Reward Expectations in Honeybees

Dissertation to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy,

Freie Universität Berlin

by

Mariana Gil

from Argentina

March 2009

1st Reviewer: Prof. Dr. Randolf Menzel

2nd Reviewer: Prof. Dr. Dorothea Eisenhardt

Date of defence: April 23rd 2009

This dissertation is based on the following articles:

1. Gil M., De Marco R.J., Menzel R. (2007) Learning Reward Expectations in Honeybees. Learning and Memory 14: 491-496.
2. Gil M., De Marco R.J. Honeybees Learn the Magnitude and Sign of Reward Variations. (Submitted)
3. Gil M., Menzel R., De Marco R.J. (2008) Does an Insect's Unconditioned Response to Sucrose Reveal Expectations of Reward? PLoS ONE 3(7): e2810.doi: 10.1371/journal.pone. 0002810.
4. Gil M., Menzel R., De Marco R.J. Side-Specific Reward Memories in Honeybees. (Submitted)

Author's contributions:

I participated in the design of all the experiments. I performed all the experiments and analysed all the data. I wrote all the papers under the guidance of RJ De Marco and R Menzel.

"The bee is above all…a creature of the crowd…isolate her, and however abundant the food or favourable the temperature, she will expire in a few days not of hunger or cold, but of loneliness."

M. Mäterlinck, The life of the Bee, 1924

"Una apis, nulla apis"

Proverb

Contents

General Introduction

Honeybees (*Apis mellifera)* live in large colonies, whose primary source of energy is nectar. During its annual cycle, a colony can collect about 120 kg of nectar. Such amount of food is gathered by forager bees, which represent only a quarter of the total population of a colony. Each forager, in addition, can collect no more than 60 µl of nectar per foraging trip. Nectar offer fluctuates continuously as a result of both variations in the rates at which flowers produce nectar and the activity of other flower visitors (e.g., Núñez 1977, Teuber and Barnes 1979, Vogel 1983, Baker and Baker 1983, Real and Rathcke 1988). Thus, within a time scale of hours, foragers face unpredictable scenarios. As many other animals, forager honeybees have evolved strategies to efficiently cope with food variability. Evidence indicates that they learn how to map situations to actions so as to maximize food gathering rates (e.g., Gould and Gould 1988, Seeley 1995). In doing so, honeybees appear to rely on their memory store to decide when, where and how to forage. They leave the hive with a relatively large and diverse amount of information. They learn the localization of food sources, the time of the day when those sources are productive, and other characteristics like the odours, colours, and shapes of the flowers (e.g., Wahl 1932, Kleber 1935, Kolterman 1969, von Frisch 1967, Menzel 1990, Menzel et al. 2006). In addition to such information, each forager leaves the hive with an estimate of how much nectar is ought to be collected (Núñez 1966). Apparently, this estimate develops throughout successive trips to the food source, and is adjusted in relation to the quality and quantity of food. It is reasonable therefore to ask whether honeybees adjust their behaviour based on the level of reward they 'expect' to find next at a given location. This dissertation addresses whether and how honeybees adjust their behaviour in relation to their past experience with variations in the level of sugar reward.

If a forager's behaviour at any given time depends to a great extent upon its past experience with variations in the level of reward, then a correlate of such variations must be present in the honeybee brain. In psychology, 'incentive' is the word defining such correlate. Incentive is a hypothetical concept referring to what might

popularly be described as a subject's expectation of reward (Logan 1960). Incentive must be learned, just as responses and stimuli are learned, and it may imperfectly represent the actual reward. Moreover, equal differences in reward do not produce equal differences in incentive. Thus, an expectation of reward, or the incentive value of reward, is a variable determined by previous reward experiences and modulating current performances (Logan 1960). When animals receive a reward, in addition to the memory arising from the contingency between a given stimulus (as a conditioned stimulus, or CS) and the offered reward (as an unconditioned stimulus, or US), a different memory about specific properties of the reward is formed (Tolman 1959, Logan 1960, Schultz 2000). Here, I will refer to a 'reward expectation' as a behavioural adjustment that depends upon the formation and subsequent activation of memories about specific properties of reward, whose recollection is eventually triggered in the absence of reward by the cues and events that predict it. Accordingly, if honeybees adjust their behaviour based on past variations in the level of reward, then they develop reward expectations.

A few studies indicate that past reward experience modulates a honeybee's behaviour both inside and outside the hive. For example, foragers returning to the hive from highly desirable food sources perform complex motor displays called 'dances' to communicate the presence of food to their nest-mates (von Frisch 1946, 1967). Evidence shows that foragers that experienced an increase in reward level dance more intensively than bees that experience a maximum but constant reward level (De Marco and Farina 2001). Likewise, forager honeybees control the way in which the offer and beg for food within the colony depending on present and past reward (De Marco and Farina 2001). It has also been shown that, within a time span of minutes, honeybees can keep track on the amount of the food offered by several flowers (Greggers and Menzel 1993). These and other studies indicate that honeybees assess the quality of sugar reward based on a reference value that develops throughout their foraging experience (Raveret-Richter and Waddington 1993, De Marco and Farina 2001, De Marco et al. 2005, Greggers and Menzel 1993). Forager honeybees, therefore, develop short-term reward memories which modulate their ongoing behaviour.

Yet, there is a lack of information on the long-lasting effects of reward memories. This is probably due to the fact that the relationship between reward variations and a honeybee's behaviour has long been evaluated in the presence of sugar reward, as opposed to a situation in which reward is entirely absent during testing. Under these circumstances, it is likely that the regulatory effects of current rewards are sufficient to control the foragers' food gathering behaviours. Moreover, foraging honeybees tend to maximize the rate of energy gain, which depends upon food availability and the energy cost associated to forage (e.g., Schmid-Hempel et al. 1985, Varjú and Núñez 1991, 1993). With sugar reward present at the feeding site, it would be difficult to distinguish between the effects of past and current rewards on a bee's ongoing behaviour, because the immediate effects of the present rewards, together with the bee's tendency to maximize its rate of energy gain, would easily overshadow the effects of past rewards. It follows that the influence of long-term reward expectations on a honeybee's behaviour would become methodologically accessible only in the absence of food. Under these circumstances, the regulatory effects of current rewards will be absent, and the energy cost associated to forage will exert a greater influence on the bee's ongoing behaviour. When honeybees on a negative energy budget invest time/energy searching for food at a feeding site, it is reasonable to assume that their investment will be influenced by their memories on past experiences at the site. It is in such a situation that a honeybee's eagerness, or persistence, to search for food in the absence of reward will rely on its already developed expectations of reward.

In the first two chapters of this dissertation, I present the results of two related experiments. In these experiments, bees first foraged on an artificial flower patch offering variable levels of reward and, then, after a long foraging pause, searched for food at the site in the absence of reward. In **chapter 1**, I asked whether honeybees are able to develop long-term memories about the sign of variations (i.e., either positive or negative) in the level of the experienced reward. To answer this question, I compared the performance of bees that experienced increasing, decreasing, or constants reward levels. In **chapter 2**, I asked whether honeybees are able to develop long-term memories about not only the sign, but also the magnitude of variations in

the level of the experienced reward. In this case, I compared the performance of bees that experienced either a large or a small increase in reward level, or, instead, a decrease in reward level. In both experiments, I evaluated the honeybees' behaviour at the patch in the absence of reward after a long foraging pause. I considered different behavioural measures as different manifestations of a honeybee's eagerness to forage for food, namely, the duration of the visits to the patch, the number of flower inspections, and/or the cumulative duration of such inspections. Also, the experimental design and behavioural measurements allowed me to uncouple signal learning and nutritional aspects of foraging from the effects of past reward experience. The results of these two experiments demonstrated that honeybees develop long-term memories based on past variations in the level of reward. The wonder arises as to how they do this.

The first step to address such question is to develop a laboratory procedure allowing reproducing the observations with free-flying bees. In doing so, I focused on honeybees' proboscis extension response (PER, Takeda 1961, Kuwabara 1957, Bitterman et al. 1983). This response allows bees to gather sugar solution, and is elicited reflexively when the gustatory receptors of their antennae, proboscis and tarsi are stimulated with sucrose (Kuwabara 1957). For the last 30 years, a honeybees' PER has successfully been used in the study of learning and memory phenomena in harnessed bees. The aim of the experiments presented in chapters 3 and 4 was to develop a laboratory procedure suitable to examine behavioural changes that depend upon memories of variations in the level of reward. Eventually, such procedure would be fruitful for further pharmacological and electrophysiological approaches to study the neural substrates underlying these memories. In **chapter 3**, I used an experimental design analogous to that of my initial experiment with free-flying bees, and asked whether harnessed bees are able to learn that reward level increases or decreases over time, so as to subsequently adjust their PERs. I trained bees by coupling the stimulation of one antenna with increasing, decreasing or constant volumes of sugar solution offered to their probosces throughout consecutive training trials. Next, I evaluated their PERs to sucrose stimulation of the antenna in the absence of reward. Interestingly, this procedure proved successful to evince

adjustments of the bees' PER depending upon past variations in the level of reward. However, because honeybees adjust its response to reward based on subjective values of it (Page et al. 1998, Scheiner et al. 2005), any laboratory procedure suitable for the analysis of behavioural, and neural correlates of reward memories should include within-animal controls. Initially, the reason behind the experiments presented in **chapter 4** was to look for a way to incorporate within-animal controls into the laboratory procedure developed in chapter 3. To do so, I made use of the fact that honeybees show different forms of side-specific learning (Masuhr and Menzel 1972, Macmillan and Mercer 1987, Sandoz and Menzel 2001, Giurfa and Malun 2004, Braun and Bicker 1992, Sandoz et al. 2002). Thus, in chapter 4, I asked whether honeybees learn side-specifically that the level of reward increases or decreases over time. To answer this question, I trained bees by coupling the stimulation of each of their antennae with either increasing or decreasing volumes of sugar solution offered to the animal's proboscis throughout consecutive training trials. Next, I evaluated their PERs to stimulation of each antenna separately, in the absence of reward. Using this procedure, I asked a number of additional questions aiming to further understand how honeybees learn and process side-specific stimuli which are linked to specific rewards. I examined the temporal dynamic and specificity of the ensuing reward memories. Moreover, because the stimulation of a honeybee's antenna as in my experiments involves input from gustatory as well as mechanosensory receptors, I also evaluated the interplay between these inputs in the formation and retrieval of side-specific reward memories.

Chapter 1

Learning Reward Expectations in Honeybees

Abstract

The aim of this study was to test whether honeybees develop reward expectations. In the experiment, bees first learned to associate colours with sugar reward in a setting closely resembling a natural foraging situation. I then evaluated whether and how the sequence of the animals' experiences with different reward levels changed their later behaviour in the absence of reinforcement and within an otherwise similar context. I found that the bees that had experienced increasing reward levels during training assigned more time to flower inspection 24 and 48 h after training. The design and behavioural measurements allowed me to uncouple the signal learning and the nutritional aspects of foraging from the effects of subjective reward values. I thus found that the animals behaved differently neither because they had more strongly associated the related predicting signals nor because they were fed more or faster. The results document for the first time that honeybees develop long-term expectations of reward; these expectations can guide their foraging behaviour after a relatively long pause and in the absence of reinforcement.

Introduction

Modern views on associative learning acknowledge that both classical and instrumental conditionings depend upon associations between external cues or behavioural responses and internal representations of reward (Rescorla 1987). Within this context, the term 'expectation', or 'expectancy', denotes an activation of an internal representation of reward in the absence of reinforcement by the cues and events predicting such a reward (Tolman 1959, Logan 1960). According to theory, the reward value associated with a stimulus is not a static, intrinsic property of the stimulus. Thus, for example, animals can assign different appetitive values to a stimulus as a function of both their internal state at the time when the stimulus is

encountered and the background of their previous experience with such stimulus. This means that specific neural mechanisms have evolved to not only detect the presence of reward but also to predict its occurrence and magnitude based on internal representations from past experiences, in turn activated by the subject's current motivational status (Schultz 2000).

Studying this form of learning is critical for understanding how reward controls behaviour, how it leads to the formation of reward expectations, and how the brain uses reward-related information to control goal-directed behaviour. Studies on reward expectations, however, sometimes appear to be paradoxical in assessing the cognitive complexity underlying such processes, as well as the basic principles of planning and decision making. The reason is probably to be found in the fact that an anticipatory imagery or idea aroused by learned associations is thought to underlie these phenomena. In principle, however, neither highly complex cognitive abilities nor consciousness phenomena are assumed to be the bases for the development of reward expectations (Hebb and Donderi 1987).

In invertebrate species, as opposed to vertebrate species, reward expectations have not been systematically addressed. Here, I ask whether and how the behaviour of a highly social insect depends upon the development of reward expectations. The focus is on *Apis mellifera* bees, animals that form large societies, appear to have evolved multiple forms of communication in the course of evolution, including the famous waggle dance (von Frisch 1967), and whose ability to associate an initially neutral stimulus (as a conditioned stimulus, or CS) with sugar reward (as an unconditioned stimulus, or US) is at the heart of the behavioural flexibility that they exhibit during foraging (Menzel 1990, 1999). For example, honeybees perform complex time-dependent sequences of actions (e.g., Zhang et al. 2006), and learn, for example, the place and time of the day when food is available (von Frisch 1967). They also adjust their foraging efforts to the quality and quantity of available resources, and it is reasonable to ask whether they 'expect' specific rewards at particular locations and times of the day, although it has not yet been proven whether they can store and retrieve multiple combinations of 'what, when and where' attributes (Menzel et al. 2006). In the present study, I addressed reward expectations in the

context of honeybee foraging, because this form of learning might be revealed under conditions mimicking natural situations as closely as possible.

The approach was straightforward: I presented bees with two variable and three constant reward schedules, and observed their later behaviour in the absence of reinforcement. In the variable schedules, the amount of reward either increased (small-medium-large) or decreased (large-medium-small), whereas in the constant schedules I used three different levels of reward (small, medium, and large) equivalent to those of the variable schedules. In the experiment, the bees first had to forage individually on a relatively large patch of flowers giving off two different colour signals, and learn which of these two colours was actually offering rewards. The set-up, in addition, did not allow the bees to have immediate access to the offered reward: each animal first needed to discriminate between the two types of flowers, then enter and walk inside a tubular flower in order to find and drink a small amount of sugar solution, and, finally, repeat this procedure several times in order to obtain a certain amount of sugar reward before returning to the hive. The use of flowers giving off two different colours demanding a certain amount of handling time allowed me to separately quantify two different, still-connected aspects of the animals' responses in the absence of reinforcement: the 'correctness' of choice and the overall length of their searches for reward, or 'persistence'. The first component is usually applied to measure learning and retention scores, whereas the latter might be capable of reflecting a reward-related component.

I predicted that (1) the bees from all series will show both high learning scores and significant retention scores, because they learn flower colours very fast (Menzel 1967), and only three learning trials are needed to form long-term colour memories, which last for a lifetime (Menzel 1968), and (2) that in the absence of reinforcement and in an otherwise similar context, the animals would search for reward more intensively after having experienced an increasing reward schedule than after having experienced a decreasing reward schedule. The first prediction refers to the 'correctness' of choice, whereas the second refers to the animals' 'persistence'. If the results fit the second prediction, they might be accounted for by means of rather simple 'stimulus-response' mechanisms, without reference to expectations of reward.

For example, if the bees from the different groups had differentially associated the related predicting signals during training, they might assign a different proportion of time to inspect the flowers that had previously yielded reward, as calculated from the total amount of time assigned to flower inspection, even if they show similar retention scores. Moreover, the bees' responses during testing might reflect their most recent experience during training. By this argument, the bees in the decreasing series might only retain information on the small amount, and the bees in the increasing series might only retain information on the large amount; next, the later behaviour during testing is controlled by this information. If this were the case, similar results must be expected between the large and the increasing series, and the small and decreasing series. Finally, had the bees differentially associated the related predicting signals because they were fed more or faster, one should expect differences in the bees' responses across the constant series, because in these groups the animals received different amounts of reward, and also experienced different rates of nectar intake.

On the other hand, if the bees from the increasing series search for reward more intensively than those from the decreasing series, and, in addition, their responses cannot be accounted for by simple 'stimulus-response' mechanisms, their later behaviour in the absence of reinforcement will only be explained by reference to reward expectations. In other words, they behaved differently because they learned that reward magnitude either increased or decreased over time, and, therefore, expected more or less reward during testing.

Methods

A colony of *Apis mellifera carnica* bees was placed indoors in a two-frame observation hive. A small group of labelled bees was trained to collect unscented 50% w/w sucrose solution at an artificial flower patch placed 145 m from the hive. These bees (henceforth, recruiting bees) were not used as experimental animals; they only recruited nest-mates to the foraging place. The newcomers arriving at the feeding place were trapped before they got in contact with any sugar reward. They were

cooled, marked with plastic tags, and released. Upon returning to the hive, these animals became potential experimental bees. Those which returned underwent a pre-training phase and became experimental bees. The artificial flower patch consisted of 24 Eppendorf tubes (4 cm-deep) (henceforth, 'flowers') regularly distributed over the surface of a foraging arena (28 cm x 28 cm) made of two superposed plastic squares, both of which presented 24 holes (1 cm diameter). The lower part of the arena was a 0.7 cm thick opaque acrylic-plastic, while the upper square was a 0.2 cm thick transparent Plexiglas. The tubes were placed inside the holes and raised 1.8 cm above the upper surface of the transparent Plexiglas. The flowers gave off one of two signalling colours, either yellow or blue. I presented 24 colour circles, 12 yellow and 12 blue, centred on the single holes holding the flowers. Each circle had a diameter of 3.8 cm. These 24 coloured circles, set onto a grey cardboard offering a homogeneous background, were visible to the bees through the upper transparent Plexiglas square. Since the flowers were held by the upper surface of the patch and the coloured circles were set below this surface, both the flowers and their corresponding visual stimuli could easily be replaced between the successive visits of the experimental bees. In between the successive visits by the experimental bees, I randomly changed the relative position of the 24 visual stimuli, thus minimizing visual orientation based on the position of the single flowers relative to the entire patch. Since 1) the patch consisted of a relatively large number of flowers whose signalling colours were regularly distributed, 2) the bees had no access to the surface of the visual stimuli, 3) all flowers were replaced between visits, and 4) the relative position of the visual stimuli changed across visits, any putative influence of chemosensory cues that bees may produce and benefit from while foraging (Núñez 1967) were minimized and restricted to the single visits by the animals.

The labelled bees had to learn how to handle the flowers in order to efficiently access the offered reward. They were allowed to forage on the patch twice before training (pre-training phase). During these two visits, each flower offered 50% w/w sugar solution (*ad libitum*), and the bees were exposed to a homogeneous grey background. After the beginning of this pre-training phase, in addition, the recruiting bees (see above) and the newcomers present at the patch, with the

exception of the single experimental bee, were captured and kept inside small cages until the end of the experiment. Training started when the experimental bee returned to the feeding place after its last pre-training visit. It consisted of 9 successive visits to the patch, always presenting 12 yellow- and 12 blue-flowers. Throughout the experiments, half of the bees were rewarded at yellow flowers, the other half at the blue flowers. Different volumes of unscented, 20% w/w sugar solution were used as sugar reward; these volumes correspond to the different reward magnitudes used during the experiment, and were defined according to the different experimental series described below. The foraging arena was removed from the feeding location after training. The volume of sugar solution (or reward magnitude) offered by the single flowers of the patch changed across the five different experimental series. The first two series presented a variable volume, either increasing or decreasing, throughout the 9 visits by the single bees. In the increasing series, the volume per flower was 2 µl during visits 1-3, 5 µl during visits 4-6, and 10 µl during visits 7-9. In the decreasing series, the volume per flower was 10 µl during visits 1-3, 5 µl during visits 4-6, and 2 µl during visits 7-9. Hence, the mean volume per flower as well as the total volume of sugar solution offered by the patch at the end of the 9 successive visits by the single bees (5.67 and 612 µl, respectively) was the same in both series. The remaining three series (henceforth, the small, medium and large series) offered a constant volume of sugar solution per flower throughout the 9 visits by the single bees: either 2 µl, 5.67 µl, or 10 µl, respectively. The total volume of sugar solution offered to the bees in the small, medium and large series was 216 µl, 612.4 µl and 1080 µl, respectively.

The behaviour of each experimental bee foraging at the patch was evaluated three times after training. The flowers offered no sugar reward during testing. The first, second and third tests took place 24, 25, and 48 h after training, respectively. The second test began when the experimental bee returned spontaneously to the patch after having performed the first test; the time elapsed between the first and the second test clearly varied across individuals, and was approximately 1 h.

The entire sequence of behaviours performed by the experimental bees at the flower patch was video-recorded during both training and testing. The following

variables were analysed: 1) Learning score: defined as the ratio between the number of inspections of the flowers signalled by the rewarded colour and the total number of inspections of both types of flowers (those of the rewarded as well as the unrewarded colour) that the single bees performed during each of their visits to the patch. The cumulative learning score, computed for the sake of comparisons across series, is the total proportion of inspections of the rewarded colour throughout the 9 successive visits; it equals the sum of the individual learning scores. 2) Retention score: defined as the ratio between the number of inspections of the rewarded colour and the total number of inspections of both colours. 3) Total number of successful inspections (henceforth, 'SI'): equals the number of times that the experimental bee found sugar reward during its multiple inspections of the flowers. 4) Total number of unsuccessful inspections of the rewarded colour (henceforth, 'UI'): it corresponds to the number of times that the experimental bee did not find sugar solution upon inspecting a flower signalled by the rewarded colour. These events occurred either when the inspected tube was already emptied or when the length of the inspection did not allow the animal to reach the offered sugar solution. 5) Cumulative inspection time (henceforth, 'CIT'), in seconds: defined as the amount of time that the experimental bee spent searching for sugar reward inside the tubes - both rewarded and unrewarded - during each test session. 6) Visit time (henceforth, 'VT'), in minutes: defined as the time the experimental bee spent foraging on the arena during each single visit. I also calculated a total VT (henceforth, 'TVT'), as the sum of the single VT values recorded during the 9 successive visits. 7) Training time (henceforth, 'TT'), in minutes: defined as the sum of the total visit time (TVT) and the time the experimental bee spent inside the hive in-between its successive foraging visits to the arena. It therefore computes the time interval between the beginning and the end of training. 8) Total volume collected during training (henceforth, 'Vol'), in µl: as the sum of the volumes of sugar solution that the experimental bee collected during each of the 9 successive visits to the patch. 9) Solution intake rate throughout the total visit time (henceforth, 'SIR_1'), in µl/min: defined as the ratio between Vol and TVT. 10) Solution intake rate throughout the training time (henceforth, 'SIR_2'), in µl/min: defined as the ratio between the Vol and TT. 11) Mean solution intake rate

per visit to the patch (henceforth, 'MSIR'), in µl/min. I computed the ratio between the collected volume and the VT for each of the 9 visits to the patch, and then averaged these values in order to calculate the mean solution intake rate.

Data were analysed by means of one-sample t-tests, one-way ANOVAs, Kruskal-Wallis tests (when the data did not fulfil the requirements of parametric tests), LSD tests, and planned comparisons. While performing planned comparisons, I used the Bonferroni adjustment to set a level per comparison so that the overall alpha level was 0.05.

Results

In the experiment, foraging bees first had to forage individually on a relatively large patch of flowers consisting of 12 yellow and 12 blue artificial feeders ('flowers'), and learn which of these two colours was actually rewarding. During the variable series, I offered either increasing (small-medium-large) or decreasing (large-medium-small) volumes of sugar solution during nine successive visits by the single bees. Hence, both series offered the same total volume of sugar solution at the end of these visits. Three additional series, called the constant series, offered the same volume (small, medium, or large) of sugar solution throughout all the visits to the patch by the single bees. The bees' foraging behaviour was then observed in the absence of reward (extinction tests) 24, 25 and 48 h after the animals finished foraging on the patch. The set-up did not allow the bees to have immediate access to the sugar reward, meaning that each bee first needed to discriminate between the two flower types, then handle a tubular flower in order to find and drink a small amount of solution, and systematically repeat this procedure in order to fill its crop as much as possible before flying back to the hive.

The bees showed similar learning scores for yellow and blue colours (data not shown), and I therefore pooled the data from both training situations. Hence, as I expected on the basis of previous results (Menzel 1967, 1968), the bees from all series showed both high learning scores (which developed even during the first visit to the patch) and significant retention scores at 24, 25 and 48 h after training (Fig. 1.1 A, B;

the proportion of correct choices was higher than that expected by random choices, one-sample t-test, $P < 0.02$). Learning scores, in addition, slightly increased throughout the successive visits, and were similar in all five experimental series (Fig. 1.1 A, one-way ANOVA, $P = 0.6$), even when the total number of successful (SI) and unsuccessful inspections (UI) differed across series (Table 1.1). Likewise, retention scores did not differ during testing across the five experimental series (Fig. 1.1 B, Kruskal-Wallis test, $P > 0.1$). Hence, colour learning as related to the animals' choices did not vary across series, meaning that any possible effect of the strength of reinforcement was saturated for this type of learning.

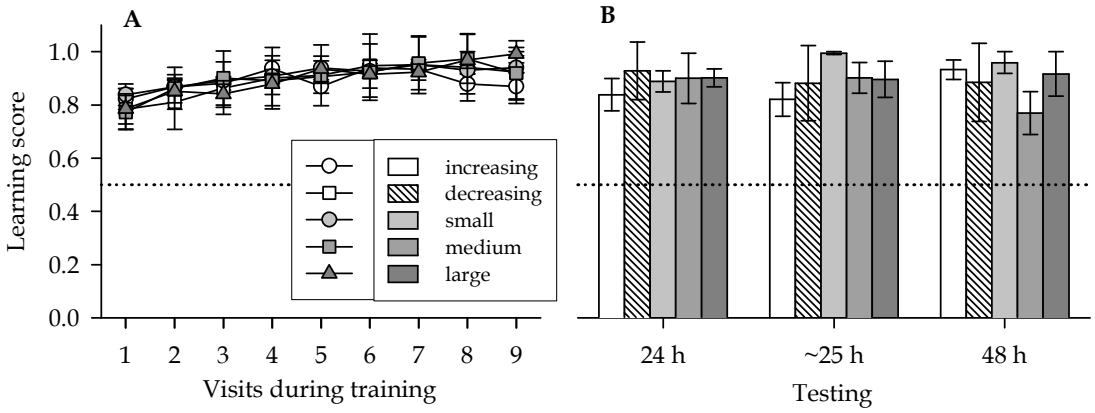

Figure 1.1 Means (± s.e.m) of the learning **(A)** and retention scores **(B)**, measured as the ratio between the number of inspections of the rewarded colour and the total number of inspections of both colours, for the increasing (white circles and bars), decreasing (white squares and dashed bars), small (light grey circles and bars), medium (grey squares and bars) and large (dark grey triangles and bars) series. Dotted lines indicate the score that would be expected via random choices: one-sample t-test, for *learning score*: increasing, $t_{(6)} = 8.3$, $P = 0.0002$, $N = 8$; decreasing, $t_{(7)} = 4.6$, $P = 0.002$, $N = 9$; small; $t_{(8)} = 8.4$, $P < 0.001$, $N = 9$; medium; $t_{(8)} = 3.8$, $P = 0.005$, $N = 9$; large; $t_{(7)} = 5.0$, $P = 0.001$, $N = 8$; for *retention score*: increasing, 24 h, $t_{(7)} = 5.6$, $P = 0.0008$, $N = 8$, ~25 h, $t_{(7)} = 5.0$, $P = 0.0015$, $N = 8$, 48 h, $t_{(6)} = 11.92$, $P < 0.0001$, $N = 7$; decreasing, 24 h, $t_{(8)} = 11.5$, $P < 0.0001$, $N = 9$, ~25 h, $t_{(6)} = 5.4$, $P = 0.002$, $N_{\sim 25h} = 7$, 48 h, $t_{(4)} = 5.2$, $P = 0.007$, $N = 5$; small, 24 h, $t_{(8)} = 9.9$, $P < 0.0001$, $N = 9$, ~25 h, $t_{(8)} = 93.5$, $P < 0.0001$, $N = 9$, 48 h, $t_{(6)} = 11.3$, $P < 0.0001$, $N = 7$; medium, 24 h, $t_{(8)} = 12.4$, $P < 0.0001$, $N = 9$, ~25 h, $t_{(7)} = 6.9$, $P = 0.0002$, $N = 8$, 48 h, $t_{(4)} = 3.3$, $P = 0.02$, $N = 5$; large, 24 h, $t_{(7)} = 12.0$, $P < 0.0001$, $N = 8$, ~25 h, $t_{(7)} = 5.8$, $P = 0.0006$, $N = 8$, 48 h: $t_{(3)} = 5.0$, $P = 0.01$, $N = 4$. One-way ANOVA for cumulative score learning (see Methods), $F_{(4, 37)} = 0.7$, $P = 0.6$. Kruskal-Wallis test for retention score, 24 h: $H = 1.9$, $P = 0.7$; ~25 h: $H = 7.8$, $P = 0.1$; 48 h: $H = 5.2$, $P = 0.3$.

I then compared the time that the bees spent inspecting both types of empty flowers during testing (cumulative inspection time, or CIT), as well as the visit time, which included the CIT, but also took into account the time that the bees spent outside the tubes while flying over the flowers (see Methods). I found a greater CIT in the increasing series than in the decreasing series during the first test, performed 24 h after training (Fig. 1.2 A, planned comparison, $t_{I\ vs.\ D} = 2.1$ $P < 0.05$). It decreased and did not differ across series during the second test, performed ~25 h after training (Fig. 1.2 A, one-way ANOVA, $P = 0.9$). Finally, I found a greater CIT in the increasing series than in the decreasing and the large series during the third test, performed 48 h after training (Fig. 1.2 A, planned comparisons, $t_{I\ vs.\ D} = 2.4$, $P < 0.05$ and $t_{I\ vs.\ L} = 2.1$, $P < 0.05$). The visit time was also greater in the increasing series than in the large series during the first test (Fig. 1.2 B, planned comparisons, $t_{I\ vs.\ L} = 2.2$, $P < 0.05$). It decreased and did not differ across series during the second test (Fig. 1.2 B, one-way ANOVA, $P = 0.6$), and, finally, it was greater in the increasing series than in the decreasing series during the third test (Fig. 1.2 B, planned comparison, $t_{I\ vs.\ D} = 2.7$, $P < 0.05$). It is important to note that the bees were not rewarded in the first test, and that, over a short period of time (i.e., between the first and the second test), extinction learning might have overridden the differences in inspection time. However, 24 hours later (i.e., during the third test) the animals' original response was partially re-established, indicating a recovery from extinction, and led to clear differences for these measures between the increasing and decreasing series. In summary, when first tested 24 and 48 h after training, the animals searched for reward more intensively after having experienced an increasing reward schedule than after having experienced a decreasing reward schedule, as revealed by the higher scores of either one or both of the two measures of 'persistence'. This result matched my second prediction (see above), and suggested that subjective reward values controlled the animals' behaviour during testing. Other studies have also found that time-based measurements seem to be more sensitive to subjective reward values than choice-based measurements (e.g., Sage and Knowlton 2000, Schönbaum et al. 2003).

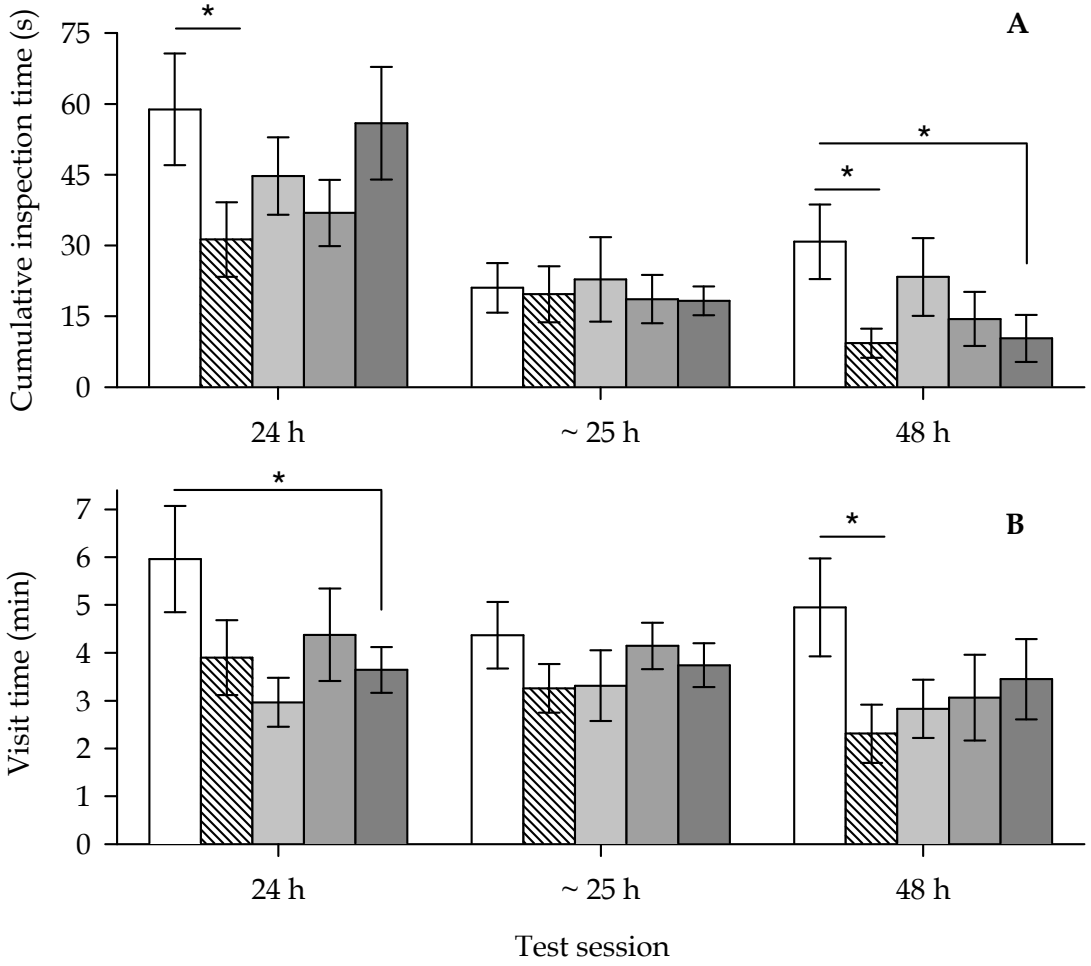

Figure 1.2 Means (± s.e.m) of the cumulative inspection time (in s) **(A)** and visit time (in min) **(B)** during testing for the increasing (white bars), decreasing (dashed bars), and the constant series: small (light grey bars), medium (grey bars) and large (dark grey bars). One-way ANOVA: for *cumulative inspection time*, 24 h, $F_{(4, 38)}$.= 1.6, P = 0.19; ~25 h, $F_{(4, 37)}$.= 0.1, P = 0.9; 48 h, $F_{(4, 26)}$.= 2.1, P = 0.11; for *visit time*: 24 h, $F_{(4, 38)}$.= 1.9, P = 0.1; ~25 h, $F_{(4, 36)}$.= 0.7, P = 0.6; 48 h, $F_{(4, 26)}$.= 1.9, P = 0.13. I made the following planned comparisons for each test: increasing vs. decreasing series, increasing vs. large series, decreasing vs. small series, small vs. medium series, small vs. large series, and medium vs. large series. Asterisks indicate statistical differences, P < 0.05. Sample size across tests: increasing series, N_{24h} = 8, $N_{\sim 25h}$ = 8, N_{48h} = 7; decreasing series, N_{24h} = 9, $N_{\sim 25h}$ = 8, N_{48h}= 8; small, N_{24h} = 9, $N_{\sim 25h}$ = 9, N_{48h} = 7; medium, N_{24h} = 9, $N_{\sim 25h}$ = 8, N_{48h} = 5; large, N_{24h} = 8, $N_{\sim 25h}$ = 8, N_{48h} = 5.

These results might be due to the fact that the bees from the increasing series had differentially associated the related predicting signals during training. Thus, they might have assigned proportionally more time to inspect the flowers that had previously yielded reward, as related to the total amount of time assigned to flower

inspection. I calculated the proportion of time that the bees assigned to inspect the flowers that had previously yielded reward, and found it similar in all groups during all three tests (One-way ANOVA, 24 h: $F_{(4, 38)} = 0.4$, $P = 0.8$; 25 h: $F_{(4, 35)} = 2.1$, $P = 0.1$; 48 h: $F_{(4, 23)} = 0.8$, $P = 0.6$). Still, the differences in performance between the increasing and decreasing series could be accounted for by assuming that the bees' behaviour on the test reflects their most recent experience during training. Behaviour controlled in this way could be learned through simple 'stimulus-response' mechanisms, without reference to reward expectations. However, the differences in the animals' 'persistence' between the increasing and the large series 24 and 48 h after training, and the similar performance of the bees from the three constant series (Fig. 1.2 A, B) argue against the results being a simple reflection of the most recent reward experience, and in favour of a learned expectation of relative reward magnitude.

Finally, the differences between the increasing and decreasing series might be due to changes in the energy balance of their foraging excursions. That is, they might have differentially associated the related predicting signals derived from the entire patch because they were fed more or faster. Hence, I also analyzed the bees' experience with the offered reward on the basis of the energy balance of their successive foraging trips during training. At the end of training, the bees from the increasing, decreasing and medium series had collected similar volumes of sugar solution; these volumes were greater than those of the small series, and smaller than those of the large series (Table 1.1). In addition, the total visit time (TVT) and training time (TT) (see Methods) gave minimal values for the large and the medium series, intermediate values for both variable series, and maximal values for the small series (Table 1.1). As a result, the bees' solution intake rate clearly varied across series. SIR_1 and SIR_2 were the ratios between the total volume collected and the TVT and the TT, respectively (see Methods). Both the SIR_1 and SIR_2 gave a series of decreasing values for the different groups (from maximum to minimum): large, medium, variable, and small series (see Table 1.1). I also computed the mean solution intake rate (MSIR) that the bees experienced throughout their single visits to the patch (see Methods), and found maximal values for the large series, intermediate values for both the increasing and the medium series, and minimal values for the decreasing and the small series

(Table 1.1). The difference observed between the increasing and the decreasing series is due to the fact that the bees from the decreasing series collected a lower volume of solution and required a larger amount of time while searching for the offered reward during their first visit to the patch. In summary, the bees from the increasing and the decreasing series collected the same amount of sugar solution and experienced the same overall intake rate during training. I found differences between these series in the mean solution intake rate per visit, although I also found differences for this variable across the constant series, where the bees behaved similarly during testing (Table 1.1, Fig. 1.2).

Taken together, the results show that simple 'stimulus-response' mechanisms cannot account for the differences in 'persistence' found in the increasing and decreasing series, meaning that these differences can only be explained by reference to reward expectations.

Table 1.1 Variables measured during training across the different series (mean ± s.e.m).

	Variable series		Constant series			
	Increasing	**Decreasing**	**Small**	**Medium**	**Large**	**One-way ANOVA**
SI	81.5 ± 3.9^{a}	88.4 ± 5.6^{a}	103.9 ± 4.0^{b}	76.7 ± 5.0^{a}	63.4 ± 3.9^{c}	$F_{(37,4)}$=12.1, P<0.0001
UI	64.4 ± 11.1^{a}	73.7 ± 9.1^{a}	174.3 ± 19.4^{b}	22.8 ± 1.7^{c}	19.7 ± 3.2^{c}	$F_{(37,4)}$=29.5, P<0.0001
Vol (µl)	351.5 ± 8.1^{a}	353.7 ± 9.9^{a}	192.4 ± 8.6^{b}	368.2 ± 22.5^{a}	471.4 ± 18.7^{c}	$F_{(37,4)}$=64.6, P<0.0001
TVT (min)	46.4 ± 2.8^{a}	48.5 ± 3.0^{a}	74.8 ± 6.1^{b}	29.7 ± 2.6^{c}	30.6 ± 2.5^{c}	$F_{(37,4)}$=24.8, P<0.0001
TT (min)	92.6 ± 9.2^{a}	81.4 ± 4.2^{a}	133.1 ± 12.7^{b}	59.6 ± 6.1^{c}	59.1 ± 4.5^{c}	$F_{(36,4)}$=14.9, P<0.0001
SIR_1 (µl/min)	7.7 ± 0.3^{a}	7.5 ± 0.4^{a}	2.6 ± 0.1^{b}	12.9 ± 1.2^{c}	15.9 ± 0.9^{d}	$F_{(37,4)}$=102.1, P<0.001
$SIFR_2$ (µl/min)	4.0 ± 0.3^{a}	4.4 ± 0.3^{a}	1.6 ± 0.2^{b}	6.8 ± 0.8^{c}	8.3 ± 0.6^{d}	$F_{(37,4)}$=43.5, P<0.0001
MSIR (µl/min)	12.9 ± 0.7^{a}	9.6 ± 0.8^{b}	2.9 ± 0.2^{c}	13.8 ± 1.1^{a}	18.6 ± 1.4^{d}	$F_{(37,4)}$=83.0, P<0.0001

Different superscript letters indicate significant LSD comparisons, $P < 0.05$. (SI) Total number of successful inspections; (UI) total number of unsuccessful inspections; (Vol) total volume collected; (TVT) total visit time; (TT) training time; (SIR1) total solution intake rate along the total visit time; (SIR2) total solution intake rate along the training time; (MSIR) mean solution intake rate per visit.

Discussion

In the experiment, bees first learned to associate colours with sucrose reward in an array of artificial flowers closely resembling a natural foraging situation. I evaluated whether and how the sequence of the bees' experience with different reward magnitudes changed their later foraging behaviour in the absence of reward and under an otherwise similar context. In addition to the usual measure of correctness of

choice, I also evaluated the bees' 'persistence' during their searches for sugar reward. I found that the animals that had experienced increasing volumes of sugar reward during training assigned more time to flower inspection (i.e., showed greater 'persistence') when tested 24 and 48 hours after training. I found that the animals behaved differently neither because they had more strongly associated the related predicting signals nor because they were fed more or faster. Instead, they appear to have changed their 'persistence' based on the variations in reward magnitude they had previously experienced during training. This becomes evident if one considers (1) the proportion of time that the bees from the different groups assigned to inspect the flowers that previously yielded sugar reward, as related to the total time assigned to flower inspection, (2) the relationship between the most recent experience with the offered reward during training and the animals' responses during testing, and, finally, (3) the results of the constant series as related to the energy balance of the animals' foraging trips during training (see Results). The latter issue, for example, is well-illustrated by comparisons across the constant series: the bees from the large series collected approximately twice as much sugar solution as the bees from the small series, and they did it in approximately half the time; both groups, however, showed similar values for their measures of 'persistence' during testing (Fig. 1.2 B, Table 1.1).

Hence, the results indicate that the animals from both variable series developed different long-term reward expectations, and that these expectations eventually led to differences in test performance in the absence of reward, and did so even 48 h after training. The term 'expectation' denotes an effect on behaviour at a later time that reflects specific past experiences with the offered reward. These variations at a later time presumably depend upon the activation of a memory about specific properties of the experienced reward, which differs from and exists in addition to a memory arising from the contingency between a given stimulus (such as the flower colour), the animal's response (such as the inspection of the flower), and the offered reward. Thus, according to theory, the bees' later behaviour at the patch must have been modulated by different subjective reward values learned during training.

The reward schedule I used somehow resembles those of experiments addressing incentive phenomena, exemplified by Crespi's (1942) early studies. He trained rats to feed at the end of a straight alley, and found that the animals shifted from a large to a small reward size ran more slowly for the small reward size than did the animals trained only with the small reward size, while the animals shifted from a small to a large reward size ran faster than did those trained only with the large reward size. Both types of responses are usually referred to as 'Crespi effects', or, more specifically, as successive 'negative' and 'positive' contrast effects, respectively (Flaherty 1982). Considering the results from my first test, for example, I found that the bees from the increasing series spent more time in the patch than the bees from the large series (Fig. 1.2 B), somehow resembling the successive positive contrast effect found in rats (Flaherty 1982). In contrast, I found no evidence of successive negative contrast effects during the first test (Fig. 1.2 A, B). This is intriguing because positive contrast effects seem to be much more elusive to reveal than negative contrast effects (Flaherty 1982). Contrast effects are often linked with reward expectations. My experiment was not designed to tackle such effects, but it unambiguously shows that bees make use of long-lasting subjective reward values. Moreover, expectations in laboratory animals are usually investigated by means of the so-called reward devaluation procedure, in which reward values are manipulated outside the learning situation by using satiation or conditioned taste aversion (e.g., Holland and Straub 1979, Rescorla 1987, Gallagher et al. 1999, Sage and Knowlton 2000). This approach might also be considered in future experiments on reward expectations with free-flying and restrained bees.

Reward expectations are a key product of acquired knowledge about reward properties. Studies on reward learning and the subsequent development of reward expectations are critical for understanding the rules controlling goal-directed behaviours, and for the assessment of the cognitive complexity underlying decision making and planning. Reward expectations and incentive phenomena have systematically been addressed only in vertebrate species, probably because such phenomena are frequently linked to complex cognitive abilities only ubiquitous in animals with large brains. Studies of rodents (e.g., Gallagher at al. 1999), nonhuman

primates (e.g., Schultz 2000), and humans (e.g. O'Doherty et al. 2001), indicate that neural interaction between the basolateral complex of the amygdala and the orbitofrontal cortex are crucial for the development and subsequent use of reward expectations involved in goal-directed behaviours (Holland and Gallagher 2004). Here I document for the first time that honeybees also develop long-term reward expectations. These expectations can guide their foraging behaviour after a relatively long pause and in the absence of reinforcement, and further experiments will aim toward an elucidation of the neural mechanisms involved.

It has been reported that foraging honeybees develop a form of short-term reward expectation (Greggers and Menzel 1993, Bitterman 1996, Greggers and Mauelshagen 1997). This form of expectation becomes evident through the analysis of an animal's intra- and inter-patch choices across its successive visits to an array of multiple feeders, and depends upon the amount and concentration of the solution offered by these feeders; bees match their choices to these properties. Moreover, they also appear to be sensitive to variance of reward (Real 1981, Shafir et al. 1999, Waddington 2001, Shapiro et al. 2001, Drezner-Levy and Shafir 2007). These short-term reward expectations seemingly help the animal in anticipating the level of reward, and suggest that the value of the appetitive stimulus depends on what the animal expects to experience next in a given situation and, therefore, on the background of its experience under a similar situation (Greggers and Menzel 1993, Fülop and Menzel 2000, Waddington and Gottlieb 1990, Real 1991, Wiegmann et al. 2001). No attempts have been made, however, to distinguish between the strength of signal learning and learning about subjective values of reward, let alone the possible development of long-term reward expectations. Interestingly, evidence has been reported indicating that honeybee dance behaviour, an intriguing example of multisensory convergence and central processing, also depends upon the magnitude of past rewards (Raveret-Richter and Waddington 1993, De Marco and Farina 2001, De Marco et al. 2005).

Honeybees seem to critically rely on their memory store in deciding when and where to forage. A honeybee's working memory can track the rewarding properties of several, simultaneous feeding stations, integrating critical components of the

animal's reward experience over a time span of several minutes (Greggers and Menzel 1993). Here I show that honeybees also develop persisting forms of subjective reward values. It might be interesting to evaluate how these persisting memories are subsequently retrieved by specific constellations of stimuli, and how their contents are appropriately integrated with a number of current conditions based on the time of the day and the animal's general motivational state. This may allow further dissociation between stimulus-response association, incentive phenomena, and basic forms of planning.

These results show that foraging honeybees are able to learn that the level of reward either increases or decreases over time. This suggests that they benefit from a built-in change detector that computes the sign of variations in reward level. Any efficient change detector should compute not only the sign, but also the magnitude of variations in the signal supplied by the corresponding sensor. In the next chapter thus, I address the question whether foraging honeybees are also able to learn the magnitude of variations in the level reward.

Chapter 2

Honeybees Learn the Magnitude and Sign of Reward Variations

Abstract

In this chapter, I asked whether honeybees learn the magnitude and sign of variations in the level of reward. I made an experiment in which bees first had to forage on a three-flower patch offering variable reward levels, and then search for food at the site after a long foraging pause and in the absence of reinforcement. During training, the bees were presented with either a large or a small increase in reward level, or, instead, with a decrease in reward level. Testing took place as soon as they visited the patch on the day following training, when I measured the bees' food searching behaviours. I found that the bees that had experienced increasing reward levels searched for food more eagerly than the bees that had experienced decreasing reward levels. Similarly, the bees that had experienced a large increase in reward level searched for food more eagerly than the bees that had experienced a small increase in reward level. These group differences could not be accounted for by the bees' energy balance during training. These results show that honeybees adjust their investment of time/energy during foraging in relation to both the magnitude and the sign of past variations in reward level. Apparently, such variations lead to the formation of reward expectations which may enhance a forager's reliance on a feeding site. This ability would make it more likely for honeybees to find food when forage is scarce.

Introduction

In the previous chapter (Gil et al. 2007), I first trained honeybees to associate colours with sucrose reward in a setting closely resembling a natural foraging situation, and then examined whether their sequence of encounters with different volumes of sugar solution changed their subsequent foraging behaviour. I did so in the absence of reward and under otherwise similar circumstances. I found that those bees that had

experienced increasing volumes of sugar reward during training assigned more time to flower inspection when tested 24 and 48 hours after training. They behaved differently neither because they were fed more or faster nor because they had more strongly associated the related predicting signals. These results showed for the first time that the behaviour of honeybees in the absence of reinforcement can be subject of changes at a later time on the basis of a specific property of reward, namely, that its magnitude increased over time.

These results suggest that honeybees have a built-in change detector which computes the sign of variations in the level of reward. This computation is followed by estimates of expected rewards. In this scheme, one does not yet know whether honeybees are able learn not only the sign, but also the magnitude of variations in reward level. This is important because flowers produce nectar at low and variable flow rates (Núñez 1977, Vogel 1983, Baker and Baker 1983), and honeybees have to adjust their selectivity among nectar sources in relation to forage abundance (Seeley 1995). The ability to adjust the investment of time/energy during food searches in relation to the magnitude and sign of past variations in the level of reward would make it more likely for them to maximize their individual and collective rates of food collection by increasing their chances to find food. Here, I present the results of an experiment addressing these issues.

In this experiment, bees had to forage individually on a flower patch which offered low flow-rates of sugar solution, thus resembling a natural foraging situation. While foraging, they experienced either a large or a small increase in reward level, or, instead, a decrease in reward level. I then examined how they searched for food at the site in the absence of reward and after a long foraging pause. In doing this, I also pondered the effect of the bees' energy balance during foraging. I had two predictions. First, that the bees will search for food during longer periods of time after having experienced increasing reward levels than after having experienced decreasing reward levels. And, second, that they will search for food during longer periods of time after having experienced a large increase in reward level than after having experienced a small increase in reward level. I discuss our findings in the context of learning and foraging behaviour.

Methods

I placed a colony of *Apis mellifera carnica* bees in a two-frame observation hive, and trained marked bees to collect unscented 50% w/w sucrose solution at a three-flower artificial patch placed 80 m from the hive. These bees were not used as experimental subjects, but to recruit nest-mates to the patch. The newcomers arriving at the patch were immediately trapped, marked with plastic tags, and released; they did not contact the offered solution. These bees became potential experimental subjects. Of them, those returning to the patch underwent a pre-training phase, thereby becoming experimental bees.

The patch consisted of a single acrylic cylinder (4.5 cm diameter, 5 cm high) with three centred holes (flowers) placed 1 cm away from each other. Each hole had a small container (40 mm diameter, 50 mm deep) connected to a specially designed feeder by means of a plastic cannula. Detailed descriptions of our feeder have been given elsewhere (Núñez 1966, 1970). Here, it will be sufficient to say that it delivered sugar solution to the bees at constant and adjustable flow rates, and that it was connected to the three flowers of the patch by means of three separate cannulas. Thus, at any given time during the experiment, the three separate flowers offered similar flow-rates of sucrose solution. The overall flow-rate offered by the patch always arose from the sum of the flow-rates offered by each of these flowers.

Each experimental bee had to introduce its head into each of the plastic containers in order to reach the sugar solution offered at the bottom of the flowers. Thus, it had to learn how to handle the flowers in order to access the offered reward efficiently. Before training, every bee was allowed to forage on the patch once, in a so-called pre-training phase, in which it was fed ad libitum with unscented 50% w/w sucrose solution. As it happened during training (see below), only one bee at the time underwent pre-training. Any other bee landing on the patch was captured and kept inside a small cage until the end of the experiment. Training began immediately after pre-training, as soon as the experimental bee returned to the patch. It involved four successive foraging excursions by that bee. The patch offered unscented 20% w/w sucrose solution at two different flow-rates during training. The experimental bee

was first presented with one of these two different flow-rates during its first two visits to the patch, and then with the other flow-rate, either larger or smaller (see below) than the preceding one, during its last two visits to the patch. I used three different flow-rates of sucrose solution: high (15 µl/min, or 5 µl/min per flower), medium (9 µl/min, or 3 µl/min per flower), and low (3 µl/min, or 1 µl/min per flower).

The experiment had four different experimental series: one decreasing series and three different increasing series. In the decreasing series (henceforth, S-15/3), I presented the bees with the highest and lowest flow-rate in their first and last two visits to the patch, respectively. In the first increasing series (henceforth, S-3/15), the bees were given the lowest and highest flow-rate in their first and last two visits to the patch, respectively. In the second increasing series (henceforth, S-3/9), they were presented with the lowest and medium flow-rate in their first and last two visits, respectively. And, finally, in the third increasing series (henceforth, S-9/15), they were given the medium and highest flow-rate in their first and last two visits to the patch, respectively. Details on these treatments are given in Table 2.1.

Table 2.1 Variables defining the different experimental series

	Experimental series			
	S-15/3	S-3/15	S-3/9	S-9/15
Flow-rate (µl/min) in visits 1-2	15	3	3	9
Flow-rate (µl/min) in visits 3-4	3	15	9	15
Mean flow-rate (µl/min)	9	9	6	12
Magnitude of reward variation (µl/min)	12	12	6	6

I recorded the behaviour of the bees during both training and testing. I removed the flower patch from the feeding site in-between training and testing, and testing began 24 h after training. The patch did not offer sugar reward during testing. Under these circumstances, I recorded the behaviour of the bees in their first two visits to the patch. I call these visits 'first' and 'second' test, or test 1 and 2.

I focused on the following variables for the analysis: 1) the total visit time during training (in minutes), as the cumulative time that each bee spent collecting food at

the patch during training; 2) the total volume of sugar solution collected during training (in μl), as the sum of the volumes of sucrose solution that each bee collected in its four successive visits to the patch; 3) the visit time per test (in seconds), as the time that each bee spent searching for food at the patch per test; 4) the overall visit time during testing (in seconds), as the sum of the visit times recorded in both tests; 5) the cumulative inspection time per test (in seconds), as the amount of time that each bee spent searching for food inside the flowers per test; 6) the overall inspection time during testing (in seconds), as the sum of the single cumulative inspection times from both tests; 7) the number of flower inspections per test, as the number of times in which each bee introduced its head into any of the three flowers of the patch in each of the single tests; 8) the overall number of inspections during testing, as the sum of the numbers of flower inspections from both tests; 9) the time elapsed in-between both tests, or inter-test time; and 10) the ratio between the overall visit time and the inter-test time.

The variables concerning the tests, i.e., visit time, cumulative inspection time and number of inspections, were analyzed by means of two-way repeated-measures ANOVA, with repeated measures on only one factor, the test; the other being the experimental series. If the interaction effect was significant, then we examined the single effects of the series and test by means of one-way ANOVAs, with LSD multiple comparisons and paired t-tests, respectively. The remaining variables were analyzed by means of one-way ANOVAs with LSD multiple comparisons (Zar 1996).

Results

I first compared the results of the different series within each test. In test 1, I found that the visit time and cumulative inspection time were maximal in S-15/3 and S-9/15, intermediate in S-3/9, and minimal in S-15/3 (Fig. 2.1 A, C). And the number of inspections was maximal in S-3/9 and S-9/15, intermediate in S-3/15 and minimal in S-15/3 (Fig. 2.1 E). In test 2, the visit time was longer in S-15/3 than in the other series (Fig. 2.1 B). And the cumulative inspection time was maximal in S-3/15, intermediate in S-3/9 and minimal in S-9/15 and S-15/3 (Fig. 2.1 D). Also, the

number of inspections was maximal in S-3/15, intermediate in S-3/9 and S-9/15, and minimal in S-15/3 (Fig. 2.1 F).

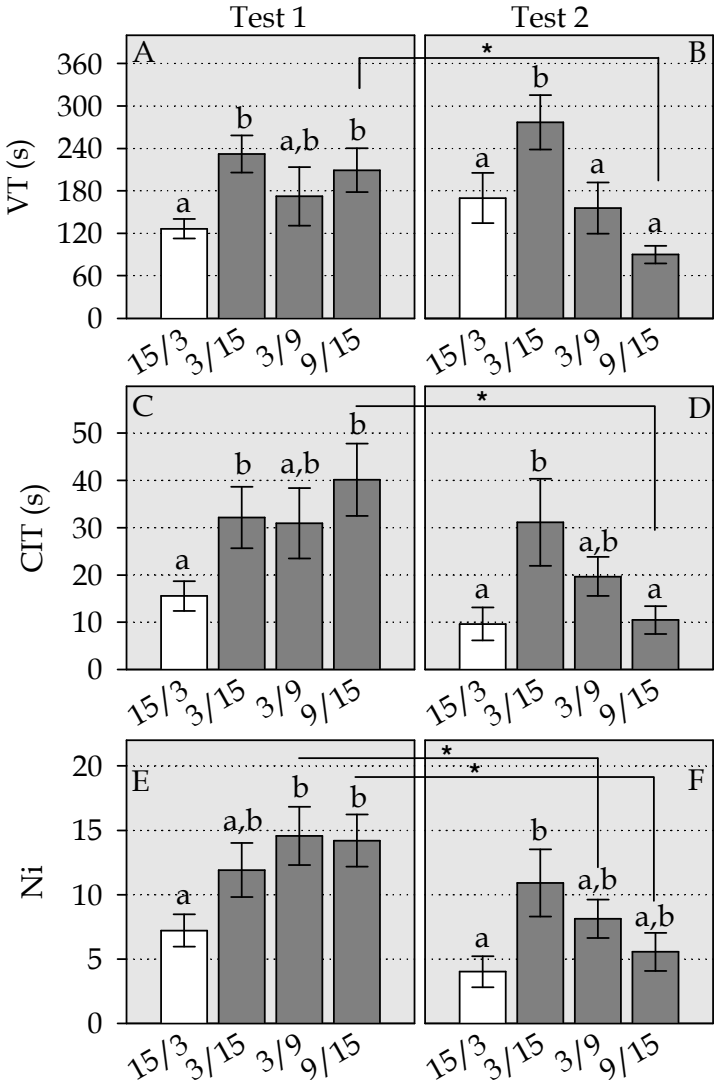

Figure 2.1 Means (± s.e.m) of the visit time (in s) **(A, B)**, cumulative inspection time (in s) **(C-D)**, and number of inspections **(E-F)** during testing for S-15/3, S-3/15, S-3/9 and S-9/15. Two-way repeated measures ANOVA: A-B) series effect $F_{(3,35)} = 4.7$, $P = 0.007$, test effect $F_{(3,35)} = 0.4$, $P = 0.5$, interaction effect $F_{(3,35)} = 3.6$, $P = 0.02$; C-D) series effect $F_{(3,35)} = 2.9$, $P = 0.04$, test effect $F_{(3,35)} = 12.4$, $P = 0.001$, interaction effect $F_{(3,35)} = 3.2$, $P = 0.03$; F-G) series effect $F_{(3,35)} = 2.8$, $P = 0.05$, test effect, $F_{(3,35)} = 23.9$, $P < 0.0001$, interaction effect, $F_{(3,35)} = 3.2$, $P = 0.03$. Different letters indicate LSD multiple comparisons $P < 0.05$ after one-way ANOVA. Asterisks indicate paired t-tests $P < 0.05$. Sample size: $N_{S\text{-}15/3} = 10$, $N_{S\text{-}3/15} = 11$, $N_{S\text{-}3/9} = 9$, $N_{S\text{-}9/15} = 10$.

Next, I compared the results of the different tests within each series. I found that the performance of the bees of S-15/3 and S-3/15 did not differ between tests (Fig. 2.1). In S-3/9, the visit time and cumulative inspection time did not differ between tests, but the number of inspections was higher in test 1 than in test 2 (Fig. 2.1). In S-9/15, the visit time, cumulative inspection time and number of inspections were higher in test 1 than in test 2 (Fig. 2.1).

In spite of the fact that we found differences between tests, I pooled data from both tests and made an analysis of the overall test performance of the bees (Table 2.2 A). Thus, I found that the overall visit time was significantly longer in S-3/15 than in the other three series. Moreover, the overall cumulative inspection time was minimal in S-15/3, intermediate in both S-3/9 and S-9/15, and maximal in S-3/15. The overall number of inspections was minimal in S-15/3, intermediate in S-9/15, and maximal in both S-3/15 and S-3/9. The time elapsed between the first and the second test varied markedly across individuals, ranging from 8 to 144 minutes. Overall, it did not differ between series, although it was significantly shorter in S-3/15 than in S-3/9 (planned comparison, P = 0.03). In addition, the ratio between the overall visit time and the inter-test time was significantly higher in S-3/15 than in the other three series (Fig. 2.2).

Table 2.2 Overall data from both tests **(A)**, and variables measured during training **(B)** across the different series (mean ± s.e.m).

		Experimental series				
		S-15/3	**S-3/15**	**S-3/9**	**S-9/15**	**One-way ANOVA**
A)	**oVT (s)**	296.1 ± 41.8^{a}	508.9 ± 57.9^{b}	327.9 ± 46.9^{a}	303.0 ± 39.0^{a}	$F_{(3,35)}=4.7$, P =0.007
	oCIT (s)	42.0 ± 9.2^{a}	63.2 ± 14.5^{b}	$50.6 \pm 8.2^{a, b}$	$52.3 \pm 8.2^{a, b}$	$F_{(3,35)}=2.6$, P=0.06
	oNi	11.2 ± 1.6^{a}	22.8 ± 4.4^{b}	22.7 ± 3.3^{b}	$20.2 \pm 2.7^{a, b}$	$F_{(3,35)}=2.8$, P=0.04
	ITT (min)	46.2 ± 8.9	38.9 ± 9.1	74.5 ± 14.8	58.2 ± 13.0	$F_{(3, 35)}=1.8$, P=0.1
B)	**Vol (µl)**	183.8 ± 15.5^{a}	189.5 ± 4.6^{a}	125.3 ± 24.9^{b}	250.2 ± 16.7^{c}	$F_{(3, 35)}=9.5$, P < 0.0001
	TVT (min)	34.2 ± 4.0^{a}	34.2 ± 1.7^{a}	46.5 ± 2.6^{b}	24.4 ± 1.7^{c}	$F_{(3, 35)}=10.9$, P < 0.0001

Superscript letters indicate significant LSD comparisons, $P < 0.05$. (oVT) overall visit time; (oCIT) overall cumulative inspection time; (oNi) overall number of inspections; (ITT) inter-test time; (Vol) the total volume of collected sugar solution during training; (TVT) the total visit time during training.

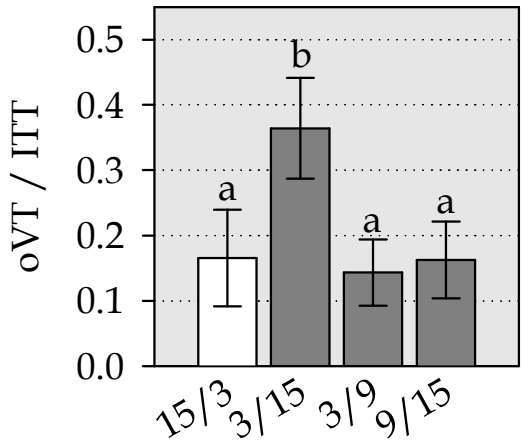

Figure 2.2 Mean (± s.e.m) of the ratio between the overall visit time (oVT) and the inter-test time (ITT) for S-15/3, S-3/15, S-3/9 and S-9/15. One-way ANOVA: $F_{(3,35)} = 3.3$, $P = 0.03$. Different letters indicate LSD multiple comparisons $P < 0.05$ after one-way ANOVA. Sample size: $N_{S\text{-}15/3} = 10$, $N_{S\text{-}3/15} = 11$, $N_{S\text{-}3/9} = 9$, $N_{S\text{-}9/15} = 10$.

Finally, I compared the total volume of sugar solution that the bees of the different groups collected during training, as well as the total amount of time that they spent foraging on the patch (Table 2.2 B). I found that the total volume of solution collected by the bees was maximal in S-9/15, intermediate in both S-15/3 and S-3/15, and minimal in S-3/9. The total visit time was maximal in S-3/9, intermediate in both S-15/3 and S-3/15, and minimal in S-9/15.

Discussion

In this chapter, I asked whether honeybees learn the magnitude and sign of variations in the level of reward. First, I found that bees that had experienced increasing reward levels searched for food more eagerly than bees that had experienced decreasing reward levels (Fig. 2.1 A-D and F, Fig. 2.2, S-15/3 vs. S-3/15). Further, when first tested, the bees of all the increasing series (S-3/15, S-3/9, and S-9/15) searched for food more eagerly than the bees of the decreasing series (Fig. 2.1 A, C, E). These bees did not behave differently because they were fed more or faster (Table 2.2 B). For example, the bees of the decreasing series had collected more food

than the bees of the increasing series S-3/9 (Table 2.2 B), but the latter searched for food more eagerly during testing (Fig. 2.1 E). These results mean that an increase in reward level induces long-term behavioural changes by itself, irrespective of the experienced levels of rewards and the amount of food collected. Moreover, in the second test, the bees that had experienced the larger increase in reward level searched for food more eagerly than the bees that had experienced the smaller increase in reward level (Fig. 2.1 B, D, Fig. 2.2, grey bars). As before, these bees did not behave differently because they were fed more or faster. For example, the bees of S-9/15 had collected more food, and faster, than the bees of S-3/15 (Table 2.2 B), but the bees of S-3/15 searched for food more eagerly (Fig. 2.1 B, D, Fig. 2.2). Also, the bees of S-9/15 had collected twice as much solution as the bees of S-3/9 in approximately half of the time (Table 2.2 B), but these two groups of bees, which had experienced a similar increase in reward level, behaved similarly in test 2 (Fig. 2.1, Fig. 2.2). To account for these results, one would also postulate a relationship between the most recent reward level that the bees experienced during training and their subsequent performance during testing. By this argument, the bees of S-3/15 and S-9/15 should only retain information about the highest reward level, and similar behaviours must be observed between these two groups of bees. But the data do not support such view (Fig. 2.1, Fig. 2.2). Taken together, these results support the view that honeybees have a built-in detector of variations in the level of reward (Chapter 1, Gil et al. 2007, 2008). Theory poses that such a detector should compute not only the sign, but also the magnitude of variations in the signal supplied by the corresponding sensor. Here, I show that honeybees learn the sign and magnitude of an increase in reward level.

The bees that experienced the large increase in reward level during training behaved similarly in both tests, but the bees that experienced the small increase in reward level searched for food less eagerly in test 2 than in test 1 (Fig. 2.1). Because this reduction was more conspicuous in the bees that received the highest reward levels during training (Fig. 2.1, S-9/15), an interaction must exist between the effect of an increase in reward level and that of reward level itself. The wonder arises as to why the bees of the increasing series behaved differently in test 2, but not in test 1

(Fig. 2.1, grey bars, A, C, E vs. B, D, F). It is likely that an increase in reward level leads to the formation of reward expectations which enhance a forager's reliance on a feeding site, and that the strength of this reliance increases with the magnitude of the experienced increase in reward level. In this line of argument, bees with positive expectations will search for food longer at an exhausted site (Fig. 1 A, C, E, white vs. grey bars). Yet, a honeybee's likelihood of searching for food at an empty site diminishes over time (Núñez 1966). Having a positive expectation of reward, the strength of a forager's reliance may become detectable only after one or more unsuccessful visits to the site. Under these circumstances, bees that have experienced a large increase in reward level would search for food more eagerly than bees that have experienced a small increase in reward level (Fig. 1 B, D, F, grey bars).

Taking into account the results of both tests, the bees that had experienced a large increase in reward level not only searched for food during longer periods of time than the bees of the other series, but also spent less time in the hive between both tests (Table 2.2 A). Thus, from the beginning of the first test until the end of the second one, the ratio between the amounts of time spent outside and inside the hive was clearly higher in the bees of S-3/15 than in the bees of the other series (Fig. 2.2). This is interesting because, inside the hive, foragers are exposed to cues and signals from other colony members, which they use to regulate their ongoing activities (e.g., von Frisch 1946, 1967, Ribbands 1954). It has been shown that, as food source profitability diminishes, honeybees extend their pauses in between foraging excursions (Núñez 1966, 1970, Grosclaude and Núñez 1998, De Marco and Farina 2001). This makes it more likely for them to become exposed to signals and cues from other colony members, and, eventually, to be recruited to a new food source. Given that honeybees cooperate by sharing newly discovered food sources, it would be interesting to examine the relationship between the magnitude and sign of past variations in the level of reward and a forager's probability of being recruited to new food sources.

It is also interesting to compare the present experiment with that presented in chapter 1 (Gil et al. 2007). In these two experiments, I used different artificial flower patches. In my previous experiment (Chapter 1), bees foraged on a relatively large

artificial patch offering both rewarding and unrewarding tubular flowers identified by two different colours. Thus, each forager first needed to discriminate between the two types of flowers, then to enter and walk inside the tubular flowers in order to drink a small volume of sugar solution. In the present experiment, bees foraged on a patch with only three semi-tubular flowers. Each forager needed to introduce its head in any of the flowers in order to find the sugar solution that was offered at low flow-rates, that is, below a honeybee's maximal intake rate (i.e., 60 µl/min for 50% w/w sucrose solution; Núñez 1966). Thus, the bees' ensuing intake rate was maximal in the experiment of chapter 1, but not in the present experiment. Moreover, in chapter 1, the foragers made nine foraging visits to the patch, whereas in this experiment they visited the patch only four times during training. In spite of these experimental differences, in both cases the bees that had experienced increasing reward levels searched for food more eagerly during the tests than the bees that had experienced decreasing reward levels. Yet, there are differences between the results of these two experiments. For example, the visit times and cumulative inspection times were clearly higher in the experiment of chapter 1. This is probably related to the fact that the patch was larger in such case. Moreover, in chapter 1, the differences between the increasing and decreasing series observed in the first test disappeared in the second test (Fig. 1.2). This did not occur in the present experiment (Fig. 2.1). One wonders whether such difference is due to the differences between the two set-ups or, instead, to other factors like the identity of the colony, the year in which the experiments were conducted, or both. Further experiments are needed to answer these questions.

The present results confirm and extend the concept that honeybees develop long-term reward expectations (Chapter 1, Gil et al. 2007). Expectations of reward are seen as behavioural adjustments which depend upon the formation and subsequent activation of memories about specific reward properties, whose retrieval is triggered in the absence of reinforcement by the cues and events which predict it (Tolman 1959, Logan 1960, Schultz 2000). Because flowers produce nectar at low and variable flow-rates (Núñez 1977, Vogel 1983, Baker and Baker 1983), one sees that a forager's ability to expect future rewards will make it more likely for it to compete with other

flower pollinators for limited resources. In this scheme, honeybees will make full use of past information about food as to finally gain an advantage in cost effectiveness during flower inspection. In a honeybee colony, each forager works in a way that optimizes the food collection of the whole group (Seeley 1995). It would be interesting to investigate how the whole colony benefits from a forager's ability to develop reward expectations; for example, by studying the behaviour of honeybees that forage on multiple feeders offering increasing, decreasing and constant reward levels.

Theory poses that foragers in general assess patch quality using an optimisation rule which tends to maximize their rates of energy gain during foraging (Charnov 1976). Accordingly, each forager first sets a threshold level of net energy gain. If it visits an above-level patch, then it forages until its level falls below expectation. By contrast, the forager abandons the patch when it is below-level. Hence, food availability will determine a forager's investments of time/energy during food collection (Charnov 1976). But optimal foraging theory does not capture how foragers control these investments in the absence of reward (Pyke 1984), although effort has been made to incorporate how learning and memory adapt to the problem of foraging (e.g., Kamil and Roitblat 1985, Devenport and Devenport 1994). A comprehensive model about how foraging decisions adapt to past reward variations is still lacking, and new observational and theoretical evidence is necessary to explain how honeybees and other animals adjust their behaviours in relation to the magnitude and sign of past variations in the level of reward.

The results of these two initial chapters bring up the question of how a honeybee's expectation of reward can be studied under laboratory conditions. In the next chapters, I present laboratory procedures that proved suitable to examine behavioural adaptations depending on memories of specific reward properties. Such procedures would be useful to further investigate how honeybees assess the sign and the magnitude of variations in reward level.

Chapter 3

Does an Insect's Unconditioned Response to Sucrose Reveal Expectations of Reward?

Abstract

In this chapter, I asked whether and how a sequence of a honeybee's experience with different reward levels changes its subsequent unconditioned proboscis extension response (PER) to sucrose stimulation of the antennae, 24 hours after training, in the absence of reward, and under otherwise similar circumstances. I found that the bees that had experienced an increasing reward schedule extended their probosces earlier and during longer periods in comparison to bees that had experienced either decreasing or constant reward schedules, and that these effects at a later time depend upon the activation of memories formed on the basis of a specific property of the experienced reward, namely, that its magnitude increased over time. An anticipatory response to reward is typically thought of as being rooted in a subject's expectations of reward. Therefore these results make me wonder to what extent a long-term 'anticipatory' adjustment of a honeybee's PER is based upon an expectation of reward.

Introduction

In the experiments presented in chapters 1 and 2, I trained honeybees to collect sugar solution in settings closely resembling natural foraging situations, and tested whether their sequence of experience with different levels of reward changed their subsequent foraging behaviour, in the absence of reward and under otherwise similar circumstances. I found that those bees that had experienced increasing reward levels during training assigned more time to flower inspection when tested 24 and 48 hours after training. These animals behaved differently neither because they were fed more or faster nor because they had more strongly associated the related predicting signals, thereby indicating that bees can develop long-term

expectations of reward, in that their behaviour in the absence of reinforcement can be the subject of changes at a later time on the basis of a specific property of an experienced reward, namely, that its magnitude increased over time. Indeed, the term 'reward expectation' refers to behavioural adaptations that depend upon the formation and subsequent activation of memories about specific properties of a given reward, whose recollection is eventually triggered in the absence of reinforcement by the cues and events predicting such a reward (Tolman 1959, Logan 1960). Eventually, an utterly important first step to elucidate the neural mechanisms underlying such a form of learning is to develop a laboratory procedure suitable to examine behavioural adaptations depending on memories of specific reward properties. This would allow experiments based on pharmacological and electrophysiological approaches. I took advantage of the honeybees' proboscis extension response, or PER (Takeda 1961, Kuwabara 1957), in order to develop such a procedure.

A honeybee's PER possesses at least two features indicating that it might constitute a suitable behavioural response to reveal memories about specific reward properties in the laboratory. First, PER in non-satiated honeybees is reflexively elicited when chemoreceptors in the animals' antennae, proboscis and tarsi are stimulated with sucrose (Kuwabara 1957). Sugar solution is a honeybee's primary source of energy, and sucrose thus acts as an appetitive stimulus; this reflects response specificity. Second, a PER's motor program consists of at least three phases: extension, repeated licking and retraction (Rehder 1987). These three phases have different thresholds and require integration of internal state conditions, evaluation of external stimuli, and muscle coordination. The variability of the temporal pattern and the strength of the motor response, in relation to both the nature of the stimulus that releases it and the subject's experience with such stimulus, have been described elsewhere (Rehder 1987, Smith and Menzel 1989, Haupt 2004). What is important here is that a honeybee's PER is a rather flexible -unconditioned- response whose innately defined parameters can subsequently be calibrated through learning. Based on these two features, response specificity and behavioural flexibility, I benefited from an experimental design analogous to that of my experiments with free-flying bees (Chapters 1 and 2, Gil et al. 2007), and asked whether and how a sequence of a

honeybee's experience with different reward magnitudes changes its subsequent proboscis extension response to sucrose stimulation of the antennae, in the absence of reward and under otherwise similar circumstances.

Methods

To this end, I caught honeybees (*Apis mellifera carnica*) at a hive's entrance and harnessed them in metal tubes by strips of tape between their head and thorax, so that they could freely move their antennae and mouthparts. After harnessing, I placed the bees in racks, fed them with 10 µl of 1.2 M sucrose solution, and kept them overnight in a dark humidified chamber. I presented the bees with three 'training' trials during the next morning. In the study of associative learning in honeybees, the term 'training' trial often refers to a CS-US presentation; here, however, it specifically refers to the sucrose stimulation of an animal's antenna and the subsequent presentation of a given volume of sugar solution to its proboscis. Such a distinction is important because my analysis focused on a honeybee's PER as an 'unconditioned' response to sucrose stimulation of the antenna. While the inter-trial interval lasted 10 minutes, each training trial lasted approximately 30 s. Removing a bee from a rack to the training site was followed by a 10 s accommodation period, after which I first stimulated one of its antennae for 2 s by touching it with a toothpick soaked in an unscented, 1 M sucrose solution, and then fed the animal for 10 s with a given volume of the same sucrose solution delivered to its proboscis by means of a micrometer syringe. After the 10 s feeding period, the bee remained in the training site for 7 s, and was then placed back in the rack. In order to leave aside possible side-specific effects of sucrose stimulation of the antenna on the development of memories about specific reward properties, I always presented only one, either left or right, of an animal's antennae with sucrose solution during both training and testing.

I performed two variable and three constant experimental series. They differed in the volume of sucrose solution that the bees received throughout the three consecutive training trials. In the variable series, I offered either increasing (small-medium-large) or decreasing (large-medium-small) volumes of sugar solution

throughout the three training trials. The bees in the increasing series received 0.4 µl, 1 µl and 1.6 µl, while the bees in the decreasing series received 1.6 µl, 1 µl and 0.4 µl in the first, second and third trial, respectively. Both series thus offered the same volume of sugar solution during training. In the constant series, I offered the same volume of sugar solution (small, medium or large) during the three successive training trials, and the bees of the 'small', 'medium' and 'large' series received 0.4 µl, 1 µl and 1.6 µl of sugar solution per trial, respectively. The evening following training, bees were fed with 5 µl of 1.2 M sucrose solution, and kept overnight inside a dark humidified chamber. To feed the bees after both harnessing and training, I released their PERs by stimulating their proboscis with sugar solution, instead of their antennae, thereby avoiding triggering PERs in a way similar to that of training. I tested the animals 24 h after training. Testing consisted of a 10 s accommodation period followed by a 2 s stimulation of the antenna similar to that of training. During testing, I video-recorded the animals' proboscis extension responses at 30 frames s^{-1}. Subsequently, I quantified measures arising from the animals' responses to sucrose stimulation by analysing the videos frame by frame. Bees that did not respond to sucrose stimulation during training were excluded from the analysis. I focused on several parameters related to the animals' PER's motor program. Thus, I measured a PER's 'reaction-time' (in ms), as the time elapsed between the onset of sucrose stimulation of the antenna and the first movement of the proboscis, provided that such movement subsequently led to a successful extension of the animal's proboscis, scored as such if the proboscis crossed an imaginary line between the tips of the opened mandibles. I also estimated a PER's strength by measuring: 1) the number of times that a bee extended its proboscis during testing, or '#PE', 2) the mean duration of the proboscis extension, or 'mean PE', 3) the cumulative duration of the proboscis extension, or 'CPE', 4) the number of licking events, or '#L', as the number of exposures of the animal's glossa, 5) the mean duration of licking, or 'mean L', and, finally, 6) the cumulative duration of licking, or 'CL'. It has been reported bees may differ with respect to their responsiveness to sucrose solution (Page et al. 1998), and that such responsiveness may influence how well a bee can learn and remember tactile stimuli (Scheiner et al. 2005). Before training, therefore, I tested the bees for

their spontaneous responsiveness to sugar solutions of different sucrose concentrations, and then assigned the subjects to the different experimental series so that each series involved a similar proportion of bees from the different sucrose responsiveness categories previously defined. Later on, however, I pooled data from animals with different sucrose responsiveness, simply because their performance during both training and testing was invariant to such responsiveness (data not shown).

Data did not fulfil the requirements of parametric tests and were then analysed by means of Kruskal-Wallis tests, Dunn's multiple comparison, and Bartlett test (with the corresponding alpha level adjustment).

Results

All the bees extended their probosces successfully during the experiments. I found a significantly shorter reaction-time in the bees of the increasing series, in comparison to that of the bees of the decreasing and the constant series (Fig. 3.1 A). Moreover, an analysis of the cumulative frequencies of the 'CPE' durations from the different series showed that the bees of the increasing series were more likely to extend their proboscis during longer periods, in comparison to the bees of the remaining series (Fig. 3.1 B). Thus, 'CPE' had a higher variance in the increasing series than in the decreasing, small and medium series, and such variance did not change across the constant series (Bartlett test, $P_{I \text{ vs. } D} < 0.0001$, $P_{I \text{ vs. } S} = 0.002$, $P_{I \text{ vs. } M} < 0.0001$, $P_{I \text{ vs. } L} = 0.02$, $P_{D \text{ vs. } S} = 0.1$, $P_{D \text{ vs. } M} = 0.3$, $P_{D \text{ vs. } L} = 0.0009$, $P_{S \text{ vs. } M} = 0.06$, $P_{S \text{ vs. } L} = 0.1$, $P_{M \text{ vs. } L} = 0.001$; differences should be taken as significant only if $P < 0.005$). The mean values of '#PE', 'mean PE', 'CPE', '#L', 'mean L', and 'CL' did not change across series (Table 3.1).

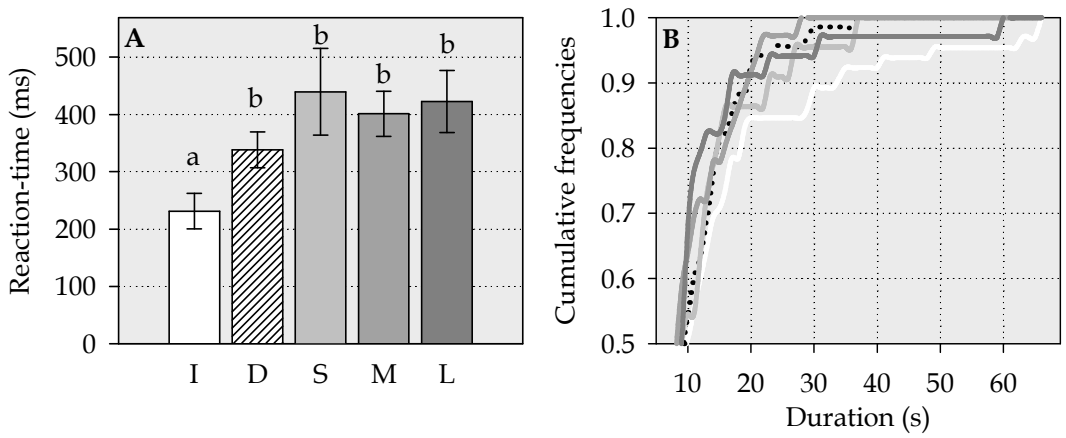

Figure 3.1 A) Means (± s.e.m) of the PE reaction-time (in ms). **B)** Cumulative frequencies of the PE cumulative duration (CPE, in seconds). The data from the different series are shown separately: white, dashed, light-grey, grey and dark-grey bars and lines correspond to the increasing ($N_I = 63$), decreasing ($N_D = 68$), small ($N_S = 22$), medium ($N_M = 35$) and large ($N_L = 34$) series, respectively. In **A**, different letters indicate statistical differences across series: Kruskal-Wallis test, $H = 26.66$, $P < 0.001$; Dunn's multiple comparisons $P < 0.001$.

Table 3.1 Mean values (± s.e.m.) of variables characterizing a PER's strength.

	Variable series		Constant series			Kruskal-Wallis test
	Increasing	**Decreasing**	**Small**	**Medium**	**Large**	
#PE	1.9 ± 0.2	1.5 ± 0.1	1.7 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	H=0.7, P=0.9
Mean PE (s)	9.9 ± 1.5	8.3 ± 0.8	7.9 ± 1.6	6.8 ± 0.8	7.8 ± 1.7	H=2.9, P=0.5
CPE (s)	14.6 ± 1.8	10.9 ± 0.8	11.2 ± 1.7	9.9 ± 1.0	10.7 ± 1.8	H=2.2, P=0.7
#L	7.4 ± 1.8	8.2 ± 1.3	4.5 ± 2.1	5.3 ± 1.0	7.2 ± 2.9	H=3.3, P=0.5
Mean L (ms)	373.6 ± 31.9	377.1 ± 25.7	445.1 ± 44.4	359.1 ± 18.5	322.1 ± 28.5	H=7.0, P=0.1
CL (s)	2.7 ± 0.7	2.9 ± 0.5	2.0 ± 0.8	2.0 ± 0.4	3.6 ± 2.1	H=3.4, P=0.5

(#PE) number of proboscis extensions; (mean PE) means duration of the proboscis extension; (CPE) cumulative duration of the proboscis extension; (#L) number of licking events; (mean L) mean duration of licking; (CL) cumulative duration of licking.

Discussion

I found that the bees that had experienced an increasing reward schedule extended their probosces earlier and during longer periods in comparison to bees that had experienced either decreasing or constant reward schedules (Fig. 3.1). The different performance of the bees of the increasing and decreasing series cannot be accounted for by assuming that their behaviour during testing reflects their most recent reward experience. By this argument, the bees of the decreasing series might only retain information on the small volume, and the bees of the increasing series might only retain information on the large volume; next, their behaviour during testing should

be controlled by this information. If this were the case, similar results must be expected between the large and the increasing series, and between the small and decreasing series, as well as differences among the constant series. Nevertheless, I found differences in the reaction-time between the animals of the increasing and the large series, and neither the reaction-time nor the CPE changed across the constant series (Fig. 3.1). Similarly, these results cannot be explained on the basis of the total amount of reward that the bees received during training. If this were the case, the bees of the constant series should have behaved differently during testing, because they had attained different volumes of sugar solution during training, and the bees of the increasing and decreasing series should have behaved similarly during testing, because they had attained similar volumes of solution during training. Clearly, this has not been the case (Fig. 3.1). In principle, multiple exposures to sucrose might provide an opportunity for habituation to such a stimulus. Therefore the increasing series could eventually be interpreted by some as less affected by habituation than the other series. However, the differences that I found among the several experimental series can not be explained in this way, simply because habituation of the sucrose response in bees requires tens of stimulation repetitions (Braun and Bicker 1992). Taken together, therefore, these results unambiguously document that an increasing reward schedule has long-term effects on the 'eagerness' and the 'strength' of a honeybee's proboscis extension response to stimulation of the antennae, and indicate that these effects at a later time depend upon the activation of memories formed on the basis of a specific property of the experienced reward, namely, that its magnitude increased over time.

These results resemble my findings with free-flying bees (Chapters 1 and 2, Gil et al. 2007) in that specific long-term reward memories lead to later behavioural adjustments in the absence of reinforcement. In principle, the experimental design might have also allowed us to reveal specific reward memories arising from a decreasing reward schedule. If the effects of such memories on a bee's PER to sucrose stimulation were symmetrically opposite to those of the memories arising from an increasing reward schedule, then the bees exposed to a decreasing reward schedule would have shown longer reaction times and shorter PE durations, in comparison to

measures from the bees that had been exposed to either increasing or constant reward schedules. The results do not support this view, however, since I found no difference among the subjects of the decreasing and the constant groups. Since all the bees included in the present analysis successfully extended their probosces during testing, one possible explanation for such a lack of differences is that the system controlling both the reaction-time and duration of a honeybee's PER is much more sensitive to positive than to negative changes in reward magnitude. If this were the case, using larger differences in reward magnitude would be useful to reveal possible effects of a decreasing reward schedule on a honeybee's PER. In the experiments with free-flying bees of chapter 1 (Gil et al. 2007), it was also an increasing reward schedule during training, and not a decreasing one, that had long term effects on the bees' subsequent behaviour during testing. Yet, because non-satiated bees extend their probosces reflexively in response to sucrose stimulation of the antenna, it might well have happened that a form of ceiling effect prevented me from detecting the effects of a decreasing reward schedule on a honeybee's PER. Characterizing the PERs of untrained honeybees would prove helpful to distinguish among these and other hypotheses. Eventually, it would also be interesting to examine whether and how a PER's reaction-time changes during training, and how the magnitude and frequency of reward variations relate to the adjustment a honeybee's PER.

The procedure I present here can be improved by increasing the spatial and temporal precision of the sucrose stimulation of the antenna. A substitution of the movements of the proboscis by the activity of a muscle responsible for such movements, called M17 (e.g., Rehder 1987, Smith and Menzel 1989, Haupt 2004), would also prove fruitful for further analyses of the neural substrates underlying long-term adjustments of a honeybee's PER. This is important because honeybees allow recording neuronal activity over long periods of time (e.g., Okada et al. 2007), making it possible to trace the neural substrates of learning related plasticity. Moreover, global and local injections of pharmaca into a honeybee's brain allow manipulating transmitter and modulator systems (Hammer and Menzel 1998). This would help in characterizing the circuitry underlying a form of reward anticipation as revealed in the present context. Interestingly, elements of the pathway mediating a

PE's response to sucrose have already been identified (Rehder 1989, Haupt 2007, Schröter and Menzel 2003), and the same holds true for its modulatory actions on additional pathways (Bicker and Menzel 1989, Hammer 1993, Schröter et al. 2007). Evidence supports the view that neurons of the VUM system of the suboesophageal ganglion (Schröter et al. 2007) encode the reinforcing function of sucrose reward in olfactory conditioning (Hammer and Menzel 1998, Hammer 1993, Hammer and Menzel 1994, Hammer 1997, Hammer and Menzel 1995), and it will be a task for future research to record and pharmacologically manipulate such neurons in order to search for neural correlates of reward memory. In addition, my experiments with free-flying bees showed that reward memories arising from increasing reward schedules are independent of classical and/or operant associations between an initially meaningless visual stimulus and the offered reward (Chapter 1, Gil et al. 2007). Further experiments combining conditioning of a honeybee's PER (Takeda 1961, Kuwabara 1957, Bitterman et al 1983, Erber et al. 1997, Hori et al 2006) and reinforcing schedules of variable reward levels would help to elucidate whether and to what extent variable reward schedules influence a conditioned PER. Moreover, Pavlovian conditioning does not require that the CS be initially neutral. It is a matter of experimental convenience that one usually uses a stimulus that does not elicit any unconditioned response because this makes it easier to demonstrate emergence of the CR to that CS. Hence, I might ask whether the application of sucrose solution on a honeybee's antennae could also serve as a CS for subsequent reward. In fact, water vapour emanating from a drop of sucrose solution may reach the antennae immediately before sucrose stimulation, and water vapour is known to act as a CS (Kuwabara 1957). This is also the case of the mechanical stimulation of the antennae (Giurfa and Malun 2004). In the present context, such forms of CS/US conditioning would have happened in all of the experimental groups, and there is no reason why the increasing group should have associated the CS component of sucrose stimulation of the antenna more strongly than the other groups. Still, it will be a task for future research to study the potential effect of Pavlovian conditioning on increasing reward schedules, and vice-versa.

Apparently, animals assign rewards with 'motivational values' (Schultz 2006) depending on the probability, quality and quantity of such reward. It is said that varying a reward's subjective value can lead to the adjustment of an animal's anticipatory response to such reward. The adjusted response is, in addition, typically thought of as being rooted in the subject's already developed expectation of reward (Schultz 2006). I suggest that when a harnessed bee extends its proboscis reflexively in response to sucrose stimulation of the antenna and receives either variable or constant volumes of sucrose solution throughout several trials, a built-in 'change detector' computes the difference in volume across trials. An internal estimate of an expected reward follows the detection of changes in reward magnitude. Such estimate is then combined with additional inputs determining a subjective evaluation of reward, and, finally, a 'motivational value' arises from such evaluation. A reward of increasing magnitude is assigned with a high motivational value, and this leads, in turn, to the adjustment of the animal's PER. Expectations of reward are thought to be part and parcel of a set of rules controlling goal-seeking behaviours, and one should ask to what extent a long-term adjustment of a honeybee's PER is rooted in a form of expectation of reward. Honeybees already proved fruitful to study how brain connectivity is eventually mapped to behaviour (e.g., Menzel 1990, 2001), meaning that, if that were the case, a rather simple unconditioned response would help to identify, and eventually also to characterize, the neural correlates of such a form of learning in the honeybee brain.

Chapter 4

Side-Specific Reward Memories in Honeybees

Abstract

Here, I report a hitherto unknown form of side-specific learning in honeybees. I trained bees individually by coupling gustatory and mechanical stimulation of each antenna with either increasing or decreasing volumes of sucrose solution offered to the animal's proboscis along successive learning trials. Next, I examined their proboscis extension response (PER) after stimulation of each antenna 1, 2, 3 and 24 h after training. I found that the bees extended their probosces earlier after stimulation of the antenna that had been coupled with increasing volumes than after stimulation of the antenna that had been coupled with decreasing volumes, thereby revealing short- and long-term side differences in the their PE reaction-time. The bees' reaction-time correlated well with the reaction-time of the muscles M17. Long-term side differences in reaction-time were prevented by repetitive antennal stimulation. Mechanosensory input was indispensable and sufficient for revealing side differences in reaction-time. Such differences were specific to the gustatory input that the bees experienced during training. These results show that side differences in the bees' PE reaction-time depend upon the activation of side-specific reward memories. These memories are formed via the combined effect of a specific property of reward, i.e., that its magnitude increases or decreases over time, and side information seemingly relying on mechanosensory input. Thus, I present a learning procedure suitable to study reward memories in honeybees which includes precise behavioural measures, physiological correlates of behaviour, and within-animal controls. This procedure will prove fruitful in pharmacological and electrophysiological analyses of the neural substrates underlying reward memories in honeybees.

Introduction

As I already mentioned in the previous chapter, honeybees extend their probosces reflexively when the gustatory receptors of their antennae, proboscis and tarsi are stimulated with sucrose (Kuwabara 1957). This behaviour allows them to gather sucrose solution, which constitutes their primary source of energy and acts as sugar reward in appetitive learning (Takeda 1961). Because it is an innate behaviour which can be calibrated through learning, the honeybees' proboscis extension response (PER) led to a well-established laboratory procedure for the study of learning and memory phenomena (Takeda 1961, Bitterman et al. 1983, Rehder 1987, Smith and Menzel 1989, Haupt 2004). In the previous chapter (Gil et al. 2008), I showed that bees that had been presented with increasing volumes of sugar solution across successive learning trials extended their probosces earlier in delayed tests, in comparison to bees that had been presented with either decreasing or constant volumes of sugar solution. It follows that harnessed bees learn that reward magnitude increases over time and adjust their PERs accordingly.

Learning phenomena limited to input from one side of the sensory system, i.e., side-specific learning, is well documented in honeybees. Honeybees learn side-specific olfactory and mechanical stimulation of their antennae (Macmillan and Mercer 1987, Sandoz and Menzel 2001, Giurfa and Malun 2004). Habituation and sensitisation can also be side-specific (Braun and Bicker 1992, Sandoz et al. 2002). Thus, the wonder arises as to whether honeybees are able to learn side-specifically that reward magnitude increases or decreases over time. Here, I asked whether honeybees associate the stimulation of each of their antenna with either increasing or decreasing volumes of sugar solution so as to subsequently adjust their PERs depending on which antenna is stimulated. To answer this question, I developed a side-specific training procedure in which gustatory and mechanical stimulation of each of a honeybee's antenna is coupled with either increasing or decreasing volumes of sugar solution offered to the animal's proboscis throughout a series of consecutive training trials. Using side-specificity, I incorporated within-individual controls into a behavioural procedure which proved suitable for the analysis of behavioural correlates of memories of specific reward properties (Chapter 3, Gil et al. 2008). By

means of such procedure, I asked a number of additional questions: Does such an association lead to short- and long-term memories? How are these side-specific reward memories extinguished? How do they develop during training? Can they be mapped to a physiological measure of behaviour? Moreover, because the stimulation of a honeybee's antenna as in my experiments involved input from both gustatory and mechanosensory receptors, I also asked: What is the role of mechanical stimulation of the antennae in the formation of these side-specific memories? Are the underlying associations specific with respect to the gustatory input? What is the interplay between mechanical and gustatory inputs in the formation and retrieval of these memories? The answers to these questions will contribute to the understanding of how honeybees learn and process side-specific stimuli which are linked to specific rewards.

Methods

I caught honeybees (*Apis mellifera carnica*) at a hive's entrance, and harnessed them in metal tubes by strips of tape between their head and thorax, so that they could freely move their antennae and mouthparts (Bitterman et al. 1983). After harnessing, I placed the bees in racks, fed them with 10 µl of unscented 1.2 M sucrose solution, and kept them overnight in a dark humidified chamber. Next, I presented the bees with six training trials on the following morning. The term 'training trial' refers to the stimulation of a bee's antenna with a toothpick soaked in sucrose solution, and the subsequent presentation of a given volume of sucrose solution delivered to the animal's proboscis. Each training trial lasted approximately 30 s. First, I moved a bee from a rack to the training site. Following a 10 s accommodation period, I stimulated one of its antennae for 2 s by touching it with a toothpick soaked in an unscented 1 M sucrose solution, and then fed the animal for 10 s with a given volume of the same sucrose solution delivered to its proboscis by means of a micrometer syringe. The bee remained in the training site for 8 s after feeding, and was then placed back in the rack. I performed a side-specific training in which the stimulation of each antenna was coupled with either increasing (small: 0.4 µl - medium: 1 µl - large: 1.6 µl) or

decreasing (large: 1.6 µl - medium: 1 µl - small: 0.4 µl) volumes of sucrose solution throughout six consecutive training trials. The inter-trial interval was 10 minutes. The total volume of sucrose solution that each bee received throughout the entire training session was 5 µl. A bee's antennae were stimulated alternately, so that one antenna, either left or right, was stimulated in the 1st, 3rd and 5th training trial, whereas the other antenna was stimulated in the 2nd, 4th and 6th training trial. When, for example, the right and left antennae were assigned to the increasing and decreasing reward schedule, respectively ('increasing antenna' and 'decreasing antenna', respectively), I stimulated the right antenna and fed the bee with 0.4 µl of sucrose solution in the 1st training trial, next, I stimulated the left antenna and fed the bee with 1.6 µl of sucrose solution in the 2nd training trial, and so on, until each antenna was stimulated three times. Half of the bees were presented with an increasing reward schedule following stimulation of the right antenna, whereas the other half with an increasing schedule following stimulation of the left antenna. Also, half of the bees were trained by starting with the small sugar solution volume (increasing reward schedule), and half by starting with the large sugar solution volume (decreasing reward schedule).

In addition, the trained bees were divided into two groups. One group was tested 1, 2, 3 and 24 h after training, while the other group was tested only 24 h after training (henceforth, test 24 $h_{(we)}$ –(we) stands for 'without extinction trials'-). Each test session consisted of a 10 s accommodation period followed by a 2 s stimulation of the antenna. Next, the bee remained in the testing site for 8 s, and was then placed back in the rack. After 20 minutes, the test procedure was repeated with the other antenna. Half of the bees were tested by stimulating first the right and then the left antenna. The other half was tested in the opposite way. Depending on the experimental group (see below), the 2 s stimulation of each antenna was performed with a toothpick soaked in either 1 M sucrose solution, 0.2 M sucrose solution or water, or with a dry toothpick. The evening following the training and the test sessions made 1, 2 and 3 h after training, the bees were fed with 5 µl of unscented 1.2 M sucrose solution and kept overnight inside a dark humidified chamber. To feed the bees after harnessing and training, I released their PERs by stimulating their

proboscis with sucrose solution, instead of their antennae, thereby avoiding triggering their PERs in a way similar to that of the training trials and tests.

I evaluated the performance of six groups of bees trained as described above. The bees of these groups differed in two ways. First, they could be tested with different stimuli, namely, 1 M sucrose, 0.2 M sucrose, water or mechanical stimulation. Second, they could have their antennae immobilized or not. Immobilization of the antennae was performed 30 min prior to either training or testing, depending on the treatment (see below). To immobilize the bees' antennae, I fixed both antennal scapes (the basal segment of the antennae) to the head using acrylic paint, so that the animals could freely move only the flagellum of the antenna. Thus, the six groups were: a) bees that had their antennae free in both training and testing, and that were tested with 1 M sucrose solution; b) bees that had their antennae immobilized in both training and testing, and that were tested with 1 M sucrose solution; c) bees trained with free antennae and tested with immobilized antennae using 1 M sucrose solution; d) bees that had their antennae free in both training and testing, and that were tested 1, 2 and 3 h after training with mechanical stimulation as well as 24 h after training with 1 M sucrose solution; e) bees that had their antennae free in both training and testing and were tested with 0.2 M sucrose solution; f) bees that had their antennae free in both training and testing and were tested with water. Additionally, two groups of untrained bees were fed with 5 µl of unscented 1 M sucrose solution, and subsequently tested with 1 M sucrose solution. One of such group was tested 1, 2, 3 and 24 h after feeding, whereas the other group was tested only 24 h after feeding (test 24 $h_{(we)}$). I also evaluated the PER to 1 M sucrose, 0.2 M sucrose, water and mechanical stimulation of the antennae of bees that were only harnessed, fed with 10 µl of 1.2 M sucrose solution and kept overnight in a dark humidified chamber. I shall refer to these bees as 'naïve bees'. Up to four groups per day were run in parallel and assayed in a semi-random way.

I video-recorded the bees' PERs at 60 frames s^{-1} during the training trials and tests, and subsequently analysed the videos frame by frame. The bees that did not respond to sucrose stimulation during training were excluded from the analysis. I characterized the bees' PER to antennal stimulation using four variables. The first of

such variables (1) was the PE reaction-time (in ms), defined as the time elapsed between the onset of antennal stimulation and the first movement of a bee's proboscis, provided that such movement subsequently led to a successful extension of the bee's proboscis (see below). In each test session, I obtained two reaction-time values per bee, those following stimulation of the bee's 'increasing' and 'decreasing' antennae. For the sake of comparison, I calculated a 'differential reaction-time', as the difference between the reaction-time following stimulation of the decreasing and the increasing antennae *(D - I)*. Next, for the sake of normalization, I divided such difference by the highest reaction-time obtained for each bee in each test *((D - I) / D, if D > I, or (D - I) / I, if I > D)*, which allowed us to express group differences in percentage. The second (2) variable was the PE probability, defined as the proportion of bees that successfully extended their probosces, as calculated from the total number of bees involved in each test. A successful extension was scored as such if the proboscis crossed an imaginary line between the tips of a bee's opened mandibles. Because each animal was tested twice in each test session, I calculated three different PE probabilities: defined as the proportions of animals that responded to a) the stimulation of the increasing antenna, b) the stimulation of the decreasing antenna, and c) the stimulation of both antennae, as calculated from the total number of bees involved in each test. The third (3) variable was the PE duration, defined as the total amount of time during which the bees remained with the proboscis extended within a 60 s time period following antennal stimulation. In parallel to the video recordings, I made electromyogram recordings (EMGs) of the bees' M17 muscles (Rehder 1987), a pair of bilaterally symmetrical muscles involved in the extension of the proboscis (Snodgrass 1956). I made two tiny holes at the level of the lateral ocellus near the dorsal rim of each compound eye, and inserted a metal wire (0.125 mm diameter silver wire) 1-2 mm into each of such holes to record from both M17 muscles. The reference electrode was inserted in one compound eye (Rehder 1987). Recordings were made using a CED micro 1401 interface and Spike2 software (Cambridge Electronic Design, Cambridge UK). In this way, following antennal stimulation I obtained EMGs of both the ipsi- and the contralateral M17 muscles. Thus, I calculated a fourth (4) variable, namely, the M17 reaction-time (in ms, either ipsi- or

contralateral), as the time elapsed between the onset of the antennal stimulation and the first spike of each muscle.

Data were analysed by means of one sample t-test, Wilcoxon signed rank test, two-ways repeated measures ANOVA and Tukey's multiple comparisons, Mann Withney test, t-test, Spearman correlation, G-test. Non parametric tests were used when data did not fulfil the requirements of parametric tests.

Results

Side-specific adjustments of a bee's PE reaction-time

When tested 1, 2 and 3 h after side-specific training, the bees showed a shorter PE reaction-time after stimulation of the antenna that had been coupled with increasing reward volumes than after stimulation of the of the antenna that had been coupled with decreasing reward volumes (Fig. 4.1 A, B, C). The differential reaction-time (i.e., the normalized difference between the reaction-times of the decreasing and the increasing antennae) was 29.9 % (± 6.78), 19.3 % (± 5.81) and 21.0 % (± 6.51) in test 1, 2 and 3 h, respectively. These values were significantly higher than zero (one sample t-test: $t_{(63)} = 4.39$, $P < 0.0001$, $t_{(65)} = 3.32$, $P = 0.001$, $t_{(71)} = 3.22$, $P = 0.002$, respectively). The same bees did not exhibit such side differences when tested 24 h after training (Fig. 4.1 D). The differential reaction-time 24 h after training was 9.31 ± 9.75 %, a value which did not differ from zero (one sample t-test: $t_{(41)} = 0.95$, $P = 0.34$). By contrast, the bees that were tested only once 24 h after training (test 24 $h_{(we)}$) did show side-specific reaction-times (Fig. 4.1 E), and the corresponding differential reaction-time was 18.0 % (± 8.0), a value which was significantly higher than zero (one sample t-test: $t_{(45)} = 2.24$, $P = 0.03$). Thus, in the tests the trained bees extended their proboscis earlier after stimulation of the increasing antenna than after stimulation of the decreasing antenna. These side-specific adjustments of the PE reaction-time can be observed 1, 2, 3 and 24 h after training and are, in the long-term (24 h after training), prevented by repetitive antennal stimulation.

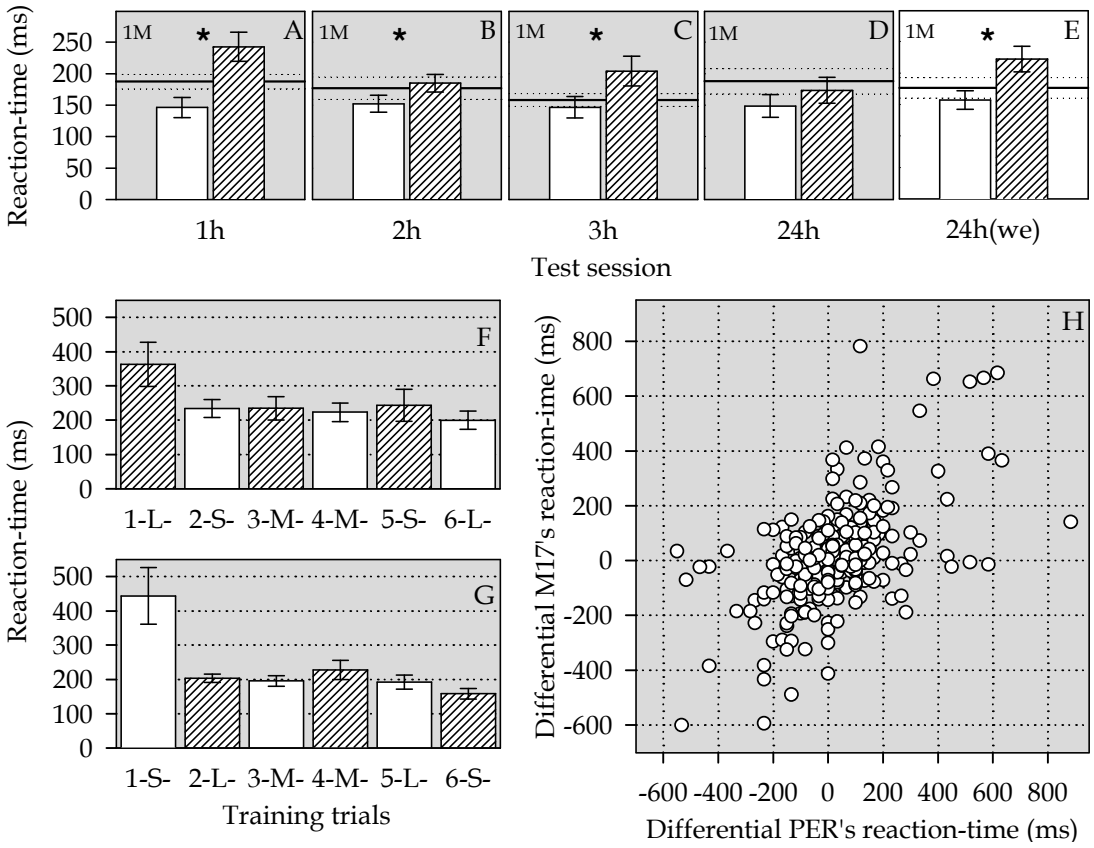

Figure 4.1 Means (± s.e.m) of the PE reaction-time (in ms) following stimulation of the increasing and the decreasing antennae (white and dashed bars, respectively). Data are shown for both testing **(A-E)** and training **(F-G)**. Bees were trained and tested with 1 M sucrose solution. In **A-E**, asterisks indicate statistical differences (Wilcoxon signed rank test) between the reaction-times following stimulation of the increasing and decreasing antennae: **A)** W = -1168, P < 0.0001, N = 64, **B)** W = -760, P = 0.009, N = 67, **C)** W = -994, P = 0.002, N = 72, **D)** W = -155, P = 0.3, N = 42, and **E)** W = -543, P = 0.001, N = 46. Reference lines correspond to the PE reaction-times of untrained bees tested simultaneously (mean ± s.e.m, solid and dotted lines, respectively). **F-G**, Side-specific training consisted of coupling stimulation of each antenna with either increasing or decreasing volumes of sugar solution. It could start either with the smallest (**F**, N = 52) or the largest volume (**G**, N = 59). S, L and M designate the small, medium and large volume of sugar solution, respectively. PE reaction-times during training were analysed by means of two-way repeated-measures ANOVA, with repeated measures on one factor, the training trials; the other being the type of training, i.e., starting with the small or the large reward volume: $effect_{S\ vs\ L}$, $F_{(1,109)} = 0.05$, P = 0.8, $effect_{tt}$, $F_{(5,545)} = 13.4$, P < 0.0001, $effect_{interaction}$, $F_{(5,545)} = 1.24$, P = 0.3. **H)** Relationship between PE and M17 differential reaction-times; each point represents an individual difference between the reaction-time of the decreasing and the increasing antennae (See Methods).

I also asked whether the bees' PE reaction-time changed during training. I did not find side-specific changes throughout training, although the bees' PE reaction-time did change across the several training trials (Fig. 4.1 G-F). The PE reaction-time in the

first trial was higher than those of the remaining trials (Tukey's multiple comparisons, $P_{tt1 \text{ vs. } ttn} < 0.0001$ in all cases). Those of the second trial were higher than those of the last trial, in addition (Tukey's multiple comparisons, $P_{tt2 \text{ vs. } tt6} = 0.005$). I found no differences between those of the remaining trials (Tukey's multiple comparisons, $P > 0.05$ in all cases). Thus, the bees' PE reaction-time diminished by 55 % during training, and I did not observe side-specific differences in PE reaction-time. Moreover, I did not find differences while comparing the data from the bees whose training started with either the small or the large volume of sugar reward (Fig. 4.1 F-G).

I also examined whether the side-specific differences in PE reaction-time arose from either a reduction in the reaction-time of the increasing antenna or, instead, an increase in the reaction-time of the decreasing antenna, or both. To this end, I compared the PE reaction-times of both trained and untrained bees. Untrained bees were fed with the same amount of sucrose solution offered to the trained bees, and their reaction-time was recorded 1, 2, 3, 24 and 24 $h_{(we)}$ after feeding (Fig. 4.2). In the first test, the PE reaction-time of the untrained bees was lower than that of the trained bees following stimulation of their increasing antenna (Fig. 4.1 A, Mann Whitney test: $P_{ut \text{ vs. } I} = 0.005$). It was also higher than that of the trained bees following stimulation of their decreasing antenna (Fig. 4.1 A, Mann Whitney test: $P_{ut \text{ vs. } D} = 0.04$). In the tests performed 2, 3 and 24 h after training, the PE reaction-times of the untrained and the trained bees did not differ from each other (Fig. 4.1 B, C and D respectively, Mann Whitney test: test 2 h: $P_{ut \text{ vs. } I} = 0.4$, $P_{ut \text{ vs. } D} = 0.2$; test 3 h: $P_{ut \text{ vs. } I} = 0.1$, $P_{ut \text{ vs. } D} = 0.2$; test 24 h: $P_{ut \text{ vs. } I} = 0.1$, $P_{ut \text{ vs. } D} = 0.5$). In the test 24 $h_{(we)}$, the PE reaction-time of the untrained bees was lower than that of the trained bees following stimulation of their decreasing antenna (Fig. 4.1 E, Mann Whitney test: $P_{ut \text{ vs. } I} = 0.5$, $P_{ut \text{ vs. } D} = 0.04$). Thus, side-specific differences in PE reaction-time of the trained bees observed in the test performed 1 h after training arose from both a reduction and an increase in the reaction-times that followed the stimulation of the increasing and the decreasing antennae, respectively (Fig. 4.1 A). In the test 24 $h_{(we)}$, such side-specific responses arose from an increase in the reaction-time associated to the stimulation of the bees' decreasing antenna (Fig. 4.1 E).

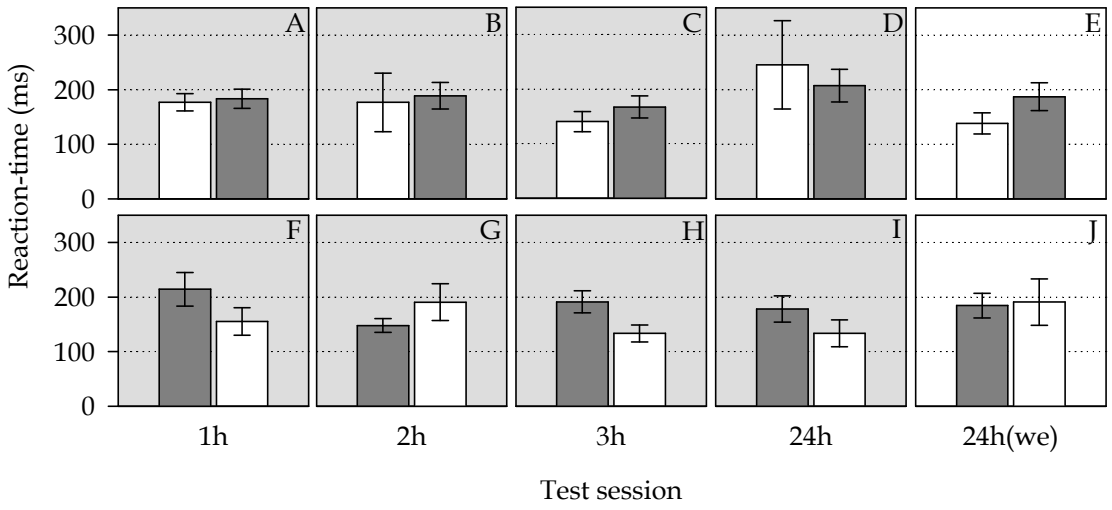

Figure 4.2 Means (± s.e.m) of the PE reaction-time of the untrained bees in ms) following stimulation of the right and the left antennae (white and grey bars, respectively). Data are shown for the different tests. Two different situations were possible during testing: to stimulate first the left and then the right antennae (**A-E,** LR situation), and to stimulate first the left and then the right antennae (**F-J,** RL situation). I made analyses using two-way repeated-measures ANOVA, with repeated measures on one factor, the identity of the antenna (right or left); the other being the test situation, i.e., LR or RL situation: Test 1 h, $effect_{LR\ vs.\ RL}$: $F_{(1,79)} = 0.034$, P = 0.8, effect $_{L\ vs.\ R}$: $F_{(1,79)} = 3.64$, P = 0.06, $effect_{interaction}$ $F_{(1,79)} = 3.59$, P = 0.06, $N_{LR} = 40$, $N_{RL} = 41$, test 2 h, $effect_{LR\ vs.\ RL}$: $F_{(1,63)} = 0.24$, P = 0.6, effect $_{L\ vs.\ R}$: $F_{(1,63)} = 2.06$, P = 0.15, $effect_{interaction}$ $F_{(1,63)} = 0.31$, P = 0.6, $N_{LR} = 35$, $N_{RL} = 30$; test 3 h, $effect_{LR\ vs.\ RL}$: $F_{(1,54)} = 0.1$, P = 0.7, effect $_{L\ vs.\ R}$: $F_{(1,54)} = 1.5$, P = 0.2, $effect_{interaction}$ $F_{(1,54)} = 2.06$, P = 0.1, $N_{LR} = 28$, $N_{RL} = 28$; test 24 h, $effect_{LR\ vs.\ RL}$: $F_{(1,25)} = 2.78$, P = 0.1, effect $_{L\ vs.\ R}$: $F_{(1,25)} = 0.63$, P = 0.4, $effect_{interaction}$ $F_{(1,25)} = 1.94$, P = 0.2, $N_{LR} = 11$, $N_{RL} = 16$; test 24 h $_{(we)}$, $effect_{LR\ vs.\ RL}$: $F_{(1,35)} = 0.3$, P = 0.6, effect $_{L\ vs.\ R}$: $F_{(1,35)} = 0.14$, P = 0.7, $effect_{interaction}$ $F_{(1,34)} = 3.63$, P = 0.07, $N_{LR} = 20$, $N_{RL} = 16$). Because the untrained bees showed similar PE reaction-times irrespective of the antenna and the sequence of sucrose stimulation, I averaged the values of both antennae for each test. These are the means (± s.e.m) of the PE reaction-times of untrained bees represented as reference lines in Fig. 4.1 A-E, Fig. 4.3 A-J and Fig. 4.5 B-F.

In parallel to the video recordings, I measured the activity of the muscles M17 (see Methods). I found that the PE reaction-time correlated well with both the ipsi- and contralateral M17's reaction-times (Spearman correlation: ipsi-: r = 0.43, P < 0.0001, n = 1028; contra-: r = 0.42, P < 0.0001, n = 1013). It also correlated with the average of the ipsi- and contralateral M17's reaction-times (Spearman correlation: r = 0.44, P < 0.0001, n = 1013). Thus, I found a positive correlation between the PE's and M17's differential reaction-times (Fig. 4.1 H, Spearman correlation: r = 0.51, P < 0.0001, n = 327).

The role of mechanosensory input

In my experiments, stimulation of a honeybee's antennae involves inputs from gustatory as well as mechanosensory receptors (Schneider 1964, Markl 1971). I examined the roles of gustatory receptors located alongside the flagellum and of mechanoreceptors located between the scapes and the head in the development of side differences in PE reaction-time. I hampered mechanical inputs by fixing a honeybee's scapes to the head, so that the animal could move only the flagellum of each antenna (see Methods). Next, I examined the behaviour of bees trained and tested with immobilized antennae, and of bees trained with free antennae and tested with immobilized antennae (group b and c respectively, see Methods). The bees did not show side-specific PE reaction-times in any of the several tests if their antennae had been immobilized prior to training (Fig. 4.3 A-E). And their reaction-times did not differ from those of the untrained bees (Fig. 4.3 A-E, Mann Whitney test: test 1 h: $P_{ut\ vs.\ I} = 0.4$, $P_{ut\ vs.\ D} = 0.1$; test 2 h: $P_{ut\ vs.\ I} = 0.5$, $P_{ut\ vs.\ D} = 0.5$; test 3 h: $P_{ut\ vs.\ I} = 0.3$, $P_{ut\ vs.\ D} = 0.4$; test 24 h: $P_{ut\ vs.\ I} = 0.3$, $P_{ut\ vs.\ D} = 0.6$; test $24h_{(we)}$: $P_{ut\ vs.\ I} = 0.9$, $P_{ut\ vs.\ D} = 0.9$). Similarly, they did not show side-specific PE reaction-times when tested 1, 2, 3 and 24 h after training if their antennae had been immobilized just before testing (Fig. 4.3 F-I), and, as before, their behaviour did not differ from that of the untrained bees (Fig. 4.3 F-I, Mann Whitney test: test 1 h: $P_{ut\ vs.\ I} = 0.9$, $P_{ut\ vs.\ D} = 0.9$; test 2 h: $P_{ut\ vs.\ I} = 0.7$, $P_{ut\ vs.\ D} = 0.6$; test 3 h: $P_{ut\ vs.\ I} = 0.3$, $P_{ut\ vs.\ D} = 0.1$; test 24 h: $P_{ut\ vs.\ I} = 0.7$). However, their PE reaction-time following stimulation of the decreasing antenna was significantly higher than that of the untrained bees when tested only once 24 h after training (Fig. 4.3 J, Mann Whitney test: test $24h_{(we)}$: $P_{ut\ vs.\ I} = 0.3$, $P_{ut\ vs.\ D} = 0.02$), although I did not find statistical differences between the corresponding reaction-times. In addition, the bees trained with immobilized and free antennae showed similar reaction-times during training (Two-ways repeated measures: $effect_{fixed\ vs.\ free}$, $F_{(1,160)} = 2.9$, $p = 0.09$, $effect_{tts}$, $F_{(5,800)} = 20.7$, $P < 0.0001$, $effect_{interaction}$, $F_{(5,800)} = 0.29$, $P = 0.9$). Overall, mechanosensory input played a key role in the formation and triggering of side differences in reaction-time. I did not observe short- and long-term side-specific reaction-times with hampered mechanosensory input in both training and testing. Yet, hampering such input only during testing did not prevent me from

recording long-term side differences in reaction-time, indicating that gustatory input was sufficient to trigger long-term side-specific responses.

I also asked whether mechanosensory input is sufficient to trigger side-specific PE reaction-times, and how repetitive stimulation with mechanosensory input affects the corresponding side differences in reaction-time. To answer these questions, I trained bees as before and tested them 1, 2 and 3 h after training with mechanical stimulation only, as well as with 1 M sucrose solution 24 h after training (group d, see Methods). Following mechanical stimulation, I found that the PE reaction-times of both antennae did not differ from each other 1 and 3 h after training (Fig. 4.3 K, M), whereas that the reaction-time of the increasing antenna was shorter than that of the decreasing antenna 2 h after training (Fig. 4.3 L). I did not find side differences in reaction-time when the same bees were tested with 1 M sucrose solution 24 h after training (Fig. 4.3 N). The bees' reaction-times following mechanical stimulation of the antennae were approximately fourfold higher than those which followed sucrose stimulation of the antennae. The percentage of bees that extended the proboscis after mechanical stimulation of both antennae was 12-19% (Fig. 4.5 A-F). Thus, mechanical input alone proved to be sufficient to evince side-specific PE reaction-times. The bees that experienced mechanical stimulation 1, 2 and 3 h after training did not exhibit long-term side differences in reaction-time when tested with sucrose solution. This happened also with the bees which had not responded to mechanical stimulation of the antennae prior to the test performed 24 h after training. Thus, repetitive mechanical input prevented long-term side differences in reaction-time.

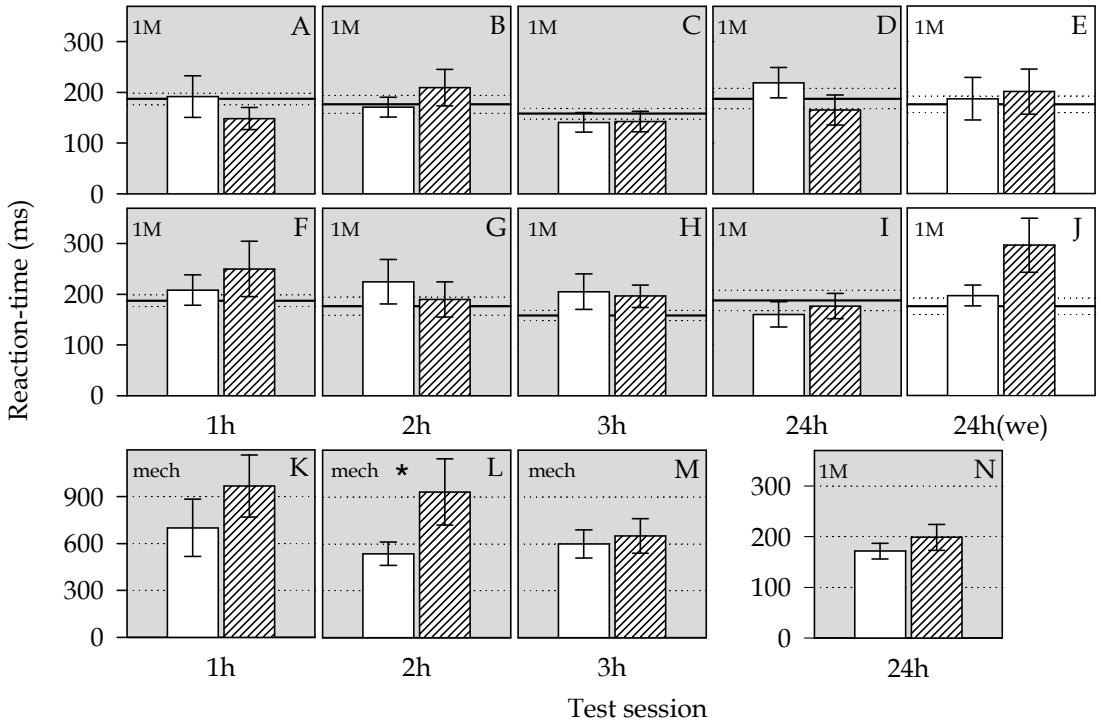

Figure 4.3 Means (± s.e.m) of the PE reaction-time (in ms) following stimulation of the increasing and the decreasing antennae (white and dashed bars, respectively). Data are shown for the different tests. **A-E**, bees with immobilized antennae in both training and testing, and tested with 1 M sucrose solution. **F-J**, bees trained with free antennae and tested with immobilized antennae; they were tested with 1 M sucrose solution. **K-N**, bees trained and tested with free antennae; they were tested 1, 2 and 3 h after training using mechanical stimulation (a dry toothpick) and 24h after training using 1 M sucrose solution. All groups were trained with 1 M sucrose solution. In **A-J**, reference lines correspond to the reaction-time of untrained bees tested simultaneously (mean ± s.e.m, solid and dotted lines, respectively). Asterisks indicate statistical differences (Wilcoxon signed rank test) between the reaction-times which followed stimulation of the increasing and decreasing antennae: **A)** W = 37, P = 0.2, N = 20; **B)** W = -40, P = 0.4, N = 20; **C)** W = -10, P = 0.8, N = 20; **D)** W = 33, P = 0.1, N = 13; **E)** W = -5, P = 0.8, N = 14; **F)** W = 0, P = 0.9, N = 28; **G)** W = 34, P = 0.6, N = 26; **H)** W = 1, P = 0.9, N = 26; **I)** W = -19, P = 0.4, N = 10; **J)** W = -117, P = 0.05, N = 23; **K)** W = -22, P = 0.1, N = 8; **L)** W = -57, P = 0.04, N = 13; **M)** W = -9, P = 0.7, N = 10; **N)** W = -82, P = 0.5, N = 36.

Specificity of the gustatory input

I also asked whether side differences in PE reaction-time can be triggered by gustatory inputs different than that of training. I trained the bees as before with 1 M sucrose solution, and tested them with either 0.2 M sucrose solution or water (group e and f, respectively, see Methods). I did not find side differences in reaction-time after stimulation with either 0.2 M sucrose solution (Fig. 4.4 A-E) or water (Fig. 4.4 F-J). This means that side differences in reaction-time are specific to the gustatory input used during training.

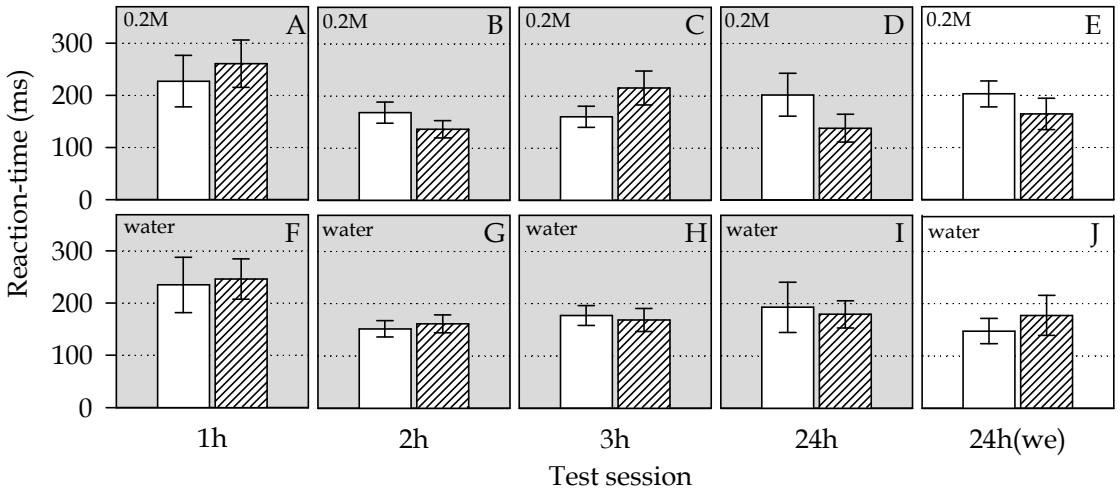

Figure 4.4 Means (± s.e.m) of the PE reaction-times (in ms) following stimulation of the increasing and the decreasing antennae (white and dashed bars, respectively) with either 0.2 M sucrose solution **(A-E)** or water **(F-J)**. Data are shown for the different tests. Bees were trained using 1 M sucrose solution. I found no differences (Wilcoxon signed rank test) in the bees' reaction-times following stimulation of either the increasing or decreasing antennae: **A)** W = -34, P = 0.7, N = 32, **B)** W = -39, P = 0.7, N = 28, **C)** W = 60, P = 0.5, N = 25, **D)** W = -14, P = 0.7, N = 12, **E)** W = -7, P = 0.8, N = 32, **F)** W = -165, P = 0.06, N = 30, **G)** W = 95, P = 0.2, N = 31, **H)** W = 99, P = 0.2, N = 29, **I)** W = 16, P = 0.6, N = 15, **J)** W = 23, P = 0.5, N = 15.

Side-specific adjustments of the PE probability and duration

In addition to the PE reaction-time, I measured the probability and the duration of the bees' PERs. For all the different groups of bees, I did not find side differences in the probability and the duration of PER in any of the tests (Table 4.1 A-B).

Table 4.1 PE probability **(A)**, and means (± s.e.m) of the PE duration **(B)** of the increasing (I) and decreasing (D) antennae for the six groups of trained bees.

A)		**Test session**				
Group		**1 h**	**2 h**	**3 h**	**24 h**	**24 h(we)**
a	**I**	0.95	0.97	1	0.95	1
	D	0.95	0.94	1	0.95	1
		P = 1	*P = 0.99*	*P = 1*	*P = 1*	*P = 1*
b	**I**	0.93	1	1	1	1
	D	1	0.96	1	0.83	1
		P = 0.72	*P = 0.92*	*P = 1*	*P = 0.70*	*P = 1*
c	**I**	1	1	1	1	0.93
	D	1	0.95	1	0.93	1
		P = 1	*P = 0.92*	*P = 1*	*P = 0.92*	*P = 0.92*
d	**I**	0.22	0.27	0.28		
	D	0.25	0.40	0.33		
		P = 0.92	*P = 0.26*	*P = 0.85*		
e	**I**	0.94	0.92	1	1	0.96
	D	0.97	1	0.94	0.95	0.76
		P = 0.99	*P = 0.51*	*P = 0.72*	*P = 0.92*	*P = 0.47*
f	**I**	0.93	0.83	0.76	0.93	0.74
	D	0.90	0.83	0.76	0.93	0.74
		P = 0.99	*P = 1*	*P = 1*	*P = 1*	*P = 1*
B)		**Test session**				
Group		**1 h**	**2 h**	**3 h**	**24 h**	**24 h(we)**
a	**I**	11.16 ± 1.43	13.64 ± 1.45	13.28 ± 1.55	18.44 ± 3.16	19.79 ± 2.52
	D	11.16 ± 1.33	12.73 ± 1.29	15.59 ± 2.04	19.39 ± 2.34	17.06 ± 2.31
		P = 0.57	*P = 0.45*	*P = 0.34*	*P = 0.39*	*P = 0.08*
b	**I**	14.83 ± 3.21	12.22 ± 3.03	15.97 ± 3.19	17.19 ± 3.39	19.75 ± 3.39
	D	12.64 ± 2.12	9.46 ± 1.69	11.10 ± 2.57	13.23 ± 2.24	24.0 ± 5.06
		P = 0.68	*P = 0.93*	*P = 0.96*	*P = 0.43*	*P = 0.18*
c	**I**	7.93 ± 0.83	12.23 ± 1.54	10.60 ± 1.34	22.93 ± 5.28	17.24 ± 2.08
	D	9.72 ± 1.54	11.77 ± 1.51	11.20 ± 1.21	17.27 ± 2.68	13.37 ± 1.99
		P = 0.64	*P = 0.90*	*P = 0.09*	*P = 0.34*	*P = 0.73*
d	**I**	2.29 ± 0.44	3.83 ± 0.86	3.23 ± 0.71		
	D	3.30 ± 1.23	2.56 ± 0.64	4.84 ± 2.10		
		P = 0.96	*P = 0.32*	*P = 0.71*		
e	**I**	4.87 ± 0.45	7.84 ± 1.12	9.30 ± 1.61	13.21 ± 3.73	11.12 ± 3.66
	D	4.97 ± 0.74	9.07 ± 0.93	9.06 ± 1.13	8.72 ± 1.52	8.49 ± 2.56
		P = 0.61	*P = 0.11*	*P = 0.42*	*P = 0.32*	*P = 0.67*
f	**I**	5.67 ± 0.71	7.76 ± 1.06	10.34 ± 2.49	6.63 ± 2.18	7.08 ± 0.95
	D	4.61 ± 0.57	7.49 ± 1.08	8.33 ± 1.60	7.05 ± 1.85	7.09 ± 1.09
		P = 0.25	*P = 0.21*	*P = 0.85*	*P = 0.91*	*P = 0.68*

P values correspond to G-tests (A) and Wilcoxon signed rank tests (B). (a) Bees trained and tested with free antennae and tested with 1 M sucrose solution; (b) bees trained with fixed antennae and tested with 1 M sucrose solution; (c) bees trained with free antennae and tested with fixed antennae and 1 M sucrose solution; (d) Bees trained and tested with free antennae and tested using mechanical stimulation 1, 2 and 3 h after training; (e) Bees trained and tested with free antennae and tested with 0.2 M sucrose solution; (f) Bees trained and tested with free antennae and tested with water.

The reaction-time, probability and duration of a bee's PER

Next, irrespective of the input side, I asked whether the reaction-time, probability and duration of a bee's PERs to the different stimuli changed before and after training. I also compared the performance of naïve and trained bees. Before testing, the naïve bees were neither trained nor fed (see Methods). The trained bees were tested with either 1 M or 0.2 M sucrose solution, water, or mechanical stimulation (groups a, e, f, d respectively, see Methods). I compared: 1) the mean PE reaction-time, as the average of the values from both antennae; 2) the PE probability, as the proportion of bees which showed PER after stimulation of both antennae, calculated from the whole amount of tested bees; and 3) the mean PE duration, as the average of the values from both antennae.

I first compared these variables across stimuli, i.e., as recorded from the bees PERs to the different stimuli, in both naïve and trained bees. I did not find differences in the mean PE reaction-time after stimulation with 1 M, 0.2 M sucrose solution and water (Fig. 4.5 A-F), and the corresponding values were lower than that recorded after mechanical stimulation of the antennae (Fig. 4.5 A-F). The PE probabilities and durations were maximal after stimulation with 1 M sucrose stimulation, intermediate with 0.2 M sucrose solution and water, and minimal with mechanosensory input alone (Fig. 4.5 G-R).

Next, for each stimulus, I made comparisons between these three measures as recorded from the naïve and trained bees. I found that, irrespective of the stimuli used during testing, training induced changes in these three variables. In all the tests, the mean PE reaction-time of the trained bees after stimulation with either 1 M, 0.2 M sucrose solution or water was lower than that of the naïve bees (Fig. 4.5 A-F, Kruskal Wallis test, 1 M: $H_6 = 128.6$, $P < 0.0001$; 0.2 M: $H_6 = 14.6$, $P = 0.01$; water: $H_6 = 11.5$, $P = 0.04$, Dunn multiple comparisons $P < 0.05$ in all cases). The mean PE reaction-time after mechanical stimulation appeared to not have changed after training, although reduced samples did not allow statistical comparisons. The PE probability following stimulation with 1 M sucrose solution was similar in the naïve and trained bees in the tests performed 1, 2 and 24 h after training, and significantly higher in the trained

bees in the tests performed 3 and 24 $h_{(we)}$ after training (Fig. 4.5 A-F, G-test: $G_{naïve\ vs.\ 1h}$ = 6.75, P = 0.2; $G_{naïve\ vs.\ 2h}$ = 3.64, P = 0.6; $G_{naïve\ vs.\ 3h}$ = 13.5, P = 0.001; $G_{naïve\ vs.\ 24h}$ = 1.9, P = 0.8; $G_{naïve\ vs.\ 24h(we)}$ = 16.3, P = 0.005; df = 5). The PE probability following stimulation with 0.2 M sucrose solution was higher in the trained than in the naïve bees in the tests performed 1, 2, 3 and 24 h after training, and did not differ between these two groups in the test performed 24 $h_{(we)}$ after training (Fig. 4.5 A-F, G-test: $G_{naïve\ vs\ 1h}$ = 27.7, P < 0.0001; $G_{naïve\ vs.\ 2h}$ = 27.7, P < 0.0001; $G_{naïve\ vs.\ 3h}$ = 25.7, P < 0.0001; $G_{naïve\ vs.\ 24h}$ = 19.9, P = 0.001; $G_{naïve\ vs.\ 24h(we)}$ = 7.85, P = 0.1; df = 5). The PE probability following either mechanical stimulation or water was higher in the trained than in the naïve bees in all the different tests (Fig. 4.5 A-F, water: G test: $G_{naïve\ vs.\ 1h}$ = 55.2, P < 0.0001; $G_{naïve\ vs.\ 2h}$ = 51.7, P < 0.0001; $G_{naïve\ vs.\ 3h}$ = 50.7, P < 0.0001; $G_{naïve\ vs.\ 24h}$ = 37.2, P < 0.0001; $G_{naïve\ vs.\ 24h(we)}$ = 36.1, P < 0.0001; df = 5; mechanical: $G_{naïve\ vs.\ 1h}$ = 14.47, P < 0.0001; $G_{naïve\ vs.\ 2h}$ = 24.42, P < 0.0001; $G_{naïve\ vs.\ 3h}$ = 19.08, P < 0.0001; df = 2). Also, the mean PE duration following stimulation with either 1 M or 0.2 M sucrose solution or water was higher in the trained bees than in the naïve bees in all the different tests (Fig. 4.5 G-L, Kruskal Wallis test: 1 M: H_6 = 41.9, P < 0.0001; 0.2 M: H_6 = 42.4, P < 0.0001; water: H_6 = 24.0, P = 0.0002, Dunn multiple comparisons P < 0.05 in all cases). I found a similar tendency while comparing the data from the trained and naïve bees which were mechanically stimulated, although reduced samples prevented statistics.

Finally, I further examined the effect of training by comparing the performance of untrained (Fig 4.2) and trained bees tested with 1 M sucrose solution. The mean PE reaction-time and the PE probability did not differ between the untrained and trained bees in all the different tests (Fig. 4.5 B-F reference lines vs. first bar, Mann Whitney test: U_{1h} = 2722, P = 0.9, U_{2h} = 2224, P = 0.8, U_{3h} = 2118, P = 0.9, U_{24h} = 494, P = 0.1, $U_{24h(we)}$ = 882, P = 0.7; Fig 4.5 H-L reference lines vs. first bar, G test, G_{1h} = 3.6, P = 0.6, G_{2h} = 0.08, P = 1, G_{3h} = 10.7, P = 0.06, G_{24h} = 0.4, P = 1, $G_{24h(we)}$ = 8.2, P = 0.1). Also, the mean PE duration was significantly lower in the untrained bees than in the trained bees in all the different tests (Fig. 4.5 G-L, reference lines vs. first bar, Mann Whitney test: U_{1h} = 1639, P = 0.0009, U_{2h} = 1140, P < 0.0001, U_{3h} = 1254, P = 0.002, U_{24h} = 355, P = 0.02, $U_{24h(we)}$ = 540, P = 0.003).

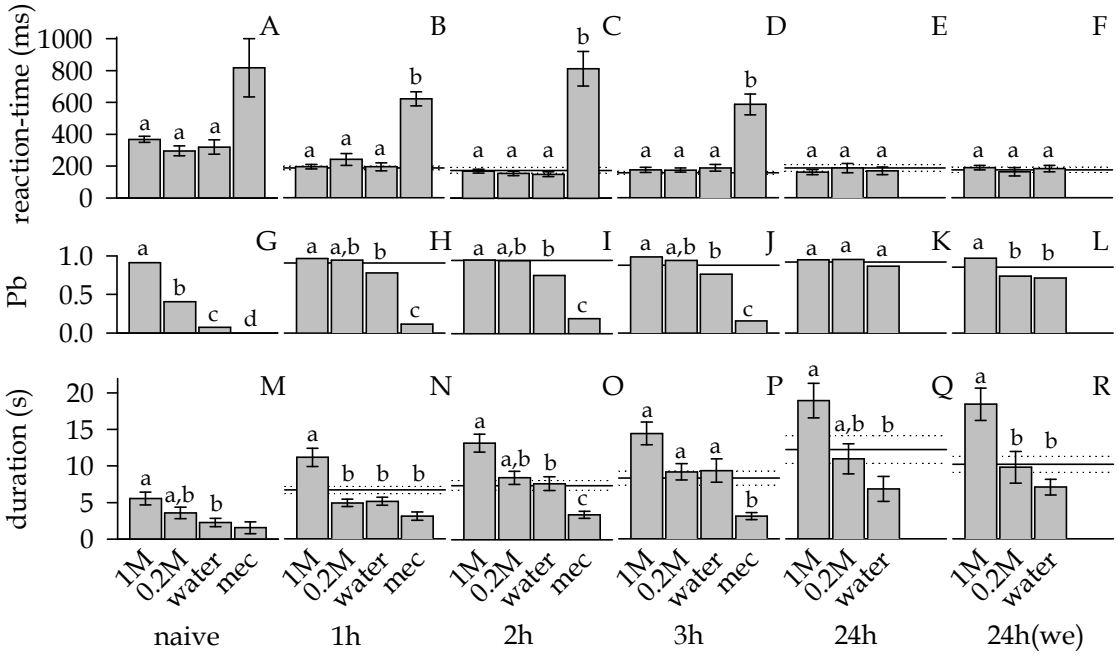

Figure 4.5 A-F) Mean PE reaction-times (in ms), as the average (± s.e.m) of the values from both antennae. **G-L)** PE probability, as the proportion of bees which showed PER after stimulation of both antennae, calculated from the total amount of tested bees. **M-R)** mean PE durations (in s), as the average (± s.e.m) of the values from both antennae following stimulation with 1 M or 0.2 M sucrose solution, water, and mechanosensory input. Data are shown for naïve and trained bees and the tests performed 1, 2, 3 24 h and 24 $h_{(we)}$ after training. Reference lines designate the values from untrained bees (mean ± s.e.m, solid and dotted lines respectively). Different letters indicate statistical differences across stimuli for naïve and trained bees (PE reaction-time and durations were analysed by Kruskal Wallis test and Dunn multiple comparisons $p < 0.05$, PE probability was analysed by G-tests): **A)** $H_3 = 0.2$, $P = 0.9$, $N_{1M} = 474$, $N_{0.2M} = 11$, $N_w = 14$, $N_m = 2$; **B)** $H_5 = 42.7$, $P < 0.0001$, $N_{1M} = 70$, $N_{0.2M} = 30$, $N_w = 30$, $N_m = 19$; **C)** $H_5 = 61.8$, $P < 0.0001$ $N_{1M} = 70$, $N_{0.2M} = 34$, $N_w = 28$, $N_m = 35$; **D)** $H_5 = 52.4$, $P < 0.0001$, $N_{1M} = 76$, $N_{0.2M} = 29$, $N_w = 25$, $N_m = 27$; **E)** $H_4 = 1.5$, $P = 0.5$, $N_{1M} = 46$, $N_{0.2M} = 19$, $N_w = 12$; **F)** $H_4 = 0.7$, $P = 0.7$, $N_{1M} = 50$, $N_{0.2M} = 8$, $N_w = 13$. **G)** $N_{1M} = 817$, $N_{0.2M} = 37$, $N_w = 55$, $N_m = 94$, df = 3, $G_{1M\ vs.\ 0.2M} = 53.9$, $P < 0.0001$, $G_{1M\ vs.\ w} = 191.6$, $P < 0.0001$, $G_{1M\ vs.\ m} = 374.4$, $P < 0.0001$, $G_{0.2M\ vs.\ w} = 15.1$, $P = 0.0005$, $G_{0.2M\ vs.\ m} = 47.3$, $P < 0.0001$, $G_{w\ vs.\ m} = 8.2$, $P = 0.04$; **H)** $N_{1M} = 150$, $N_{0.2M} = 37$, $N_w = 41$, $N_m = 68$, df = 3, $G_{1M\ vs.\ 0.2M} = 0.3$, $P = 0.8$, $G_{1M\ vs.\ w} = 13.1$, $P = 0.001$, $G_{1M\ vs.\ m} = 172.5$, $P < 0.0001$, $G_{0.2M\ vs.\ w} = 4.7$, $P = 0.0$, $G_{0.2M\ vs.\ m} = 77.3$, $P < 0.0001$ $G_{w\ vs.\ m} = 50.9$, $P < 0.0001$; **I)** $N_{1M} = 150$, $N_{0.2M} = 37$, $N_w = 41$, $N_m = 67$, df =3, $G_{1M\ vs.\ 0.2M} = 0.03$, $P = 0.9$, $G_{1M\ vs.\ w} = 12.6$, $P = 0.005$, $G_{1M\ vs.\ m} = 131.0$, $P < 0.0001$, $G_{0.2M\ vs.\ w} = 5.9$, $P = 0.1$, $G_{0.2M\ vs.\ m} = 60.4$, $P < 0.0001$, $G_{w\ vs.\ m} = 33.3$, $P < 0.0001$; **J)** $N_{1M} = 141$, $N_{0.2M} = 34$, $N_w = 38$, $N_m = 64$, df =3, $G_{1M\ vs.\ 0.2M} = 1.9$, $P = 0.6$, $G_{1M\ vs.\ w} = 20.1$, $P = 0.0002$, $G_{1M\ vs.\ m} = 163.9$, $P < 0.0001$,$G_{0.2M\ vs.\ w} = 4.7$, $P = 0.2$, $G_{0.2M\ vs.\ m} = 63.2$, $P < 0.0001$, $G_{w\ vs.\ m} = 36.6$, $P < 0.0001$; **K)** $N_{1M} = 82$, $N_{0.2M} = 21$, $N_w = 15$, df =2, $G_{1M\ vs.\ 0.2M} = 0$, $P = 0.9$, $G_{1M\ vs.\ w} = 1.3$, $P = 0.5$, $G_{0.2M\ vs.\ w} = 0.8$, $P = 0.6$; **L)** $N_{1M} = 91$, $N_{0.2M} = 25$, $N_w = 19$,df =3, $G_{1M\ vs.\ 0.2M} = 19.7$, $P < 0.0001$, $G_{1M\ vs.\ w} = 14.8$, $P = 0.0006$, $G_{0.2M\ vs.\ water} = 0.05$, $P = 0.9$; **M)** $H_3 = 10.14$, $P = 0.006$, $N_{1M} = 25$, $N_{0.2M} = 28$, $N_w = 14$, $N_m = 2$; **N)** $H_4 = 47.1$, $P < 0.0001$, $N_{1M} = 63$, $N_{0.2M} = 33$, $N_w = 31$, $N_m = 23$; **O)** $H_4 = 52.8$, $P < 0.0001$ $N_{1M} = 69$, $N_{0.2M} = 30$, $N_w = 27$, $N_m = 33$; **P)** $H_4 = 45.5$, $P < 0.0001$, $N_{1M} = 72$, $N_{0.2M} = 29$, $N_w = 25$, $N_m = 29$; **Q)** $H_3 = 13.0$, $P = 0.001$, $N_{1M} = 41$, $N_{0.2M} = 15$, $N_w = 41$; **R)** $H_3 = 13.7$, $P = 0.001$, $N_{1M} = 47$, $N_{0.2M} = 14$, $N_w = 13$.

Discussion

I report a hitherto unknown form of side-specific learning in honeybees. In these experiments, training involved coupling gustatory and mechanical stimulation of each of a honeybee's antennae with either increasing or decreasing volumes of sucrose solution offered to the animal's proboscis throughout a series of consecutive training trials. Testing involved stimulating each antenna separately. Such procedure allowed us to compare several PER measures, as elicited by gustatory and/or mechanical stimulation of each of a bee's antennae. When tested, the trained bees extended their probosces earlier after stimulation of the antenna that had been linked to increasing volumes of sugar solution than after stimulation of the antenna that had been linked to decreasing volumes of sugar solution. This happened 1, 2, and 3 h after training (Fig. 4.1 A-C), as well as 24 h after training (Fig. 4.1 E). Thus, training led to both short- and long-term side differences in the bees' PE reaction-time. This long-term side-specific responses could be prevented by repeatedly stimulating the bees' antennae after training (Fig. 4.1 D), which reveals an extinction effect on the side-specific association. Furthermore, I did not find side differences in the bees' reaction-time during training (Fig. 4.1 F-G), which suggests that both integration over the six training trials and a subsequent consolidation period are necessary for the side-specific responses to be evinced. A comparison between the reaction-times of the trained and untrained bees showed that the short-term side-specific responses arose from the joint effects of the increasing and decreasing reward schedules (Fig. 4.1 A), whereas the long-term side-specific responses arose from the effect of the decreasing reward schedule alone (Fig. 4.1 E). Mechanosensory input played an important role in the development and triggering of these side-specific behavioural adjustments. I did not observe short- and long-term side-specific responses when mechanosensory input was absent during both training and testing (Fig. 4.3 A-E). However, the use of such input during training and not during testing led to long-term side differences only, which means that gustatory input alone is sufficient to trigger long-term side-specific responses (Fig. 4.3F-J). Mechanosensory input alone also proved sufficient to evince and to extinguish side differences in a bee's reaction-time (Fig. 4.3 L and N). Finally, the bees tested with either 0.2 M sucrose solution or

water did not show short- and long-term side differences in their reaction-times (Fig. 4.4), demonstrating that side-specific responses are also specific regarding the nature of the gustatory input.

Hence, these results show that honeybees learn to associate the gustatory and mechanical stimulation of each antenna with either increasing or decreasing reward magnitudes (Fig. 4.1 A-E). I propose that a built-in change detector allows honeybees to compute differences in reward magnitude across feeding events, and that such computations can be side-specific. As a result, estimates of expected magnitudes of a given reward can be linked to each antennal input. I shall refer to such estimates to as 'side-specific reward memories'. These memories underlie a form of side-specific learning based on a measurable property of the experienced reward, namely, that its magnitude increases or decreases over time. The activation of such memories is necessary to reveal persistent side differences in a honeybee's PE reaction-time. One wonders whether the side-specificity of such memories derives from an association between the mechanical input that the bees experience during training and the animals' expected magnitudes of a given reward. This view is consistent with the fact that (1) short- and long-term side-specific responses are not observed if mechanosensory input is absent during both training and testing (Fig. 4.3 A-E), (2) mechanosensory input alone is sufficient to evince and to extinguish side-specific responses (Fig. 4.3 L and N), and (3) short-term side-specific responses are not observed if mechanosensory input is absent during tests (Fig. 4.3 F-I). However, in the latter situation the gustatory input alone is sufficient to evince long-term side-specific responses (Fig. 4.3 J). These results suggest that mechanical and gustatory inputs interact during the formation and retrieval of side-specific reward memories, so that the gustatory input contributes in the development of side-specificity and acquires the capacity to retrieve these memories only in the long-term. Still, further experiments are required to ponder the relative involvement of mechanical and gustatory inputs in the development of side-specificity.

In addition to the side differences in the bees' PE reaction-time, I found that training also exerts an overall effect on the reaction-time, the probability and duration of the bees' PERs to different stimuli (Fig. 4.5, Fig. 4.6; see also Results).

Such overall effect becomes evident if one compares the average of both antennae data for each of such three measures between trained and naïve bees. I found that training decreased the average reaction-time and increased the probability and duration of the bees' PERs. This effect was invariant to the test stimulus, with two exceptions: training changed neither the reaction-time following mechanical stimulation nor the PE probability following 1 M sucrose stimulation of the antennae (Fig. 4.5, Fig. 4.6). The increase of PE probability after training suggests that stimulation of the antenna, which involves gustatory and mechanical inputs, serves not only as an unconditioned stimulus (US), but also as a conditioned stimulus (CS) for subsequent rewards. This interpretation is consistent by previous reports indicating that a honeybee's PER can be conditioned to mechanical stimulation of the antenna (Giurfa and Malun 2004, Menzel et al. 2001), and to the water vapour emanating from a drop of sucrose solution (Kuwabara 1957).

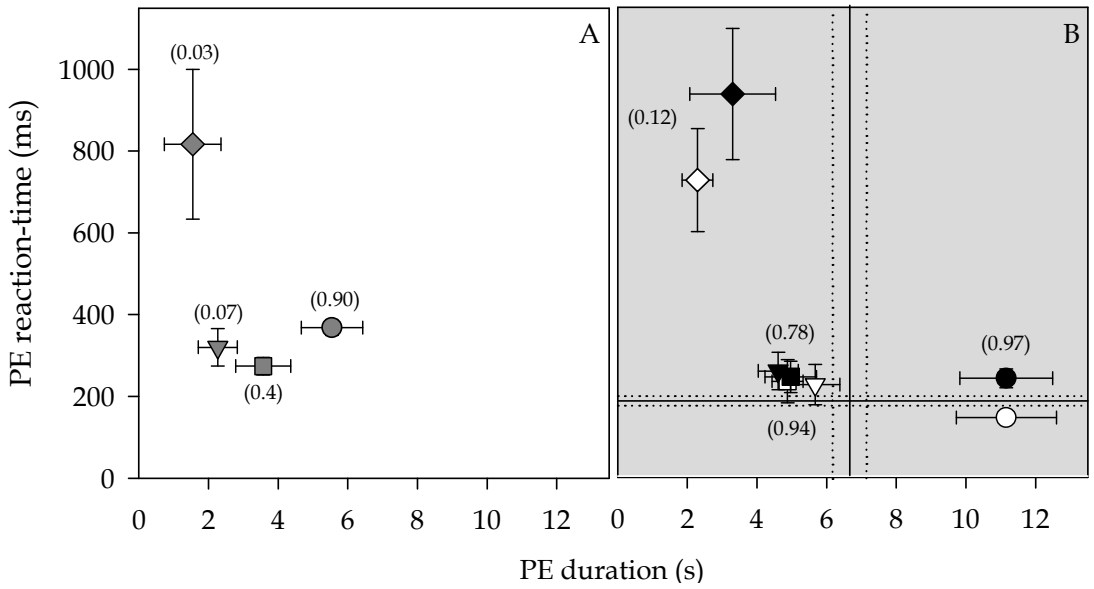

Figure 4.6 Depicted is the relationship between PE reaction-times (in ms) and PE durations (in s) of naïve **(A)** and trained bees **(B)** in the test performed 1 h after training. Data are shown for the bees stimulated with 1 M sucrose solution (circles), 0.2 M sucrose solution (squares), water (triangles) and mechanical input (diamond). In **B**, white and black symbols indicate values from the increasing and the decreasing antennae, respectively. The reference lines designate the PE reaction-time and duration of the untrained bees (mean ± s.e.m, solid and dotted lines, respectively). PE probability is given within parentheses.

These results also document that the regulation of the probability, the reaction-time and the duration of a honeybee's PE involves not only a series of common interacting elements but also separate ones which are specific for each of these three measures. I found training-related changes in the overall probability, reaction-time and duration of the bees' PERs which did not necessarily correlate with each other (Fig. 4.5, Fig. 4.6). Moreover, I found side differences in the reaction-time but not in the probability or the duration of the bees' PER (Fig. 4.1, Table 4.1 A-B). Further, I found that side differences in the reaction-time were specific to the gustatory stimulus (Fig. 4.4), and largely independent of the overall probability of the bees' PER. Thus, the tests with either 1 M or 0.2 M sucrose solution or water gave similar PER probabilities, but only those with 1 M sucrose solution led to side differences in reaction-time. Moreover, mechanical stimulation led to both very low PE probabilities and side differences in reaction-time (Fig. 4.5, Fig. 4.6). These results are consistent with the idea that side differences in the PE reaction-time depend upon the activation of side-specific reward memories formed on the basis of a specific property of the offered reward, namely, that its magnitude changed over time. They are also consistent with the idea that such memories are formed in parallel to those arising from a contingency between the stimulation of the antennae (as a CS) and the offered reward (as a US). This is important because it indicates that PE reaction-time can be a measure of a honeybee's anticipatory response to specific rewards. The adjustment of a subject's anticipatory response to reward is typically thought of as being rooted in the subject's expectations of reward (Schultz 2006). Along the same line, one might conclude from these observations that honeybees learn to 'expect' at least two different reward magnitudes.

In chapters 1 and 2 (Gil et al. 2007), I showed that free-flying honeybees adjust their eagerness to forage for food based on a specific property of a previously experienced reward, namely, that its magnitude changed over time. Next, in chapter 3 (Gil et al. 2008), I showed that harnessed honeybees adjust their PE reaction-times based on the same specific property of a previously experienced reward. Here, I report that the adjustments in the PE reaction-time can also be side-specific. Thus, I have developed a laboratory procedure suitable to examine behavioural correlates of

memories about specific reward properties which includes within-animal controls. This is important because honeybees assign sugar solutions with subjective values of reward (Page et al. 1998, Scheiner et al. 2005). Furthermore, because the PE reaction-time correlates well with the reaction-time of muscles involved in the movements of a honeybee's proboscis (Fig. 4.1 H), this preparation also includes a physiological correlate of behaviour. The substitution of a behavioural response like a honeybee's PER by such a physiological measure may be an important contribution to future studies using pharmacological, electrophysiological and optophysiological techniques. Such studies would focus on brain areas where projections of gustatory receptors from the antennae and proboscis, and mechanosensory receptors from the antennae converge. Evidence points towards the dorsal lobe and the suboesophageal ganglion as neuropils where the processing of both mechanosensory input from the antennae and gustatory input from both the antennae and the proboscis actually occurs (Suzuki 1975, Haupt 2005, Maronde 1991). The present results may guide future anatomical and physiological studies aiming to characterize the neural correlates of memories on specific reward properties.

General Discussion

The goal of this thesis was to investigate whether and how honeybees adjust their food gathering behaviour in relation to their past experience with variations in the level of sugar reward. I first performed a series of experiments with free-flying bees under conditions closely mimicking natural foraging situations (Chapters 1 and 2), and then a series of laboratory experiments with harnessed bees using a honeybee's proboscis extension response (Chapters 3 and 4). In all these experiments, I used the same general approach: I first presented the bees with increasing, decreasing or constant reward levels, and then evaluated their subsequent behaviour in the absence of reward. The general conclusions of these experiments are the following:

1) Foraging bees adjust their eagerness to search for food in relation to the sign and magnitude of past variations in the level of reward. This form of learning is independent of the bees' energy balance during foraging, and of classical and/or operant associations between the reward and its related predicting signals.

2) Harnessed bees adjust their proboscis extension responses (PERs) in relation to the sign of past variations in the level of reward. This learning can be side-specific, so that they show side differences in their PERs depending on past side-specific variations in reward level.

Taken together, these results indicate that honeybees have a built-in 'change detector' that computes the sign and magnitude of reward variations across feeding events. Such computation is seemingly followed by internal estimates of expected rewards stored as 'reward memories'. The behavioural adjustments which depend upon the formation and subsequent activation of these reward memories reveal that honeybees develop expectations of reward.

Reward Expectations in Foraging Bees

In the first series of experiments, I asked whether honeybees are able to learn the sign of variations in the level of reward that they experience during their foraging

excursions to a food source (Chapter 1). I made an experiment in which bees foraged on a relatively large artificial flower patch. This patch presented the bees with rewarded and unrewarded flowers identified by different colours. During training, the bees experienced increasing, decreasing or constant volumes of sugar solution distributed between the rewarded flowers. After a long foraging pause, I evaluated the honeybees' foraging behaviour at the patch in the absence of reward. I found that the bees that had experienced increasing reward levels subsequently searched for food more eagerly than the bees that had experienced decreasing or constant reward levels, either large or small (Fig. 1.2). The bees behaved differently neither because they had more strongly associated the signals predicting the reward nor because they were fed more or faster (Fig. 1.1, Table 1.1). These results documented for the first time that honeybees develop long-term expectations of reward, which can guide their foraging behaviour in the absence of reward and after a long foraging pause.

Next, I asked whether honeybees are able to learn not only the sign but also the magnitude of variations in the level of reward (Chapter 2). In this experiment, bees foraged on a three-flower patch offering low flow-rates of sugar solution. During their foraging excursions to this patch, the bees experienced either a large or a small increase in reward level, or, instead, a decreasing reward level. Like in the first experiment, I evaluated their foraging behaviour at the patch in the absence of food after a long foraging pause. I found that the bees that had experienced increasing reward levels subsequently searched for food more eagerly than the bees that had experienced decreasing reward levels. This result matched those of chapter 1. Moreover, I found that the bees that had experienced a large increase in reward level searched for food more eagerly than the bees that had experienced a small increase in reward level (Fig. 2.1, 2.2). These group differences could not be accounted for by the bees' energy balance during foraging (Table 2.2). Interestingly, the effect of the magnitude of an increase in reward level became detectable only after the bees' initial attempts to find food at the feeding site (Fig. 2.1). Taken together the results of chapters 1 and 2, it is likely that an increase in reward level leads to the formation of expectations of reward which enhance a forager's reliance on a food source, and that

the strength of this reliance increases together with the magnitude of the past increase in the level of reward.

In Fig. 5.1, I illustrate schematically the results and conclusions of these two first chapters. When honeybees forage in a flower patch offering variable reward levels, two parallel learning processes take place. On the one hand, bees learn the sign and magnitude of the variations in the level of reward that they experience across successive foraging excursions. They seemingly do this using a build-in 'change detector' which computes differences in reward magnitude across feeding events. Such computation leads to an internal estimate of an expected reward. I refer to this estimate as to a 'reward memory'. On the other hand, bees associate the reward (as the US) with signals and cues present at the feeding site like flower colour (as the CS), and an associative memory is formed. When a bee visits the feeding site after a long foraging pause and in the absence of reward, these two kinds of memories are retrieved by the signals related with the reward. Accordingly, associative memories are revealed through the bee's choice behaviour, while reward memories are revealed through its 'persistence' or 'eagerness' to forage for food, namely, the duration of its visit to the feeding site (Fig. 1.2 B, 2.1 A, B), the number of flower inspections it makes (Fig. 2.1 E, F), the cumulative duration of such inspections (Fig. 1.2, 2.1 C, D), and, eventually, the frequency of its visits to the site (Fig. 2.2). Taken together, these results demonstrate that foraging honeybees adjust their investment of time/energy during food searches in relation to both the sign and magnitude of past variations in the level of reward. This ability might make it more likely for them to compete with other flower pollinators for limited resources, and to maximize their individual rates of food collection by increasing their chances of finding food when forage is scarce. Moreover, this ability might help the colony as a whole to adjust its selectivity among nectar sources in relation to forage abundance. It would be interesting to incorporate these results and hypotheses to a model of individual and collective foraging in honeybees.

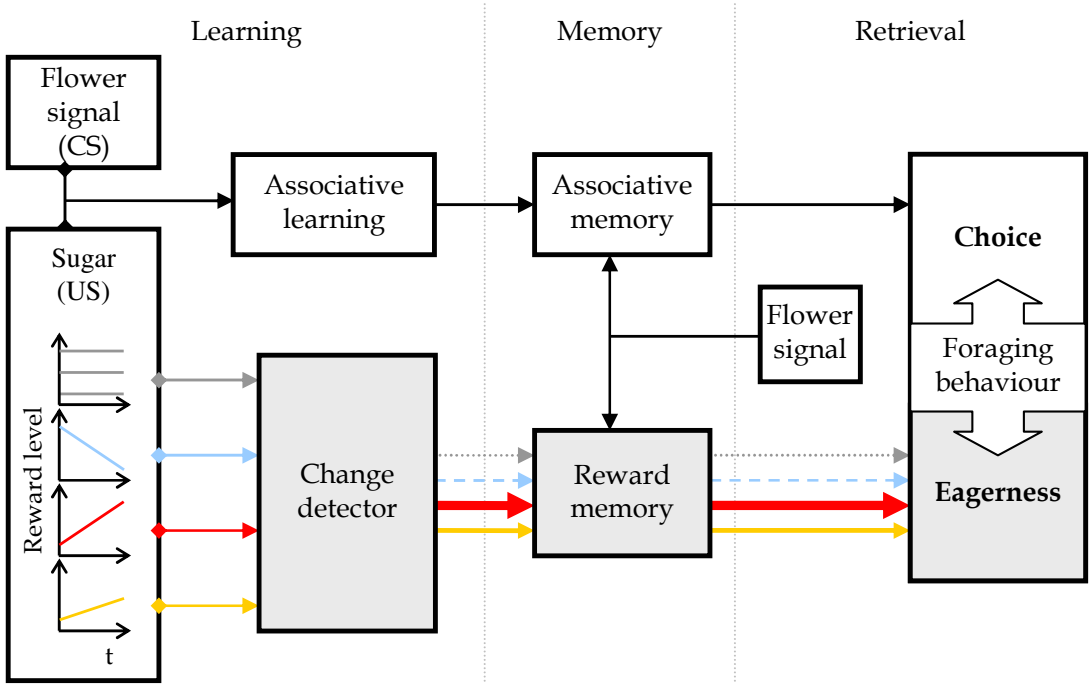

Figure 5.1 Depicted is a schematic representation of the effects of constant (grey lines), decreasing (blue lines), and increasing sugar reward levels (red and orange lines for either a large or small increase in reward level, respectively) on a honeybee's foraging behaviour. A built-in 'change detector' in the honeybee brain computes differences in reward level over time. This leads to the formation of a specific reward memory. In parallel, bees associate the reward with signals present at the food source (as the CS), and an associative memory is formed. After a long foraging pause, the reward's related signals can retrieve these two forms of memory. Associative and reward memories are evinced through honeybees' choice behaviour and foraging eagerness, respectively. In this scheme, differences in eagerness are interpreted as evidence of different expectations of reward.

The theory of optimal foraging attempts to predict the behaviour of animals while they collect food on sources of variable quality distributed heterogeneously in space (for a review see Pyke 1984). One hypothesis of this theory proposes that foragers assess the quality of a feeding site through an optimisation rule that tend to maximize the rate of net energy intake (Charnov 1976, Hodges 1981). According to this hypothesis, a forager first estimates the rate of energy intake for an average food source in their habitat and then, when visiting any given food source, it forage until the rate of energy intake falls below the estimated rate. Accordingly, a forager should invest more time foraging at an above-average food source than at a below-average food source. Thus, a forager's investment of time/energy while collecting food at a

given patch is positively correlated to the food availability of such patch (Charnov 1976, Hodges 1981). This is consistent with the fact that honeybees tend to maximize their rates of energy gain, (e.g., Schmid-Hempel et al. 1985, Varjú and Núñez 1991, 1993). But, optimal foraging theory does not capture how foragers control their time/energy investments in the absence of reward (Pyke 1984). When a honeybee searches for food at an unrewarding source, it is expected that the energy cost associated to forage exerts a large influence on its ongoing behaviour. Under this situation, it is reasonable to assume that the bee's decision about whether to continue searching at such source will be influenced by its memories on past reward experiences at the site. Thus, these memories would help the forager to determine how much time/energy is ought to be invested in the ongoing task. My results support this hypothesis. I found that a honeybee's 'eagerness' to search for food on a negative energy budget relies on its already developed expectations of reward (Chapters 1 and 2). A comprehensive theory aiming to explain how honeybees (and probably also other animals) forage for food should include the animals' ability to make use of their past reward experience. Further, in the case of honeybees, the question remains as to how such ability relates to the honeybees well-know tendency to visit repeatedly their sources of food (their so called flower constancy, Winston 1987).

Some effort has been made to create models that incorporate how learning and memory are adapted to the problems of foraging. Some models, for example, incorporate the variability of the reward level into the animal's evaluation of the quality of a patch (Harley 1981, Devenport and Devenport 1994). One of these models predicts that the foraging behaviour of animals that experienced variable rewards at a given patch depends on their memories about either the more recently experienced reward level, or the average reward level experienced at the patch, depending on the time elapsed since such experience (Devenport and Devenport 1994). My results do not match the predictions from this model. As I discussed in chapters 1 and 2, the differences observed during the tests between the bees that experienced increasing, decreasing and constant reward levels could not be account for by differences in the reward level experienced in their last visit to the patch, or by

differences in the average reward level experienced during the whole training (Table 1.1, Table 2.2). Therefore, an alternative model is needed to explain how honeybees use their memories about the sign and magnitude of past variations in the level of reward during foraging.

The results of chapters 1 and 2 pose new questions. For example, how does a colony benefits from an individual honeybee's ability to develop expectations of reward? Or, what is the relationship between the sign and magnitude of past variations in the level of reward and a forager's probability of being recruited to new food sources? To answer these and other related questions, it would be fruitful to simultaneously investigate the individual behaviour of bees inside the hive and the pattern of collective foraging over time of honeybees foraging on multiple feeders offering increasing, decreasing and constant reward levels. The comparison of the ability to develop and use reward memories between races, and even species, of social bees, and of how such ability relates to the particular characteristics of their environment, might be fruitful to better understand the biological significance of reward expectations in this social insects.

Reward Expectations in Harnessed Bees

Using an approach analogous to that of the experiments with free-flying bees described in chapters 1 and 2, I performed a series of experiments in the laboratory using the honeybee proboscis extension response (PER). In these experiments, I first asked whether harnessed bees also learn the sign of variations in reward level, so as to adjust its subsequent PER to sucrose stimulation of the antennae (Chapter 3). I trained bees by coupling the stimulation of one antenna with increasing, decreasing or constant volumes of sugar solution offered to their probosces. I evaluated their PERs to sucrose stimulation of the antenna 24 h after training and in the absence of reward. I found that the bees that had experienced increasing rewards during training extended their probosces earlier and during longer periods in comparison to the bees that had experienced decreasing or constant rewards, either large or small (Fig. 3.1). These group differences could not be accounted for by the bees' energy

balance during training. Thus, harnessed bees learn that reward level increases or decreases over time.

Next, in a new series of experiments (Chapter 4), I asked whether honeybees can learn side-specifically that the level of reward increases or decreases over time. I trained bees by coupling the stimulation of each antenna with either increasing or decreasing volumes of sucrose solution offered to their probosces. I evaluated the bees' PERs after stimulation of each antenna, 1, 2, 3 and 24 h after training in the absence of reward. I found that the bees extended their probosces earlier after stimulation of the antenna that had been linked to increasing rewards than after stimulation of the antenna that had been linked to decreasing rewards, thereby revealing short- and long-term side differences in the reaction-time of their PER (Fig. 4.1 A-F). Long-term side differences were prevented by repetitive antennal stimulation (Fig. 4.1 E). I also found that PER reaction-time correlated well with the reaction-time of the muscles M17 (Fig. 4.1 H). Mechanosensory input was necessary and sufficient for revealing side-specific responses (Fig. 4.3), which were also specific regarding the sucrose concentration of the offered reward (Fig. 4.4). Thus, chapter 4 presents a hitherto unknown form of side-specific learning in honeybees. The events that might be involved in such side-specific learning are summarized schematically in Fig. 5.2. When, throughout a series of consecutive trials, a bee experiences gustatory and mechanical stimulation of each antenna coupled with either increasing or decreasing rewards offered to its proboscis, a built-in 'change detector' computes the differences in reward level linked to each antenna. This computation leads to the formation of an internal estimate of an expected reward associated with each input side, and then, to the formation of 'side-specific reward memories'. After training, the joint effect of the gustatory and mechanosensory receptors of each antenna leads to the retrieval of both short- and long-term side-specific reward memories. Mechanosensory input alone leads to the retrieval of short-term side-specific reward memories (I have not tested whether it also leads to the retrieval of long-term side-specific reward memories, but the results suggest that this might be the case). Specific gustatory input alone leads to the retrieval of long-term side-specific reward

memories only. The activation of such memories leads to side differences in a honeybee's PE reaction-time, also evinced by the activity of the muscles M17s.

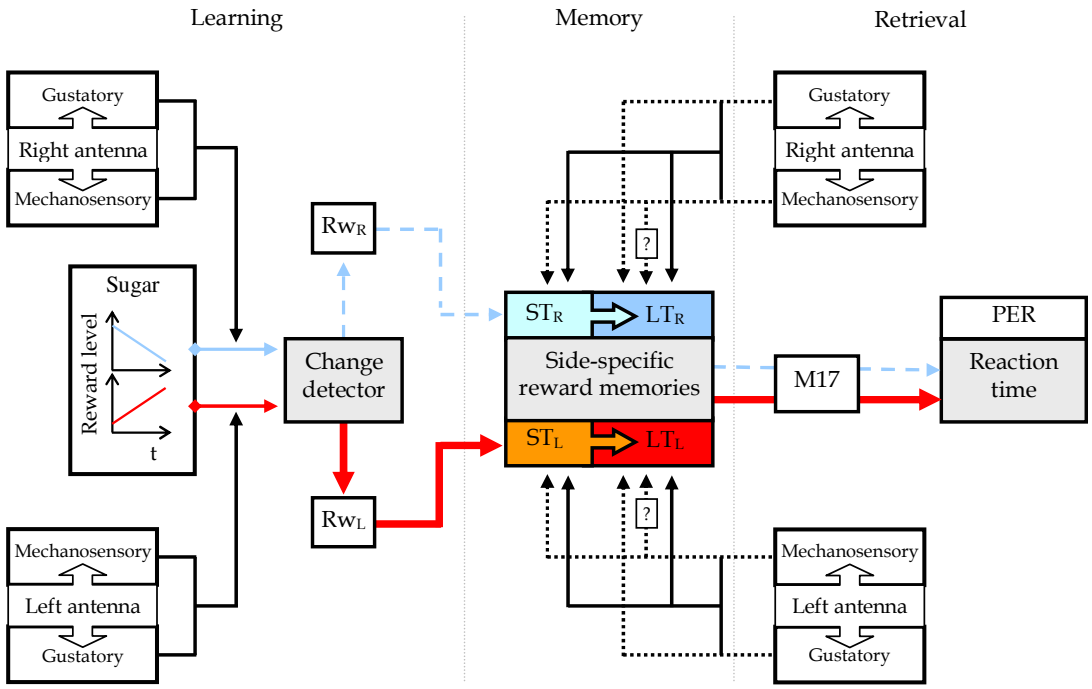

Fig. 5.2 Schematic representation of the events involved in the development of side-specific memories as reported in chapter 4. Side-specific training consisted of coupling gustatory and mechanical stimulation of each antenna with either increasing or decreasing rewards offered to a bee's proboscis (red and blue lines, respectively). During training, a built-in 'change detector' computes the differences in reward level associated with each antenna. This computation leads first to internal estimate of an expected reward associated to the input side (Rw_L, Rw_R), and then to 'side-specific reward memories'. During memory retrieval, the combined stimulation of gustatory and mechanosensory receptors of the antenna (solid lines) activates both short- and long-term (ST, LT) side-specific reward memories Mechanosensory stimulation of the antennae retrieves short- and probably also long-term side-specific reward memories (dashed lines). Specific gustatory stimulation of the antenna retrieves only long-term side-specific reward memories (dashed lines). The activation of such memories leads to side differences in a honeybee's PE reaction-time, also evinced by the activity of the muscles M17s.

When comparing the effect of increasing and decreasing rewards between bees (Chapter 3), I found differences in both, the reaction-time and duration of their PERs. However, when comparing the effect of increasing and decreasing rewards within bees (Chapter 4), I found differences only in their PE reaction-times. Further research is needed to understand the processes and mechanisms that control a honeybee's PER in these two situations, that is, when a bee experiences either increasing or

decreasing rewards, and when a bee experiences both increasing and decreasing rewards linked to the stimulation of each of its antennae. What becomes clear through within-animals comparisons is that the regulation of the probability, the reaction-time and the duration of a honeybee's PER involves not only a series of common interacting elements, but also separate ones which are specific for each of these three measures (Fig. 4.5, Fig. 4.6). The results of chapter 4 suggest that the reaction-time of a honeybee's PER represents a measure of an anticipatory response to a specific reward. Because adjustments of anticipatory responses are typically thought of as being rooted in a subject's expectations of reward, one wonders to what extent the adjustments of a honeybee's PER are based on reward expectations. Yet, further experiments are needed to determine what the connection is between the reward expectations that lead to the modulation of a honeybee's eagerness to forage for food and the modulation of the reaction-time of its proboscis extension response.

The results of these laboratory experiments pose new questions about how honeybees learn and process variations in the level of reward. For example, it would be interesting to investigate how the magnitude and frequency of reward variations relate to the adjustment of a honeybee's PER. It would also be interesting to perform additional experiments to determine the relative involvement of mechanical and gustatory inputs in side-specific learning. In addition, because the laboratory procedure presented in chapter 4 proved suitable for the analysis of within-animal behavioural correlates of reward memories, it becomes appropriate for pharmacological, electrophysiological and optophysiological studies aiming to elucidate the neural substrates underlying these memories. These studies would be combined with neuro-anatomical studies designed to identify more precisely the brain areas where projections of gustatory receptors from the antenna and proboscis, on the one hand, and mechanosensory receptors from the antenna, on the other, actually converge. Previous studies show that the gustatory receptors of the antenna project into the ipsilateral antennal lobe and dorsal lobe, and into the suboesophageal ganglion (Suzuki 1975). The gustatory receptors of the proboscis project into the suboesophageal ganglion, and ascend to the dorsal lobes (Haupt 2005). The mechanoreceptors of the antenna project into the ipsilateral dorsal lobe, and into the

suboesophageal ganglion (Suzuki 1975, Maronde 1991). Hence, the dorsal lobe and the suboesophageal ganglion seem to be the first-order neuropils for processing mechanosensory and gustatory input from the antennae and the proboscis. One can ask whether electrophysiological and/or optophysiological recordings of the neural activity of these neuropils correlates with the adjustments of a honeybee's PER that occur following the activation of reward memories. A pharmacological approach would also be fruitful in this context. For example, the bioamine octopamine (OA) appears to be involved in associative learning, memory retrieval, and food arousal in honeybees (Hammer 1993, 1997, Hammer and Menzel 1998, Bicker and Menzel 1989, Erber at al. 1993, Menzel et al. 1999, Mercer and Menzel 1982, Braun and Bicker 1992). It would be interesting to evaluate the role of octopamine in the context of these experiments by injecting or feeding the bees with either octopamine or antagonists of octopamine receptors before or after training, and then compare the performance of the treated and non-treated bees during testing.

Reward Expectations in General

The term expectation is used in many different contexts, from psychology to statistics, and that is why its meaning remains ambiguous. The dictionary defines an expectation as *"a strong hope or belief that something that you want will happen; to anticipate or look forward to the coming or occurrence of an event"*. Because the notions of expectation and anticipation are linked to each other, it is frequently taken for granted that these two words are synonymous. However, although one needs to expect in order to anticipate, the existence of an expectation does not imply by itself that anticipation will occur. Thus, an expectation of reward can be thought as a desire for a particular reward. Early investigations used general observations of behaviour to show that non-human animals expect outcomes, and that these expectations are linked to specific magnitudes or kinds of rewards. For example, monkeys train in a simple choice task show "*disappointment, hesitation, and searching behaviour*" when they find a non-preferred food item where a preferred food item used to be (Tinklepaugh 1928). Other classic studies by Crespi (1942) and Logan

(1960) reported that the running time of rats in a runway changes dramatically when they experience a sudden shift in reward magnitude. Trapold (1970) developed another method to test reward expectations of rats: the so-called 'differential outcome procedure'. In this procedure, animals learn instrumental (or Pavlovian) discrimination tasks in which their actions yield different outcomes. Thus, if one action (or stimuli) produces one kind of reward and another action (or stimuli) produces a different kind of reward, it is assumed that different expectations develop for different outcomes and, then, the animal can anticipate the outcome appropriate to each kind of trial at the moment the trial begins (Trapold 1970). A posterior modification of this procedure is the 'reward devaluation procedure' (Rescorla 1987). In this procedure, animals are first trained with a differential outcome procedure and then the value of one of the rewards is manipulated outside the learning situation by using satiation or taste aversion. When the animals are tested in the absence of reward after this manipulation, they show a reduction in the frequency of the action that predicts the devaluated food. These and other experimental procedures are meant to reveal the activation of memories about specific properties of a given reward, whose recollection is triggered by the cues and/or events that predict that reward. It is important to emphasize that the activation of these reward memories is assumed to exist in addition to any stimulus-response association, even when the experimental design and/or the behavioural measures do not allow a distinction between them. Thus, it is assumed that reward memories are active regardless of the particular behavioural procedures used to detect them. Reward expectations are frequently linked to complex cognitive abilities and have been systematically addressed in vertebrates (e.g., pigeons: Peterson et al. 1978; rodents: Holland and Straub 1979; nonhuman primates: Watanabe et al. 2001; humans: O'Doherty et al. 2001). These studies are critical for understanding the rules controlling goal-directed behaviours, and for the assessment of the cognitive complexity underlying decision making and planning. Previous studies have reported that honeybees and bumblebees are able to develop short-term reward expectations while foraging (Greggers and Menzel 1993, Bitterman 1996, Greggers and Mauelshagen 1997, Waddington and Gottlieb 1990, Real 1991, Wiegmann et al. 2001). The results

presented in this dissertation, however, are the first evidence that honeybees develop long-term reward expectations that can be evinced in the absence of reward. The experimental design presented in chapter 1, in addition, allows a distinction between reward expectations and associative memories. Furthermore, the experimental procedures presented in the chapters 3 and 4 represent the first attempts to test an insect's expectations of reward in the laboratory.

Summary

In this work, I asked whether and how honeybees (*Apis mellifera*) adjust their behaviour in relation to their past experience with reward variations. First, I performed a series of experiments with free-flying bees under conditions closely mimicking a natural foraging situation (Chapters 1 and 2). Next, I performed a series of laboratory experiments focused on a honeybee's proboscis extension response (PER) (Chapters 3 and 4). In all these experiments, I used the same general approach: First, I offered bees with increasing, decreasing or constant reward levels, and then recorded their subsequent behaviour in the absence of reward. I found that foraging bees learn both the sign and magnitude of reward variations. This form of learning is manifested through several measures of the bees' 'eagerness' to search for food. For example, bees that experience increasing rewards across foraging excursions subsequently search for food during longer periods, when compared to bees that experience either decreasing or constant rewards. This type of behavioural adjustments can not be accounted for by classical and/or operant associations between the reward and its related predicting signals or by the bees' energy balance during foraging. In the laboratory, I also found that harnessed bees learn that the level of reward increases or decreases over time. This form of learning is manifested through measures of a bee's PER. Such response allows bees gathering sugar solution, and is reflexively elicited when gustatory receptors of the antennae, proboscis and tarsi are stimulated with sugar solution. Bees that experience increasing rewards across feeding events subsequently extend their proboscis earlier and during longer periods in response to sucrose and/or mechanical stimulation of the antennae, in comparison to bees that experience either decreasing or constant rewards. Furthermore, this form of learning can be side-specific, in that bees show short- and long-term side differences in the reaction-time of their PER depending on past side-specific variations in the reward level. These side-specific behavioural adjustments involve an interplay between gustatory and mechanosensory input, and correlate well with the activity of muscles responsible for controlling the movements of the proboscis. I discuss these results in the context of the formation of reward memories and expectations of reward, individual and collective foraging strategies,

and further approaches to the study of the neural substrates underlying memories on specific properties of reward.

Zusammenfassung

In der vorliegenden Arbeit untersuchte ich, ob und wie Honigbienen (*Apis mellifera*) ihr Futtersammelverhalten in Bezug auf ihre Erfahrungen mit variierenden Belohnungen anpassen. Zuerst führte ich eine Reihe von Experimenten mit frei fliegenden Bienen unter ähnlichen Konditionen wie bei der natürlichen Futtersuche (Kapitel 1 und 2) und danach Laborexperimente zum Rüsselstreckreflexe (PER, Proboscis Extension Response) der Honigbienen durch (Kapitel 3 und 4). Allen diesen Experimenten liegt die gleiche Herangehensweise zu Grunde: Zunächst bot ich den Bienen Belohnungen in Form von steigenden, sinkenden oder konstanten Futtermengen an und nahm dann ihr Verhalten in Abwesenheit einer Belohnung auf. Ich fand heraus, dass Sammelbienen sowohl das Vorzeichen (d.h. die Zu- oder Abnahme) als auch die Stärke der Variation von Belohnungen lernen. Diese Form des Lernens zeigt sich durch verschiedene Messungen des „Eifers" der Bienen bei der Futtersuche. So suchen zum Beispiel Bienen, die während der Futtersuche zunehmende Belohnungsmengen erhalten, anschließend länger nach Futter als Bienen, die während der Futtersuche Erfahrungen mit entweder abnehmenden oder konstanten Belohnungen sammeln. Diese Art der Verhaltensanpassung kann weder durch die klassische und/oder operante Assoziation zwischen der Belohnung und dem Signal, das mit der Belohnung verbunden ist und ihr vorausgeht, noch durch die Energiebilanz während der Futtersuche erklärt werden. Meine Untersuchungen im Labor zeigten, dass auch in Röhrchen eingespannte Bienen lernen, ob das Belohnungsniveau im Verlauf der Zeit zu- oder abnimmt. Diese Art des Lernens offenbart sich durch Messungen des Rüsselstreckreflexes der Bienen. Das Ausstrecken des Rüssels ermöglicht den Bienen, Zuckerlösung zu sammeln und wird als Verhaltensantwort reflexiv ausgelöst, wenn Geschmacksrezeptoren der Antennen, des Saugrüssels und der Tarsi durch Zuckerlösung stimuliert werden. Bienen, die während der Fütterungen mit steigenden Belohnungen Erfahrungen sammeln, strecken nachfolgend als Verhaltensantwort auf die Stimulation mit

Saccharose und/oder eine mechanische Stimulation der Antennen ihren Rüssel früher und länger aus als Bienen, die während der Fütterungen Belohnungen sinkender oder konstanter Quantität erhalten. Des Weiteren kann diese Form des Lernens insofern seitenspezifisch sein, dass Bienen kurz- und langfristige Unterschiede in der PER-Reaktionszeit bis zum Ausstrecken des Rüssels als Verhaltensantwort auf einen Reiz, in Abhängigkeit von vorherigen seitenspezifischen Variationen in der Belohnungsphase, zeigen. Diese seitenspezifischen Verhaltensanpassungen umfassen ein Zusammenspiel zwischen gustatorischen und mechanosensorischen Eingängen und korrelieren positiv mit der Aktivität der für das Ausstrecken des Rüssels verantwortlichen Muskeln. Ich diskutiere diese Ergebnisse im Zusammenhang mit der Bildung von Erinnerungen an und Erwartungen von Belohnungen, individuellen und kollektiven Strategien bei der Futtersuche und weiteren Ansätzen zur Erforschung der neuronalen Grundlagen, die für das Erinnern an bestimmte Eigenschaften von Belohnungen eine wichtige Rolle spielen.

I would like to thank the Free University of Berlin and Prof. R. Menzel for giving me the opportunity to conduct my thesis at the Institute of Neurobiology. Prof. R. Menzel provided me with the materials and equipments I needed during my experiments, made helpful comments on the manuscripts included in this thesis, and helped me to receive financial support. My work was funded by the Deutsche Forschungsgemeinschaft (DFG Me 365/36-1 'Expectations and planning in honeybees', granted to Prof. Menzel).

I am indebted to Josué Núñez for his permanent encouragement, fruitful discussions and valuable comments. I would also like to thank him for designing, constructing and sending from Buenos Aires the rate-feeders that I used in the experiments of chapter 2.

I am grateful to Heidi Nickel and her family for their hospitality during the three months I stayed at their home performing the experiments of chapter 2. I also would like to thank Ravit Hadar for her assistance during the experiments of chapter 3, and Anke Schumann for patiently translating my summary into German.

My most deep and enduring acknowledgement is to Rodrigo De Marco, my companion and teacher. Without his unconditional support, selfless dedication and constant companionship, this thesis could have never been possible. He encouraged me to begin with this project, discussed with me the design of the experiments and their results, helped me to decide what to write, and then polished my horrible texts. Not only he patiently supervised my work, but also has shared with me his passion for biology over the last eight years. Basically, he has taught me everything I know about science. No one could wish for a more passionate, honest and bright guidance. I am proud of being part of his 'team'.

References

Baker H.G., Baker I. (1983) A Brief Historical Review of the Chemistry of Floral Nectar. In: The Biology of Nectaries (eds. B. Bentley and T. Elias), pp. 126-152. New York: Columbia University Press.

Bicker G., Menzel R. (1989) Chemical codes for the control of behaviour in arthropods. Nature 337: 33-39.

Bitterman M.E. (1996) Comparative analysis of learning in honeybees. Anim. Learn. Behav. 24: 123-141.

Bitterman M.E., Menzel R., Fietz A., Schäfer S. (1983) Classical conditioning of proboscis extension in honeybees (Apis mellifera). J. Comp. Psychol. 97: 107-119.

Braun G., Bicker G. (1992) Habituation of an appetitive reflex in the honeybee. J. Neurophysiol. 67: 588-598.

Charnov E.L. (1976) Optimal foraging, the marginal value theorem. Theor. Pop. Biol. 9: 129-136.

Crespi L.P. (1942) Quantitative variation in incentive and performance in the white rat. Am. J. Psychol. 40: 467-517.

De Marco R.J., Farina W.M. (2001) Changes in food source profitability affect the trophallactic and dance behavior of forager honeybees (*Apis mellifera* L.). Behav. Ecol. Sociobiol. 50: 441-449.

De Marco R.J., Gil M., Farina W.M. (2005) Does an increase in reward affect the precision of the encoding of directional information in the honeybee waggle dance? J. Comp. Physiol. (A) 191: 413-419.

Devenport L.D., Devenport J.A. (1994) Time-dependent averaging of foraging information in least chipmunks and golden-mantled ground squirrels. Anim. Behav. 47: 787-802.

Drezner-Levy T., Shafir S. (2007) Parameters of variable reward distributions that affect risk sensitivity of honey bees. J. Exp. Biol. 210: 269-277.

Erber E., Pribbenow B., Grandy K., Kierzek S. (1997) Tactile motor learning in the antennal system of the honeybee (*Apis mellifera* L.). J. Comp. Physiol. 181: 1432-1351.

Flaherty C.F. (1982) Incentive contrast: A review of behavioral changes following shifts in reward. Anim. Learn. Behav. 10: 409-440.

Frisch K. von (1946) Die Tänze der Bienen. Österr Zool Z 1:.1-48

Frisch K. von (1967) The Dance Language and Orientation of Bees. Cambridge, MA: Harvard University Press.

Fülöp A., Menzel R. (2000) Risk-indifferent foraging behaviour in honeybees. Anim. Behav. 60: 657-666.

Gallagher M., McMahan R.W., Schoenbaum G. (1999) Orbitofrontal cortex and representation of incentive value in associative learning. J. Neurosc. 19: 6610-6614.

Gil M., De Marco R.J., Menzel R. (2007) Learning reward expectations in honeybees. Learn. Mem. 14: 491-496.

Gil M., Menzel R., De Marco R.J. (2008) Does an Insect's Unconditioned Response to Sucrose Reveal Expectations of Reward? PLoS ONE 3(7): e2810. doi: 10.1371/journal.pone.0002810.

Giurfa M., Malun D. (2004) Associative Mechanosensory Conditioning of the Proboscis Extension Reflex in Honeybees. Learn. Mem. 11: 294-302.

Gould J.L., Gould C.G. (1988) The Honey Bee. New York: Scientific American Library.

Greggers U., Menzel R. (1993) Memory dynamics and foraging strategies of honeybees. Behav. Ecol. Sociobiol. 32: 17-29.

Greggers U., Mauelshagen J. (1997) Matching behavior of honeybees in a multiple-choice situation: The differential effect of environmental stimuli on the choice process. Anim. Learn. Behav. 25: 458-472.

Grosclaude F., Núñez J.A. (1998) Foraging pauses and their meaning as an economic strategy in the honeybee *Apis mellifera* L. J. Comp. Physiol. (A) 183: 61-68.

Hammer M. (1993) An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. Nature 366: 59-63.

Hammer M. (1997) The neural basis of associative reward learning in honeybees. TINS 20: 245-252.

Hammer M., Menzel R. (1994) Octopamine local injections into the mushroom body calyces and the antennal lobe substitute for the unconditioned stimulus (US) in honeybee olfactory conditioning. Soc. Neurosc. Abstr. 20: 258.

Hammer M., Menzel R. (1995) Learning and memory in the honeybee. J. Neurosci. 15: 1617-1630.

Hammer M., Menzel R. (1998) Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. Learn. Mem. 5: 146-156.

Harley C.B. (1981) Learning the evolutionarily stable strategy. J. Theor. Biol. 89: 611-633.

Haupt S.S. (2004) Antennal sucrose perception in the honey bee (*Apis mellifera* L.): behaviour and electrophysiology. J. Comp. Physiol. (A) 190: 735-745.

Haupt S.S. (2005) Das Gustatorische System und antennales Lernen in der Honigbiene (*Apis mellifera* L.). PhD Thesis, Technischen Universität Berlin, Germany.

Haupt S.S. (2007) Central gustatory projections and side-specificity of operant antennal muscle conditioning in the honeybee. J. Comp. Physiol. (A) 193: 523-535.

Hebb D.O., Donderi D.C. (1987) Textbook of Psychology. London: Lawrence Erlbaum Ass. Hilldale.

Hodges C.M. (1981) Optimal foraging in bumblebees: hunting by expectation. Anim. Behav. 29: 1166-1171.

Holland P.C., Straub J.J. (1979) Differential effects of two ways of devaluing the unconditioned stimulus after Pavlovian appetitive conditioning. J. Exp. Psychol. 5: 65-78.

Holland P.C., Gallagher M. (2004) Amygdala-frontal interactions and reward expectancy. Curr. Op. Neurobiol. 14: 148-155.

Hori S., Takeuchi H., Arikawa K., Kinoshita M., Ichikawa N., Sasaki M., Kubo T. (2006) Associative visual learning, color discrimination, and chromatic adaptation in the harnessed honeybee *Apis mellifera* L. J. Comp. Physiol. (A) 192: 1432-1351.

Kamil AC, Roitblat HL (1985) The ecology of foraging behaviour: implications for animal learning. Ann. Rev. Psychol. 36, 141-169.

Kleber E. (1935) Hat das Zeitgedächtnis der Bienen biologische Bedeutung? Z. vergl. Physiol. 22: 221-262.

Kolterman R. (1969) Lern- und Vergessensprozesse bei der Honigbiene – aufgezeigt anhand von Duftdressuren. Z. vergl. Physiol. 63: 310-334.

Kuwabara M. (1957) Bildung des bedingten Reflexes von Pavlovs Typus bei der Honigbiene, *Apis mellifica*. J. Fac. Hokkaido Univ. Ser. VI Zol. 13: 458-464.

Logan F.A. (1960) Incentive. New Haven: Yale University.

Macmillan C.S., Mercer A.R. (1987) An investigation of the role of dopamine in the antennal lobes of the honeybee, Apis mellifera. J. Comp. Physiol. 160: 359-366.

Markl H. (1971) Propioceptive Gravity Perception in Hymenoptera. In: Gravity and the Organism (eds. S. Gordon and M. Cohen), pp. 185-194. Chicago: University of Chicago Press.

Maronde U. (1991) Common projection areas of antennal and visual pathways in the honeybee brain, *Apis mellifera*. J. Comp. Neurol. 309: 328-340.

Masuhr T., Menzel R. (1972) Learning Experiments on the Use of Side-Specific Information in the Olfactory and Visual System in the Honeybee (*Apis mellifica*). In: Information Processing in the Visual Systems of Arthropods (ed. R. Wehner), pp. 315-322. Berlin-Heidelberg-New York: Springer.

Menzel R. (1967) Untersuchengen zum Erlernen von Spektralfarbe durch die Honigbiene (*Apis mellifera*). Z. verg. Physiol. 56: 22-62.

Menzel R. (1968) Das Gedächtnis der Honigbiene fur Spektralöfarben. I. Kurzzeitiges und langzeitiges Behalten. Z. verg. Physiol. 60: 82-102.

Menzel R. (1990) Learning, Memory, and 'Cognition' in Honey Bees. In: Neurobiology of Comparative Cognition (ed. R.P. Kesner and D.S. Olten), pp. 237-292. Erlbaum, Hillsdale, NJ.

Menzel R. (1999) Memory dynamics in the honeybee. J. Comp. Physiol. (A) 185: 323-340.

Menzel R. (2001) Searching for the memory trace in a mini-brain, the honeybee. Learn. Mem. 8: 53-62.

Menzel R., Heyne A., Kinzel C., Gerber B., Fiala A. (1999) Pharmacological dissociation between the reinforcing, sensitizing and response releasing functions of reward in honeybee classical conditioning. Behav. Neurosc. 113: 744-754.

Menzel R., Manz G., Menzel R.M., Greggers U. (2001) Massed and spaced learning in honeybees: The role of CS, US, the inter-trial interval and the test interval. Learn. Mem. 8: 198-208.

Menzel R., De Marco R.J., Greggers U. (2006) Spatial memory, navigation and dance behaviour in *Apis mellifera*. J. Comp. Physiol. (A) 192: 889-903.

Mercer A.R., Menzel R. (1982) The effects of biogenic amines on conditioned and unconditioned responses to olfactory stimuli in the honeybee, *Apis mellifera*. J. Comp. Physiol. 145: 363-368.

Núñez J.A. (1966) Quantitative Beziehungen zwischen den Eigenschaften von Futterquellen und dem Verhalten von Sammelbienen. Z. Vergl. Physiol. 53: 142–164.

Núñez J.A. (1967) Sammelbienen markieren versiegte Futterquellen durch Duft. Naturwiss. 54: 322.

Núñez J.A. (1970) The relationship between sugar flow and foraging and recruiting behavior of honeybees (*Apis mellifera* L.). Anim. Behav. 18: 527–538

Núñez J.A. (1977) Nectar flow by melliferous flora and gathering flow by *Apis mellifera ligustica*. J. Insect. Physiol. 23: 265–275.

O'Doherty J., Kringelbach M.L., Rolls E.T., Hornak J., Andrews C. (2001) Abstract reward and punishment representations in the human orbitofrontal cortex. Nat. Neurosci. 4: 95-102.

Okada R., Rybak J., Manz G., Menzel R. (2007) Learning-related plasticity in PE1 and other mushroom body-extrinsic neurons in the honeybee brain. J. Neurosci. 27: 11736-11747.

Page R.E. Jr., Erber J., Fondrk M.K. (1998) The effect of genotype on response thresholds to sucrose and foraging behavior of honey bee (*Apis mellifera* L.). J. Comp. Physiol. (A) 182: 489-500.

Peterson G.B., Wheeler R.L., Armstrong G.D. (1978) Expectancies as mediators in the differential reward conditional discrimination performance of pigeons. Anim. Learn. Behav. 6: 279–285.

Pyke G.H. (1984) Optimal foraging theory: a critical review. Annu. Rev. Ecol. Syst. 15: 523-575.

Raveret-Richter M., Waddington K.D. (1993) Past foraging experience influences honeybee dance behavior. Anim. Behav. 46: 123-128.

Real L.A. (1981) Uncertainty and pollinator-plant interactions: the foraging behavior of bees and wasps on artificial flowers. Ecol. 62: 20-26.

Real L.A. (1991) Animal choice behavior and the evolution of cognitive architecture. Science 253: 980-986.

Real L.A., Rathcke B.J. (1988) Patterns of individual variability in floral resources. Ecol. 69: 728-735.

Rehder V. (1987) Quantification of the honeybee's proboscis reflex by electromyographic recordings. Ins. Physiol. 33: 501-507.

Rehder V. (1989) Sensory pathways and motor neurons of the proboscis reflex in the suboesophageal ganglion of the honeybee. J. Comp. Neurol. 279: 499-513.

Rescorla R.A. (1987) A Pavlovian analysis of goal-directed behavior. Am. Psychol. 42: 119-129.

Ribbands C.R. (1954) Communication between honeybees, I: the response of crop-attached bees to the scent of their crop. Proc. R. Entomol. Soc. Lond. (A) 29: 10–12.

Sage J.R., Knowlton B.J. (2000) Effects of US devaluation on win-stay and win-shift radial maze performance in rats. Behav. Neurosci. 114: 295-306.

Sandoz J-C., Menzel R. (2001) Side-specificity of olfactory learning in the honeybee: generalization between odors and sides. Learn. Mem. 8: 286-294.

Sandoz J-C., Hammer M., Menzel R. (2002) Side-specificity of olfactory learning in the honeybee: US input side. Learn. Mem. 9: 337-348.

Schmid-Hempel P., Kacelnik A., Houston A.J. (1985) Honeybees maximize efficiency by not filling their crop. Behav. Ecol, Sociobiol. 17: 61-66.

Scheiner R., Kuritz-Kaiser A., Menzel R., Erber J. (2005) Sensory responsiveness and the effects of equal subjective rewards on tactile learning and memory of honeybees. Learn. Mem. 12: 626-635.

Schneider D. (1964) Insect antennae. Ann. Rev. Entomol. 9: 103-122.

Schönbaum G., Setlow B., Nugent S.L., Saddoris M.P., Gallagher, M. (2003) Lesions of orbitofrontal cortex and basolateral amygdala complex disrupt acquisition of odor-guided discriminations and reversals. Learn. Mem. 10: 129-140.

Schröter U., Menzel R. (2003) A New Ascending sensory tract to the calyces of the honeybee mushroom body, the subesophageal-calycal tract. J. Comp. Neurol. 465: 168-178.

Schröter U., Malum D., Menzel R. (2007) Innervation pattern of suboesophageal VUM neurons in the honeybee brain. Cell Tissue Res. 327: 647-667.

Schultz W. (2000) Multiple reward signals in the brain. Nature Rev. Neurosc. 1: 199-207.

Schultz W. (2006) Behavioral theories and the neurophysiology of reward. Ann. Rev. Psychol. 57: 87-115.

Seeley T.D. (1995) The Wisdom of the Hive. Cambridge, Mass: Harvard University Press.

Shafir S., Wiegmann D.D., Smith B.H., Real L.A. (1999) Risk-sensitivity of honey bees to variability in volume of reward. Anim. Behav. 57: 1055-1061.

Shapiro M.S., Couvillon P.A., Bitterman M.E. (2001) Quantitative tests of an associative theory of risk-sensitivity in honeybees. J. Exp. Biol. 204: 565-573.

Smith B.H., Menzel R. (1989) An analysis of variability in the feeding motor program of the honey bee; the role of learning in releasing a modal action pattern. Ethol. 82: 68-81.

Snodgrass R.E. (1956) Anatomy of the Honey Bee. Ithaca: Comstock.

Suzuki H. (1975) Antennal movements induced by odour and central projection of the antennal neurons in the honeybee. J. Insect Physiol. 21: 831–847.

Takeda K. (1961) Classical conditioned response in the honey bee. J. Insect. Physiol. 6: 168-179.

Teuber L.R., Barnes D.K. (1979) Environmental and genetic influences on quantity and quality of alfalfa nectar. Crop Sci. 19: 874-878.

Tinklepaugh O.L. (1928) An experimental study of representative factors in monkeys. J. Comp. Psychol. 8(3): 197-236.

Tolman E.C. (1959) Principles of Purposive Behaviour. In: A Study of a Science (ed. S. Koch), pp. 92-157, Psychology Vol. 2, New York: McGraw-Hill.

Trapold M.A. (1970) Are expectancies based upon different positive reinforcing events discriminably different? Learn. Motiv. 1: 129–140.

Varjú D., Núñez J.A. (1991) What do foraging honeybees optimize? J. Comp. Physiol. (A) 169: 729–736.

Varjú D., Núñez J.A. (1993) Energy balance versus information exchange in foraging honeybees. J. Comp. Physiol. (A) 172: 257-261.

Vogel S. (1983) Ecophysiology of Zoophilic Pollination. In: Physiological Plant Ecology III (eds. O.L. Lange, P.S. Nobel, C.B. Osmond and H. Ziegler), pp. 559–624. Berlin, Heidelberg, New York: Springer.

Waddington K.D. (2001) Subjective Evaluation and Choice Behavior by Nectar- and Pollen-Collecting Bees. In: Cognitive Ecology of Pollination: Animal Behavior and Floral Evolution (ed. L. Chittka and J.D. Thompson), pp. 41-60. Cambridge: Cambridge University Press.

Waddington K.D., Gottlieb N. (1990) Actual vs. perceived profitability: a study of floral choice of honey bees. J. Ins. Behav. 3: 429:441.

Wahl O. (1932) Neue Untersuchungen über das Zeitgedächtnis der Bienen. Z. vergl. Physiol. 16: 529-589.

Watanabe M., Cromwell H., Tremblay L., Hollerman J.R., Hikosaka K., Schultz W. (2001) Behavioral reactions reflecting differential reward expectations in monkeys. Exp. Brain Res. 140: 511-518.

Wiegmann D.D., Wiegmann D.A., Waldron F.A. (2003) Effects of a reward downshift on the consummatory behavior and flower choices of bumblebee foragers. Physiol. Behav. 79: 561-566.

Winston M.L. (1987) The Biology of the Honey Bee. Cambridge, MA: Harvard University Press.

Zar J.H. (1996) Biostatistical Analysis, 3rd edn. New Jersey: Prentice-Hall.

Zhang S.W., Schwarz S., Pahl M., Zhu H., Tautz T. (2006) Honeybee memory: a honeybee knows what to do and when. J. Exp. Biol. 209: 4420-4428.

Publications in peer-reviewed journals

Gil M., Menzel R., De Marco R.J. (2008) Does an Insect's Unconditioned Response to Sucrose Reveal Expectations of Reward? PLoS ONE 3(7):e2810.doi: 10.1371/journal.pone.0002810.

Gil M., De Marco R.J., Menzel R. (2007) Learning Reward Expectations in Honeybees. Learning and Memory 14: 491-496.

Gil M., De Marco R.J. (2006) *Apis mellifera* bees Acquire Long-term Olfactory Memories within the Colony. Biology Letters 2: 98-100.

Gil M., De Marco R.J. (2005) Olfactory Learning by means of Trophallaxis in *Apis mellifera*. Journal of Experimental Biology 208: 671-680.

De Marco R.J., Gil M., Farina W.M. (2005) Does an Increase in Reward Affect the Precision of the Encoding of Directional Information in the Honeybee Waggle Dance? Journal of Comparative Physiology (A) 191: 413-419.

Gil M., Farina W.M. (2003) Crop Scents Affect the Occurrence of Trophallaxis among Forager Honeybees. Journal of Comparative Physiology (A) 189: 379-382.

Fernández P.C., Gil M., Farina W.M. (2003) Reward Rate and Forager Activation in Honeybee: Recruiting Mechanisms and Temporal Distribution of Arrivals. Behavioural Ecology and Sociobiology 54: 80-87.

Gil M., Farina W.M. (2002) The Resumption of Foraging at a Known Nectar Source in the Honeybee *Apis mellifera* L.: Social Interactions between Unemployed and Employed Foragers. Naturwissenchaften 87: 322-325.

Manuscripts submitted

Gil M., De Marco R.J. Dance Communication in Honeybees: Re-Examining the Behaviour of the Dance Followers. (In revision)

Gil M., De Marco R.J. Honeybees Learn the Magnitude and Sign of Reward Variations.

Gil M., Menzel R., De Marco R.J. Side-Specific Reward Memories in Honeybees.