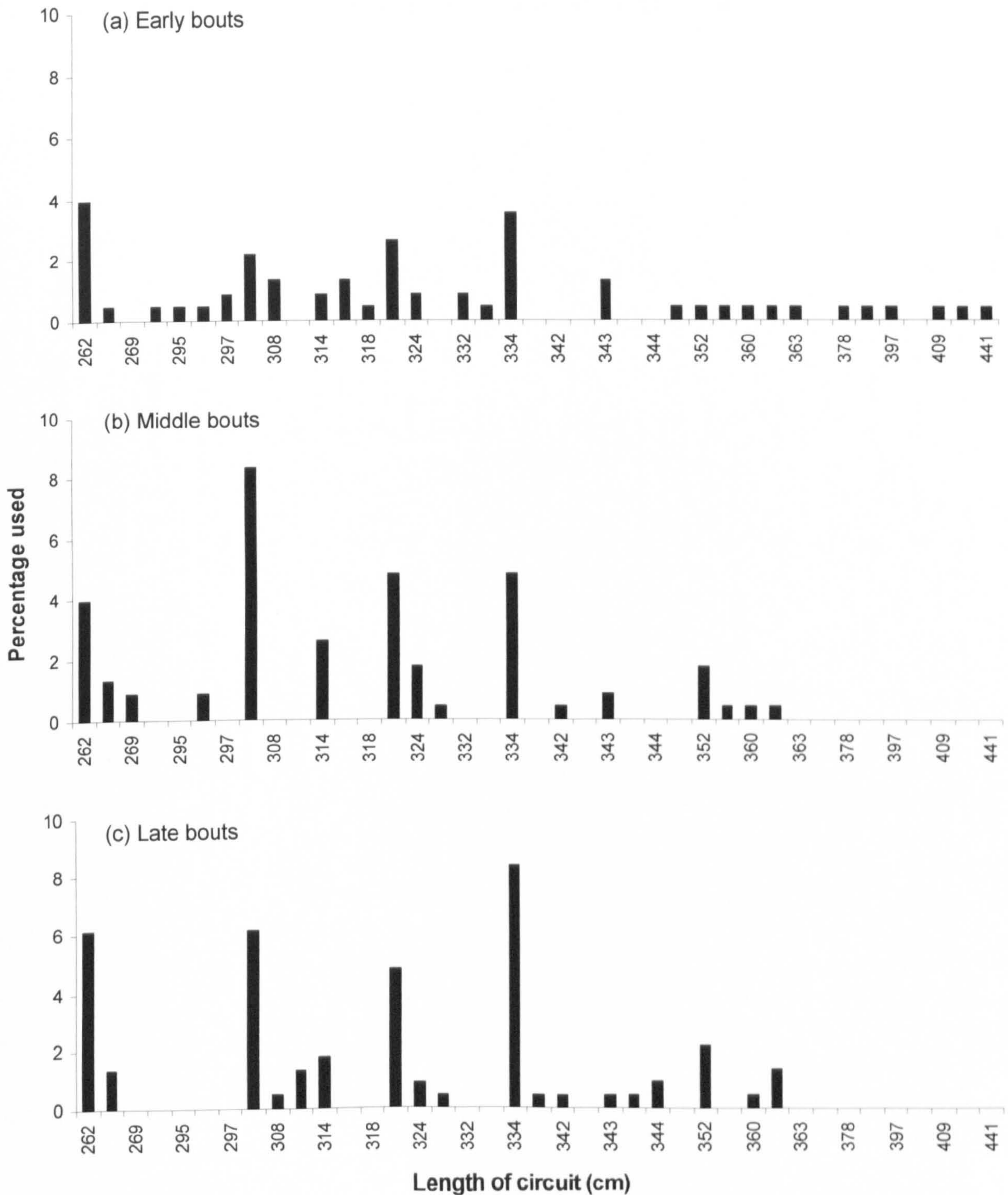


FIGURE 5.5 (a) Mean number of revisits per bout as a function of experience. Traplining bees consistently had lower revisitation rates. (b) Mean time spent in flight per bout as a function of experience. The non-traplining bees performed better than the stable bees in only 5 of 40 bouts. Error bars represent standard deviation. The continuous line with open circles represents data for bees foraging on random arrays and the dashed lined with filled circles represents data for bees foraging on the stable arrays. Vertical bars indicate standard deviations; where the grey bars below the data point are for stable array treatment bees and black bars above the data point are for random array treatments bees.

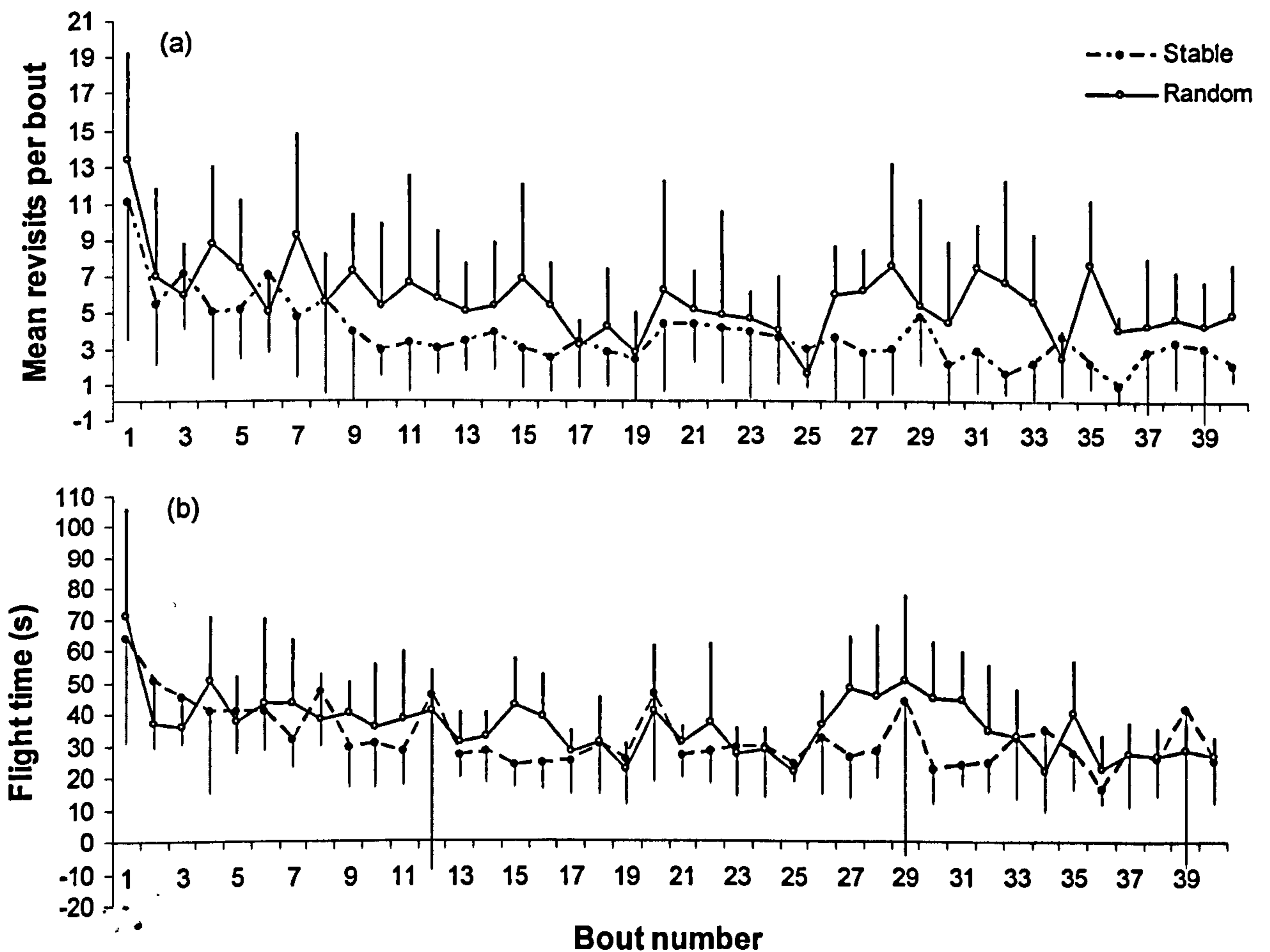


FIGURE 5.6 Mean number of revisits to same and different flowers for bees foraging on stable (top) and random (bottom) foraging arrays. The continuous line with open circles represents same revisits and the dashed lined with filled circles represents different revisits. Vertical bars indicate standard deviations. Grey bars pointing down are for same flower revisits and black bars pointing up are for different flower revisits.

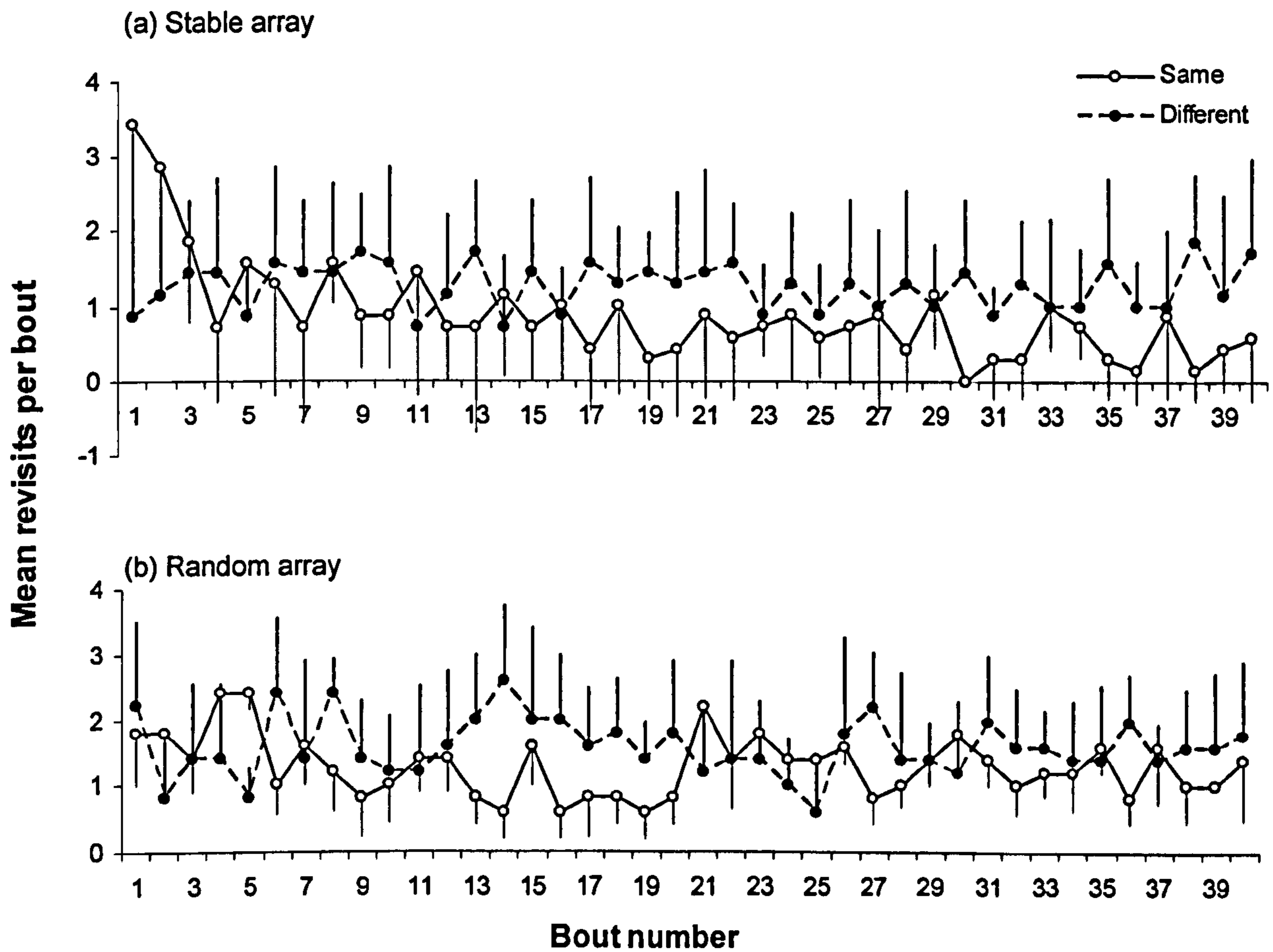


TABLE 5.1 Flower visitation sequences for each traplining bee. These sequences were determined by excluding revisits. Bees found their own unique solutions, but did share some visitation sequences. Each number represents a flower position (see Fig. 5.2a and b). All flowers were located in the same positions for all bees. Sequences with the same colour are identical and numbers within brackets indicate the number of times the sequence was performed in 40 bouts. Sequences with no number beside them were only used once. The values below each sequence indicate the distance a bee would travel to visit the flowers in the order given.

Bee	Sequence										
A	123456 (7)	234561 (7)	124563 (5)	145623 (5)	245631 (3)	154623 (2)	324516	316452	456231	213456	234516
	319.2cm	334.0cm	262.0cm	267.0cm	306.6cm	268.7cm	370.8cm	336.0cm	323.8cm	354.1cm	400.9cm
B	245631 (15)	124563 (8)	234561 (4)	236451	123456	264531	321465	245163	245613	245316	214563
	306.6cm	262.0cm	334.0cm	308.3cm	319.2cm	332.0cm	347.9cm	343.6cm	313.6cm	408.9cm	296.9cm
C	245631 (11)	234561 (9)	245613 (4)	246513 (2)	246315	124563	123465	236451	246153	234651	456312
	306.6cm	334.0cm	313.6cm	313.5cm	397.2cm	262.0cm	343.0cm	308.3cm	378.9cm	333.8cm	341.9cm
D	123456 (17)	124563 (7)	123465 (2)	124653 (2)	123645						
	319.2cm	262.0cm	343.0cm	297.3cm	317.5cm						
E	263451 (10)	126345 (5)	123465 (4)	263541 (3)	234561 (3)	123456 (3)	123546 (2)	234165 (2)	213465	263415	
	352.3cm	361.5cm	343.0cm	360.3cm	334.0cm	319.2cm	328.9cm	423.0cm	377.9cm	441.2cm	
F	234561 (14)	245631 (8)	245613 (4)	214563 (2)	236451 (2)	245163	213456	126543	234516	231456	246531
	334.0cm	306.6cm	313.6cm	296.9cm	308.3cm	343.6cm	354.1cm	277.7cm	400.9 cm	343.4cm	313.5cm
G	124563 (8)	456231 (7)	456213 (3)	245613 (3)	245631(2)	123456 (2)	145623	145632	452163		
	262.0cm	323.8cm	314.2cm	313.6cm	306.6cm	319.2cm	267.0cm	294.8cm	363.2cm		

6

Remote detection of reward

6.1 ABSTRACT

Scientists have assumed that bees cannot discriminate between water and sucrose solutions except through direct antennal or proboscis contact. For the first time, I show that this assumption is erroneous; bees are able to remotely detect the difference between these two liquids. Bees discriminated between 1ml volumes of water and concentrated sucrose solution made of purified ingredients. I found consistent differences in relative humidity above these two liquids. Therefore, I tested if bees use these differences to make their distinction. Although there is a small effect of humidity, this factor does not fully explain the bees' behaviour. I suggest further tests that will help determine what cues the bees are using.

6.2 INTRODUCTION

Scientists have assumed that bees cannot tell the difference between water and sugar solutions (e.g. Marden, 1984; Burns & Thomson, 2006). This has resulted in the use of water to control for remote effects elicited by the presence of a sucrose solution in experimental flowers (e.g. Cruse, 1974; Waddington & Heinrich, 1979; Marden, 1984; Macuda et al., 2001; Thivierge et al., 2002; Burns & Thomson, 2006). The main argument supporting this assumption is that sucrose molecules are relatively non-volatile (Kambara & Hishida, 1981; Moreira & De Maria, 2005) and not detectable through GC-MS headspace analysis (Raguso, 2004). Therefore, direct antennal or proboscis contact is thought to be required for bees to differentiate between the two liquids (Haupt, 2004; Haupt & Klemm, 2005). However, no consideration has been given to the fact that the bees can use other chemical or optical differences.

Water and sucrose play different roles for bees. Sucrose is the energy source of the bee and its colony, but we know far less about the role water plays. Honey bees (Kühnholz & Seeley, 1997; Pankiw, 2003) and some stingless bees (Cauch et al., 2004) use water to thermoregulate their nest. During periods of intense heat, specialised foragers collect water in the form of dew droplets or splashes on rocks off river banks (Kühnholz & Seeley, 1997). This water is placed in honeypots (Kühnholz & Seeley, 1997) and its evaporation is thought to reduce the hive temperature (Karsai & Wenzel, 2000; Cauch et al., 2004). Water may also be used by bees in the nest to adjust their water balance (Visscher et al., 1996) or dilute nectar that has become too thick due to evaporation (Corbet et al., 1979). In bumblebees, the group used here, this type of foraging seems rare. For example, in contrast to honeybees and stingless bees, they do not respond to induced transient heat by collecting water in the laboratory (Saleh, personal observations). Yet, there is one report in the literature where bumblebees were observed foraging for water (Ferry & Corbet, 1996). This behaviour is thought to be due to exceptionally high temperature and humidity during the summer season. This evidence suggests that bumblebees will forage for water when temperature and humidity are high for prolonged periods of time.

Thus theoretically, there may be an adaptive need to detect water remotely and differentiate it from other liquids such as nectar [composed mainly of sucrose (Chalcoff et al., 2006)]. For example, a bee will notice when she

passes over a water source and, if necessary, can distinguish between the two liquids on the surface of shallow flowers while in flight, thereby reducing the need to land and examine the solution by contact chemoreception. One way they may be able to make such distinctions is through hygrometers located on their antennae.

Insects, including bees, possess hygrometers that respond to changes in relative humidity (Yokohari, 1983; Altner & Loftus, 1985). For social insects these receptors are most likely used to detect changes in relative humidity within the hive (Potts et al., 1984; Roces & Kleineidam, 2000; Walters & Mackay, 2003) and select nesting sites with high moisture content (Potts & Willmer, 1997; Wuellner, 1999). In addition to detecting the presence of water, hygrometers may permit nectar foraging bees, for example, to detect the presence or absence of nectar. Humidity gradients within tubular flowers (Corbet et al., 1979; Corbet & Willmer, 1981) may also allow these bees to detect the presence of nectar of different concentrations. Thus, an ability to detect differences in relative humidity can be used to avoid visiting emptied flowers or to focus the bees' visits on flowers containing concentrated nectar, [which they prefer (Dupont et al., 2004)]; ultimately improving their foraging efficiency.

The aim of this study was to 1) establish if bees can discriminate between water and sugar solutions 2) test if bees use differences in relative humidity above the two solutions to identify them.

6.3 MATERIALS AND METHODS

6.3.1 Test animals and flight arenas

Colonies of *Bombus terrestris dalmaninus* were obtained from Koppert Ltd. (The Netherlands). Each colony was connected to a flight arena [100 (L) x 40 (S) x 70 (H) cm] by means of a transparent Plexiglas tube. This tube contained moveable cardboard flaps to allow only selected foragers into the flight arena. Approximately, 4 g of pollen were fed directly into the nest each day. A green Bristol board was taped to the entire floor of the arena to mimic the green foliage encountered in nature.

6.3.2 Artificial flowers

The flowers used in this study were the same design as those used in Chapter 3 (Figure 3.1).

6.3.3 Experimental procedures and data analysis

Bees (n= 17) were allowed to forage on two of these flowers for 20 bouts. The flowers were placed side by side (wooden blocks touching) and their position randomised between bouts. The distance the flowers were placed away from the hive entrance was also randomly changed between bouts. New flowers were used for each bout to prevent the bees from relying on scent marks to distinguish between the flowers (Saleh et al., 2006).

6.3.3.1 Behavioural test on detection ability

Each bee was faced with one flower that contained 1ml 50% (w/w) purified sucrose solution and 1ml of once distilled water. The sucrose solution was made with reagent grade sucrose (HPLC, Fluka, Buchs, Switzerland) and once distilled water.

I investigated how bees behaved towards each flower type at every approach. An approach is defined as an interaction with the flower where the bee performs one of the following behaviours: a) Acceptance b) Crawling-in c) Landing d) Hovering. Crawling-in, landing and hovering are considered rejection behaviours.

A bee learned to detect the difference between water and sucrose solution when her acceptance rate of 'sucrose' flowers as well as her rejection rate of 'water' flowers (demonstrated through crawling-in behaviours) reached and remained at over 80% accuracy (i.e. the bee accepted over 80% of the sucrose flowers and rejected over 80% of the water flowers). I determined which bees reached this threshold and at what approach number this threshold was reached.

6.3.3.2 Relative humidity data collection

I measured the relative humidity above water and sucrose solution to determine if there was a difference. This was done in the flowers using a Vaisala HUMICAP[®] humidity indicator (HMI41 with HMP42 probe, Helsinki, Finland), which measures both temperature and humidity. The indicator was attached, via a clamp, to a retort stand positioned horizontally to fit into the arena. The

indicator's probe was inserted into the flowers at three different locations: the entrance (0.5 cm below the top), middle (3.5 cm below the top) and bottom (6.5 cm below the top). I compared the relative humidity above the sucrose solution and distilled water (n=10). Ambient temperature and humidity can influence the relative humidity above any solution exposed to the atmosphere. Therefore, each set of data shown in the results was taken at the same time with the order randomised.

6.3.3.3 Behavioural effects of relative humidity

To test the possibility that bees were using humidity differences I needed solutions made from other chemicals, e.g. salts that bees do not voluntarily ingest but could mimic the humidity above the water and sucrose solutions. I used the sucrose solution and distilled water as 'models' to generate sucrose and water 'mimics'. I measured the relative humidity above the mimics and compared them to the models in the same manner as before. The sucrose mimic was composed of 0.905 ml 25% (w/w) potassium dihydrogen phosphate (KH_2PO_4) and 0.095 ml 15% (w/w) sodium chloride (NaCl). The water mimic was composed of 1 ml 25% (w/w) KH_2PO_4 solution. All volumes added to a total of 1ml.

The behavioural tests involved training bees on two flowers; one that contained 1ml 50% purified sucrose solution and the other contained 1ml distilled water as described earlier. Then I tested the bees' reaction (n=11) to the mimics by replacing the models with the mimics on the 21st bout (mimic bout) and noting the bees' behaviour. If bees were indeed using relative humidity I expect them to accept the sugar solution mimic and reject the water mimic. After the 'mimic' test bout I allowed the bees to forage on the sucrose and water model flowers for one more bout (model test bout). I compared the bees' first approach to each flower type in this model bout to the first approach to each flower type in the mimic bout to determine if the bees were behaving in the same way towards the models and the mimics. This is a conservative comparison because I expect bees to be less likely to accept the model flowers after their interaction with the mimics. Values in the results are reported as mean \pm standard error.

6.4 RESULTS

The two behaviours that changed dramatically with experience were acceptance and crawling-in, therefore I concentrated on these behaviours for our subsequent analyses. This also suggests that bees needed to be at least a couple of centimetres from the liquids to detect the cue they are using.

Only one out of the 11 bees tested did not reach the 80% threshold of correct choices for acceptance and crawling-in behaviours. Bees learned to distinguish between water and sugar solutions at approach number 28.9 ± 3.9 . Thus the majority of the bees learned to distinguish between water and sucrose flowers without direct contact. The number of bees performing acceptance behaviour decreased for water flowers with experience (see Figure 6.1b). In addition, more bees performed crawling-in behaviours towards water flowers with experience, but this number remained low for sucrose flowers (see Figure 6.1a).

There were differences in relative humidity above water and the sugar solution (see Figure 6.2). The water was on average 3% more humid than the sucrose solution at the bottom of the flowers. This difference decreased to approximately 2% in the middle of the flowers and ceased to exist at the top of the flowers. These results also show that there was an increasing humidity gradient going deeper into the flowers.

The solution mimics that were used to test the effects of humidity had very similar relative humidity to the models (see Figure 6.3). At the middle of the flower, the sucrose solution had an average relative humidity of 63.82 ± 0.09 % and its mimic had an average relative humidity of 63.90 ± 0.10 % (N=20). Water had an average relative humidity of 65.09 ± 0.10 % and its mimic 64.80 ± 0.12 % (N=10). Therefore, the relative humidity values of the mimics fall well within the range of the models.

The number of acceptance and crawling-in behaviours did not differ between water and sucrose mimics (binomial test: $p < 0.23$ and 0.17 for acceptance and crawling-in respectively). Therefore, when faced with the mimics the bees were behaving in a similar manner towards both flower types (see Figure 6.4). However, bees foraging on the 50% purified sucrose solution and distilled water were more likely to accept sucrose flowers and crawl-in to water flowers, this difference is significant (binomial test: $p < 0.01$ and 0.031 for acceptance and crawling-in respectively).

6.5 DISCUSSION

For the first time, I provide evidence that bees are able to discriminate between water and sucrose solutions without direct contact i.e. when they are half way into the flowers. I show that there are consistent differences in relative humidity above water and concentrated sugar solutions. However, the behavioural tests reveal that this is not the cue bees rely on the most when making their distinction.

Marden (1984) examined if bees are able to detect the difference between small volumes of water and sucrose solutions. His flowers were upright vials that were 4.5 cm high and 1.3 cm in diameter. In his experiment, bees were allowed to forage in clumped arrays of rewarding and unrewarding flowers. Rewarding flowers contained 2 μ l 40% sucrose solution and unrewarding flowers contained 2 μ l distilled water. Even with training bees could not remotely detect the difference between the two flower types. This result caused Marden to conclude that bees could not remotely distinguish between water and sucrose solutions. One major difference between the two studies is the volume of the liquids used (1ml versus 2 μ l). It is possible that the bees are not able to remotely detect the difference between these two liquids when the volumes are very small. This remains to be tested.

Although the trend of the bees' response to the water and sucrose mimics was similar to those of the model solutions, the mimics did not elicit the same degree of acceptance and rejection expected if bees were solely relying on humidity differences. I, therefore, conclude that the bees are relying on another cue(s) to make their distinction.

My flowers were similar to some natural flowers in that they had increasing relative humidity gradients deeper into the test tubes (Corbet & Willmer, 1981). However, they differed from natural flowers because they were opaque thereby allowing light to penetrate the liquids. This opens up the possibility that bees may have been using visual cues, which are not present on natural flowers. Thus our ability to extrapolate the usage of this ability in the field will depend on whether the bees are using visual or chemical cues.

In order to identify the cues being used I need to first establish if the bees are using visual or olfactory cues. To do this I can remove the bees' visual ability by forcing them to forage on dark tubes. If I find that bees are still able to

discriminate between the two liquids before contact then they are using olfactory cues. It is unlikely that the bees are detecting sucrose molecules above the solutions. Sucrose is a relatively non-volatile substance (Kambara & Hishida, 1981; Moreira & De Maria, 2005), therefore any sucrose molecules that evaporate from the solution (Roger Nix, personal communication) are likely to be present right above the solution and not in the middle of the flowers, where I find the bees detecting the cue.

The addition of sucrose has been shown to cause a change in the volatility of other compounds present in the solution so that some evaporate more and others less (Massaldi & King, 1973; Franzen & Kinsella, 1974; Covarrubias-Cervantes et al., 2004). Once distilled water, as used in this experiment, contains volatile impurities such as short chained hydrocarbons (Yuan et al., 2000) as well as gaseous substances (Bunkin & Lobeyev, 1997; Bunkin & Bakum, 2006). Therefore it is possible that the presence of sucrose molecules in the water changes the evaporation rate of some of these impurities or carbon dioxide in the solutions. This can be tested by investigating the compounds present in the air above the liquids using headspace gas-chromatography.

If I find that when foraging in dark flowers bees are no longer able to discriminate between the two solutions then they are most likely using visual cues. There are several visual aspects that differ between water and sugar solutions that I can investigate. Firstly, sugar solutions rotate the plane of polarized light by up to 66° (Pennington & Baker, 1990). Although the major receptors for this light are present at the dorsal rim of the bees' eyes, there is a weak ability to detect polarized light in UV receptors outside this area (Horváth & Varjú, 2003). Secondly, there is a slight difference in the refractive index of light between water and sugar solutions (e.g. distilled water = 1.33 and 50% pure sucrose solution = 1.42 at 20°C and 589.3nm)(Bubník et al., 1995). Thirdly, there are slight differences in the amount of light transmission between the two solutions, although most of these differences are in the Infra Red (wavelengths greater than 750 nm) and more extreme Ultra Violet range (wavelengths below 300nm), (Saleh, unpublished preliminary data), which bees are not able to detect.

In conclusion, I have shown that bees are able to remotely distinguish between water and sugar solutions. However, I have still not identified the primary cue these animals are using. Future tests will first determine what

sensory modality, visual or chemical, the bees are using to make their distinction. Then I will attempt to determine what cue the bees' are using within this modality. Further research on this topic will increase our understanding of these insects' sensory capabilities and have an impact on our current experimental practices.

FIGURE 6.1 Percentage of bees ($n= 17$) performing behaviours as a function of experience. (a) Percentage of bees performing crawling-in behaviours towards water flowers, but not sucrose flowers, increased with experience (b) percentage of bees performing acceptance behaviours decreased towards water flowers with experience, but remained high for sucrose flowers. Crawling-in and acceptance number refers to the first, second etc time the bee performed this particular behaviour.

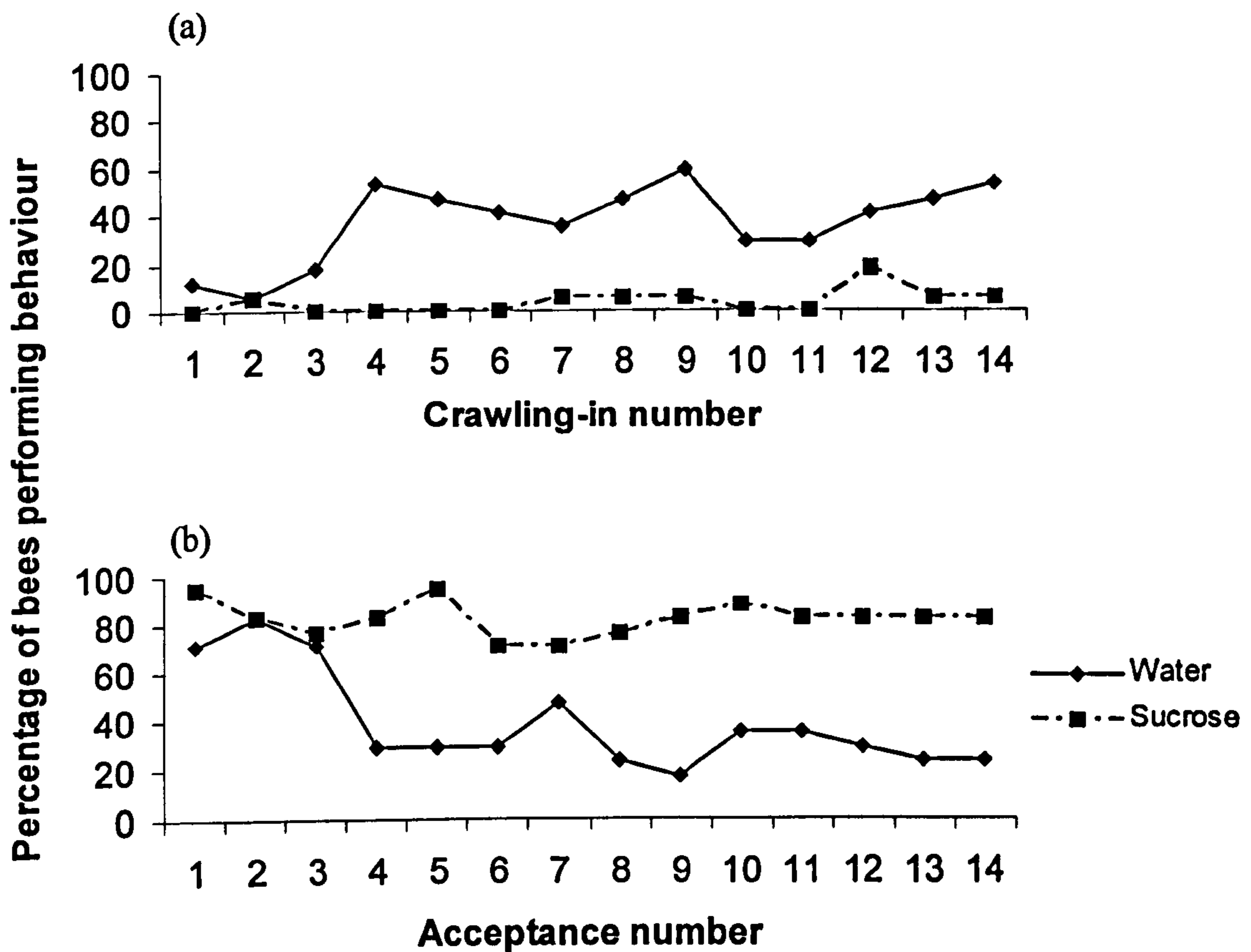


FIGURE 6.2 Mean relative humidity above water and sucrose solution measured in the top, middle and bottom of the flowers at 23°C. The relative humidity above water was generally higher than that above the sucrose solution in the middle and bottom of the flowers but not at the top. Errors bars indicate standard error. A humidity reading was taken every 30 seconds for 2 minutes.

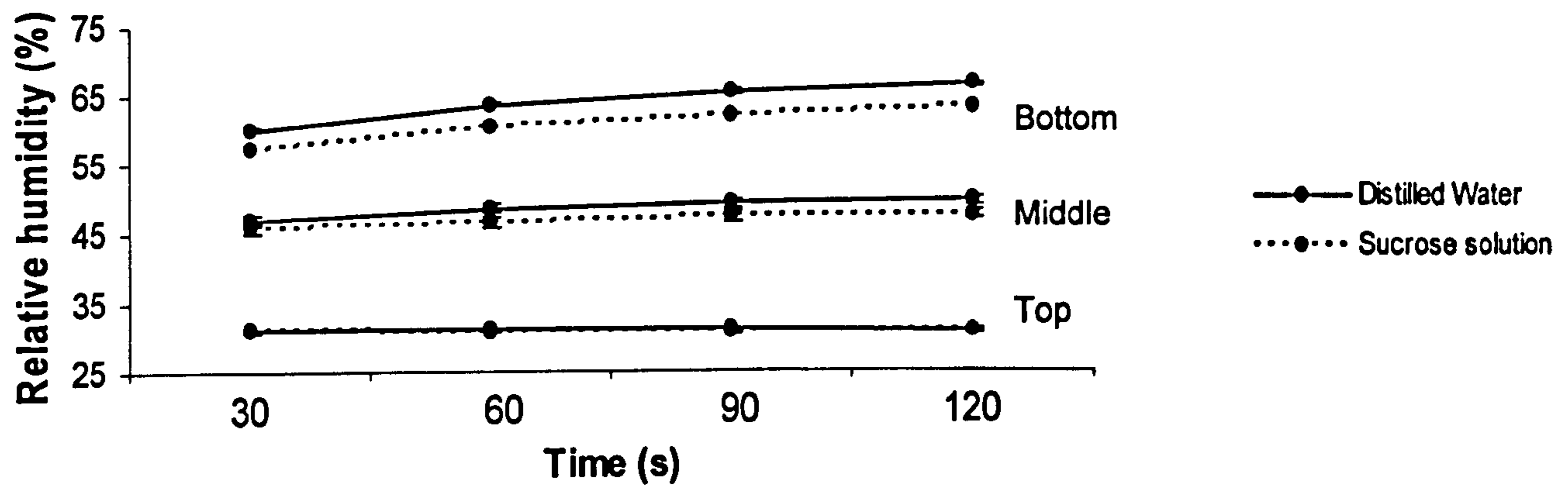


FIGURE 6.3 Mean relative humidity of mimics compared to models taken in the middle of the flowers at 23°C. The mimics produced a relative humidity that was within the range of those produced by the models. Errors bars indicate standard error. A humidity reading was taken every 30 seconds for 2 minutes.

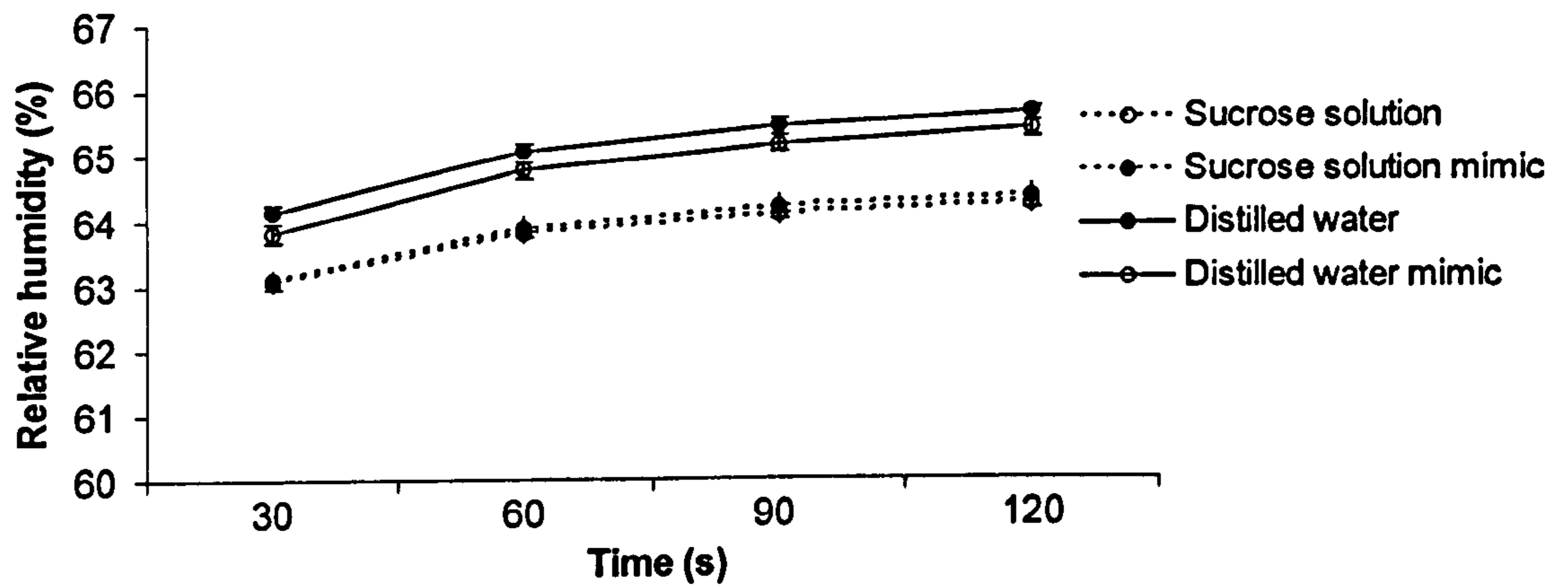
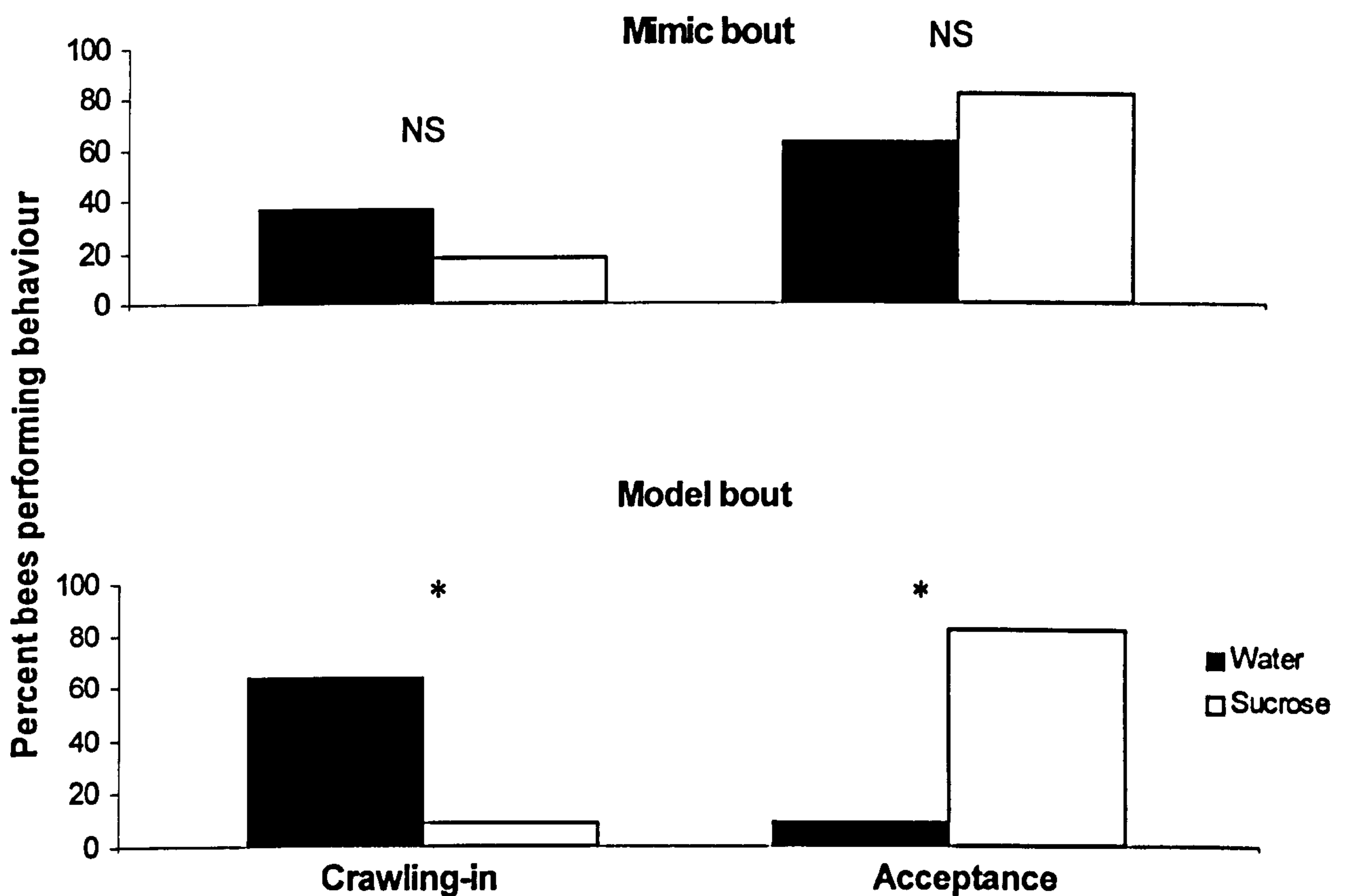


FIGURE 6.4 Comparison of bees' behaviour (n= 11) towards the models and mimics. Bees foraging on the mimics did not behave significantly differently towards the two flower types. However, those foraging on the models were more likely to accept sucrose and crawl-in to water flowers. Thus, bees are not using humidity to distinguish between the two flowers. NS= not statistically significant at p=0.05 and *= statistically significant at p=0.05.



7

Discussion

The importance of learning
and long term memory

CHAPTER 7:

DISCUSSION: The importance of learning and long term memory

Foraging strategies are used by insects to improve their chances of maximising reward intake per unit time. I investigated the properties of two foraging strategies used by bumblebees: scent marking and traplining. Specifically, I investigated how experience can change the meaning of the scent marks (Saleh & Chittka, 2006; Saleh et al., 2006) and contribute to the formation of traplines (Saleh & Chittka, 2007). I examined whether the scent marks bumblebees leave on food sources to improve foraging efficiency are evolved signals or incidental cues (Saleh et al., submitted). I also investigated some of the benefits traplining behaviour gives to a foraging bee (Saleh & Chittka, 2007). The results point to the importance of learning and memory in generating adaptive behaviour. In this chapter I will summarise the main findings of the thesis before I move on to briefly discuss the implications of this work and make suggestions for future research.

7.1 MAIN FINDINGS

7.1.1 Chapter 2: Facultative use of scent marks: flower handling time

In this chapter I compared the bees' responses to scent marks left on flowers with long and short handling times. Bees rejected scent marked flowers with a long handling time more than those with a short handling time, and when they did accept the long handling time flowers they took longer to do so. This effect is not due to scent mark concentration. Bees continued to reject more long handling time flowers when both flower types contained the same amount of scent. These results indicate that bees are relying on memory of previously stored information to make their decisions. Indeed, this memory is triggered by the visual input of the flower, rather than by performing part of the motor pattern. (Saleh et al., 2006)

7.1.2 Chapter 3: Facultative use of scent marks: flower reward level

I investigated the perplexing literature reports in which different studies found the same chemicals elicit opposite effects i.e. promote and suppress acceptance of scent marked flowers (Schmitt et al., 1991; Goulson et al., 2000). I find that bees are neither responding to two different scent marks (Stout et al.,

1998) nor interpreting 'fresh' and 'old' scent marks differently (Giurfa & Núñez, 1992; Stout et al., 1998). Bees are learning to make these two opposite associations depending on whether the food source offers them a reward at every visit. This chapter extends the findings in Chapter 2 and confirms the idea that the meaning of the scent mark is not hardwired and can change with experience. (Saleh & Chittka, 2006)

7.1.3 Chapter 4: Are scent marks pheromones?

This chapter uses GC-MS to determine if the scent mark is a communication signal or cue. To this end, I compared scent marks left at the food and nest sites to those left at a neutral site. I found that bees leave behind the same chemicals in the three sites, suggesting that the scent marks are footprints left everywhere they walk. Previous work has shown that bumblebees leave long lasting chemical trails at their nest entrance (Cederberg, 1977; Foster & Gamboa, 1989; Pouvreau, 1996). Here I show that these chemicals are general chemicals left where bees walk. This chapter highlights the importance of conspecific cues in generating adaptive behaviour and provides a convenient means of determining whether a chemical bouquet is a signal or cue. (Saleh et al., submitted)

7.1.4 Chapter 5: Traplining: Ontogeny and importance of long term spatial memory

Here I show that bees form traplining routes after they have gained familiarity with the positions of reward sources. They experience several paths before settling on preferred traplining routes. I also show that, at least for small spatial scales, bees minimise distance between near neighbour flowers rather than overall flight distance. This behaviour improves their foraging performance from their initial bouts, but does not result in using the optimal route. I also show that traplining routes are not fixed. Bees had two variations to a primary traplining sequence, despite the fixed position and reward value of the experimental flowers. Comparing the performance of traplining bees to those not capable of forming traplines shows that this strategy can help bees avoid revisits and reduce search time. (Saleh & Chittka, 2007)

7.1.5 Chapter 6: Remote detection of reward

I show, for the first time, that bees can remotely distinguish between water and sucrose solution without contact chemoreception. I also show that, although humidity may be a minor component of the cues bees are using, it does not fully explain the bees' behaviour.

7.2 IMPLICATIONS AND FUTURE WORK

7.2.1 Scent marking

The use of scent marks when foraging has been described in numerous social and solitary insects, such as ants (Hölldobler & Wilson, 1990), beetles (Fitzgerald et al., 2004), caterpillars (Colasurdo & Despland, 2005) and termites (Arab et al., 2004; Smith & Koehler, 2006). Within bees they have been described in bumblebees (Cameron, 1981; Schmitt & Bertsch, 1990; Williams, 1998; Stout & Goulson, 2001; Gawleta et al., 2005; Reader et al., 2005), honeybees (Giurfa & Núñez, 1992; Williams & Poppy, 1997; Williams, 1998), stingless (Nieh, 1999) and solitary bees (Gilbert et al., 2001; Gawleta et al., 2005). Thus, scent marking is a widespread foraging strategy in insects. We know that honeybees (Frisch, 1967) and some stingless bees (Nieh, 1999) perform specific behaviours indicative of active scent marking of rewarding food sites. However, have all such scent marks evolved to play a specific role in foraging? The results of my thesis suggest that, at least for some insect species, they have not.

The use of scent marks by bumblebees attracted researchers' attention because bumblebees are solitary foragers and are therefore not expected to communicate the value of a food sources to others (Esch, 1967; Dornhaus & Chittka, 1999). A proposed explanation was that the scent marks are evolved 'self-use' signals that help bees avoid revisiting flowers (Giurfa & Núñez, 1992; Stout et al., 1998). However, this hypothesis is not supported by the available evidence. Other insects, including non-nest mate conspecifics and heterospecifics, can detect and use scent marks deposited by other individuals (Stout et al., 1998; Williams, 1998; Gawleta et al., 2005; Reader et al., 2005). This ability allows them to gather information about the location of high and low rewarding food sites. Therefore, they can exploit these food sites at the expense of the scent marker. This is especially true given the long lasting nature of the scent marks (Schmitt et al., 1991; Stout & Goulson, 2002).

Therefore, in order to speculate what role, if any, evolution has played in generating these 'foraging' scent marks, we first needed to show that the scent marks are indeed signals left exclusively at food sites and not cues left everywhere bees walk.

Using GC-MS I identified the chemicals bumblebees leave behind at the nest and food sites and compared them to those left at a neutral site. The results show that the same chemicals were left behind at all three sites (Saleh et al., submitted). The compounds at these sites were non-polar long chained hydrocarbons similar to those found in other studies (Schmitt et al 1991, Goulson et al 2002). The fact that these 'hydrocarbon footprints' are used by bumblebees to forage *and* locate their nest entrance is very interesting. It means that bees can have multiple associations with the scent marks in different contexts. Indeed, my other work suggests that contextual learning plays an important role in the interpretation of these scent marks (Saleh & Chittka, 2006/Chapter 3; Saleh et al., 2006/Chapter 2).

Bees forage on flowers that can differ drastically in their handling times (Laverty, 1994b; Chittka et al., 1997; Ohashi, 2002) and can handle several hundred flowers per foraging bout (Ribbands, 1949). Therefore, we expect them to use cues that help avoid revisiting long handling time flowers. Indeed, I show that bumblebees accept fewer scent marked long handling time flowers and when they accept them they take longer to do so (Saleh et al., 2006/Chapter 2). There is suggestive evidence that this behaviour can occur in the field (Goulson et al., 2001). Interestingly, we find that bees do not need to perform part of the motor pattern in order to recall the memory of the flower handling time; they can do this with only the visual input of the flower type (Saleh et al., 2006/Chapter 2). They then integrate the olfactory cue of the presence or absence of the scent marks with this visual cue to decide whether they will accept or reject the flower. This behaviour is impressive for an animal considered to have 'minimal neuronal hardware' (Giurfa, 2003) and suggests that we have yet to uncover the true extent of their cognitive capabilities.

For nearly 20 years scientists have been debating the nature of the attractive and repellent scent marks in bees. Some have speculated the use of two different chemical bouquets (Stout et al., 1998) or of 'old' and 'fresh' scent marks (Giurfa & Núñez, 1992; Stout et al., 1998) in generating these opposite behaviours. However, given that the same chemicals have been shown to elicit

both attractive and repellent effects (Schmitt et al., 1991; Goulson et al., 2000), it is unlikely that bees are responding to two different chemicals. It is also unlikely that bees are responding to old and fresh scent marks because both the attractive and repellent effects last for over 20 hours (Schmitt & Bertsch, 1990; Stout & Goulson, 2002). I show that the scent marks can have these opposite meanings depending on whether a food source is rewarding at every visit (Saleh & Chittka, 2006/Chapter 3). Thus the meaning of the scent marks is dependent, once again, on contextual learning. Learning is likely to explain the mechanism by which bumblebees use other insect species' scent marks (Williams, 1998; Gawleta et al., 2005; Reader et al., 2005). A learning mechanism would also explain why some reports have found honeybees use bumblebee scent marks and others have not (Williams, 1998; Stout & Goulson, 2001). Williams (1998) reports that bumblebees were rare in her field study area, thus the fact that honeybees did not use bumblebee scent marks may be because they did not interact frequently with these scent marks and thus did not learn to use them to avoid emptied flowers. This would need to be investigated.

Thus, I show that the scent marks are not signals that have evolved a specific role in foraging. Rather, they are incidental cues that bees learn to use adaptively in a variety of contexts. Firstly, scent marks improve the efficiency of bees foraging from flowers with different handling times. Secondly, they help a bee locate high and low rewarding food sites. Lastly, the scent marks are present at the nest entrance, suggesting they are used to help bumblebees locate their nest. It would be interesting to investigate how many different associations an individual bumblebee can make with these hydrocarbon footprints and in what other contexts they use them.

7.2.2 Traplining

Traplining is another foraging strategy that is widespread in the animal kingdom. It is used by animals from insects (Ribbands, 1949; Manning, 1956; Janzen, 1971; Gilbert, 1980; Ackerman et al., 1982; Thomson et al., 1982; Williams & Thomson, 1998; Comba, 1999; Makino & Sakai, 2004) to birds (Davies & Houston, 1981; Gill, 1988; Garrison & Gass, 1999) to mammals (Lemke, 1984; Garber, 1988; Reid & Reid, 2005), yet its significance and ontogeny have not been clearly demonstrated. I show that, as with scent marking, experience plays an important role in developing traplining routes

(Saleh & Chittka, 2007/Chapter 5). Bees need to return to rewarding food sites multiple times before attempting to link them in a repeatable order. Their reliance on specific routes increases with experience until it reaches saturation. Individual experience is likely an important factor in determining what plants bees will link in their traplines. Indeed, large individual variation in traplining routes have been reported in the literature (Thomson, 1996; Comba, 1999; Makino & Sakai, 2004). However, I show here that some variations can occur even when flowers are fixed in position and reward level. Bees typically had two variations to a primary sequence, despite the constant position and reward value of the flowers in the stable array (Saleh & Chittka, 2007/Chapter 5). This finding cautions us against assuming that it is solely reward level that predicts the nature of traplining routes.

Traplining was believed to be a strategy that reduces travel distance between rewarding food sites (Janzen, 1971; Thomson et al., 1982). Therefore, we would expect bees to link plants in the most optimal sequence. However, I find that bees were using the near neighbour strategy to link flowers in the array (Saleh & Chittka, 2007/Chapter 5), indicating they may use simple rules of thumb to generate seemingly complex behaviour, at least when foraging on small spatial scales. It would be interesting to determine if these simple rules of thumb are also used on a large foraging scale.

We knew very little about what advantages traplining gives foraging bumblebees. Previous work had shown that traplining bees are likely to remove more reward from plants than 'non-resident' bees (Williams & Thomson, 1998). However, we still do not know how they do this. Here, I show that traplining can improve a bumblebees' foraging efficiency by helping it avoid revisits and reduce search time. I also highlight the importance of long term spatial memory for forming traplines and, thereby, improving foraging efficiency. This finding emphasises the importance of long term spatial memory for foraging animals, and gives clues to why animals that have it are favoured by natural selection.

7.2.3 Remote detection of water and sucrose solution

The role water plays to foraging bumblebees is not known. However, I show that bees can distinguish between water and sucrose solution remotely and this ability does not depend on differences in relative humidity above the two liquids. Marden (1984) investigated whether bees can remotely detect the difference

between very small volumes of water and sucrose. He concluded that bees were unable to perform this feat, and thus researchers have continued to use water as a control for the remote effects of sucrose. It is highly likely that he was unable to observe differences in behaviour because the volumes were too small. It would be interesting to determine at what volume the bees' ability to tell distinguish between the two liquids ceases, and how this compares to nectar volumes present in natural flowers.

The first step to identifying what cue(s) bees are using is to determine if they are using chemical or visual cues. This can be done by forcing bees to forage on dark flowers. Once I determine the sensory modality used, I can test for specific cues. For example, if bees are using chemoreception then I would examine the chemicals above the solutions using headspace GC-MS analysis. This will show if the bees are using impurities or gases above the solution. If, on the other hand, bees are using visual cues, then I would examine the possibility that bees are detecting differences in polarised light and/or refractive index. This work should provide insight into the sensory capabilities of these insects and also impact our current experimental practises.

7.3 CONCLUSION

Animal behaviour is greatly influenced by experience. So far, research has focused on the role chemical *signals* play in regulating behaviour. Less emphasis has been given to the role conspecific chemical *cues* play in shaping behaviour. My work has shown that researchers often assume chemical bouquets are signals and this, for example, has lead to many erroneous hypotheses regarding the nature of scent marking in bumblebees. My work highlights that chemical cues are just as important for generating adaptive behaviour. Therefore, future work should first establish whether chemical bouquets are signals or cues, and then establish how learning may influence their meaning.

The effect of experience in generating adaptive behaviour is also found in traplining bumblebees. When bees interact with food sources repeatedly they use their long term spatial memory to generate traplines.

In summary, my results strongly support the idea that bees are not 'pre-programmed entities'. They are able to learn from previous experience and this

learning is vital to improving their foraging efficiency and ultimately their survival.

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