

AN ABSTRACT OF THE THESIS OF

Peter B. Wetherwax for the degree of Doctor of Philosophy
in Entomology presented on July 23, 1993.

Title: Scent-marking by Nectar-collecting Honey Bees

Abstract approved: Redacted for Privacy
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Honey bees mark artificial flowers with scents that advertise about the previous history of the flower to subsequent foragers. Unrewarding flowers are marked with a scent, after a single visit, that makes the flower less attractive to subsequent foragers. Previously rewarding flowers are initially less attractive than unvisited flowers but become more and more attractive with each rewarding visit. Flowers that have rewarded bees four times are more attractive than unvisited flowers. This attractant is applied by the bees in response to the presence of nectar and is not, as has been suggested by other researchers, inadvertently applied to anything on which the bee lands.

Similar scent-markings are applied to a real flower, *Lotus corniculatus*. One visit was enough to make a flower less attractive to subsequent foragers but flowers that consistently offered high amounts of nectar became more

attractive than unvisited flowers. Repellents may be used by bees to avoid revisiting recently emptied flowers while attractants may be applied to flowers within a patch that consistently offer high rewards.

The possible selective pressures responsible for the evolution of scent-marking was investigated by doing an energetic analysis. The presence of scent-markings in a patch results in a 33% increase, over an unmarked patch, in the amount of sugar obtained per time.

The attractive scent-marking was extracted from a glass flower and maintained its biological activity when applied to a clean glass flower. The extract was chemically analyzed using gas chromatography-mass spectrometry. Four chemicals were identified; none of the chemicals has previously been found in honey bees. Mandibular glands were analyzed as a possible source of the attractant. Although none of the components was found in the gland extracts, two previously unidentified chemicals were found.

Scent-marking by Nectar-collecting Honey Bees

by

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A Thesis

Submitted to

Oregon State University

in partial fulfillment of
the requirements
for the
degree of

Doctor of Philosophy

Completed July 23, 1993

Commencement June 1994

APPROVED:

Redacted for Privacy-

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Date thesis is presented July 23, 1993

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ACKNOWLEDGMENT

I would like to thank my major advisor, Michael Burgett for his assistance throughout this project. The members of my committee: Bill Stephen, Phil Rossignol, Bob Mason, Art Boucot and Joe Beatty provided helpful comments on my proposal and final paper. Lynn Royce helped in the dissection of the mandibular glands. Lou Pannell ran the samples on the GC-MS and Bob Mason helped me to interpret the chromatograms and spectra. Jim Jenkins provided dependable bee-sitting and *Lotus* feeding services. I received funding assistance from the Northwest Scientific Association and Sigma Xi Grant-In-Aid of Research. I am especially grateful to Nancy Warshawer for her drawings and emotional support.

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SCENT-MARKING BY NECTAR-COLLECTING HONEY BEES

CHAPTER 1

Introduction

Levels of Decision Making

Decision making for nectar-collecting honey bees can be viewed as occurring at several levels (Fig. 1. 1). At the level of the colony (Seeley, 1985; Seeley, 1986), bees decide whether to forage or stay in the hive. These decisions are made based on the conditions in the nest, such as amount of brood and honey stores, as well as conditions outside of the hive, such as temperature and available forage. A potential forager then has to decide where to forage based on information gained from dancers indicating the location and amount of forage (von Frisch, 1967). Once in a patch, a forager decides whether to stay in the patch or to leave. At the level of the individual flower, bees either land and probe or reject the flower.

Most of the studies on the foraging behavior of eusocial nectar-collecting bees have looked at the decisions made and rewards gained on a patch-wise (Hodges, 1985) or species-wise (Inouye, 1978; Sowig, 1989) level while largely ignoring decisions made at the individual flower (Harder, 1988). (A patch is defined here as

anything between an individual flower and a group of plants e.g. inflorescence, group of inflorescences, single plant.) When bees are shown to forage more efficiently than can be attributed to random foraging it is usually concluded that the increase is due to area-restricted foraging or a stereotypic foraging pattern that takes advantage of a naturally occurring pattern in nectar rewards. Pyke (1979) found that the foraging pattern on vertical inflorescences, starting at the bottom of the inflorescence and working up, is in response to the nectar gradient found in many of these inflorescences. In area-restricted foraging, bees stay in an area of a patch that is high in rewards by basing their foraging decisions on the amount of nectar received at the previous flower. If a bee receives a high reward then she responds by traveling short distances (Waddington, 1980), having high turning angles, and remaining on the inflorescence (Hartling and Plowright, 1979; Heinrich, 1979) all of which have the effect of keeping the bee within the highly rewarding area.

Bees are assumed to make their foraging decisions based on the nectar content of the last flower visited (Pyke, 1978; Heinrich, 1979; Hodges and Wolf, 1981; Zimmerman, 1981). However, if a bee has some ability to distinguish between good and bad flowers prior to landing and probing then some of the conclusions of the above mentioned studies may be incorrect. If a bee remotely

senses that a particular flower has little nectar, then she may decide to leave that area.

Decisions at the Individual Flower

Various sensing abilities of bees have been hypothesized to play a role in choosing individual flowers. Visual cues, which have been studied the most, take various forms. There are several post-pollination changes that occur in flowers that make them less attractive to pollinators (Gori, 1983; Weiss, 1991). These include changes in color, changes in orientation, collapsed flower parts and corolla abscission. These changes are often accompanied by termination of odor and nectar. Zimmerman (1982) found that pollen-collecting bumble bees reject flowers that do not have enough pollen visible to make collecting profitable. Bell et al. (1984) showed that while visiting *Impatiens capensis*, honey bees, bumble bees and wasps more frequently visited the male-stage flowers. Flowers in the male stage contained more nectar and it was hypothesized that flower-preference results from the bee's ability to sense the amount of nectar in the flower. When the androecium was removed, bees still visited male flowers more often so it is not clear if visual cues are the mechanism involved. Marden (1984) found that bumble bees could discriminate between artificial flowers that were touched by humans and ones that were untouched. He assumed

that the bees were sensing fingerprints visually but it is possible that scent was involved. In another test, bumble bees were able to pick artificial flowers that contained visible amounts of nectar from flowers with no nectar. Thorp et al. (1975) found that nectar in some flowers fluoresces and postulated that bees may be able to see the fluorescent nectar and thus increase foraging efficiency. However, Kevan (1976) feels that the insect's eye physiology is unsuited to receive fluorescent light.

Two less intuitive mechanisms include the sensing of humidity and electrostatic charges. Erickson and Buchmann (1983) speculated on the possibility of electrical footprints left by bees that can be detected by subsequent foragers. The rejection-behavior bees exhibit while visiting flowers may be a response to this footprint. Corbet et al. (1979) found that sugar concentration is correlated with humidity and suggested that bees may be able to respond to intrafloral gradients in humidity. Neither of these mechanisms has been tested.

Scent has been shown by many investigators to be an important cue in choosing between flowers. The ability of bees to discriminate between minute quantities of odoriferous substances is well known (e.g. Ribbands, 1955). Some (Marden, 1984; Heinrich, 1979) have suggested that nectar may have a scent that bees can use to discriminate between rewarding and non-rewarding flowers. Heinrich

(1979) showed that humans can smell the difference between visited and unvisited clover but I know of no studies that have demonstrated that bees respond to odoriferous nectar. Marden (1984) found that bumble bees discriminated between flowers that differed in nectar volume and postulated that the discrimination was due to nectar scent, without considering the possibility of scent-marking by the bees.

The use of scent-markings by bees has been supported by studies on carpenter bees, bumble bees and honey bees. The studies fall into two major categories, field and experimental, each with their strengths and shortcomings.

Scent-Marking: Field Studies

Field studies have shown that bees discriminate between rewarding and less rewarding flowers but the precise role of scent-marking is still unknown. Frankie and Vinson (1977) found that *Xylocopa virginica* often rejected flowers that were recently visited. They discount nectar smell as the cue because bees sometimes visit flowers after the nectar was removed by the researchers; this evidence is anecdotal and no data are given. They showed that extracts from Dufour's gland will repel bees when placed on flowers and suggest that scent from the Dufour's gland may be used to mark recently visited flowers with a repellent. In a second study (Vinson et al. 1978), several components of Dufour's gland extract were found to

deter *Xylocopa virginica* bees. For bumble bees, Kato (1988) found that foragers were more likely to visit *Impatiens* spp. that had been previously visited greater than 10 minutes but less than 45 minutes earlier. He concluded that the bumble bees were scent-marking the flowers. Corbet et al. (1984) found that bumble bees and honey bees avoided visiting recently-probed flowers. However, most of their results are based on one individual bumble bee. They postulate that bees may be leaving 2 types of scents: attractants that are long lasting and short lasting repellents. Wetherwax (1986) found that honey bees foraging on *Lotus corniculatus* rejected empty flowers and thus increased their foraging efficiency. His results were more supportive, but inconclusive, of scent-marking than of nectar scent.

Scent-Marking: Experimental Studies

Experimental studies have been more successful at showing that bees will scent-mark food sources, however extrapolating to their possible use with real flowers is problematic. All of the studies suffer in that the condition in which scent-marking is demonstrated are unrealistic. The shortcomings include: extremely high levels of reward (rewards much higher than would be found in flowers), low numbers of "flowers" from which to choose (between 2 and 16), improper controls (non-rewarding

flowers may look different than rewarding flowers - see below). Despite these problems, they have been important in demonstrating that bumble bees and honey bees have the ability to scent-mark food sources.

Cameron (1981) showed that bumble bees mark constantly rewarding artificial flowers with scents that attract subsequent foragers. The attractant was removed with hexane but not water or alcohol. Schmitt and Bertsch (1990) devised elaborate artificial flowers that could measure when bumble bees probed a flower and automatically refilled the flower with a small amount of nectar. This study is the best experimental study to date because it uses a relatively large patch size (16 flowers) and offers a small reward per probe (about 1 μ l of nectar), although individual flowers rewarded bees at a much faster rate than would be found with real flowers. They concluded that the bumble bees were leaving an attractant that other bees used to find rewarding flowers.

Honey bees also have been shown to leave scent-markings at food sources. When allowed to forage in dishes containing a large amount of sugar water, *Apis mellifera* (Ferguson and Free, 1979) and *Apis florea* (Free and Williams, 1979) leave scent-markings that attract other bees. In a later study, Free and Williams (1983) found that when offered large amounts of sugar water in artificial flowers, *Apis mellifera* will mark rewarding

flowers with an attractant and apparently mark non-rewarding sources with a repellent. Nùñez (1967) found that honey bees avoided visiting artificial flowers that stopped producing reward. Since the bees stopped avoiding the non-rewarding flowers when air was blown over the top, he concluded that the bees were leaving repellent scent-markings. While the evidence from this study was inconclusive, a more recent study has substantiated the presence of repellents (Giurfa and Nùñez, 1992).

One problem with many of the experimental studies is the type of non-rewarding flowers. An empty flower may not be the appropriate non-rewarding flower. Marden (1984) found that bumble bees were able to distinguish between empty and rewarding flowers by sight. Honey bees behave differently when foraging on empty flowers than on flowers containing water (Wetherwax, personal observation). Presumably they are able to see if the flowers contain a liquid.

Objectives

Honey bees do not attempt to extract nectar from every flower that they encounter. They will sometimes briefly touch or hover-over a flower and then fly away. While bees may be able to respond to nectar scent, this discrimination behavior is probably due to scent-markings. Scent-markings may be a way for bees to advertise to their sisters which

flowers are empty and/or a cue used to avoid revisiting a recently probed flower.

In the chapters to follow are several studies that look at various aspects of scent-marking in honey bees. There are several reasons why an understanding of scent-marking is valuable. (1) An understanding of the evolution of foraging behavior requires that the investigator knows the constraints that the animal faces. If bees are using scent-markings then the assumptions about constraints implicit in many optimal foraging studies are incorrect. It may be necessary to reevaluate previous findings taking into account this additional information. (2) Honey bees are perhaps the most studied organism in animal behavior. The more we know about honey bee behavior, the better we understand behavior in general. (3) There may be some practical applications associated with an understanding of scent-marking. The predominant economic value of honey bees is as a pollinator of crops. Identification of forage pheromones may lead to increased crop yields.

The specific objectives of this study were:

1. to determine if honey bees scent-mark flowers;
2. to determine the types of response to the scent-markings, attraction and/or repellency;
3. to determine if scent-marking requires bees to apply scent to flowers or if it is inadvertently applied whenever a bee lands;

4. to explain the sequence of events that occurs during foraging that results in bees ability to discriminate between rewarding and unrewarding flowers;

5. to examine scent-marking using an artificial patch that realistically mimics real flowers;

6. to isolate the pheromone and to test its activity using a behavioral assay;

7. to isolate and chemically analyze the pheromone;

8. to determine if bees scent-mark real flowers and to explain how the marking takes place;

9. to find out if the use of scent-markings results in an increase in foraging efficiency and, if so, to quantify that increase;

10. to determine the source of the pheromones used to mark flowers.

NEST	Forage or stay in hive? -conditions in hive -conditions outside Which patch? -dancers
PATCH	Stay or leave? -nectar received at previously visited flowers
INDIVIDUAL FLOWER	Probe or not? -visual cues -olfactory cues

Fig. 1. 1. Levels of decision making for nectar-collecting honey bees.

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CHAPTER 2

The Use of Attractants and Repellents on Artificial Flowers

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Abstract

Honey bees have the ability to discriminate between flowers before landing on them. One possible explanation for this behavior is the presence of pheromone scent-markings left by previous conspecific foragers to that flower. An artificial flower patch was used to see if honey bees have the ability to scent-mark flowers with attractants and/or repellents. Foragers leave attractants at previously rewarding flowers and another marking at previously unrewarding flowers. The marks are applied to the flowers and are not the result of scents that are left by bees on any substrate on which they land.

Introduction

Several genera of bees can discriminate between individual flowers without landing (e.g. *Xylocopa*, Frankie and Vinson, 1977; *Bombus*, Heinrich, 1979; and Corbet et al., 1984; *Apis*, Wetherwax, 1986). The discrimination-behavior is evident because the bees often hover over or briefly land on a flower without probing the flower for nectar. At least two mechanisms have been suggested to explain this behavior. One possibility is that the forager is smelling something that causes the bee to reject or accept the flower. Some have argued that bees can smell the nectar and choose those flowers that have a strong enough nectar smell to make landing and probing profitable

(Heinrich, 1979; Marden, 1984). Others have suggested that the bees are smelling scent-markings left by previous visitors (Corbet et al., 1984; Wetherwax, 1986; Kato, 1988; Schmitt and Bertsch, 1990). It is still not known whether or not bees leave scent-markings at flowers and whether the markings act as repellents or attractants.

A convenient system for finding out if bees have the ability to leave scent-markings is to work with artificial food sources. It has long been known that honey bees will leave an attractant at sources of sugar-water (Ribbands, 1955). All of the studies to date have poorly modeled real systems because the artificial patches have offered unrealistically high quantities of reward, either offering the reward in a large dish (Ferguson and Free, 1979) or offering a continuous supply of nectar from a tube (Free and Williams, 1983; Giurfa and Nùñez, 1992). Additionally, the studies have allowed several bees at a time to visit the food source, which complicates analysis of the bees' behavior. Some of these studies have indicated that bees will leave an attractant (Ferguson and Free, 1979; Free and Williams, 1983) at these food sources while other studies indicate bees leave a repellent (Nùñez, 1971; Giurfa and Nùñez, 1992).

In this study, I devised a more realistic artificial flower patch that was used to test whether or not honey bees can leave attractants and/or repellents. The patch is

more realistic because (1) it does not supply a continuous amount of sugar-water, (2) one bee at a time is allowed to forage on the patch to reduce the effect of conspecifics on choices, (3) a realistic amount of reward per visit is offered (1 μ l of sugar-water) and (4) the bee is forced to fly between several flowers while foraging.

Several questions are addressed including:

- (1) Can bees leave scent-markings at food sources?
- (2) Do these scent-markings act as repellents and/or attractants?
- (3) Are the scent-markings the result of the bees applying a substance on particular types of flowers or do they leave these scents on anything on which they land (i.e. are the scents merely footprints)?

Materials and Methods

The flowers were made of disposable materials since it was critical that, with each new experiment, the flowers were not contaminated with bee-scents. The 'flower' was a plastic 0.3 ml sample cup for an auto analyzer (Starstedt, Inc.) with a hole drilled into the middle of the closure (Fig. 2. 1a). A piece of 10 μ l capillary tube (Drummond) was inserted through the hole so that the tube opening was flush with the top of the closure (landing platform). A hole was drilled in the bottom through which a rubber tube (Tygon) was attached to the capillary tube. The other end

of the tubing was attached to a 3-way stopcock. The second end of the stopcock was attached to a 3 ml syringe which was used as a reservoir to fill the system with fluid and to replenish the 50 μ l Hamilton syringe that was attached to the third end of the stopcock. After the system was filled with fluid, the stopcock was positioned so that fluid could only be injected from the 50 μ l syringe into the capillary tube. The 50 μ l Hamilton syringe was equipped with a repeating dispenser (Hamilton PB6000) so that the tube could be easily refilled. After the bee probed the flower and flew away, 1 μ l of fluid was injected into the capillary tube. The flowers (n=6) were mounted on hollow wooden dowels (with the tube threaded through the opening in the dowel) in a circle about 10 cm above a round wooden base (diameter=0.5 m) that was mounted on a turntable (Fig. 2. 1b). This design forced the bees to fly between flowers and the turntable was used to change the position of flowers between visits. A round piece of canvas with six holes was placed over the entire patch so that only the wooden dowels with the flowers could be seen by the bees. This was necessary so that the bees would not try to forage around the tubing and syringes.

Flowers were filled with either ultrafiltrated distilled water (water-flower) or sugar water (nectar-flower). The nectar consisted of 25% sucrose (EM Industries) that was measured using a handheld

refractometer. All of the experiments were conducted on warm, mostly-sunny days between May and October of 1990 and 1991 near Eugene, Oregon. Experiments were conducted in a 2 x 2 x 2 m cage placed approximately 100 m from a standard honey bee colony. Bees were trained to forage in the cage by placing a feeder with nectar at the entrance to the hive and then slowly moving the feeder until it was inside the cage. Before each new experiment, bees were allowed to forage for at least 1 h on the artificial patch that would be used in that experiment. The bees visiting the patch during this time were collected, cooled for 3 minutes and marked on the thorax with acrylic paint or small plastic numbers. Only one bee at a time was allowed to forage within the cage during the experiments.

Data were collected by noting which flower the bee was visiting and the type of visit (see below) using a tape recorder. The data were transcribed into a computer and analyzed using a custom spreadsheet macro program (Excel) and statistics package (Statview).

Visits were one of four types:

1. touch - the bee hovers over or briefly touches the flower but does not land on it.
2. land - the bee briefly lands on the flower and then flies away without extending her proboscis.

3. examine - the bee lands on the flower and extends her proboscis but does not place her proboscis near the opening of the tube or insert it into the tube.

4. probe - the bee lands and extends her tongue into or on the opening of the tube.

Experiment #1

The first experiment was designed to test whether or not bees leave scent-markings on the artificial flowers. It was not designed to test whether the markings were attractants or repellents. The patch consisted of one nectar-flower and five water-flowers, the positions of the flowers were chosen at random. Before each experiment, bees were allowed to forage for 1 hour on the patch at which time scent-markings could be left on the flowers; this period is called the 'charging-time'. After the charging-time, the patch consisted of one visited nectar-flower and five visited water-flowers. One bee at a time was allowed to enter the cage and the flower number and type of visit was recorded. After each probe visit the flower was replenished and the patch was rotated. The bee was removed from the cage after she had visited 50 flowers (i.e. 50 visits = 1 bout) or when she tried to leave the cage. A total of 22 bouts were recorded representing 12 individual bees for a total of 1094 visits.

Experiment #2

The second experiment was designed to test for the kinds of scents left on the flowers, attractants or repellents. Bees were allowed to forage for 1 hour on a patch with one nectar-flower and five water-flowers. Just prior to recording the visits, one of the visited water-flowers was replaced with an unvisited water-flower. The patch then consisted of one visited nectar-flower, one unvisited water-flower and four visited water-flowers. The positions of the flowers were randomly chosen for each bout. One bee at a time was allowed to enter the cage and the flower number and type of visit was recorded. After each probe visit the flower was replenished by pushing the button and the patch was rotated. A total of 26 bouts were recorded representing 14 individual bees for a total of 1268 visits.

Experiment #3

A third small experiment was designed to substantiate that honey bees can not distinguish between unvisited water-flowers and unvisited nectar-flowers. Bees were allowed to forage for 1 hour on a patch with one nectar-flower and five water-flowers. The experiment then began with the patch consisting of one visited nectar-flower, one unvisited nectar-flower and four unvisited water-flowers.

The results were unambiguous so the experiment was stopped after 2 bouts with two different bees.

Results

Honey bees were able to discriminate between previously rewarding and unrewarding flowers (Fig. 2. 2; experiment #1). An overall Chi-square showed that the type of behavior is independent of the type of flower ($n=1094$, $\chi^2=887$, $p<0.0001$). The bees probed the visited nectar-flower almost every time (99.5%) whereas visits to the visited water-flower were chiefly touches and lands.

Experiment #2 was designed to test which flowers were being marked. The foragers showed three types of behavior on the three types of flowers (Fig. 2. 3). An overall Chi-square test showed that the type of behavior is independent of the type of flower ($n=1268$, $\chi^2=1367$, $p<0.0001$). A comparison just between visited and unvisited water-flowers shows that behavior is independent of the type of flower ($n=1037$, $G=106$, $p<0.0001$). Visited and unvisited water-flowers were analyzed more closely to see where the two types of flowers differed. An *a posteriori* test using STP analysis (Sokal and Rohlf, 1969) indicated that probes and examines were not independent of type of flower but both lands and touches were independent of flower type.

In experiment #3, all of the visits to the visited nectar-flower were probes and all of the visits to the

unvisited nectar-flowers and unvisited water-flowers were touches (Fig. 2. 4) which indicates that the bees were not discriminating between unvisited nectar-flowers and unvisited water-flowers. This is in agreement with Ribbands (1955) who demonstrated that honey bees can not detect a sucrose solution in the vapor phase. In the analysis of the other experiments, any differences in foraging behavior between nectar-flowers and water-flowers can be attributed to markings left by previous visitors.

There is an assumption in recording the types of visits that these types accurately show the level at which the bee is discriminating. A test of this assumption is to see if the total number of visits (i.e. probes + lands + examines + touches) to a particular flower can be attributed to chance. In experiment #1, one out of six flowers was a nectar-flower so 16.7% of the total visits should have been to the nectar flower. Slightly more visits, 19.5%, than expected were to nectar-flowers ($n=1094$, $\chi^2=6.2$, $p=0.013$). In experiment #2, slightly more visits than expected were to the visited nectar-flower and slightly less visits than expected were to the visited water-flower but these differences were insignificant ($n=1268$, $\chi^2=4.7$, $p=0.097$). Thus, the types of visits used in these experiments were fairly good at documenting the level at which discrimination takes place.

Discussion

Experiment #1 indicated that honey bees were scent-marking flowers but did not indicate which flowers were being marked: nectar-flowers, water-flowers or both. Experiment #2 was designed to determine which types of flowers were being marked. Honey bees scent-marked rewarding and unrewarding flowers such that they were able to discriminate among these types of flowers and unvisited flowers. Previously rewarding flowers were more attractive than any other kind of flower. In fact, the bees probed visited nectar-flowers almost every time.

Rejected flowers fall into one of three categories: touch, land or examine. The amount of time per flower visit, and presumably energy expended, increases from touch to land to examine visits. In experiment #2, visited water-flowers received more examine and land visits and less touch visits than unvisited water-flowers. In other words, bees spent more time per rejected flower if it had already been visited. While it is clear that nectar-flowers were marked with an attractant, it is difficult to name the type of scent left on water-flowers. Scents left on water-flowers can not be called repellents because the scent caused the bees to spend more time on these flowers than on unvisited flowers.

How do these results compare to what one would expect when bees visit real flowers? Most real flowers are

emptied after being visited and do not immediately replenish with nectar. One would expect that if bees can mark flowers, they would mark any visited flower with a repellent to indicate that the flower is empty (as suggested by Corbet et al., 1984). My expectations were that any visited flower, nectar or water, would be less attractive than an unvisited flower. However, results from this study indicate that visited nectar-flowers became more attractive and bees spent more time rejecting visited water-flowers than unvisited flowers.

A possible explanation for some of this behavior is that bees have flexibility in the use of scent-markings. The flowers in this study were immediately filled after a bee visit so bees may have learned that a rewarding flower was likely to be rewarding in the future. In fact, rewarding flowers were always rewarding. All of the measurements were taken after bees had learned how to forage on the flowers and it is possible that during this time the bees learned how to maximize their foraging. It is more difficult to explain why bees spent more time rejecting visited water-flowers than unvisited flowers. Since the bees were able to take advantage of the fact that rewarding flowers are always rewarding, why didn't they use the fact that nonrewarding flowers are always nonrewarding? Optimal foraging theory would predict that the bees would

only touch visited water-flowers rather than land or examine, which takes more time and energy.

A question regarding the origin of the scent-markings can be indirectly addressed from these experiments. The legs of bees contain odoriferous materials from a variety of sources including waxes from the nest and floral fragrances. It is likely that these materials are deposited on anything that is contacted by the bees (Butler, 1969). Free (1987) has suggested that forage marking attractants may be footprints that are "inadvertently" left on flowers. One possible explanation for the differences in behavior at visited nectar-flowers versus visited water-flowers could be that the bees have landed on the nectar-flowers more than the water-flowers and so have incidentally left more scents on nectar-flowers. This hypothesis can be investigated by looking at the sequence of visits at the beginning of the one hour training period (charging-time), at which time the flowers start out as unvisited.

On four days the visits during the charging-time were recorded until at least 50 visits were made. I counted the number of visits involving landing on a flower (i.e. the sum of probe, examine and land visits) until the bees consistently probed that flower. I define consistent probing as four consecutive probing visits to a flower. A comparison was made between the nectar-flower and the

water-flowers to see how many times the bee landed on the flower before there was evidence of scent-marking. On three days it took 4 landings on the nectar-flower until the forager consistently probed; on the fourth day it took 6 landings. Once a bee consistently probed the nectar-flower, 100% of the visits to that flower were probes. During the same time period there was a minimum of 6 visits to each water-flower without consistent probes. The bees occasionally probed the water-flowers but consistently probed only one out of the 20 water-flowers measured on the four days. This one exceptional water-flower had a string of 4 probes and then other types of visits took place. Therefore, the bees did not probe nectar-flowers just because they landed on the nectar-flowers more than the water-flowers. Bees are performing some behavior that is depositing a scent.

Two possibly sources of the scents are the Arnhart gland or the mandibular gland. The mandibular gland is known to produce 2-heptanone, an alarm pheromone (Shearer and Boch, 1965). It is possible that the bees deposit this pheromone or others while probing a flower. The mandible is in an ideal position for depositing scents while probing. The Arnhart glands (Chauvin, 1962) are located on the tarsi of all of the legs. Their function is unknown but has been suggested as a possible source for 'footprint scents' (Free, 1987; Winston, 1987) and could also be used

to mark flowers as bees walk on them. A third possible candidate for the source of the scents is the Nasonov gland which produces several compounds that act as an attractant at the hive entrance and in swarms (Boch and Shearer, 1962). Bees expose their Nasonov gland when releasing scents and this would be visible to the researcher. On very rare occasions, honey bees have been seen to expose their Nasonov gland while foraging on flowers (Frisch, 1967; Free and Racey, 1966). It is unlikely that the Nasonov gland was the source of the scent-markings in this experiment as I never saw the bees expose the gland while foraging on the artificial flowers.

It is not possible from this study to say what kinds of scents are being left on nectar-flowers and water-flowers. It is possible that two different kinds of scents are used or there may merely be a difference in the amount of scent left at the two types of flowers. One possibility is that frequently rewarding flowers received a large dose while nonrewarding flowers received a small dose (Corbet et al., 1984). The small dose would dissipate faster and the flower would then be indistinguishable from unvisited flowers.

It is unclear if the scent-marking behavior is fixed or if the bees can be flexible depending on the resources. A future study will use a more realistic model (i.e. a larger patch where flowers are not immediately refilled

with nectar) to see if the behavior is flexible.

Additional studies will try to analyze the composition of the scents using gas chromatography-mass spectrometry.

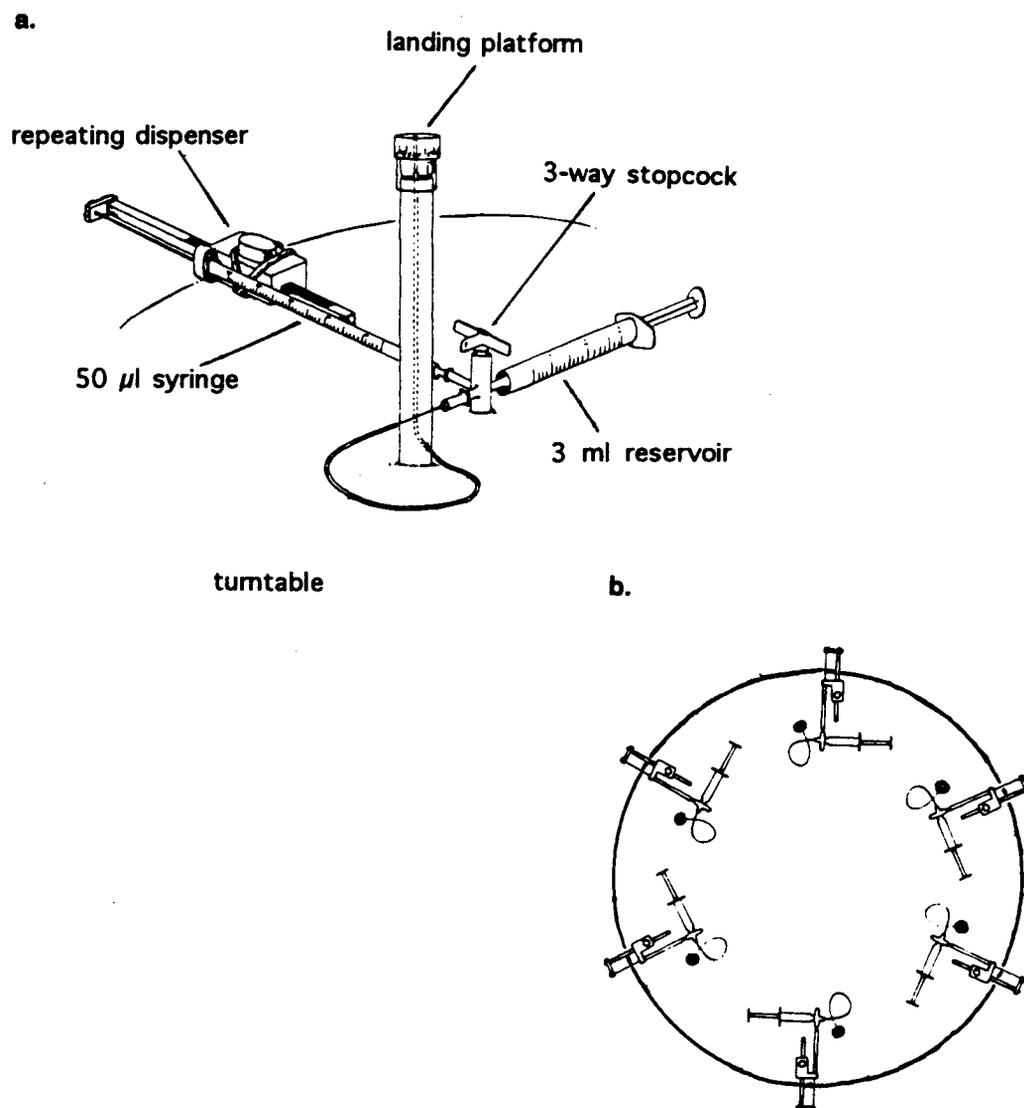


Fig. 2. 1a-b. Artificial flower patch with six flowers.

a. View of one flower unit; b. overhead view of the patch.

During the experiments, the patch was covered with a piece of cloth with six holes in it so that only the flowers and dowels were visible.

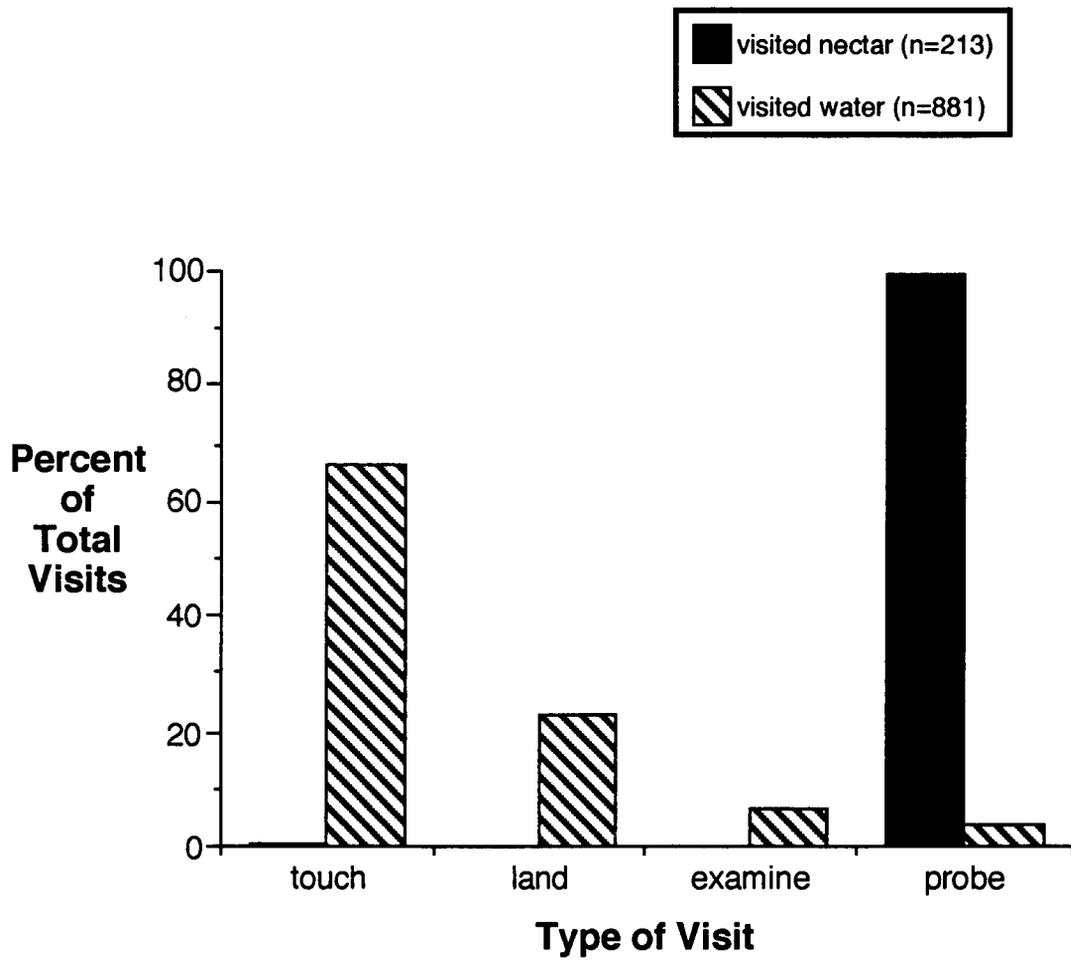


Fig. 2. 2. Experiment #1. Percent of total visits to visited nectar-flowers and visited water-flowers broken down by type of visit.

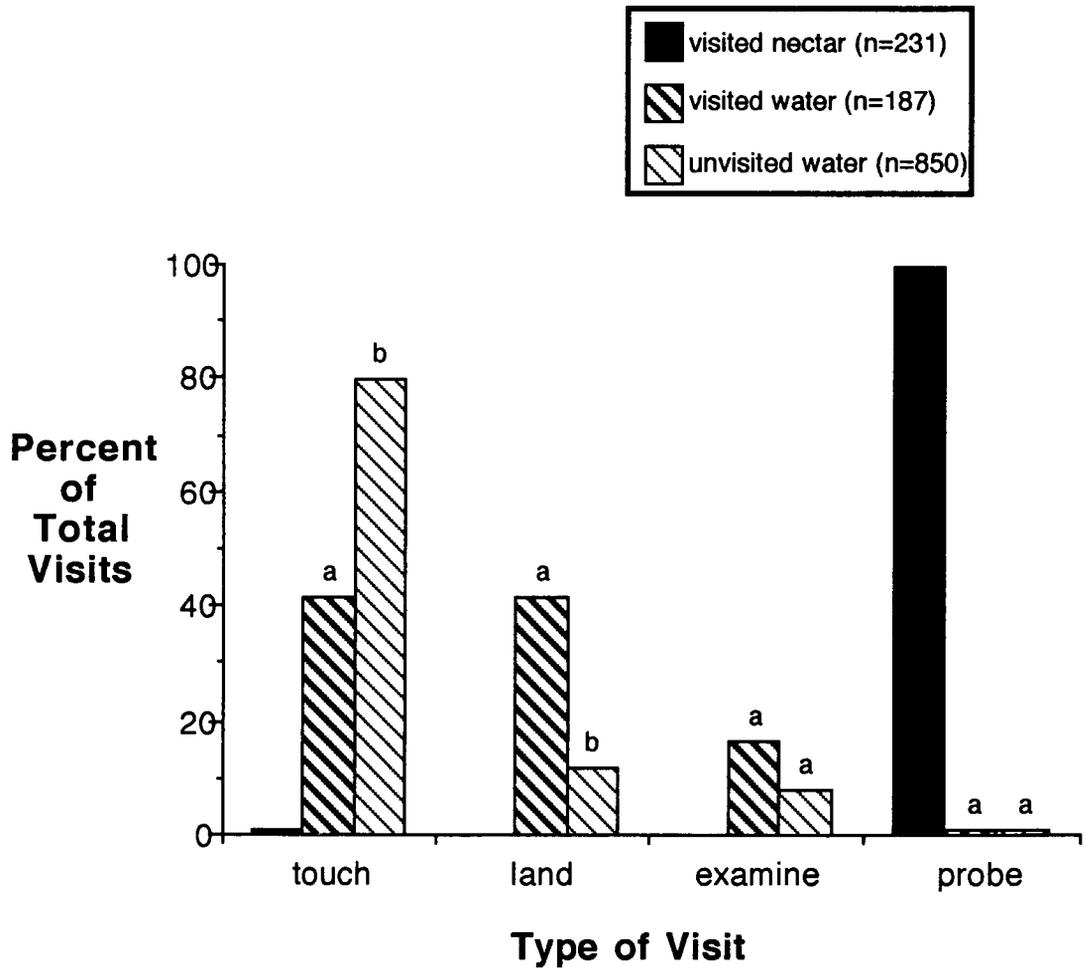


Fig. 2. 3. Experiment #2. Percent of total visits to visited nectar-flowers, visited water-flowers and unvisited water-flowers broken down by type of visit. Different letters, within a type of visit, indicate that the two flower types are significantly different.

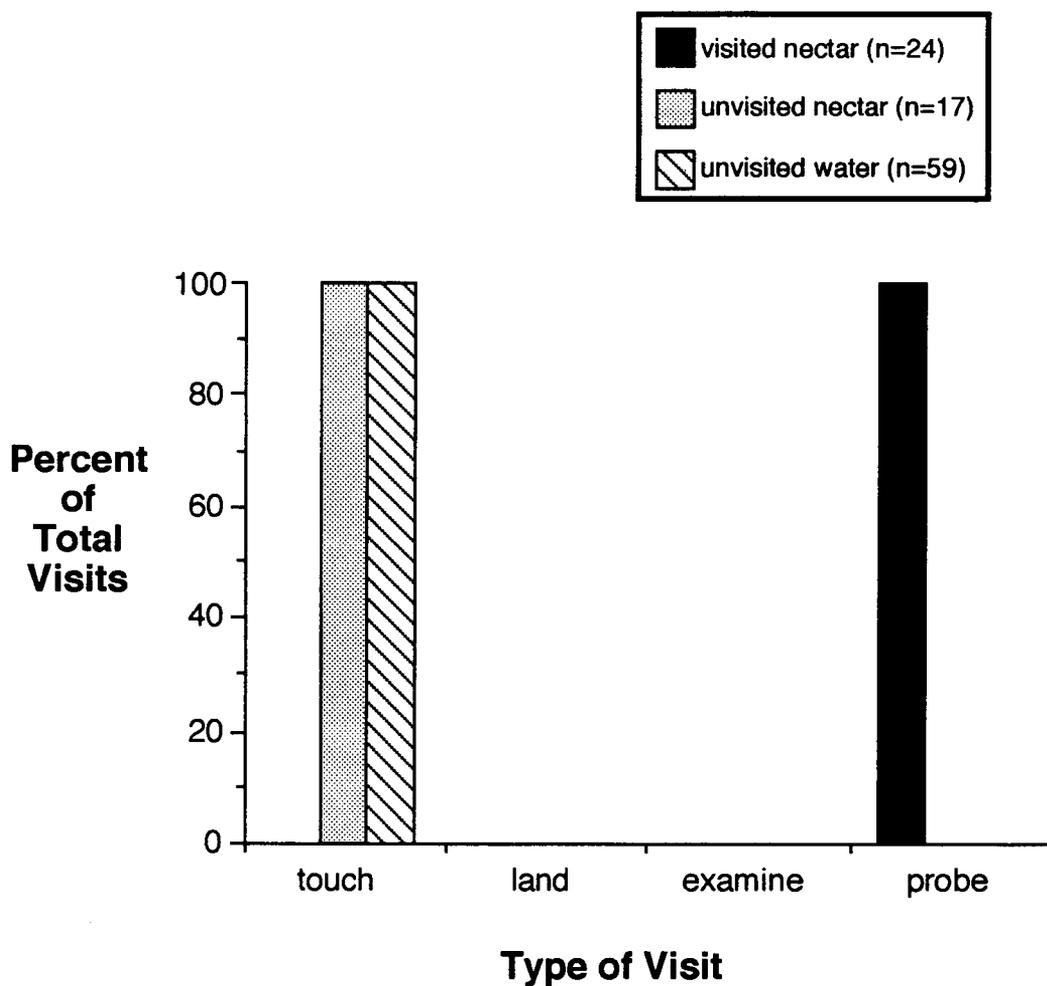


Fig. 2. 4. Experiment #3. Percent of total visits to visited nectar-flowers, unvisited nectar-flowers and unvisited water-flowers broken down by type of visit.

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CHAPTER 3

Scent-marking by Honey Bees with a Realistic Model

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Abstract

Experiments using a large realistic artificial flower patch indicated that honey bees use scent-markings on both rewarding and unrewarding flowers. Unrewarding flowers were marked so that they were less attractive than unmarked flowers. Rewarding flowers were marked so that initially they were less attractive than unvisited flowers but then, after several rewarding visits, they were more attractive than unvisited flowers. These results are more applicable to real flowers than previous studies and show that terms such as attractant and repellent may hide the intricacies of the use of scent-markings.

Introduction

Honey bees have the ability to use scent-marks to communicate with conspecifics. Butler et al. (1969) demonstrated that returning foragers leave 'footprints' at the nest entrance that leads other bees to the entrance and they suggested that bees may also use footprints to mark flowers. Ribbands (1955) found that honey bees leave scent-markings at large food sources that attract other bees. Since then other researchers have demonstrated honey bees' ability to leave scent-markings at artificial food sources (Nùñez, 1967; Ferguson and Free, 1979; Free and Williams, 1983; Giurfa and Nùñez, 1992; Wetherwax, Ch 2). The chief aim of those studies was to demonstrate that

honey bees have the ability to leave both attractants and repellents at food sources but the models used were poor mimics of real flowers. A question still exists: Are the scent-markings something honey bees use while foraging on real flowers or is it an artifact of unusually rich sources of food? One question, in particular, is why bees would mark a flower that has just been emptied with an attractant?

The purpose of this study was to develop a better understanding of how honey bees scent-mark flowers in real patches. As it is difficult to answer this with real flowers, a large flower patch that mimics a real patch was designed. It presents a large number of flowers (90), offers a small amount of reward per flower (1 μ l) and contains flowers that are not immediately replenished with nectar after a bee-visit so the bee may visit a previously-rewarding flower that is empty. By presenting a model of real flowers I hoped to understand what kinds of scents are used, whether or not the use of scent-markings is an artifact of large food sources and how scent-markings, or responses to markings, change over time.

Materials and Methods

The artificial patch was a 1 x 1 m board with 100 plastic flowers mounted on wooden dowels (approximately 10 cm high) in a 10 x 10 design (Fig. 3. 1). The board was

attached to a turntable so the patch could be easily rotated. The flowers were made of 0.3 ml disposable plastic analyzer cups (Sarstedt, Inc.) with 2 holes drilled through the closure. Through one hole was placed a small piece of 10 μ l capillary tube (Drummond), which could be filled with fluid using a repeating dispenser (Hamilton). The second hole allowed air to enter the cup as the bee sucked fluid from the capillary tube. Disposable flowers were chosen so that I could be certain of starting with unmarked flowers with each new experiment. Flowers were filled with either ultrafiltrated distilled water (water-flower) or sugar water (nectar-flower). The nectar consisted of 25% sucrose (EM Industries) that was measured using a handheld refractometer.

All of the experiments were conducted on warm, mostly-sunny days between May and October of 1990 and 1991 near Eugene, Oregon. Experiments were conducted in a 2 x 2 x 2 m cage placed approximately 100 m from a standard honey bee colony. Bees were trained to forage in the cage by placing a feeder with nectar at the entrance to the hive and then slowly moving the feeder until it was inside the cage. The bees visiting the patch during this time were collected, cooled for 3 minutes and marked on the thorax with acrylic paint or small plastic numbers. The bee was then released at the patch. Marking was necessary to insure that all of the bees came from a single colony.

Bees were allowed to forage on the large patch for at least 1 hour at the start of each day but only one bee at a time was allowed to forage within the cage during the experiments. The experimental set-up consisted of flowers randomly assigned to 90 of the 100 wooden dowels: 30 nectar-flowers, 30 water-flowers and 30 empty-flowers; the other 10 dowels were left without flowers (Fig. 3. 2). At the beginning of the experiment (i.e. "set"), all of the flowers were unvisited. One bee was allowed to forage on the patch until she visited 100 flowers (i.e. a "bout") or tried to escape from the cage. The bee was then destroyed. Half of the flowers were refilled and half of the now visited flowers were replaced with unvisited flowers of the same type. A second bee was then allowed to forage. This procedure was repeated until five bouts were completed. Between each bout the same flowers were replaced so that the overall set-up was:

Bout #1 started with 30 unvisited nectar-flowers, 30 unvisited water-flowers and 30 unvisited empty-flowers. Bouts #2-5 started with 15 unvisited nectar-flowers, 15 unvisited water-flowers and 15 unvisited empty-flowers plus 15 visited nectar-flowers, 15 visited water-flowers and 15 visited-empty flowers.

The 5 bouts made up 1 set. Ten sets were performed so the overall experiment consisted of 10 sets of 5 bouts of 100 visits. A different bee was used for each bout but no

attempt was made to control which bee was allowed to forage.

Visits were recorded as one of four types:

1. touch - the bee hovers over or briefly touches the flower but does not land on it.
2. land - the bee briefly lands on the flower and then flies away without extending her proboscis.
3. examine - the bee lands on the flower and extends her proboscis but does not place her proboscis near the opening of the tube or insert it into the tube.
4. probe - the bee lands and extends her tongue into or on the opening of the tube.

The data were entered into a computer and a spreadsheet (Excel) with a custom macro program was used to identify which visits were the first visit to a particular flower for that bout (1st- visits) and which visits occurred after the first visit (repeat-visits). Various comparisons were then made to determine what kinds of scent-markings were being used.

Results and Discussion

The experimental design used in this study allowed several kinds of comparisons to understand what kinds of scent-markings are used and when bees use them.

Initially it needed to be shown that honey bees do not distinguish water from sugar water. Honey bees were not

able to discriminate between unvisited nectar-flowers and unvisited water-flowers (Fig. 3. 3). A goodness of fit test indicated that these two groups of flowers were homogeneous ($G=0.868$, $p=0.83$). Therefore, any differences in behavior at nectar-flowers and water-flowers is not due to the bees ability to sense sugar in the vapor phase.

Bees were able to discriminate between empty-flowers and water-flowers based on something other than scent-markings: probably by sight. There was significant heterogeneity in a comparison of unvisited empty-flowers and unvisited water-flowers (Fig. 3. 4; $G=671$, $p<0.0001$). These results limit the kinds of comparisons that can be made. All of the subsequent comparisons within this study were between groups of flowers that look the same: flowers that contain liquid can be compared as can flowers that are empty.

Two initial comparisons can show whether the bees used scent-markings but don't give a clue as to the type(s) of markings, attractants, repellents or both. One comparison is the 1st-visits (bouts 2-5) of visited nectar-flowers and visited water-flowers (Fig. 3. 5). Flower type was independent of the type of visit ($G=46$; $p<0.0001$). Further analysis using an *a posteriori* test using STP analysis (Sokal and Rohlf, 1969) indicated that the number of probes and the number of examines differed between the two flower types but touch and land visits did not. Visits to visited

nectar-flowers were probes 80% of the time while 59% of the visits to visited water-flowers were probes.

A second comparison is between the repeat-visits (bouts #1-5) of visited nectar-flowers and visited empty-flowers (Fig. 3. 6). The results are consistent with the previous comparison; the type of visit was independent of the type of flower ($G=105$; $p<0.0001$). STP analysis indicated that only the probes were different between the flower types while touch, land and examine visits were homogeneous. The difference between the percent of visits that were probes is even greater when comparing these two groups: 39% of visits were probes on visited nectar-flowers while only 5% of visits were probes to visited empty-flowers. The two previous comparisons indicate that bees discriminated between previously rewarding flowers and previously unrewarding flowers but this analysis doesn't indicate which type(s) of flower(s) contained the markings.

It is possible that the bees only marked rewarding flowers, the bees only marked unrewarding flowers or they marked every flower that they visited. A comparison between the 1st-visits (bout #2-5) of visited water-flowers and unvisited water-flowers indicates that water-flowers were marked with a repellent (Fig. 3. 7). Visited water-flowers received less probe visits than unvisited water-flowers ($G=82.0$; $p<0.0001$) but STP analysis showed no differences between the three types of rejection visits.

Bees probed unvisited water-flowers on 87% of their visits while they only probed visited water-flowers 59% of the time.

These results are the opposite from what was found in an earlier study (Wetherwax, Ch 2). Bees foraging on a smaller patch (6 flowers), where the flowers were immediately refilled with nectar, exhibited a higher percent of probes to visited water-flowers than to unvisited water-flowers. An explanation for this behavior was not given. The results from the present study, however, can be justified based on optimal foraging. It is possible that the bees increased their foraging efficiency by rejecting previously visited unrewarding flowers as it is likely that less time and energy is used in a touch visit than in a probe visit. A future study (Wetherwax, Ch 7) will investigate the energetic consequences of scent-marking.

The comparison between water-flowers indicated that bees were marking unrewarding flowers. A comparison between nectar-flowers will indicate whether the bees were also marking rewarding flowers. The 1st-visits (bouts #2-5) of visited nectar-flowers were different from unvisited nectar-flowers (Fig. 3. 8; $G=18.0$; $p=0.0006$). The overall difference was due to differences between the number of probes and number of touches (*a posteriori* STP test). Bees probed unvisited nectar-flowers (89%) more than visited

nectar-flowers (80%) but touched unvisited nectar-flowers (4%) less than visited nectar-flowers (11%). These results seem to indicate that the bees marked visited nectar-flowers with a repellent too. A more detailed analysis of the probe visits to nectar-flowers shows that the behavior is more complicated.

Figure 3. 9 shows the probe visits broken down by bout number. The percent of visited nectar-flower visits that were probes increased from about 74% to 88% in bouts #2-5 ($y=4.6X + 63.5$; $r^2=0.93$; $p=0.036$). Coincidentally, the number of visits to a particular visited nectar-flower increased from bouts #2 to #5. Unvisited nectar-flowers were probed 85% of the time so visited nectar-flowers did not become more acceptable than unvisited nectar-flowers until they received about 4 visits. The markings left on the nectar-flowers acted as repellents until the flower had been visited a number of times and then the markings acted as attractants. While visited nectar-flowers were more attractive than visited water-flowers (Fig. 3. 5), they were not more attractive than unvisited flowers until they received several visits.

Most other studies indicate that honey bees mark rewarding food sources with an attractant (Ribbands, 1955; Ferguson and Free, 1979; Free and Williams, 1983). All of these studies looked at the attractiveness of rewarding food sources only after many bee visits. One study

(Wetherwax, Ch 2) looked at the initial visits to rewarding flowers on an artificial small patch and found that flowers become attractive after 1-4 visits. The results of this present study may differ from the small patch study because the flowers were not immediately refilled with nectar as they were in the small patch study. The bees may be able to alter: (1) when they mark flowers, (2) how they mark flowers (both amount and kind of scent), and/or (3) how they respond to markings, all in response to the qualities of the resource. In the small patch study, flowers were either always rewarding or always unrewarding and the bees responded by probing marked rewarding flowers. In this large patch study, the flowers were not immediately refilled after a visit and the bees responded by rejecting marked visited flowers. However, after a flower had been rewarding a number of times, the bee was more attracted to that flower.

The intricacies of scent-marking make the use of the terms attractant and repellent problematic. It is uncertain whether the bees are varying the chemical composition of scents or varying the amount of scents or varying how they respond to the scents. It is likely that all three of these take place. A scent that acts as an attractant in one context may illicit no response or act as a repellent in a different context.

To fully understand the use of scent-markings it will be necessary to identify the compounds. A future study will use gas chromatography-mass spectrometry to identify the scents. It will also be necessary to vary the conditions of the patch in order to see how scent-markings are used. Parameters such as nectar concentration and amount, the number of flowers in the patch, the distance between flowers, the distance between the nest and the flowers, the availability of other nectar sources and conditions in the nest are only a few of the factors that may influence how scent-marking is utilized.

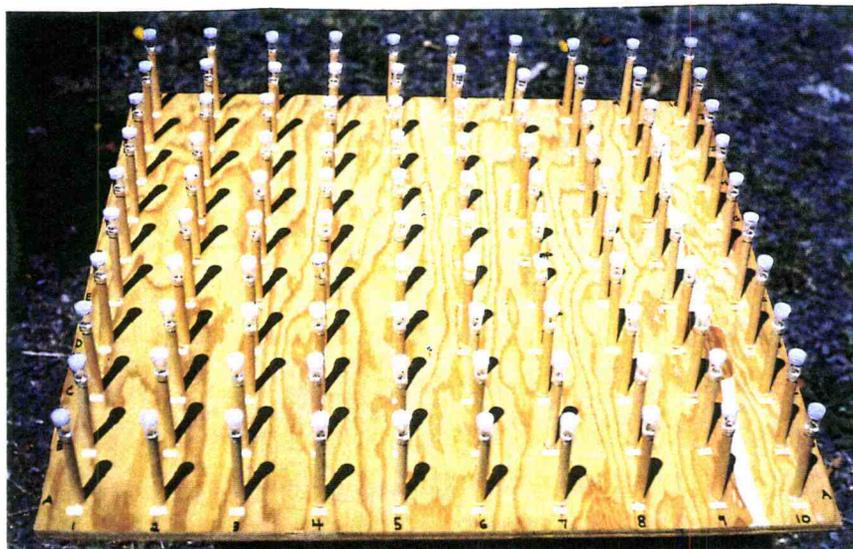


Fig. 3. 1. Artificial flower patch consisting of 90 flowers. Initially the patch consists of 30 nectar-flowers, 30 water-flowers and 30 empty-flowers.

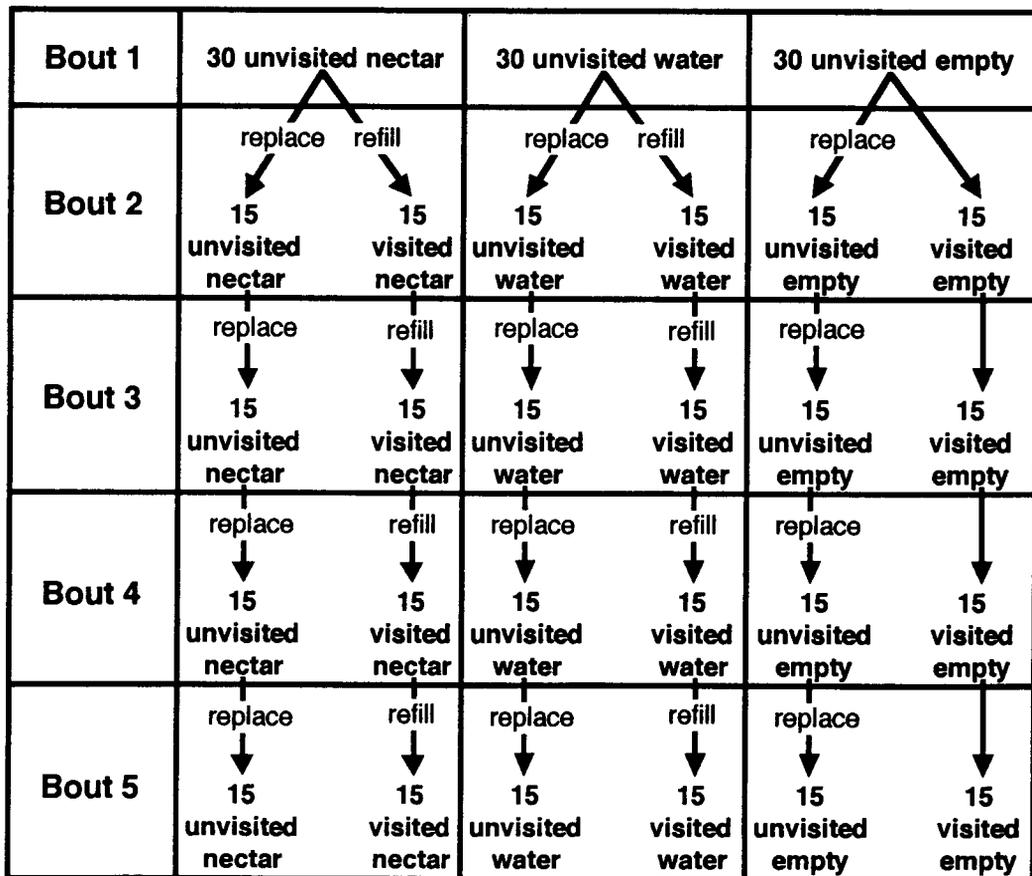


Fig. 3. 2. Experimental design. Each bout represents 100 visits. The entire set was repeated 10 times.

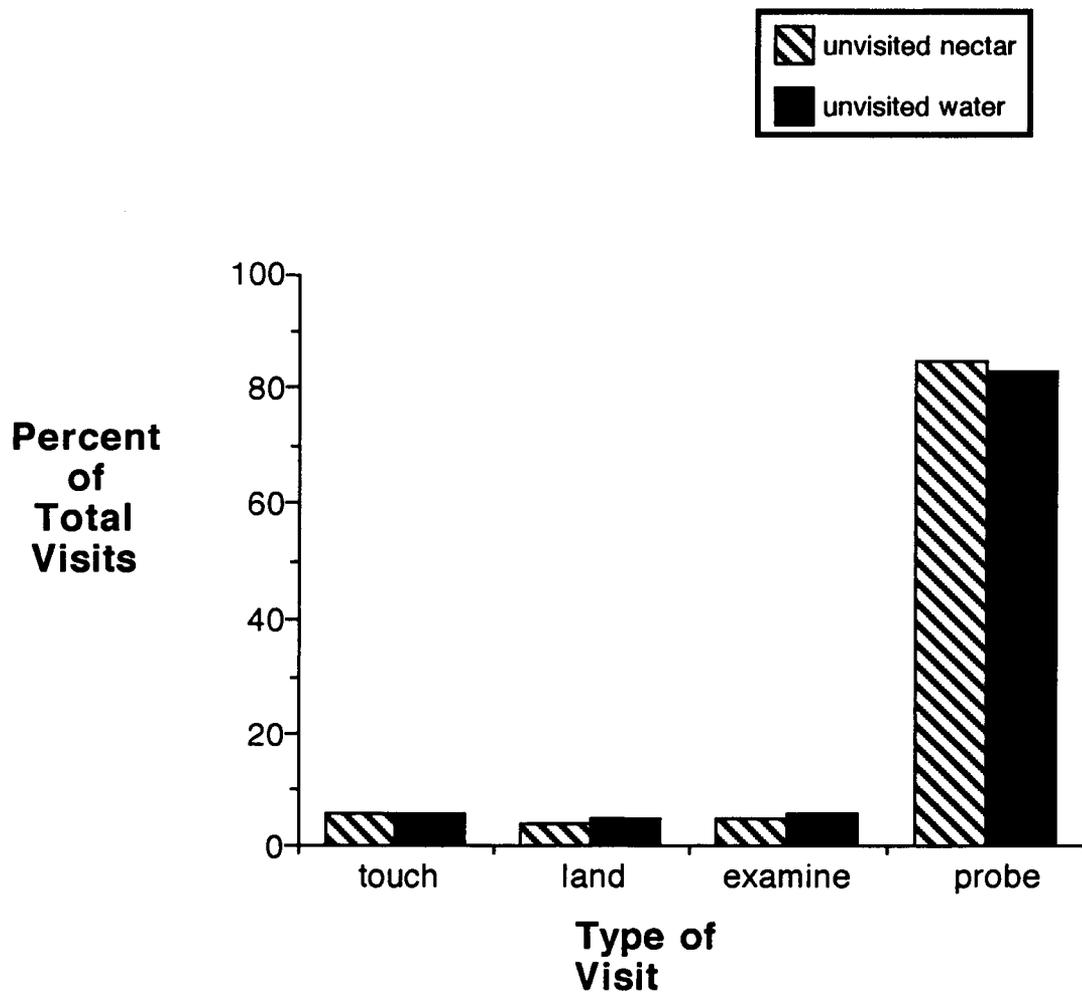


Fig. 3. 3. Unvisited nectar-flowers versus unvisited water-flowers. Honey bees did not discriminate between these two groups of flowers.

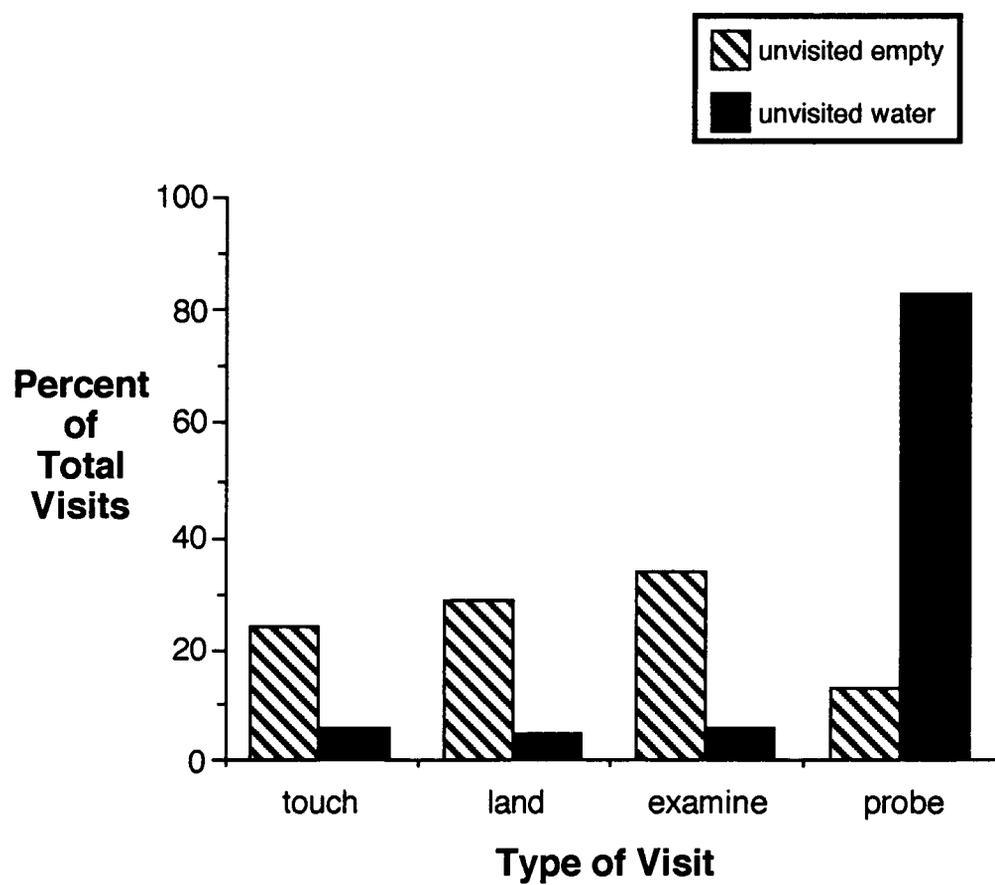


Fig. 3. 4. Unvisited empty-flowers versus unvisited water-flowers.

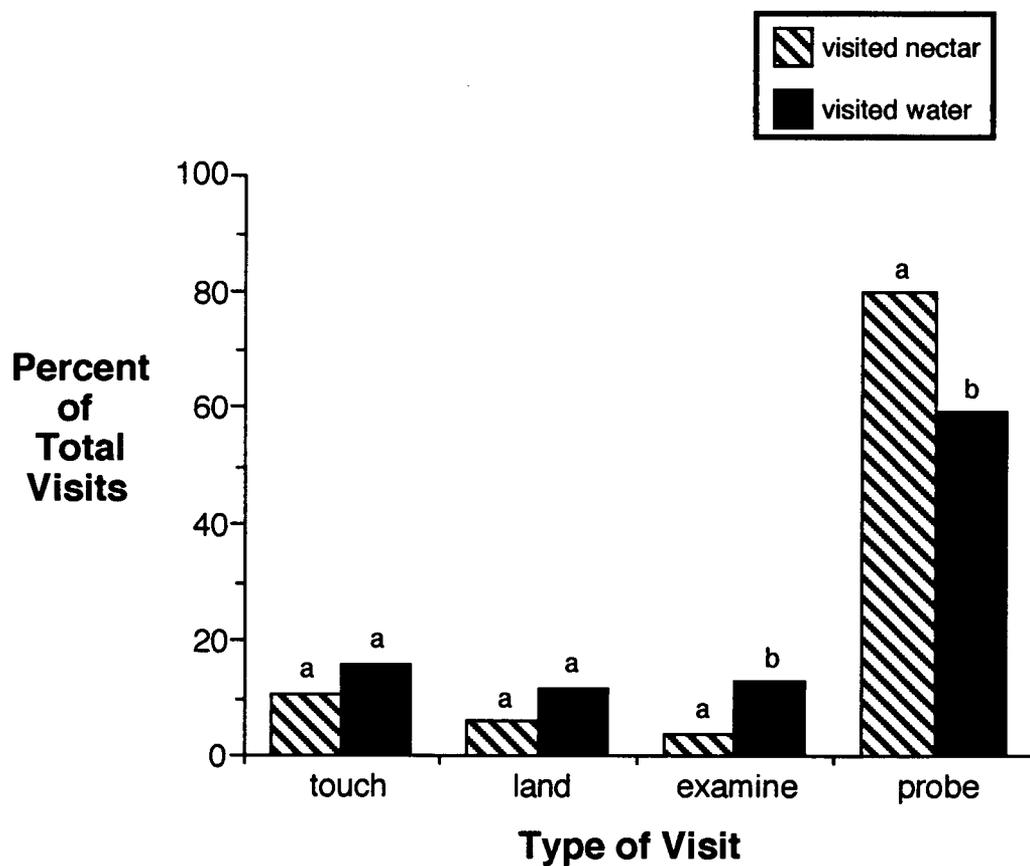


Fig. 3. 5. 1st-visits of visited nectar-flowers versus visited water-flowers bouts #2-5. Identical letters within a type of visit indicate that the 2 groups were not significantly different.

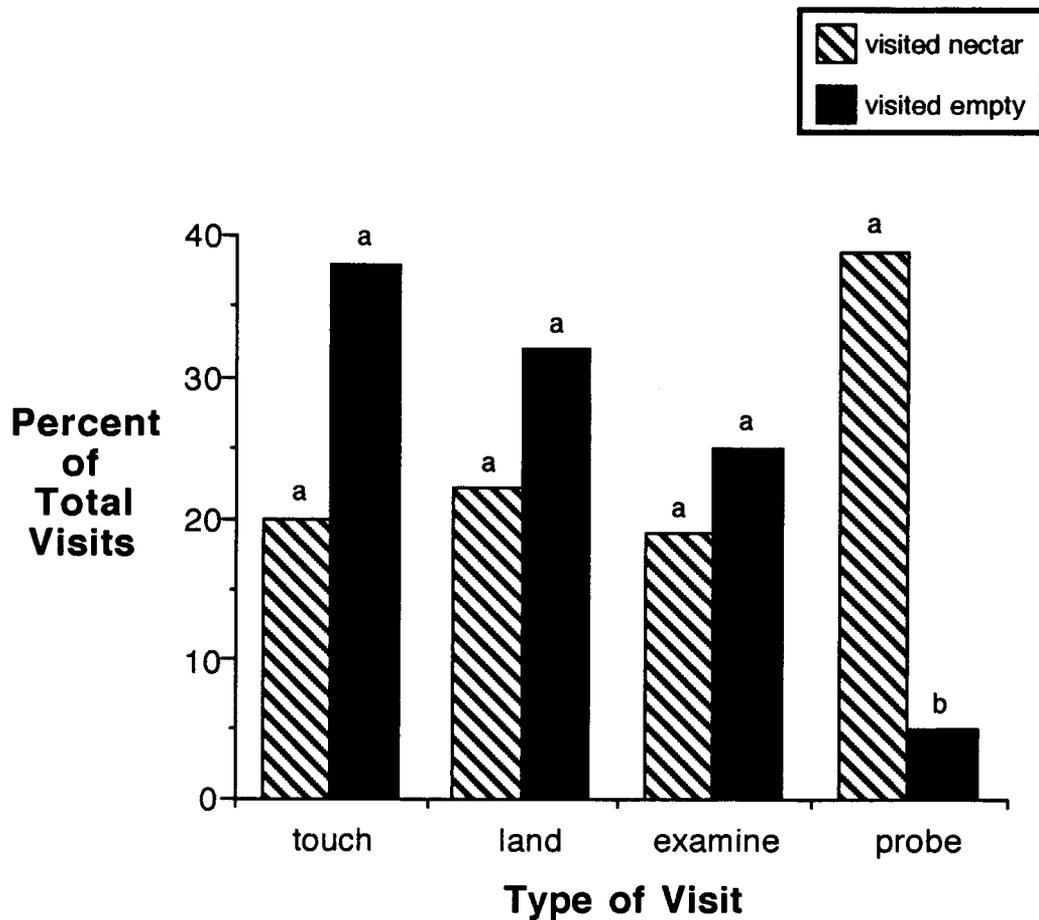


Fig. 3. 6. Repeat-visits of visited nectar-flowers versus visited empty-flowers bouts #1-5. Identical letters within a type of visit indicate that the 2 groups were not significantly different.

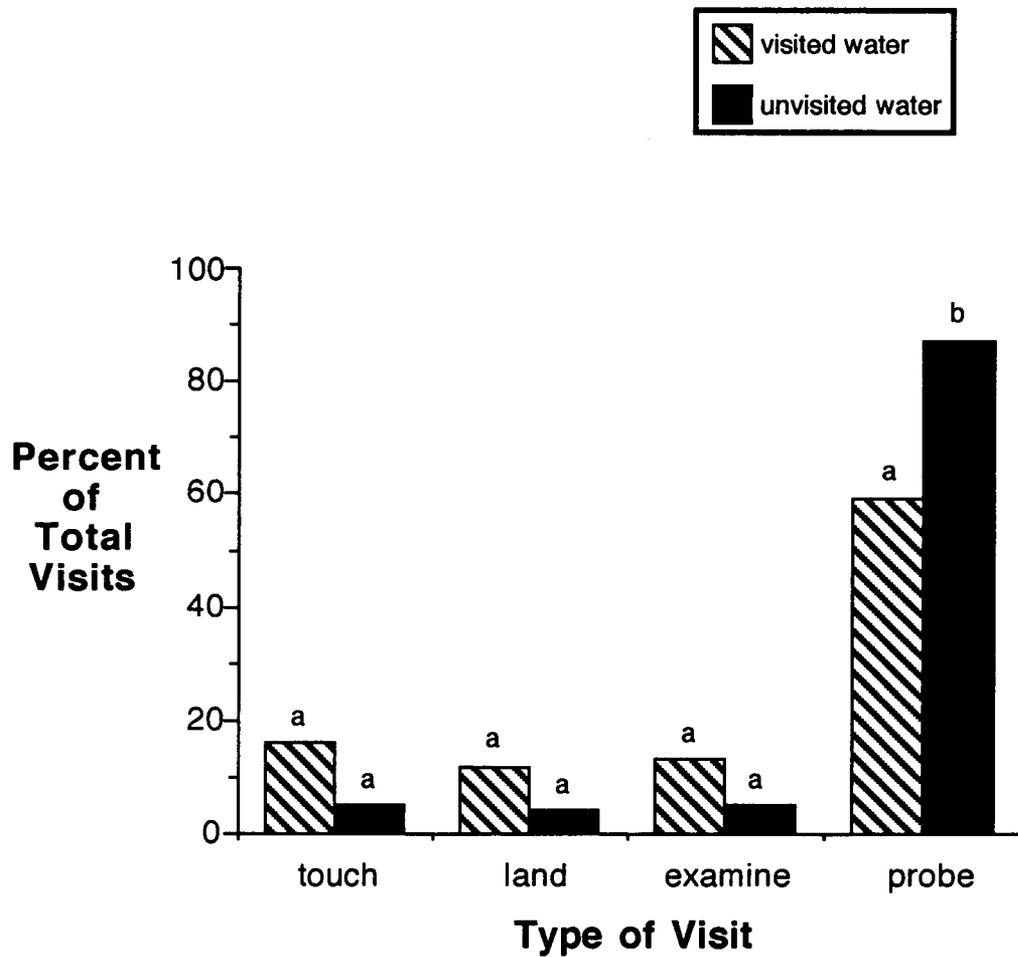


Fig. 3. 7. 1st-visits of visited water-flowers versus unvisited water-flowers bouts #2-5. Identical letters within a type of visit indicate that the 2 groups were not significantly different.

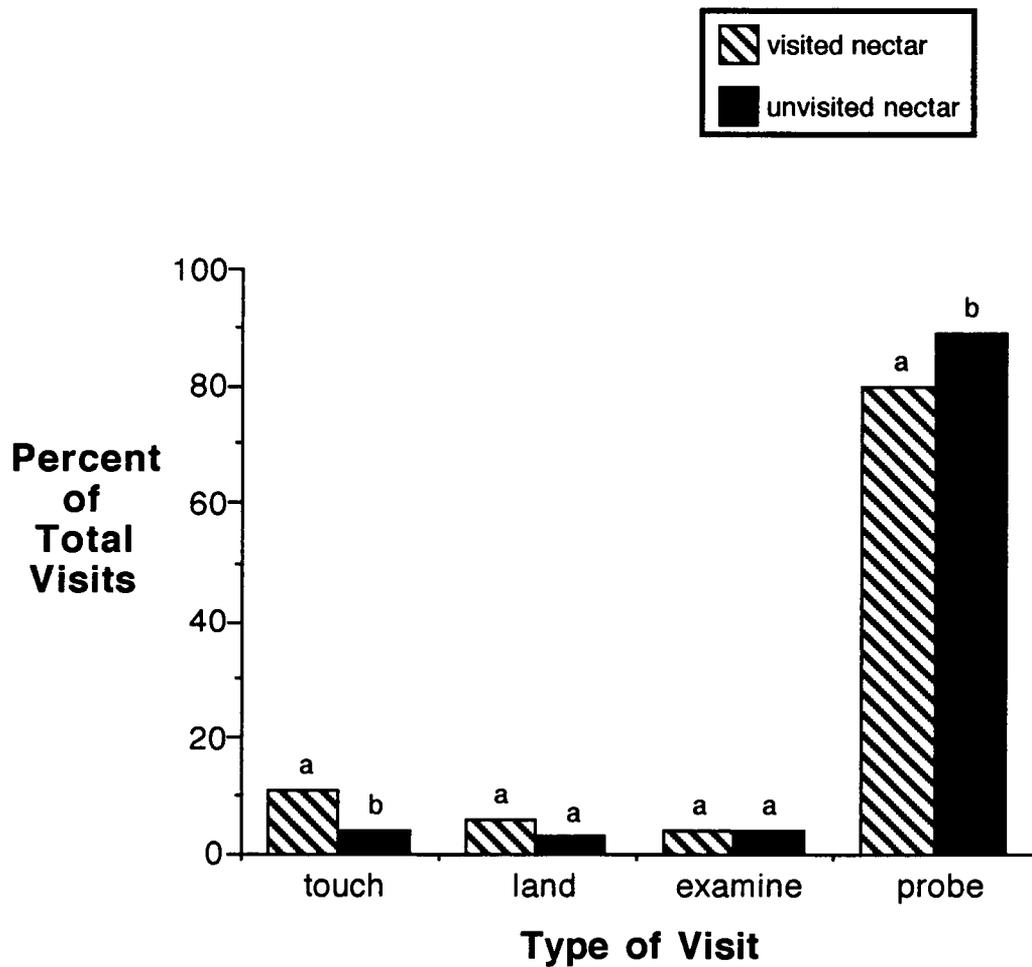


Fig. 3. 8. 1st-visits of visited nectar-flowers versus unvisited nectar-flowers bouts #2-5. Identical letters within a type of visit indicate that the 2 groups were not significantly different.

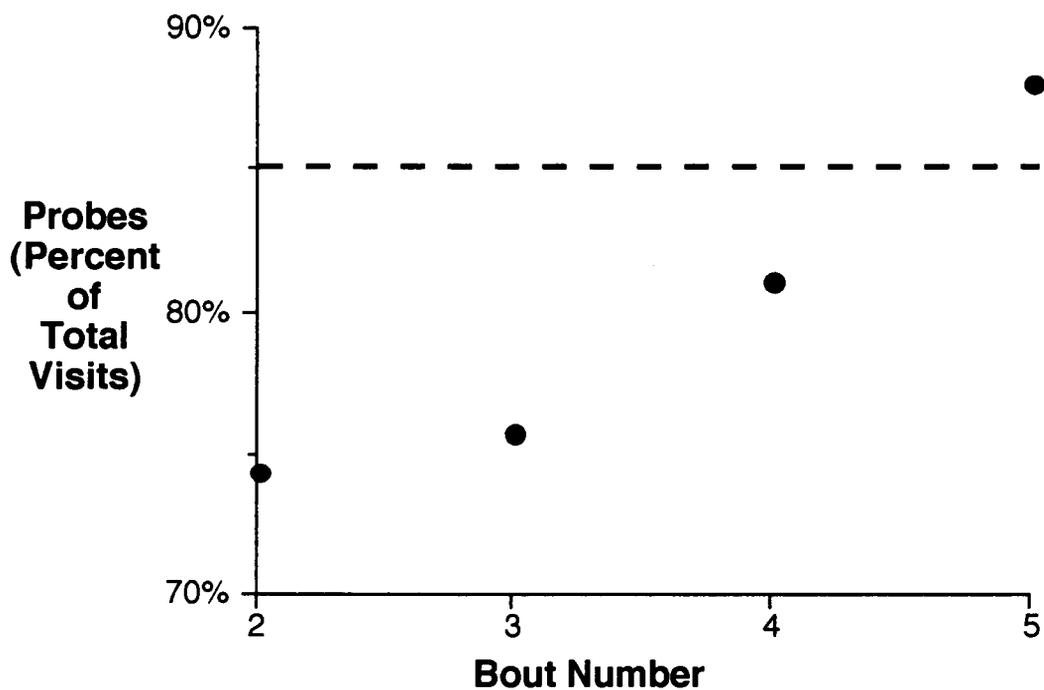


Fig. 3. 9. Nectar probes as a percent of total visits for bouts #2-5. The dotted line represents the average percent of probes for unvisited nectar flowers.

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CHAPTER 4

Bioassay Using Extracts of Attractant Scents Left by
Honey Bee Foragers

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Abstract

Honey bees left attractive scent-markings on artificial glass flowers. The scent was not soluble in water, partially soluble in hexane and acetone and completely soluble in dichloromethane. Dichloromethane extracts were found to be attractive to foragers when placed on clean glass flowers.

Introduction

Honey bees leave scent-markings at artificial flowers that attract or repel subsequent foragers (Nùñez, 1967; Ferguson and Free, 1979; Free and Williams, 1983; Giurfa and Nùñez, 1992; Wetherwax, Ch 2; Wetherwax, Ch 3). A crucial test to confirm the presence of scent-markings is to extract the scents and use them in a bioassay to determine their ability to affect behavior.

Repellents are more difficult to isolate than attractants because it is hard to get a large amount of the substance; once a repellent is applied to a flower, additional bees will not continue to land and apply more repellent. Wetherwax (Ch 3) found that nonrewarding artificial flowers did not become less attractive with more visits. An additional problem with the repellent used by foraging honey bees is that the chemical seems to be very volatile and lasts a short time (Giurfa and Nùñez, 1992).

Attractants are more easily extracted because of the positive feedback of the substance. Once an attractant is applied, additional bees land on a rewarding flower and may continue to apply more attractant. Wetherwax (Ch 3) found that rewarding artificial flowers became more attractive as they received more visits.

This study examined the solubility of attractants left at artificial rewarding flowers. An extract of the scent was tested for its ability to attract foragers.

Materials and Methods

Artificial flowers were made of glass so that the flower would not dissolve in the solvents. The flower consisted of a glass flask top with a hole drilled through the center placed over a small plastic cup that was filled with 25% sugar-water (sucrose, EM Industries) (Fig. 4. 1). A piece of 5 μ l capillary tube (Drummond) was placed through the hole into the plastic cup so that the bees could stick their proboscis into the opening of the tube and imbibe the sugar-water.

The experiments took place in September and October of 1991 near Eugene, Oregon on sunny days. The flowers were placed ca. 100 m from a standard honey bee colony. Bees were trained to forage on the flowers two days prior to the experiments. On the days of the experiments, bees foraged on a single flower for about 20 minutes. The plastic cup

was then removed and an empty clean-flower was placed along side of the empty visited nectar-flower. The number of bees landing on each flower was noted. Various solvents were then used to rinse the nectar-flower. The rinsed nectar-flower and a clean flower, that was rinsed with the same solvent, were tested for their ability to attract bees. The solvents used were water, hexane, acetone and dichloromethane.

A second study consisted of rinsing the visited nectar-flower with dichloromethane and using the extract in a bioassay. Three drops of the extract were placed on a clean flower (extract-flower) and three drops of dichloromethane were placed on a second clean flower (blank-flower). After the solvent dried, the number of bees landing on the flowers was noted for three minutes. If more than three bees congregated on one flower, they were brushed away from the flower and the position of the flowers was altered. The entire procedure, starting with clean flowers, was conducted 10 times.

Results

Many bees landed on the empty visited nectar-flower and no bee landed on the empty clean-flower therefore the bees left something on the flower which attracted subsequent visitors. After rinsing with water, the flower was still attractive to bees. Rinsing with acetone or

hexane reduced the attractiveness a little.

Dichloromethane completely removed the attractiveness of the flowers therefore it was chosen as the solvent in the extraction experiment.

The extract-flower was more attractive to bees than the blank-flower (Fig. 4. 2; paired t-test, $t=2.88$, $p=0.018$). Of a total of 266 landings, 164 were on the extract-flower.

Discussion

The attractive scent-marking was not soluble in water; this rules out the possibility that the bees were attracted to sugar-water left on the flower by previous foragers. The scent was only partially soluble in hexane or acetone but was completely soluble in dichloromethane.

The dichloromethane extract still contained some component(s) that was attractive to foragers. However, a significant number of landings were on the blank-flower so the extract-flower was not as attractive as the flower from which the extract came. This may be due to the small amount of extract applied to the flower or because some component of the scent evaporated. A future study will use this extract in a gas chromatograph-mass spectrometer to try to determine the chemical composition.



Fig. 4. 1. Artificial glass flowers used during the extraction test.

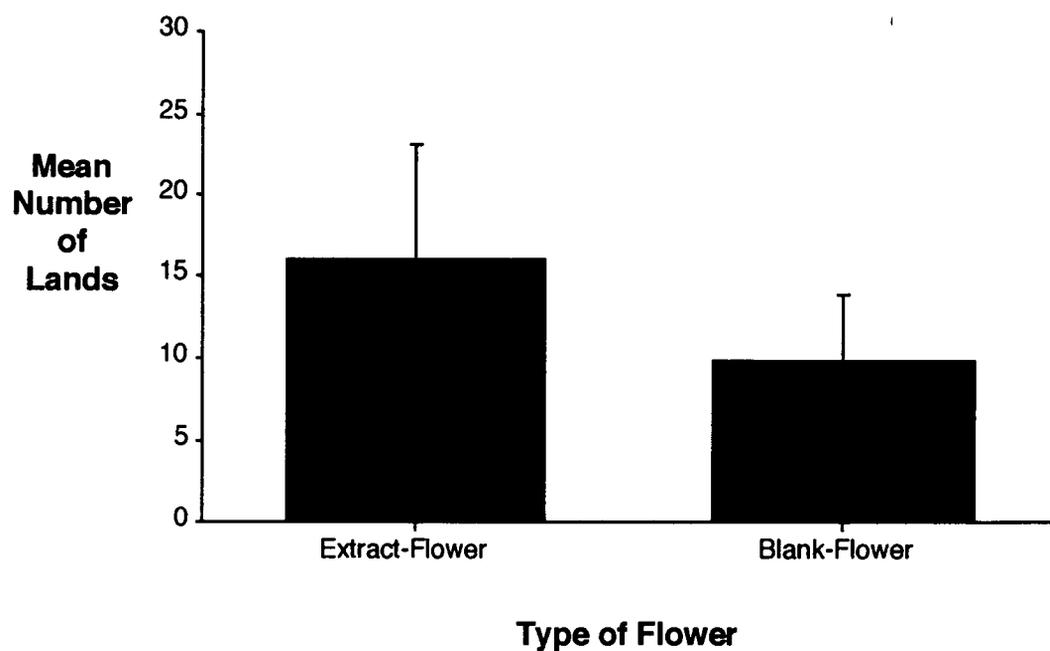


Fig. 4. 2. The number of landing visits by honey bee foragers on glass flowers containing an extract of an attractant compared to a blank-flower containing only the solvent.

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CHAPTER 5

Chemical Analysis of Honey Bee Scent-markings

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Abstract

Honey bees leave attractive and repellent scent-markings when foraging on artificial and real flowers. Attractive extracts from rewarding glass flowers were collected and analyzed using gas chromatography-mass spectrometry. Five compounds were identified in the extract. None of the compounds has previously been found in honey bees.

Introduction

Honey bees mark artificial (Wetherwax, Ch 2 and Ch 3) and real flowers (Wetherwax, Ch 6) with scents that act as attractants and repellents to subsequent foragers. Extracts collected from rewarding artificial flowers acted as an attractant when applied to clean glass flowers (Wetherwax, Ch 4). In this study, extracts from rewarding artificial flowers were collected and chemically analyzed using gas chromatography-mass spectrometry.

Materials and Methods

Artificial flowers were used to collect honey bee scent-markings in September and October of 1991 and 1992 near Eugene, Oregon. The flowers were placed ca. 100 m from a standard honey bee colony. Bees were trained to forage on the flowers for a minimum of two days before any scents were collected.

Two types of glass artificial flowers were used. One type ("flask-top flower") was made of glass flask tops with a hole drilled through the center (Fig. 5. 1). The top was placed over a small plastic cup that contained 2 ml of either 25% sugar water (nectar-flower) or ultrafiltrated water (water-flower). A small piece of capillary tube was inserted through the hole in the top of the flask into the plastic cup so that bees could obtain sugar-water from the opening of the capillary tube. Both a nectar-flower and a water-flower were placed on a board and honey bees were allowed to forage until they emptied the nectar-flower. From 5-15 bees were foraging at a time and it took about 10 minutes to empty the nectar-flower. Three samples were collected on three separate occasions.

A second type of flower was made of small glass vials ("glass-vial flower") with a hole drilled through the bottom. The vials were placed upside down on wooden dowels that were attached to a round board on a turntable (Fig. 5. 2). A piece of capillary tube was inserted through the hole and the other end was attached to a rubber tubing connected to a repeating dispenser (For a complete description of the flower patch, see Ch 2). The repeating dispenser injected 1 μ l at a time into the capillary tube. This set-up forced the bee to forage on all of the flowers rather than sitting on one flower and drinking until full, as with the flask-top flowers. One bee at a time foraged

on the patch and alternated between the nectar-flower and the water-flower until 40 visits were made on the nectar-flower. Samples were obtained for three different bees, each time starting with clean flowers.

Extracts of the bee scents were collected by extracting the flask-top or the glass-vial with dichloromethane (HPLC grade) and collecting the extract in glass vials. A separate blank was collected at the same time as each sample by rinsing a clean flower with solvent and collecting the extract. A sugar-blank was obtained by obtaining a dichloromethane extract of the sugar solution. The extracts were then analyzed using gas chromatography-mass spectrometry (GC-MS) with a high resolution mass spectrometer coupled to a GC fitted with a Supelco SPB-1 15 m*, 0.25 um column programmed at 60° C for 2 minutes then 10° C/min to 280° C where it was held for 10 minutes. Extracts collected in 1991 were analyzed on a different machine than those collected in 1992. The results were similar so only data from 1992 will be presented.

Results

Two types of flowers were used to obtain two different sources, and possibly amounts, of scent-markings. The flask-top flowers received many bees visits for about 10 uninterrupted minutes while the glass-vial flowers received a known number of visits (40) by a single bee. The

chromatograms did not differ significantly between the two flowers; the results reported are for only the flask-top flowers. The sugar-blank had no peaks in common with any of the other samples.

Figures 5. 3 - 5. 5 shows the chromatograms of a blank, water-flower and nectar-flower. There were no prominent peaks that were unique to the water-flower. Several peaks were found in the nectar-flower extract but not in the water-flower extract or the blank. These peaks were analyzed using MS.

Four compounds were identified from the nectar-flowers: clclohexanol, 2-cyclohexen-1-ol, 2-ethyl-1-hexanol and nonanal (Fig. 5. 6 - 5. 9). The analysis carried out in 1991 was not as extensive as in 1992 and only one compound, nonanal, was positively identified in the nectar-flower extract.

Discussion

Five compounds were identified in the nectar-flowers that were not found in the water-flowers, indicating that the chemicals are not deposited whenever a bee lands. This is consistent with finding by Wetherwax (Ch 2) who found that attractant and repellent scent-markings left on artificial flowers are not merely footprints (as suggested by other researchers e.g. Free, 1987). None of these compounds has been identified in honey bees. One of the

compounds, nonanal, is found in the mandibular gland of the stingless bee *Trigona gribodoi* (Keeping et al., 1982). *T. gribodoi* foragers were attracted to low concentrations of mandibular gland extracts containing nonanal.

These results are very preliminary and much more work is needed to verify their role in scent-marking. Future work will include: running known standards to verify retention times on the GC-MS, bioassays using the compounds in various mixtures to see if they attract honey bees and examination of various glands to find the source of these chemicals. Two obvious sources for these pheromones are the mandibular gland and the tarsal glands. Both of these glands have been suggested by other researchers as the possible source of forage-marking pheromones.



Fig. 5. 1. Glass flask-top flower used to collect scent-mark extracts.

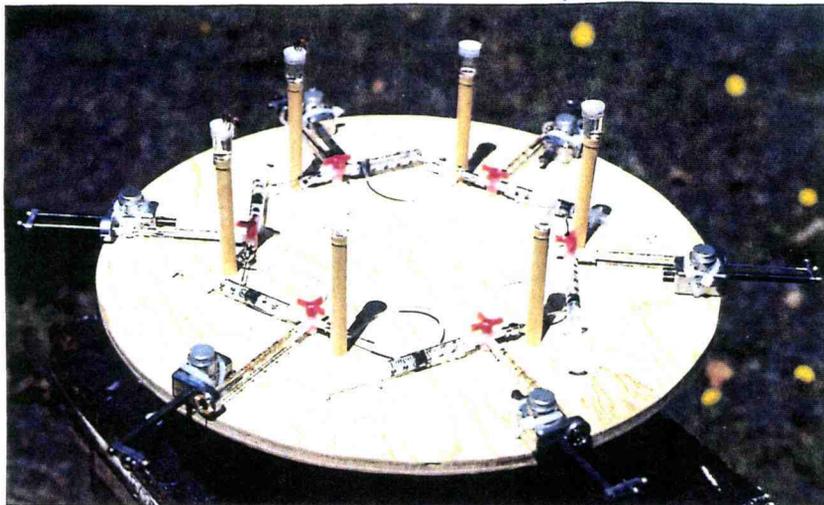


Fig. 5. 2. Glass-vial flower patch used to collect scent-mark extracts. The flowers were on a turntable so that the bees could not learn the locations of flowers.

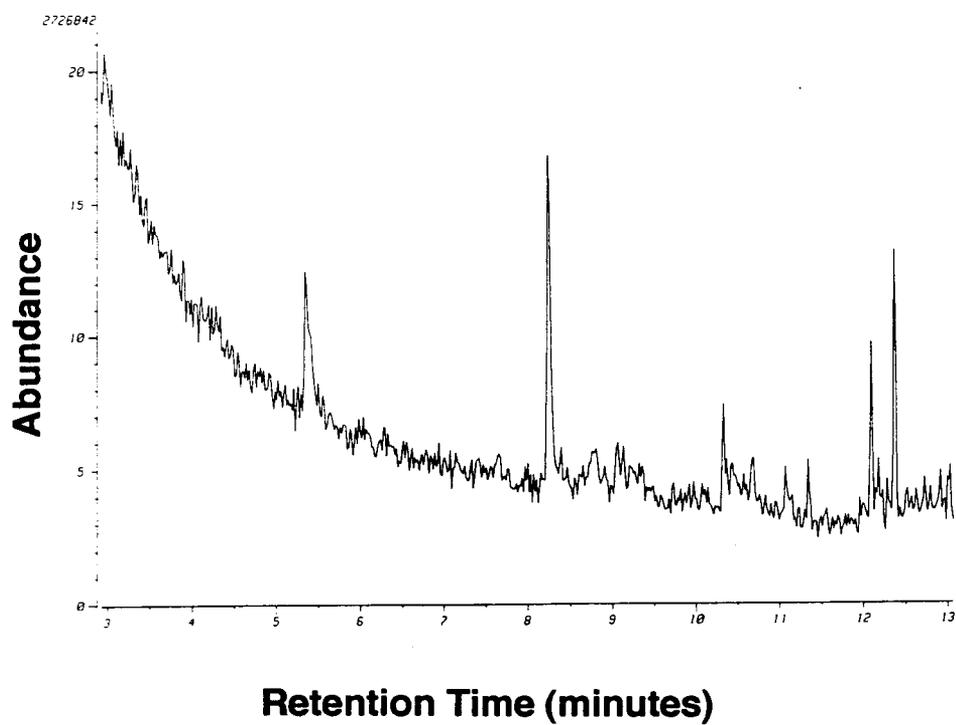


Fig. 5. 3. Gas chromatogram of the blank.

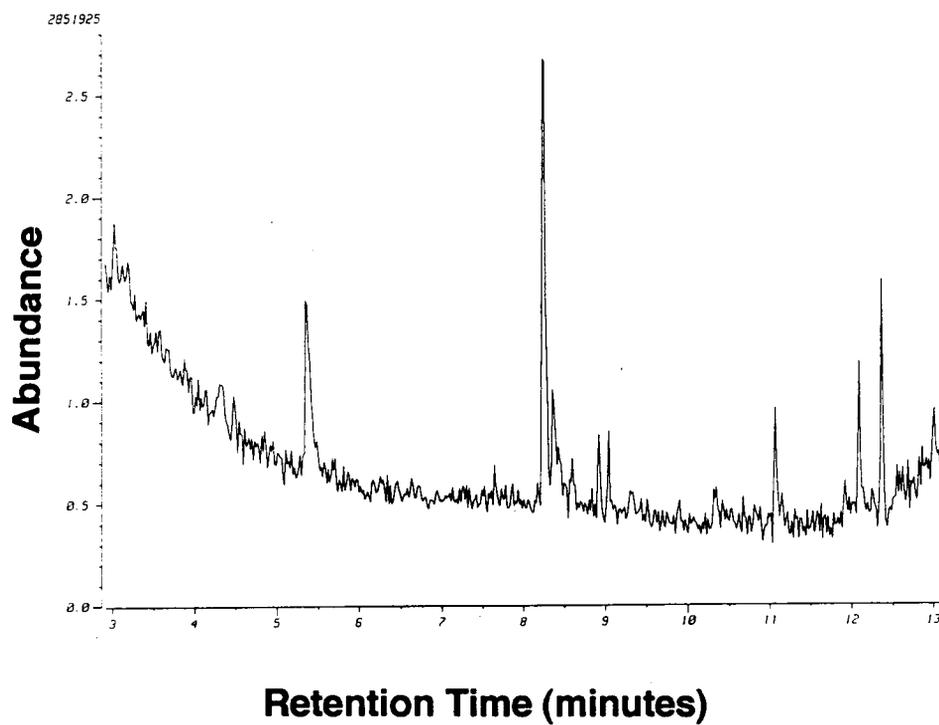


Fig. 5. 4. Gas chromatogram of the water-flower.

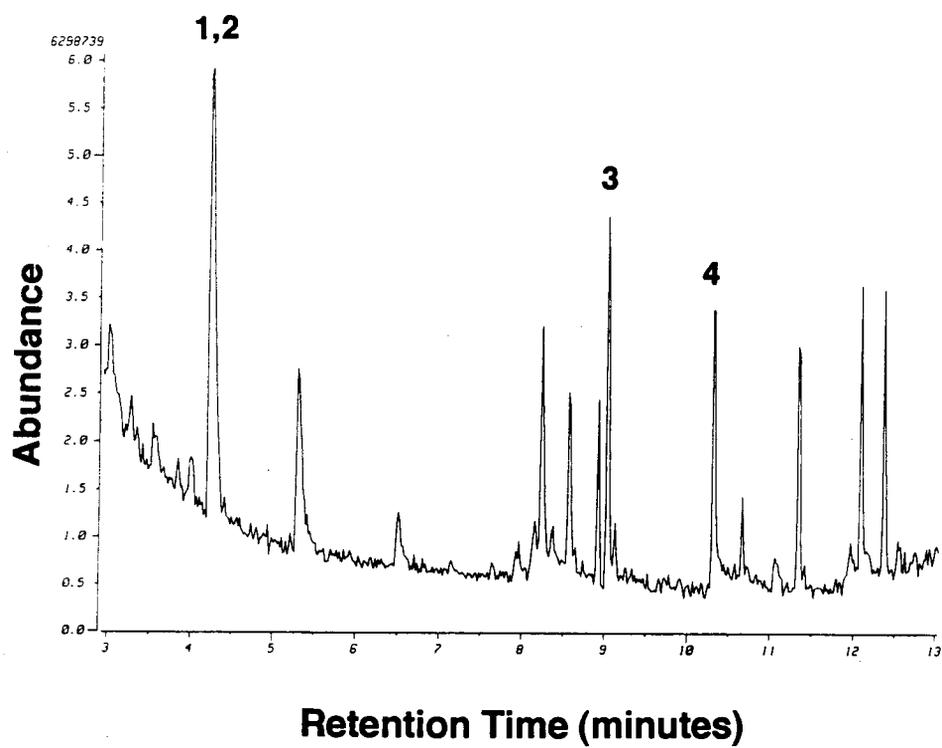


Fig. 5. 5. Gas chromatogram of the nectar-flower.

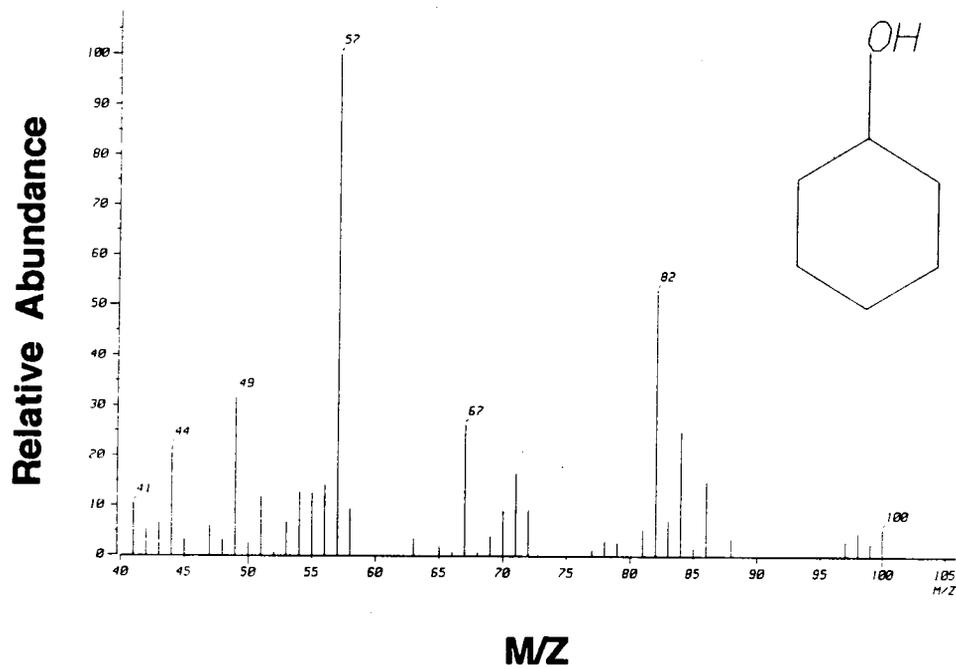


Fig. 5. 6. Mass spectrum of cyclohexanol (peak #1 in Fig. 5. 5).

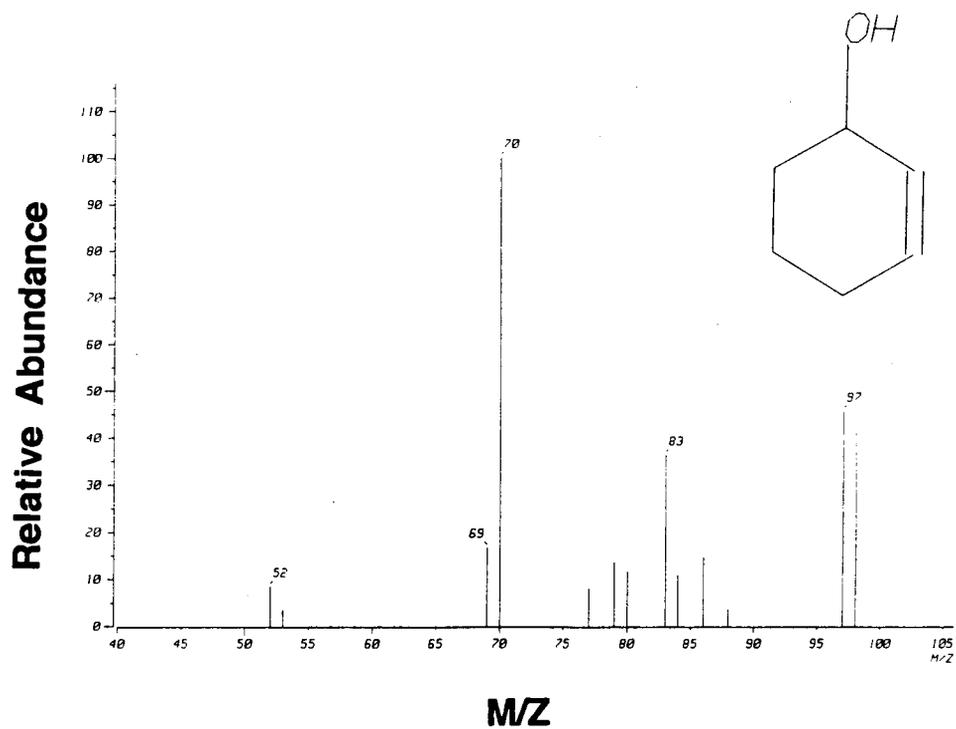


Fig. 5. 7. Mass spectrum of 2-cyclohexen-1-ol (peak #2 in Fig. 5. 5).

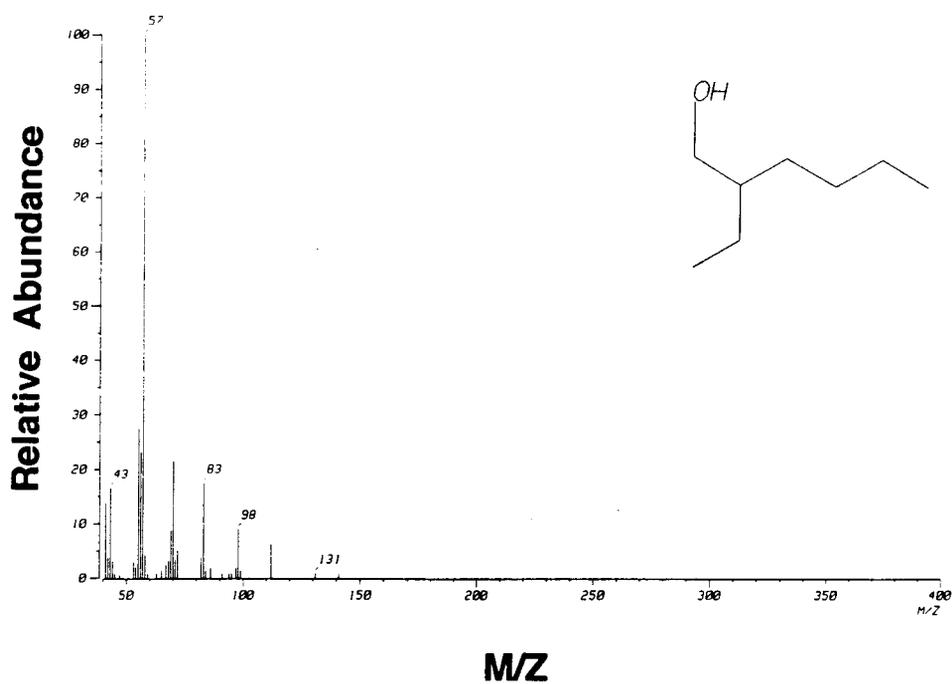


Fig. 5. 8. Mass spectrum of 2-ethyl-1-hexanol (peak #3 in Fig. 5. 5).

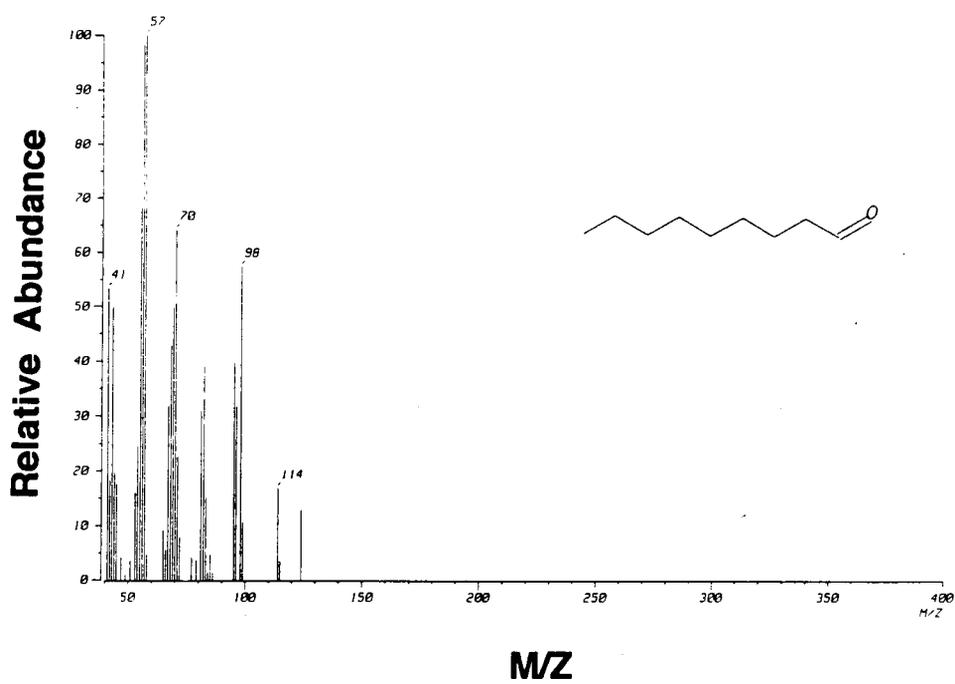


Fig. 5. 9. Mass spectrum of nonanal (peak #4 in Fig. 5. 5).

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- Wetherwax, P.B. 1993. Chapter 6: Scent-marking on *Lotus corniculatus* by nectar-collecting honey bees.

CHAPTER 6

Scent-marking on *Lotus corniculatus* by
Nectar-collecting Honey Bees

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Abstract

Honey bees will mark artificial flowers with scent that will result in an increase or decrease in the acceptance of that flower to subsequent visitors. This is the first study to demonstrate that honey bees use scent-markings on real flowers. The amount of nectar in *Lotus corniculatus* flowers was manipulated to see if and when scent-markings were used by foraging honey bees. Only one visit was necessary to make low-rewarding flowers less attractive to subsequent visitors but consistently-rewarding flowers required several visits before they were more attractive than unvisited flowers. In a natural flower patch, bees may mark recently visited flowers with a repellent to help them avoid recently visited flowers and mark consistently rewarding flowers with an attractant to indicate a "super flower".

Introduction

Honey bees will often hover-over or briefly touch flowers of *Lotus corniculatus* L. and then fly away without attempting to extract nectar. Presumably this is a form of discriminating behavior whereby the forager is considering the flower as a possible source of food but for some reason decides to reject it. Other workers have noted similar behavior for bumble bees foraging on white clover and (Heinrich, 1979) and impatiens (Kato, 1988); carpenter bees

foraging on passion flowers (Frankie and Vinson, 1977); and honey bees foraging on fireweed and blackberry (Corbet et al., 1984) Wetherwax (1986) demonstrated that, while foraging on *L. corniculatus*, this discrimination behavior pays-off energetically as accepted flowers contain more nectar than rejected flowers. The results of his study suggested that bee-scent, rather than nectar-scent, was the most likely cue used by honey bees when they decided to accept or reject particular flowers.

More recent studies have demonstrated that honey bees will mark artificial flowers with scents (Wetherwax, Ch 2; Wetherwax, Ch 3). Bees react differently to previously rewarding flowers and previously unrewarding flowers which indicates that the use and/or response to scent-markings is more complex than a single scent or single response.

While there is ample evidence that honey bees use scent-markings at artificial flowers, there is no direct evidence that scent-markings are used on real flowers. This study will investigate the use of scent-markings by honey bees foraging on *L. corniculatus*. The types of visits at unvisited (i.e. unmarked) patches and previously visited (i.e. marked) patches will be compared to determine if and when scent-markings are used.

Materials and Methods

Lotus corniculatus plants were grown from seed in a greenhouse. Inflorescences were cut off of the plant and all but one flower was cut from the inflorescence so that what remained was a short stem and a single petiole attached to a single flower. The stem was placed in a plastic analyzer cup (Sarstedt, Inc.) through a hole that was drilled in the middle of the closure. The cup was filled with water to keep the flower from wilting. Flowers manipulated like this would stay fresh looking for several days but were always prepared just prior to use.

The cups were placed on top of wooden dowels (about 10 cm high) that were attached to a wooden board (1 x 1 m) in a ten by ten design (Fig. 6. 1). The flowers were separated so that the bees had to fly between flowers. The board was attached to a turntable, allowing it to be rotated during the experiment so that the bees could not learn the position of the flowers. At the beginning of each experiment, 50 flowers were randomly assigned to one of the 100 dowels. The other 50 dowels were left empty.

All of the experiments were conducted on warm, mostly sunny days near Eugene, Oregon from August-October, 1992. The flower patch was placed within a 2 x 2 x 2 m cage approximately 100 m from a standard honey bee colony. Bees were trained to forage on the flowers for several days before data were collected. During this training, bees

were collected, cooled and marked with small plastic numbers to insure that only experienced bees were used during the experiments.

Bees were allowed to forage on flowers for at least 1 h before each experiment. At the beginning of each unmarked (i.e. control) bout, 50 new flowers were randomly assigned to 50 positions on the board. Twenty-five of the flowers (enhanced-flowers) were injected with 1 μ l of 25% sucrose (EM Industries) with a repeating dispenser (Hamilton PB6000) and the other 25 (natural-flowers) were stuck with the syringe but not injected. The needle was inserted, without puncturing the corolla, between the keel petals to the base of the flower, which is where nectar is normally found. One marked bee was allowed to forage on the patch until 50 visits were made and then that bee was destroyed. The type of visit and the number of the flower were tape-recorded. Five unmarked bouts were performed using five different bees.

For each marked bout, bees were allowed to forage on a patch of 25 enhanced-flowers and 25 natural-flowers for about 30 minutes. The flowers were refilled 3 times during this "charging" phase: the enhanced-flowers received 1 μ l of sugar-water and the natural-flowers were stuck with the syringe. All of the enhanced flowers were replenished with nectar and then one marked bee was allowed to forage. The flower number and type of visit were recorded. The bee was

then removed and destroyed. The marked set was repeated ten times using ten different bees.

Three types of flower visits were recorded:

- (1) touch - bees briefly touched flower while flying.
- (2) land - bees briefly land on flower and then fly away without attempting to insert proboscis into corolla.
- (3) probe - bees insert proboscis into corolla.

The data were transcribed into a computer and a spreadsheet program with a custom macro was used to identify which visits were the first visits to a particular flower (1st-visits) and which visits occurred after the 1st-visits (repeat-visits).

Results

It was determined prior to the experiments that honey bees always emptied both types of flower with a single visit so that no measurable nectar remained. *L. corniculatus* flowers contained an average of 0.05 μ l prior to manipulation.

Most of the 1st-visits to unmarked natural-flowers and unmarked enhanced-flowers were probes (Fig. 6. 2). The number of probes in the 1st-visits to the two flower groups were not different (paired $t=0.95$, $p=0.40$; Fig. 6. 3). However, after one visit, the number of probes was greater in the enhanced-flowers than in the natural-flowers (paired $t=4.6$, $p=0.0097$; Fig. 6. 3). There was no difference

between the percent of the total visits that were probes in the 1st-visit and repeat-visits to enhanced flowers (arcsin transformation with Bartlett correction (Zar, 1974) paired $t=2.0$, $p=0.11$, Fig. 6. 4) but there was a difference between the 1st-visits and repeat-visits of natural flowers (arcsin transformed paired $t=4.6$, $p=0.0097$), with repeat-visits receiving less probes than 1st-visits.

Marked flowers showed differences in the types of visits made by foragers (Fig. 6. 2). The mean number of probes per bout for the 1st-visits was greater for the enhanced-flowers than the natural-flowers (paired $t= 14.8$, $p<0.0001$, Fig. 6. 6). After bees had visited these flowers one time during the bout, the number of probes was still greater in the enhanced-flowers than in the natural-flowers (paired $t=6.5$, $p<0.0001$, Fig. 6. 6). When 1st-visits are compared to repeat-visits, the percent of probes is greater for 1st-visits than repeat-visits for enhanced-flowers (arcsin transformed with Bartlett correction paired $t=12.2$, $p<0.0001$) and for natural-flowers (arcsin transformed with Bartlett correction paired $t=4.1$, $p=0.0027$).

A comparison between the unmarked bouts and the marked bouts indicates that the bees probed the marked enhanced-flowers more than the unmarked enhanced-flowers (unpaired $t=3.3$, $p=0.0054$) and they probed the marked natural-flowers more than the unmarked natural-flowers (unpaired $t=4.6$, $p=0.0005$).

An assumption of this study is that I am measuring discrimination at the level at which it occurs for the bees. A test of this assumption is to compare the total number of visits to enhance-flowers vs natural-flowers. If I am accurately measuring when discrimination occurs, then there should be no difference between the total number of visits to the two flower groups. The number of visits was not different between the two flower groups for either the unmarked bouts ($\chi^2=0.784$, $p=0.38$) or the marked bouts ($\chi^2=1.15$, $p=0.28$).

Discussion

The foragers could not tell the difference between unmarked natural-flowers and unmarked enhanced-flowers (Fig. 6. 3, 1st-visits). About 80% of the first visits to unmarked flowers were probes, regardless of whether they were enhanced or not. Therefore any differences in behavior between the types of visited flowers was due to something the bees did to the flowers when they visited them. Presumably they left scent-markings on the flowers and responded to those markings on subsequent visits.

Discrimination between the two types of flowers was evident after even a single visit (Fig. 6. 3, repeat-visits). While enhanced-flowers received the same proportion of probes, natural-flowers became much less attractive to the foragers (Fig. 6. 4). Enough scent was

left after one visit to cue the same forager when that flower was visited during the same bout (i.e. within about 5 minutes). A patch becomes a mixture of visited and unvisited flowers as the bee visits the patch. Since the bees didn't discriminate between recently visited (i.e. repeat-visits) enhanced-flowers and unvisited (i.e. 1st-visits) enhanced-flowers the simplest explanation is that they were responding to a repellent left on visited natural-flowers. In a real patch bees may mark low rewarding flowers with a repellent so that they do not immediately re-visit them.

Although once-visited enhanced-flowers did not evoke a change in behavior, after several visits enhanced-flowers became more attractive. Unvisited enhanced-flowers were probed about 80% of the time but after several rewarding visits they were probed on 96% of the visits. It seems that the enhanced-flowers were marked with an attractant but only after they rewarded a forager several times. This is consistent to what was found with artificial flowers (Wetherwax, Ch 3) where it took about 4 visits before rewarding flowers were more attractive than unvisited flowers. In a real flower patch this type of marking may be used on flowers that consistently provide large amounts of nectar to a forager. Although I know of no studies that have shown this, it seems reasonable that there is variability in the amount of nectar a flower produces

during the life of the flower, relative to other flowers in the patch. This study indicates that bees may be able to exploit these highly rewarding flowers by marking them with an attractant.

In a patch where all flowers had received several visits either the bees increased their responses to the scent-markings or there were more scents present. In the marked patch the foragers discriminated between rewarding and non-rewarding flowers from previous visitors as well as between flowers that had been visited and unvisited during that bout. The flowers can be put into four categories from the most attractive to the least attractive: enhanced-flowers 1st-visits, natural-flowers 1st-visits, enhanced-flowers repeat-visits, and natural-flowers repeat-visits. For this type of discrimination to occur the bees must be responding to a suite of types of scent-markings and/or amounts of scent-markings. The marked patch is probably more similar to a real lotus patch where many of the flowers in the patch have been visited several times (Wetherwax, 1986; and personal observation).

The reason for the behavior at the natural-flowers is less clear. Natural-flowers that had been visited several times were more attractive than natural-flowers that had never been visited. Since natural-flowers were marked with a repellent after a single visit, one might expect the flowers to be at least as unattractive after several

unrewarding visits. There are at least two possible explanations for why this was not the case. (1) The response to the scent-markings may be dependent on the other flowers in the patch. If other flowers in the patch contain attractant scents then bees may probe all flowers more often. Attractants on some flowers may indicate that the patch as a whole is a good place to forage. Area restricted foraging in honey bees and bumble bees has been demonstrated in other studies (Heinrich, 1979; Waddington, 1980). Bees may be foraging not just based on the amount of nectar in the flowers but the amount of attractant in the patch. (2) The attractant left on the enhanced-flowers diffuses through the air so that the forager can not always tell exactly which flower contains the scent. Either of these explanations means that from the plant's perspective, attractants in a patch make the entire patch more attractive.

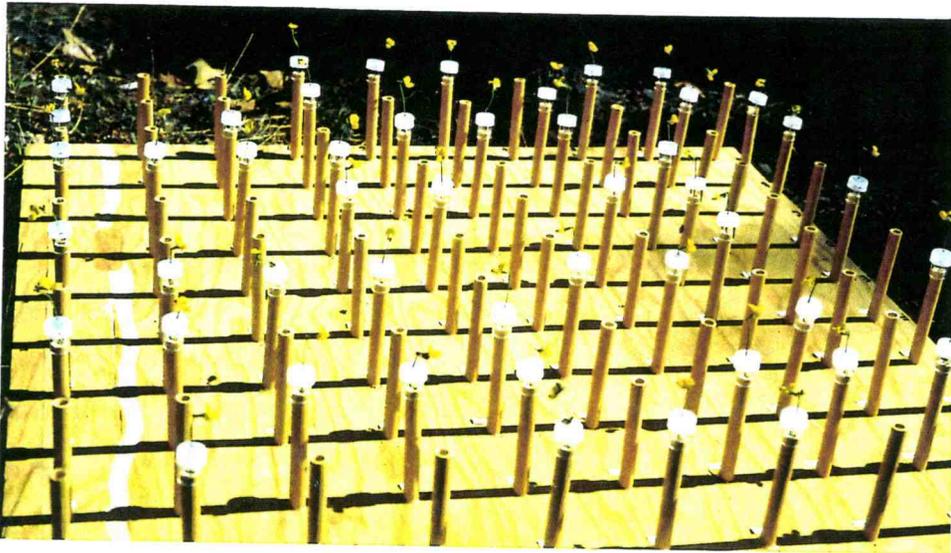


Fig. 6. 1. Honey bees foraging on artificial
L. corniculatus patch.

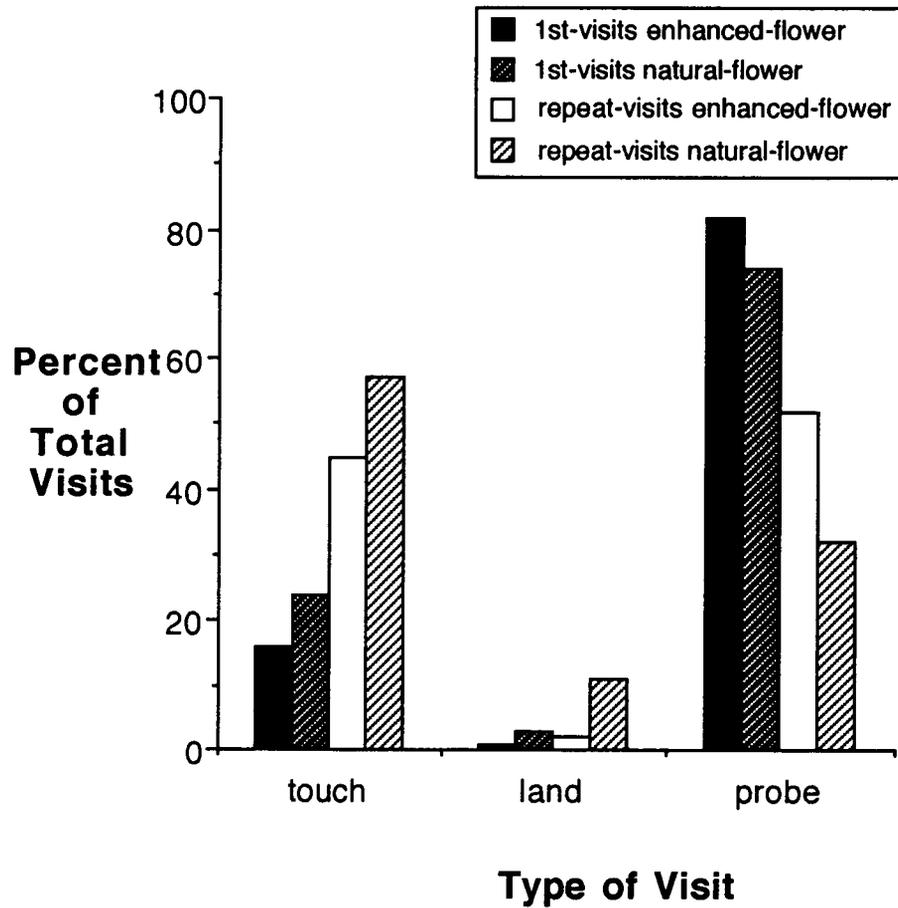


Fig. 6. 2. The percent of total visits for each flower type broken down by type of visit on the unmarked patch.

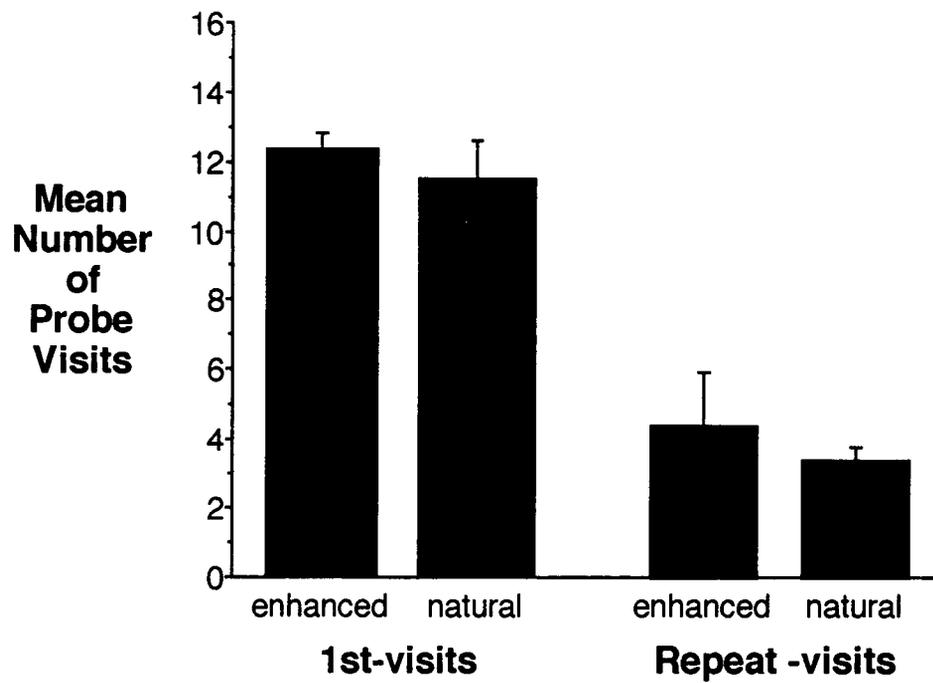


Fig. 6. 3. Mean number of probes (+se) in the unmarked patch.

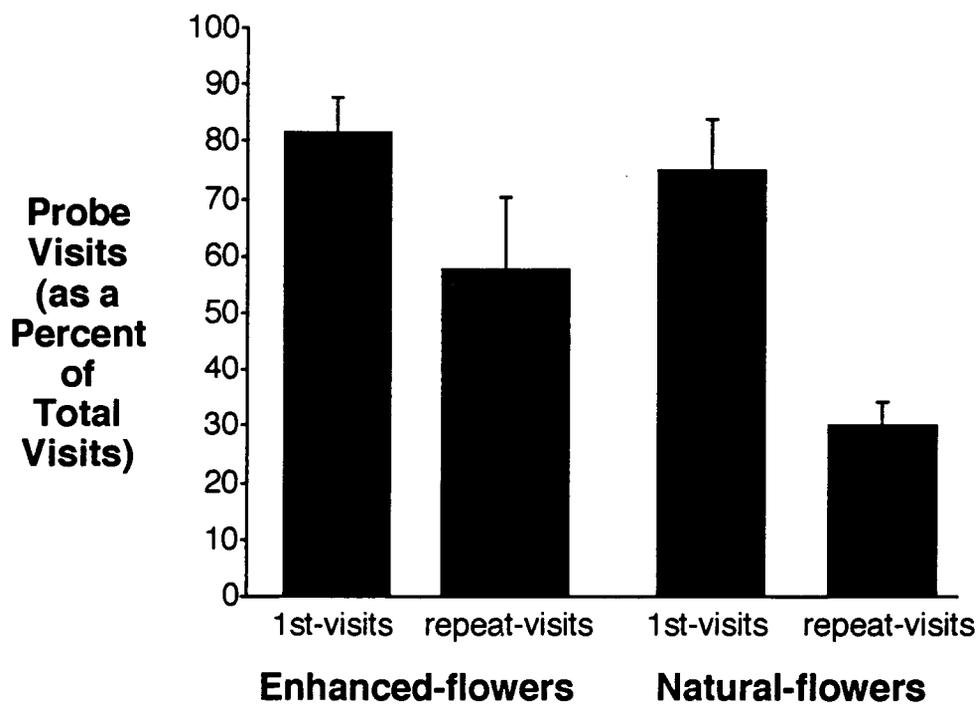


Fig. 6. 4. Probe visits (+se), as a percent of total visits, in the unmarked patch.

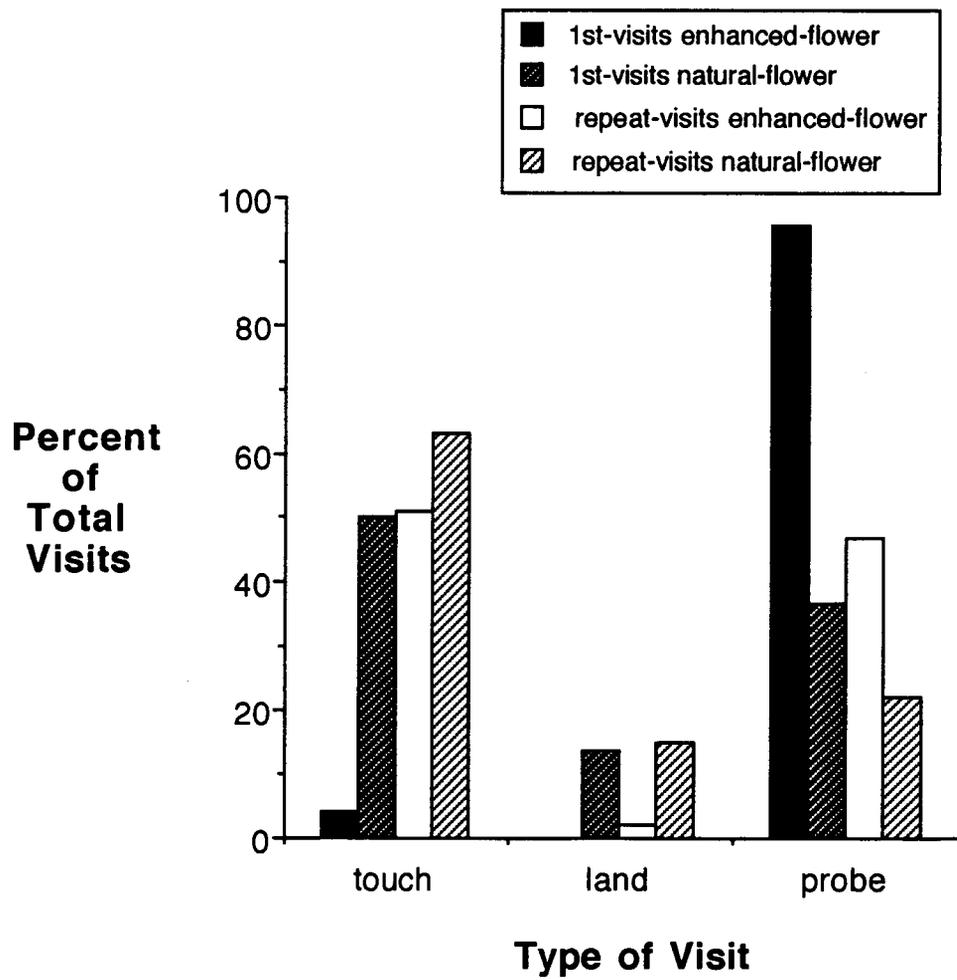


Fig. 6. 5. The percent of total visits for each flower type broken down by type of visit on the marked patch.

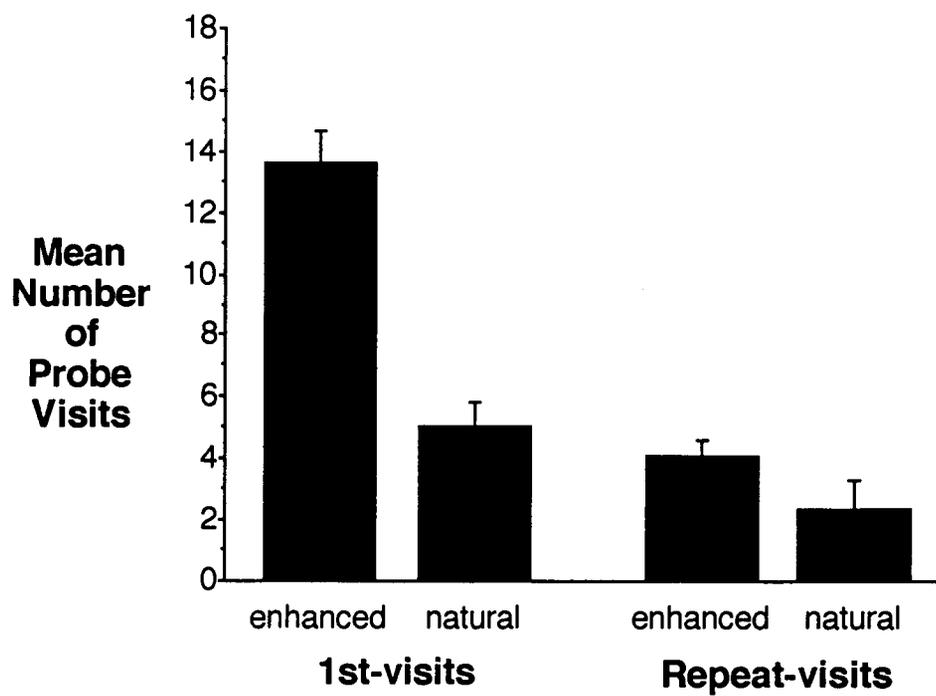


Fig. 6. 6. Mean number of probes (+se) in the marked patch.

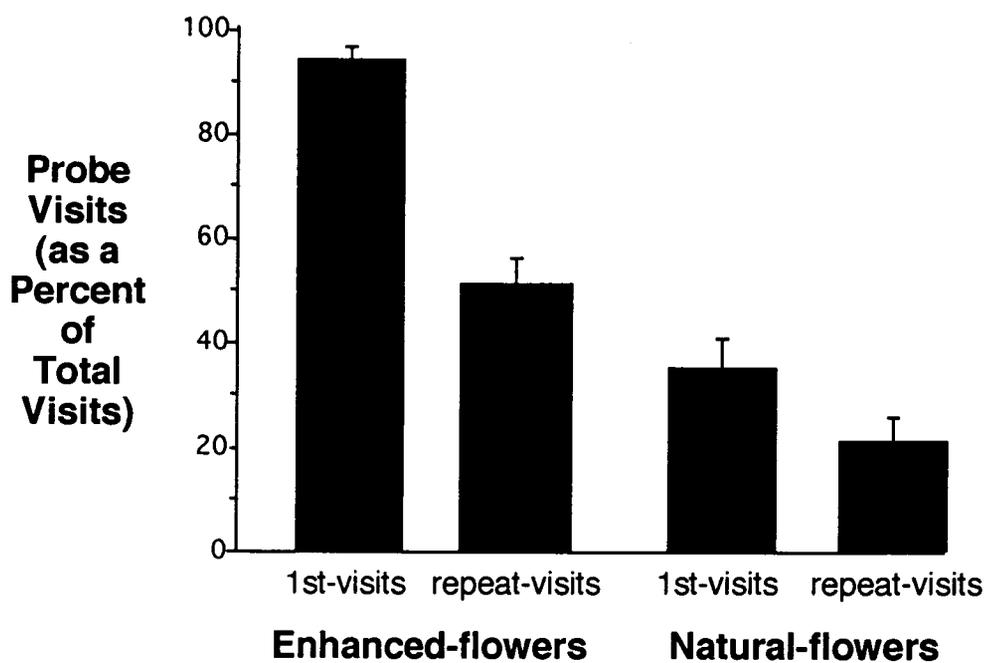


Fig. 6. 7. Probe visits (+se), as a percent of total visits, in the unmarked patch.

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CHAPTER 7

Why Honey Bees Scent-mark Flowers

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Abstract

Honey bees respond to scent-markings left by previous visitors to flowers by rejecting or accepting certain flowers. This study was designed to test whether this mechanism results in an increase in foraging efficiency. The amount of nectar obtained per unit time was compared in two types of artificial flower patches: a patch that received previous bee visits (marked patch) and a patch that had received no visits (unmarked patch). Honey bees obtained about 33% more nectar per time on marked than on unmarked patches. The foraging activities within each type of patch differed. When the different amount of energy expended on both patches was considered, the value of increased energy efficiency changed very little.

Introduction

Honey bees mark flowers with scents that they then use to discriminate between previously visited and unvisited flowers (Wetherwax, Ch 2; Wetherwax, Ch 3). Consistently rewarding flowers become more attractive with each rewarding visit while previously unrewarding flowers become less attractive after a single unrewarding visit. While this seems to be a means of increasing foraging efficiency, it has not been demonstrated that scent-marking helps bees to increase their nectar uptake.

The reproductive output of the colony is directly related to the amount of nectar obtained. Although larvae feed chiefly on pollen, nectar is necessary to feed the pollen-foraging workers as well as the workers within the hive. Bees fit the criteria for energy-maximizers (Schroener, 1971) and should forage to obtain the maximum amount of energy per unit time.

Many previous studies have shown that honey bees will alter their behavior to increase the uptake of nectar per energy expenditure. Workers will change directions more frequently and move shorter distances in a profitable patch than in patches offering smaller rewards (Waddington, 1980). Flower handling time can also affect foraging decisions. Foragers change directions more often as the amount of time spent handling a flower decreases (Schmid-Hempel, 1984). These and many other studies have assumed that honey bee foraging choices are based on information gained from sampling flowers within a patch (i.e. decisions on where to forage next are based on the rewards of the previous flower). However, other evidence (Wetherwax, 1986; Wetherwax, Ch 2; Wetherwax, Ch 3) demonstrates that honey bees have the ability to assess the quality of a flower before probing. This study is designed to test if this ability results in an increase in foraging efficiency.

In this study I compared the amount of energy obtained per time in a patch that has been foraged, and therefore

contains scent-markings, to a patch that was unmarked. The experiments were designed to accurately measure both the caloric reward and the time required to obtain that reward once the bee is foraging within the patch. The types of visits to the flowers will be further analyzed to see how time, within a patch, is allocated to various foraging activities.

Materials and Methods

The artificial patch consisted of 100 wooden dowels arranged in a 10 x 10 pattern on a 1 x 1 m wooden board which was attached to a turntable. A small plastic flower was placed on top of each dowel. (A complete description of the apparatus is detailed in Wetherwax, Ch 3.) Fifty of the flowers contained 1 μ l of 25% sucrose (nectar-flowers) and the other fifty contained 1 μ l of water (water-flowers). Previous studies demonstrated that honey bees could not discriminate, before probing, between unvisited water-flowers and unvisited nectar-flowers (Wetherwax, Ch 3). The positions of the flowers were randomly arranged and a different random arrangement was used for each experimental bout.

All experiments were conducted during September and October of 1992 on warm, sunny days near Eugene, Oregon. The patch was placed in a 2 x 2 x 2 m cage ca. 100 m from a standard honey bee colony. Bees were trained to forage on

the patch 1 to 3 days before the actual experiments. During the training the bees were captured, cooled for 3 minutes and marked with small plastic numbers. The bees were then released near the patch.

The marked bees were allowed to forage on the patch for at least 1 hr before an experiment. Each bout consisted of allowing one marked bee to enter the cage and then noting the sequence and type of visits for two minutes. At that time the bee was removed from the cage and destroyed. Bouts were limited to two minutes because of changes that take place within the patch during the bout. As the number of visits increases within a bout, the probability of finding a rewarding flower (i.e. unvisited nectar-flower) decreases. Previous studies showed that the number of repeat visits in a patch of 100 flowers was small within the first two minutes of a bout. A second problem is that as bees visit flowers in the unmarked patch, flowers within the patch become marked. Previous work demonstrated that most of the decisions made by the bees are made at the level of the visits recorded in this study. Since most visits within the first 2 minutes are not repeat visits, the majority of the flowers visited are unmarked.

There were two treatments: marked and unmarked. The unmarked treatment consisted of allowing a bee to forage on a patch with 100 new flowers: 50 nectar-flowers and 50 water-flowers. The marked treatment was a patch that had

been visited by several bees for 1 hr; during this "charging" time the patch was marked by visiting bees. The flowers were replenished with nectar and/or water every 10 minutes during the charging time. The patch was rotated during the charging time and at the beginning of each bout. At the beginning of each marked bout, every flower contained either nectar or water. There were a total of 10 bouts for each treatment using 20 different bees.

Three types of flower visits were recorded:

- (1) touch - bees briefly touched flower while flying.
- (2) land - bees briefly land on flower and then fly away without attempting to insert proboscis into corolla.
- (3) probe - bees insert proboscis into corolla.

The amount of time spent for each visit was noted on a tape recorder and transcribed at a later time.

The data were transcribed into a computer and a spreadsheet program with a custom macro was used to identify which visits were the first visits to a particular flower (1st-visits) and which visits occurred after the 1st-visits (repeat-visits).

Results

Honey bees obtained more nectar/time on the marked patch than on the unmarked patch ($t=4.47$; $p=0.0003$). This difference represents a 33% increase in the amount of nectar obtained per time (Fig. 7. 1).

There were differences in the amount of time spent for each type of visit (Fig. 7. 2), which resulted in differences between the two patches in the mean number of visits per bout ($t=2.2$; $p=0.04$). In the marked patch, most of the visits to nectar-flowers were probes and most of the visits to water-flowers were lands or touches, while in the unmarked patch most of the visits to both types of flowers were probes. Touch visit times could not be measured because they were very brief. Land visits to water-flowers in the marked patch took less time than probe visits on nectar-flowers ($t=7.11$; $p<0.0001$). These land visits were also shorter than probe visits to water-flowers in the unmarked patch ($t=5.32$; $p<0.0001$).

Discussion

In order to explain the evolution of scent-marking by natural selection, it is necessary to demonstrate an increase in fitness as a result of the scent-marking ability. Honey bees were more efficient foragers on patches that had been previously visited and marked than on unmarked patches. More calories were obtained per time spent foraging because bees were able to spend less time on non-rewarding flowers. Touch and land visits take less time than probe visits so bees should use these types of visits on flowers marked with scent that indicates that they are non-rewarding. Touch visits take much less time

than land visits so honey bees should maximize energy uptake by using touch visits whenever possible. When bees rejected water-flowers they used touch visits 29% of the time, therefore 71% of rejected visits were lands. This may indicate that there are additional constraints on the bees ability to detect scent-markings from a quick touch. Bees may be able to detect a weaker signal in a landing visit than in a touch visit since they spend more time on the flower on landing visits. Bees may also be able to detect markings without touching flowers and in this study it was difficult to determine if all touch visits involved contact. Some of the touch visits may be visits where the bee came close to the flower but did not actually make contact. A previous study (Wetherwax, 1986) indicated that bees discriminate between flowers of *Lotus corniculatus* L. by briefly hovering over the flower.

Foragers on marked patches obtained more energy per time than on unmarked patches because they were able to visit more rewarding flowers. This assumes that the bees expended the same amount of energy per time when foraging on the two types of patches. The activities within a patch can be divided into two categories: handling time, which includes walking on the flower and imbibing nectar, and flying between flowers. The amount of energy required for these two activities differs. Workers that are resting or walking and occasionally fanning their wings use about 0.7

mg sugar/h whereas flying workers use about 11.5 mg/h (Olaerts, 1956; Heinrich, 1979). This means that it takes about 16 times more energy to fly than it does to handle a flower. A comparison between the two patches indicates that there is no difference between the amount of time spent handling flowers during a bout ($t=2.02$; $p=0.06$).

The following are rough estimates of the amount of energy gained and used during foraging on the two types of patches using mean values. The total energy obtained per bee per time equals the amount of reward minus the energy expended flying and handling:

$$\text{total energy} = VC - (T_f E_f + T_h E_h), \text{ where}$$

V = volume of nectar per time ($\mu\text{l}/\text{minute}$)

C = concentration of nectar ($\text{mg}/\mu\text{l}$)

T_f = time flying per time foraging

T_h = time handling per time foraging

E_f = energy expended flying (mg/minute)

E_h = energy expended handling (mg/minute)

Total energy of unmarked patch = $(7.1 \mu\text{l}/\text{min})(0.25 \text{ mg}/\mu\text{l}) - (0.266 \text{ time flying}/\text{time foraging})(0.192 \text{ mg}/\text{min flying}) + (0.734 \text{ time handling}/\text{time foraging})(0.012 \text{ mg}/\text{min handling})$.

Total energy of marked patch = $(9.5 \mu\text{l}/\text{min})(0.25 \text{ mg}/\mu\text{l}) - (0.388 \text{ time flying}/\text{time foraging})(0.192 \text{ mg}/\text{min flying}) + (0.612 \text{ time handling}/\text{time foraging})(0.012 \text{ mg}/\text{min handling})$.

The mean amount of energy obtained at the unmarked patch was 1.72 mg/minute while the amount obtained at the marked patch was 2.29 mg/minute. Scent-marking resulted in a 33.1% increase in the amount of energy obtained per time. This increase is similar to the value of another study (Wetherwax, 1986) that found that honey bees were able to obtain 24% more nectar per visit than they would if they foraged at random and suggested that the increase was partially due to scent-marking.

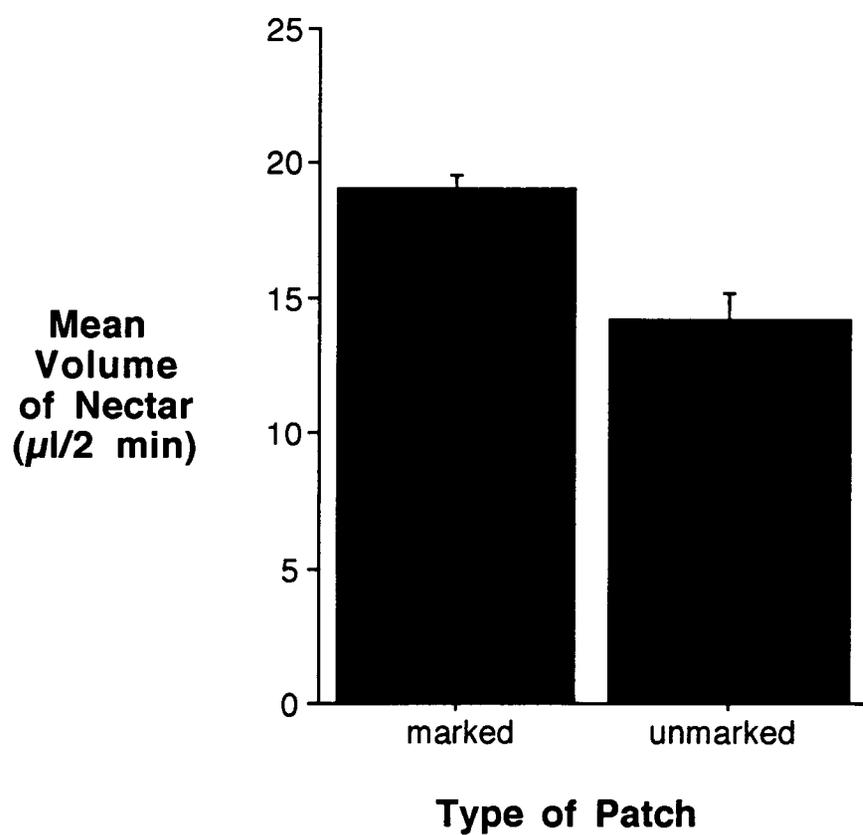


Fig. 7. 1. Mean volume of nectar (+se) per bout for both types of patch.

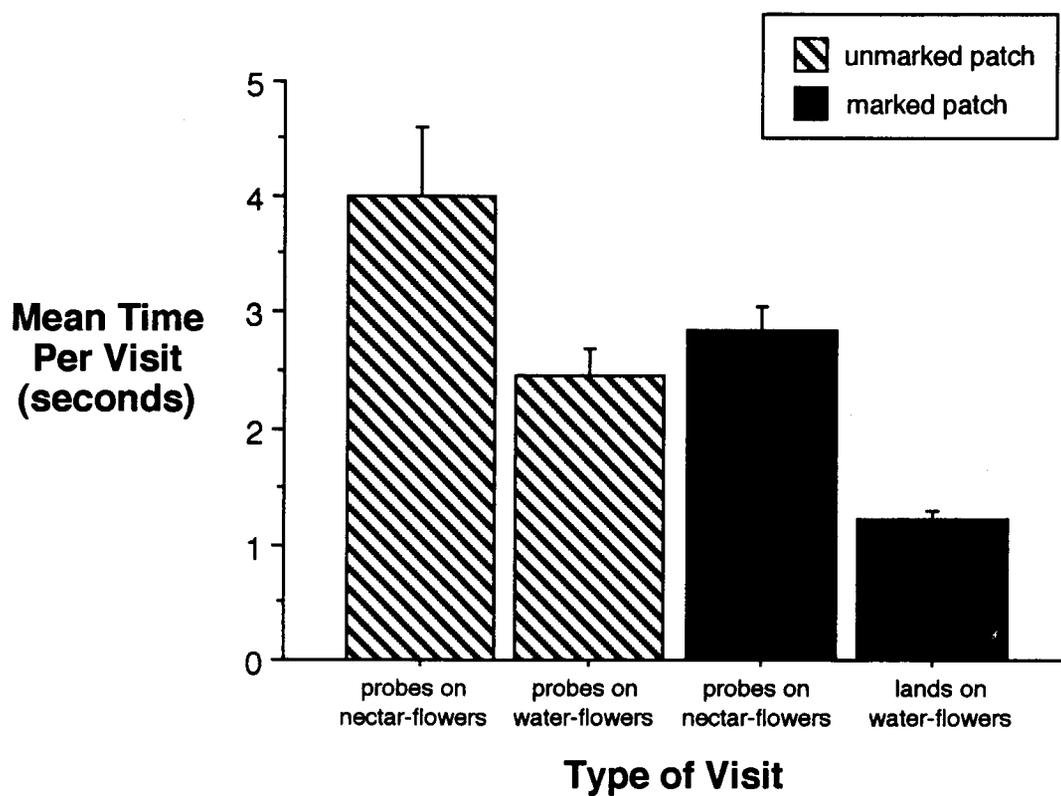


Fig. 7. 2. Mean amount of time (+se) for the most common types of visits on both marked and unmarked patches.

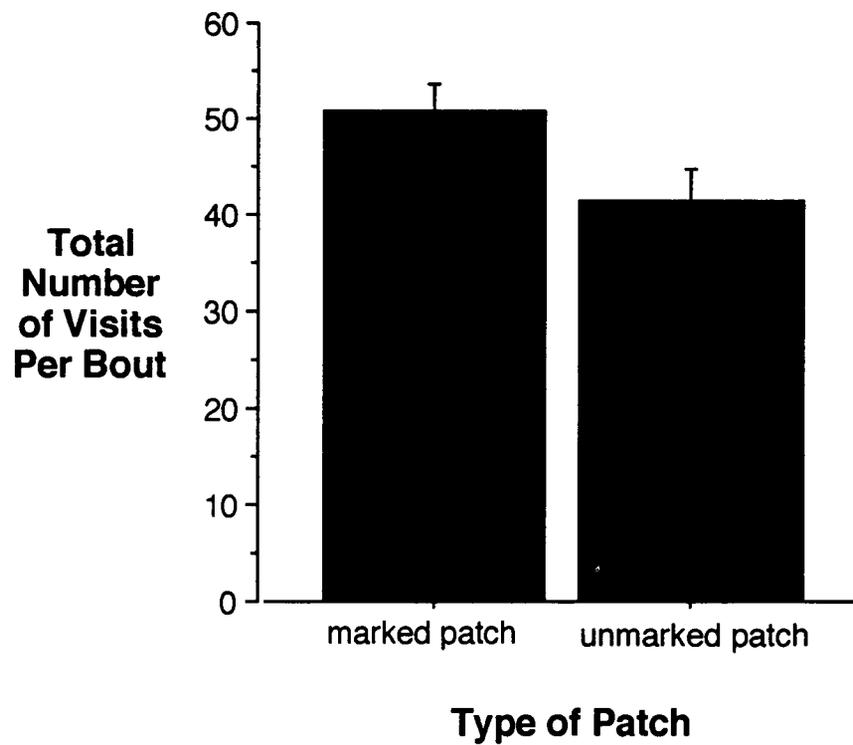


Fig. 7. 3. The number of visits (+se) per bout for both types of patch.

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CHAPTER 8

Chemical Analysis of Honey Bee
Mandibular and Tarsal Glands

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Abstract

Honey bees mark flowers with a scent that attracts subsequent foragers to the flower. Wetherwax (Ch 5) identified several of the components of the attractant. Mandibular glands and tarsal glands were dissected from foraging honey bees and analyzed using GC-MS for the presence of the attraction-components. The tarsal gland extract yielded no chemicals. The mandibular gland extract contained two chemicals that have not been previously identified in mandibular gland extract. However, neither of these chemicals was found in the scent-marking.

Introduction

Honey bees mark artificial (Ferguson and Free, 1979; Free and Williams, 1983; Wetherwax, Ch 2 and Ch 3) and real (Wetherwax, Ch 6) flowers with scents that make the flowers more attractive to subsequent foragers. Wetherwax (Ch 5) collected scent-markings from artificial flowers and identified four components as possible attractants: nonanal, cyclohexanol, 2-cyclohexen-1-one, and 2-ethyl-1-hexanol. This study will attempt to identify the source of these compounds.

Although the source of forage marking pheromones is not known, several researchers have made suggestions. Some (Ferguson and Free, 1979; Corbet et al., 1984) have suggested that a repellent forage marking pheromone may be

2-heptanone, which is an alarm pheromone secreted by the mandibular gland. Free (1987) has suggested that the attractant left at food may be the same as the trail pheromone first described by Butler et al. (1969). Free reports that the attractant is probably left inadvertently by the bee but other evidence (Wetherwax, Ch 2) suggests that the bees can control the deposition of the scent. Chauvin (1962) thought that the trail pheromone described by Butler et al. (1969) may be produced by the Arnhart's gland (tarsal glands)(Arnhart, 1923) located on the tarsi.

There are no studies on the components or function of the Arnhart's gland. Components of the mandibular glands previously reported include: (E)-10-hydroxy-2-decenoic acid which is a part of brood food (Butenandt and Rembold, 1957); hexanoic acid and octanoic acid which may have bactericidal and fungicidal properties, and (e)-oct-2-enoic acid (Boch et al., 1979); 2-heptanone, an alarm pheromone (Shearer and Boch, 1965); and in the absence of a queen, workers mandibular glands increase in size (Costa-Leonardo, 1985) and produce 10-hydroxy decenoic acid, (E)-9-hydroxy-2-decenoic acid and (E)-9-oxo-2-decenoic acid.

This study used gas chromatography-mass spectrometry (GC-MS) to identify the components of the mandibular and tarsal glands to see if they are the source of the attractive chemicals identified by Wetherwax (Ch 5).

Materials and Methods

Honey bees foraging on artificial flowers were collected and frozen. Mandibular glands were dissected (Dade, 1962) and placed in dichloromethane (HPLC grade). Each sample contained the glands of 6 bees (i.e. 12 glands). The last tarsal segments from all six legs of three bees were collected, placed in dichloromethane and pulverized using a glass rod. All extracts were concentrated under nitrogen gas prior to chemical analysis. Extracts from the glands were then analyzed using GC-MS with a high resolution mass spectrometer coupled to a GC fitted with a Supelco SPB-1 15 m*, 0.25 um column programmed at 60° C for 2 minutes then 10° C/min to 280° C where it was held for 10 minutes.

Results

Chromatograms from the tarsal glands showed no major peaks and were not analyzed any further. Future attempts will try to dissect the glands from the tarsi.

Figure 8. 1 shows the chromatogram from the mandibular gland extract. Five compounds were identified in the mandibular gland extract that were not found in the blank: 2-heptanone (Fig. 8. 2), 4-hydroxy-benzoic acid (Fig. 8. 3), octanoic acid (Fig. 8. 4), 8-nonen-2-one (Fig. 8. 5) and tetradecane (Fig. 8.6).

Discussion

None of the identified compounds was identical to the ones found by Wetherwax (Ch 5) in the attractive scent-mark. Two of the compounds have been previously identified in mandibular gland extracts: 2-heptanone, which is an alarm pheromone, and octanoic acid, which probably functions as a bacteriacide and fungicide. One of the components, tetradecane, is probably a cuticular hydrocarbon and not a part of a substance secreted by the gland. Two compounds were found that have not been previously identified in mandibular gland extracts: 4-hydroxy-benzoic acid and 8-nonen-2-one. Neither of these compounds has previously been found in honey bees. The function of these components is unknown.

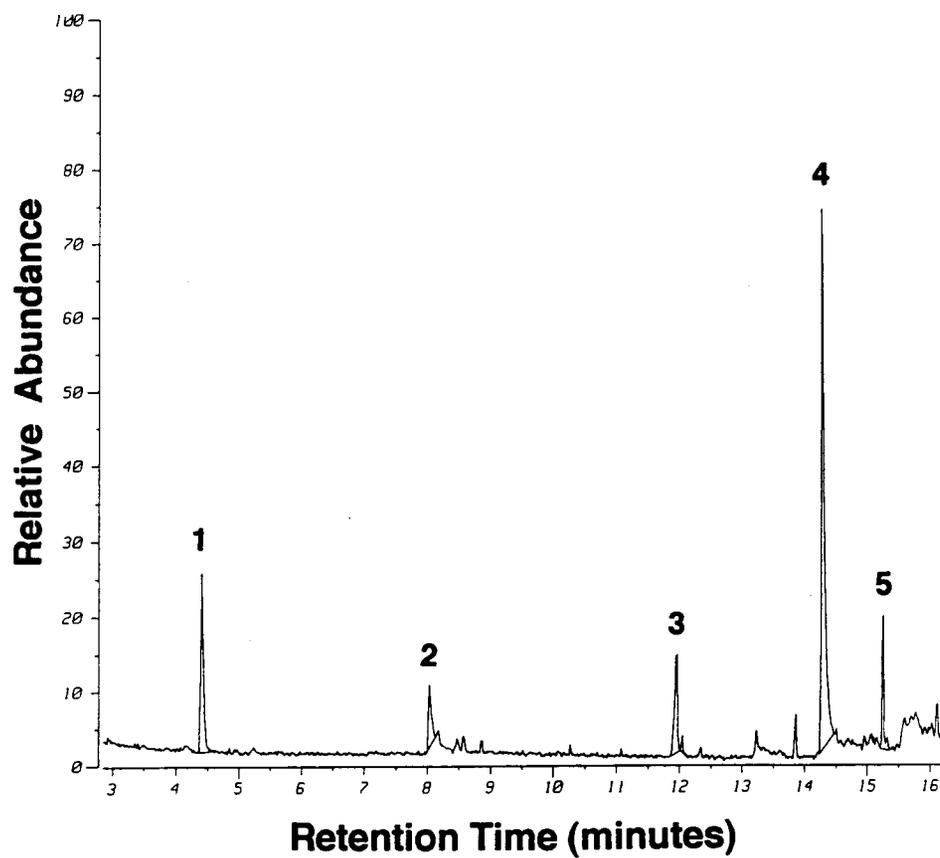


Fig. 8. 1. Gas chromatogram of the mandibular gland extract.

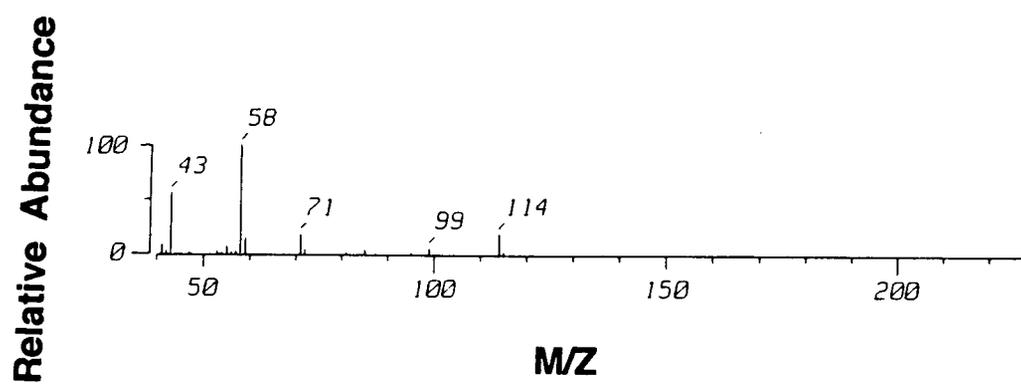


Fig. 8. 2. Mass spectrum of 2-heptanone (peak #1 in Fig. 8. 1).

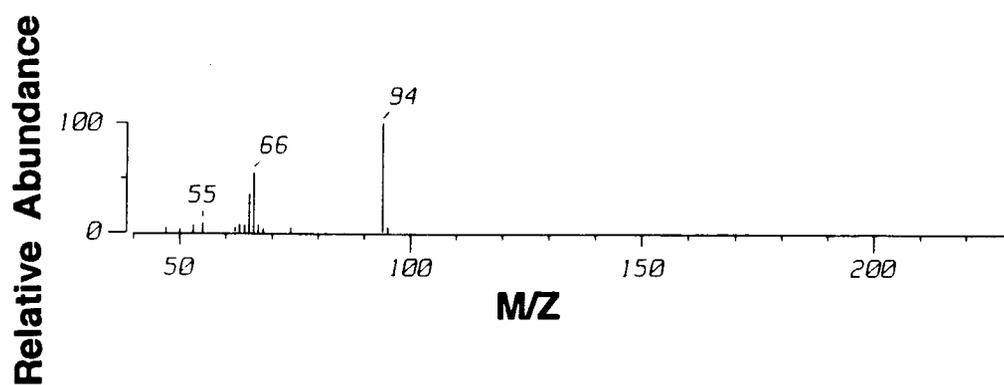


Fig. 8. 3. Mass spectrum of 4-hydroxy-benzoic acid (peak #2 in Fig. 8. 1).

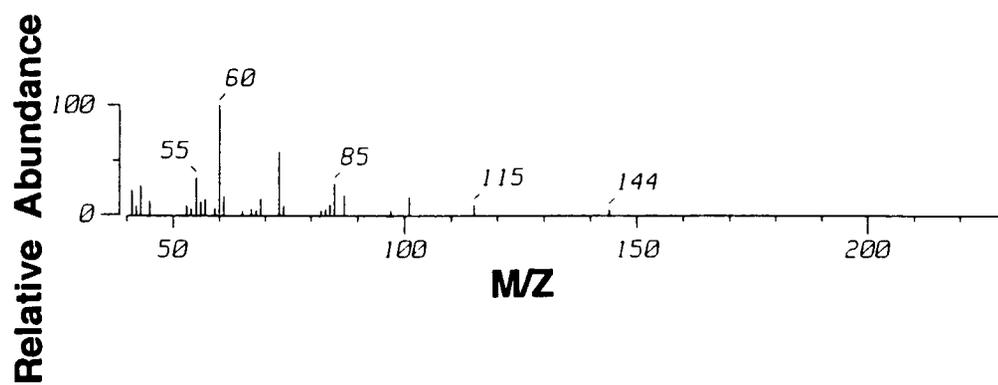


Fig. 8. 4. Mass spectrum of octanoic acid (peak #3 in Fig. 8. 1).

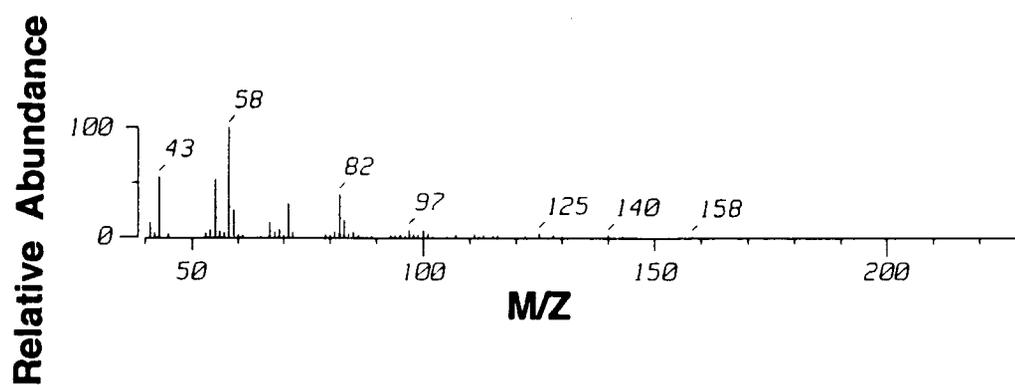


Fig. 8. 5. Mass spectrum of 8-nonen-2-one (peak #4 in Fig. 8. 1).

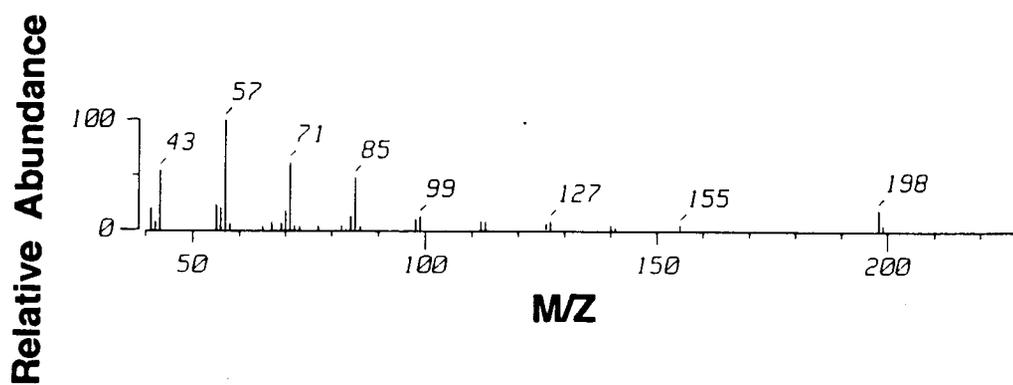


Fig. 8. 6. Mass spectrum of tetradecane (peak #5 in Fig. 8. 1).

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Wetherwax, P.B. 1993. Chapter 3: Scent-marking by honey bees on a realistic model.

Wetherwax, P.B. 1993. Chapter 6: Scent-marking on *Lotus corniculatus* by nectar-collecting honey bees.

Summary

Honey bees are recruited to nectar sources by following the directions of dancers within the colony. However, once in the patch, the bee needs to make a decision at each flower that she encounters: to probe the flower for nectar or to reject the flower and move on. The forager has many cues that she can use that indicate the amount of reward she may gain from probing, before she inserts her proboscis. This ability to assess the quality of the flower before probing pays off energetically. It costs time and energy to land and probe a flower that offers no reward. Some of the cues are provided by the flower: color and scent. Another cue is left by previous visitors to the flower.

Honey bees mark flowers with scents that act as attractants or repellents to subsequent foragers. If the next visitor is the same bee or her sister, then the cost of producing the scent pays off by increasing the foraging efficiency of the colony. Flowers are initially marked with a scent that makes the flower less attractive to the next visitor. Since flowers are usually emptied, the next visitor profits by rejecting recently visited flowers. When a flower has provided high amounts of nectar several times, the flower is marked with a scent that makes it more

attractive to subsequent visitors. This may allow bees to pick out the super flowers within a patch.

It is not known if the scents that make flowers attractive are different than the ones that make a flower less attractive. It is possible that a single scent is applied and bees are merely responding to the amount of scent. Small quantities may indicate that the flower has been marked once and probably has been recently emptied. Large quantities indicate that the flower has been visited numerous times. A bee may continue to apply scent to a flower that already contains scent only if the flower contains nectar. This explanation is possible but is not as satisfying as a two scent system. A two scents system could consist of a very volatile repellent scent and a longer lasting attractive scent. In this system foragers respond to small quantities of repellent scent but only respond to the attractive scent after a certain threshold is achieved.

This project has provided many insights about the foraging behavior of honey bees but it offers more questions than answers. While several components of the attractant extract have been identified, it is still unclear which of those components acts as an attractant. Further GC-MS analysis is needed, including running standards to confirm the identification of the components. Behavioral assays need to be done using commercial sources

of the components in different quantities and combinations. Identification of the source can start by looking at the Arnhart's glands again as well as other possible glands, such as the Dufour's gland.

Another study could compare scent-marking in related taxa. Bumble bees produce attractants but it is not known if they produce repellents. While it is easy to justify the evolution of scent-marking in honey bees, it is less obvious why bumble bees scent-mark flowers. Honey bee foraging involves recruiting colony-mates to relatively large sources of food. Since bumble bees do not recruit, it is less likely than with honey bees that the next visitor to a flower will be related. While there is some probability that the next potential visitor is the same bee or a sister (especially if the nectar source is close to the colony), the pressures to evolve scent-marking were probably not as strong as they were in honey bees. It seems more likely that repellents, since they may help to avoid revisiting flowers, rather than attractants would be used by bumble bees. Attractants may be helpful if bumble bees forage in a manner similar to the traplining of other bees.

Finally it remains to be determined how scent-marking affects the fitness of the plant. There is some evidence that attractants in a patch may make the patch as a whole more attractive and therefore increase the pollinator

visits. However, repellents probably reduce the number of visits to a particular individual.

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