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Unlearned and Learned Behaviour of Bumble Bees in the Absence of Reward

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. The bee garden is blue because blue is the 'perfect colour' and bees are the most perfect of any insect in the way they live, and the most valuable on account of the work they do, and so blue would be the colour they love best, and it is.' . If you don't believe it, watch them.

Gene Stratton-Porter, Keeper of the Bees, 1925

To my three loving, steadfast, and resilient daughters,

Laura, Mary, and Beth

and

In loving memory of my parents, Robert and Alice Simonds

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Acknowledgements

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Abstract

In the absence of previous experience with flowers, how do bees distinguish between possible food sources and non-rewarding objects? Unrewarding stimuli (colours in Experiment 1 and patterns in Experiment 2) were presented in a radial arm maze. The unlearned approach responses of bumble bees (*Bombus impatiens*) were recorded and significant preferences were obtained: the bees chose yellow and blue over other colours, and radial patterns over concentric patterns or unpatterned discs. Habituation was demonstrated when choices proportions for the same pattern by the same bees decreased over two test sessions. When an attractive novel pattern was presented in the third session the trend was reversed. These data confirm both that truly naive bees have unlearned colour and pattern preferences and that learning not to approach these stimuli occurs in the absence of reward.

Two further experiments tested the length of time that habituation is maintained by bumble bees and the degree to which contextual cues contribute to maintaining habituation. In Experiment 3 once bees habituated to a radial pattern, response decrement was maintained after a stimulus presentation delay of two hours. After a 24 hour delay, however, the preference was restored. In Experiment 4 exposure to the context without stimulus presentation did not affect the retention of the habituated response. These findings confirm bumble bees' ability to habituate to a stimulus and delineate its time course as lasting in the range of minutes and hours but not days. They call into question the associative role of contextual cues in habituation for bumble bees.

Introduction

Curiosity and observation, the historical impetus for the pursuit of knowledge, provide the beginning of this story. During a recital, a young woman stood singing at the front of a church while a panel of judges critiqued. The situation was tense enough but someone had left the front door open to allow the spring air to circulate. A large queen bee had found its way in and was particularly "attracted" to the floral print of the soloist's skirt. It flew from one large flower to the next stopping briefly at each one. A second observation occurred in a greenhouse filled with bedding plants. Real flowers ripe for foraging were in abundance but a bee landed on a brightly coloured package of flower seeds. These observations provoked enough questions about behaviour to want to observe more in the controlled setting of a laboratory. In the lab, watching bees on their first excursion outside the colony I saw some not only fly toward and land on pictures of flowers from a calendar, but even circle and extend the proboscis to the centre of the floral picture (see Plate 1).

Learned vs Unlearned Behaviour

What draws the bee to the flower in the first place? The observations noted above and common sense both suggest that some behaviours suited to foraging must be present before the bee has the benefit of experience with reward. Assessment of the distinction between learned and unlearned behaviour is dangerous territory and some would say outdated. The basic nature-nurture question has been around for at least 200 years (cf. Reviews Cravens, 1978; Pastore, 1949/84). Although psychologists have historically focussed on learning from experience, Kant challenged this way of thinking during the 18th Century (Bolles, 1988). The original contention that all behaviour derives from experience

was debated with one simple question by Kant which holds some irony for the scientist: "Why do we infer causation? We all do it, but the inference itself does not arise from experience. Why do people make it?" (p. 2, as cited in Bolles, 1988). He suggested that this was something that everyone does because the human mind is somehow equipped to do so: there is a pre-existing framework into which experience fits. These questions were the beginnings of the nativist position which posed an alternative to the empiricists and spawned a philosophical and experimental body of literature devoted to settling the controversy that would last for centuries (Cravens, 1978; Pastore, 1949/84). Most would say that the debate is no longer relevant. The debate is relevant to this thesis insomuch as the direction or emphasis of the research has been dependent on which end of the continuum the pendulum rests. In animal behaviour, by the 1920s learning theory had taken a firm hold. Bolles (1988) wrote of the 1920s "Comparative psychology was degenerating into rat psychology. Learning, whether by conditioning or reinforcement, was the only thing that mattered" (p. 7). Although this emphasis on learning did not receive exclusive attention during the 20th Century (cf. Reviews Lehrman, 1970; Lorenz, 1965; Gould & Gould, 1994), the majority of animal research continued to maintain this focus.

This has been especially true in bee research. So much has been observed, studied, and written about bees' ability to engage in associative learning (e.g., Bitterman, 1996; Couvillon & Bitterman, 1980; Hammer, 1997;). In addition to the influence of the nature-nurture debate on the path of research, a highly practical problem pushed bee research to concentrate on learning. When presented with a particular stimulus in conjunction with reward bees are very adept at associating specific stimulus features with the food source

(Ney-Nifle, Keasar, & Shmida, 2001). Because of the ease of studying associative learning in bees vast amounts of information have been amassed, but is there foundational "knowledge" or behaviour that is present before any experience with visual stimuli has occurred? If so, how much of what we already think we know about bee behaviour is truly learned and what is unlearned? Tinbergen (1951) criticized psychologists, for essentially ignoring innate[1] behaviour and specializing in what he termed higher types of behaviour. He states:

> In my opinion, this disregard of innate behaviour is due to the fact that it is not generally understood that learning and many other higher processes are secondary modifications of innate mechanisms, and that therefore a study of learning processes has to be preceded by a study of the innate foundations of behaviour (p. 6).

Some bee researchers that have studied learning temper their conclusions with the need for more information about behaviour that exists prior to experience with stimuli (e.g., Manning, 1956). Clearly, there is a need to build a foundation of knowledge of bee behaviour that exists prior to experience with flowers so that we can then more confidently know which behaviours represent true learning. The realization that the questions arising from our anecdotal and pilot observations had received little consideration in the literature, combined with the stated need for research of this type provided the basis for the initial thesis question: Which responses to visual stimuli are present in bees before contact with a food source? Asking this question carries the formidable challenge of finding a way to accurately measure unlearned responses in bees. For this thesis, a restricted definition of unlearned will be used

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[1]The term innate can be defined in many ways and has caused significant confusion (for discussion see Bateson (1984)), hence, this thesis avoids the term.

as outlined by Hogan (1988). The term unlearned is used here to refer to behaviour that has not been learned during the experiment. It is possible that the behaviours associated with the questions asked in this document have been learned over the course of development. Perhaps a better term, though seldom used, is "pre-functional" (Hogan, 1988): Behaviour shown prior to functional experience (in this case, experience with flowers).

Methods for testing unlearned responses in bees

As described above, a specific problem to the investigation of unlearned responses in bees is the difficulty in provoking responses without the use of reward. Lunau and Maier (1995) state " . . . as a consequence of the great learning ability of honey bees, it is difficult, if not impossible, to study initial colour preferences by means of training experiments" (p. 5). A few researchers have attempted methods in the past (Giurfa, Núñez, Chittka, & Menzel, 1995; Lehrer, Horridge, Zhang, & Gadagkar, 1995; Lunau, 1990, 1992; Gumbert, 2000). A detailed discussion of these methods is given in Manuscript #1 (Introduction, p. 26) of this thesis. These studies and others before them have used methods which potentially interfere with an accurate assessment of unlearned responses. As mentioned earlier, bees have an excellent capacity to learn by association and, in some of these experiments there was reward involved and so the potential for learning of the rewarded stimuli in training to have carried to the test situation is a concern. The other method controlled for associative learning from training stimuli, but the dependent measure was an approach flight from a distance of 1 metre. This method lacks precision in that it may be difficult to discern whether a bee is truly approaching the test stimuli or whether it is simply in free flight.

The need to develop an efficient and effective method initiated a 12 month period of trial and error where our main quest was to induce responses to visual floral-like stimuli from the bees at a rate great enough to facilitate collection of data and precise enough to ensure the accuracy of that data collection. Through learning from our unsuccessful (if not embarrassing) ideas, useless apparatuses, and failed pilots, a method was developed that seemed to meet the criteria. An early version of the apparatus developed for the current experiments was a Y-maze developed by a group of researchers to assess use of global and local features of a pattern (Zhang, Srinivasan, and Horridge, 1992). It was then modified by Lehrer et al. (1995) by expanding it to 12 compartments to form a 12 arm radial maze with an open portion in the middle. The maze was modified again for this series of experiments by size, construction materials, and availability of reward (See Manuscript #1, Method, p 28, and Appendix A, Figure 1). This apparatus allows for free flight but confines the bees' movement to a defined and easily observable area. It also provides enough distance (subtending an angle of more than 15° (Giurfa et al., 1996, 1997)) for the bee to recognize the stimulus as a whole.

Design and construction of the apparatus was not in itself sufficient to provoke responses from the bees. Lehrer et al. (1995) had used the apparatus in conjunction with reward. With no reward available it was necessary to test bees that were ready to forage (See Manuscript #1, Method, p. 23) and to use stimuli that would be salient enough to provoke a response in the absence of reward. The first use of the method determined that the first criterion was met in that the bees often flew toward the stimuli provided. The next step was to make sure that the responses were frequent *and* were not random. There were several

possible routes to follow but the most logical seemed to test what already had been claimed as either 'innately' attractive or learned in colour and pattern.

Colour and pattern preferences in bees

Colour and pattern are important cues for bees in finding flowers. They tend to learn both colour and pattern easily when they are associated with reward, but colour seems to be the more salient of the two (Gould & Marler, 1987). Through a series of tests on stimuli that were blue or yellow and round or triangular, bees chose the target stimuli based on colour over shape if colours and shapes were different, but resorted to shape when colours were kept constant. In order to test the viability of the method used here, the visual cue that was expected to be the most salient was chosen. Therefore, coloured stimuli were chosen for the first experiment. The next concern was which colours to choose. Note that the prior experiment used blue and yellow. In 1915 Von Frisch used blue and yellow to determine whether bees could distinguish colours (as cited in Menzel, 1990). This is no coincidence. As far back as 1881, Müller found that bumble bees predominantly visited blue flowers in alpine regions (as cited in Lunau & Maier, 1995). Tests designed to determine colour preference produced mixed results but the discrepancy was between blue and yellow. That is, some researchers found bees preferred yellow and others found they preferred blue. More recently, studies found bumble bees showed colour preferences for wavelengths that correspond to violet and dark blue for humans (Giurfa et al., 1995; Gumbert, 2000; Lunau, 1990). It should be acknowledged here that the bumble bees' visual system is not the same as humans (cf. Reviews Lunau & Maier, 1995; Menzel, 1990). In other words they do not see colours as we see them, but a detailed analysis of that system and a description of how

they see colour is not the focus of this paper. We know that bees can distinguish colour and that they seem to be attracted to what we see as blue and yellow. Based on this previous research I chose four colours, among them blue and yellow, to test the viability of the method. It was expected that blue and yellow would be chosen more frequently than the other two colours (white and red).

The second visual cue, pattern, has been investigated through training to a particular pattern and then tested with a choice of the training pattern and novel patterns, or by training to a site without pattern and then testing at the same site with pattern choices (For review see Lehrer et al., 1995). The results were consistent across methods and found that bees prefer disrupted patterns (high frequency) and that these patterns were more readily learned. Other patterns less attractive were not learned even over a long period of training. This resistance to learning suggests that response to certain characteristics of pattern are present prior to experience with floral stimuli. Lehrer and her colleagues (1995) investigated "innate" preferences for floral patterns. Radial patterns, particularly of six sectors, were chosen more frequently than random, asymmetrical, and circular patterns. According to the authors, this research represents the first evidence of innate pattern preference. Thus, these patterns were chosen as the second set of stimuli for this series of experiments (For details see Manuscript #1, Experiment #2, Method, p 36). It was expected that a radial pattern would be chosen more frequently than a circular pattern.

Habituation: Experimental evidence

This introduction is structured to follow the events as they unfolded. As described, out of anecdotal observations came questions about what draws the bee to the flower in the

first place before contact with reward offered by any flower. A large part of answering the questions was the development of a unique method to assess unlearned responses in bumble bees. When this task was accomplished, a few behaviours that were thought to be unlearned according to previous research were tested. Once again curiosity and observation lead this research down an unexpected path. While watching bees' responses to the stimuli it appeared that their choices for the initially preferred stimuli gradually waned. Were they experiencing fatigue? Or, were they habituating to the stimuli? In other words, was learning taking place even without the presence of reward? Intuitively it makes sense that bees would eventually "abandon" initially attractive flowers when no food was available. These observations and speculations formed the basis of the second part of the colour and pattern experiments.

Habituation has been studied in depth across almost all forms of invertebrate and vertebrate organisms (c.f. Reviews Harris, 1943; Peeke & Herz, 1973). Habituation is described as "response decrement as a result of repeated stimulation" (Harris, 1943, p. 385). The response decrement that is usually measured is a decrement in intensity (e.g., a decrease in the startle response). Here, the response decrement is a decrease in approach behaviour, which translates into a decrease in choice proportion for a stimulus in the maze. Habituation does not include response decrement that occurs as a result of, for example, receptor adaptation, fatigue, trauma, or aging (Thompson & Spencer, 1966). In 1943 when Harris wrote his comprehensive review of the current literature on habituation, the concept of habituation was not well understood and just on the cusp of being recognized as a form of learning. Thirty years later habituation was well established as an important form of learning

and physiological and behavioural research had come together with the mutual goal of understanding this phenomenon. However, of the *Hymenoptera* order Harris (1943) states "Habituation in this important class is as yet not systematically studied" (p. 403). In the ensuing years the study of habituation in this order has remained all but untouched.

Methodology has been honed over the years to better assess habituation and distinguish it from response decrement occurring for other reasons. The basic method is to present a stimulus repeated over several trials. Once the organism has habituated to the stimulus a novel situation (e.g., novel stimulus, change in location of stimulus, time delay) is introduced which was expected to show renewed responding. Petrinovich (1984) states "If there is an increment in responsiveness to a novel stimulus, as compared to the habituated stimulus, this will permit an inference regarding the extent to which the observed effects are the results of habituation and the extent they result from motor fatigue or sensory adaptation" (p. 33). This paradigm has been shown to work successfully across many species, even in unicellular organisms such as the amoeba. For example, *Amoeba proteus* showed a response decrement to varying lengths of exposure to light (Harris, 1943). After a period of darkness lasting from 10 to 20 seconds the organisms showed an increased responsivity to light. Similarly, once the hindlimb flexion reflex of cats was habituated to a single shock pulse, a shock train was delivered to the animals (Thompson & Spencer, 1966). There was an increase in response followed by a rapid rehabituation. Habituation of the startle response of rats was accomplished by presentation of tones separated by intervals (Davis, 1972). The introduction of a light prompted responding. Eikmanns (as cited in Peeke & Herz, 1973), found toads' orienting response habituated to a dummy placed repeatedly in the same spot of

the visual field, however, when the dummy was placed in a different position the toads oriented to the dummy in the new position.

Based on the success of this experimental model, the habituation portion of Experiments1and 2 was designed in a similar manner. The attractive colours and pattern observed in Part A (unlearned responses) of each experiment were used in Part B (habituation). The stimuli were presented over a combination of massed (20 or 30 responses in the maze) and distributed trials (over two separate sessions) to create conditions conducive to habituation. Then an attractive novel stimulus determined through pilot studies was introduced in a third session of 30 responses. It was expected that initially the bees would choose the preferred stimulus from Part A but that habituation would occur over the first two test sessions. To distinguish this response decrement from fatigue or other explanations besides habituation, it was expected that increased responding would occur when bees were presented with the novel stimulus. This portion of Experiments1 and 2 holds the potential to establish the existence of simple recognition learning for bumble bees in the form of habituation.

Habituation: Theories

Over time researchers began to identify the effects on habituation of stimulus intensity (e.g., Groves, Lee, & Thompson, 1969), frequency (massed vs distributed trials, interstimulus and intertrial intervals) (e.g., Davis, 1970), stimulus specificity, and time course and recovery (Eikmanns and Ewert studies as cited in Peeke & Herz, 1973). Once the empirical evidence for some of these phenomena were consistent, theories were developed to explain them. Sokolov (1963) developed an early theory of habituation based on a series of experiments

with humans. He suggested that a model of the stimulus is formed in the brain when it is first presented to the subject. If a stimulus is presented again it is compared with this model and if it matches, behaviour is inhibited. If a new stimulus is presented which does not match the model, a response is generated. This explains the response decrement over time to a repeated stimulus and increased responding to the test stimuli designed to disrupt habituation. Sokolov's (1963) focus was on the neurophysiological events that occur to explain this stimulus-model comparator theory. Because of the theory's sophisticated explanation it seemed implausible that it could be applied to other species besides humans. Over time, however, the theoretical research culminated in two major groups of theories. The first group states that repeated presentation of a stimulus results in a degeneration in the transmission in a stimulus-response (S-R) pathway, and that another process can be generated by the introduction of a strong novel stimulus which increases transmitter release causing disruption of habituation (e.g., Castellucci & Kandel, 1976; Groves & Thompson, 1970; Thompson & Spencer, 1966). The second group, advanced by learning theorists, states that when the stimulus is received in the sensory register a comparator matches the stimulus with those in memory and then generates a response based on the extent to which it is a familiar or an unfamiliar stimulus (e.g., Konorski, 1967; Sokolov, 1963; Stein, 1966; Wagner, 1976; 1978; 1979).

The most prominent theory to emerge after Sokolov was Groves and Thompson's (1970) dual process theory which suggested that habituation occurs in the S-R pathway and sensitization in the state system. According to dual process theory, these two processes are independent but not mutually exclusive and, in fact, "interact to produce the net response to

repeated stimulation" (p. 421). This theory challenged the established paradigm that used a novel stimulus to disrupt the habituation process to show that the response decrement was not due to fatigue, and instead suggested that responses to a novel stimulus had nothing to do with habituation but were a result of a completely separate process, i.e. sensitization. The authors presented neurophysiological evidence suggesting that two different neural systems were involved for habituation and sensitization. In addition, they presented behavioural evidence to further support their theory and provided an argument to explain the effects of intensity and frequency on habituation. This theory has gained wide acceptance with some researchers and is still tested today (Bee, 2001).

The dual process theory fell short of explaining some complex results in the habituation research. In a series of experiments using the acoustic startle reflex in rats it appeared that the interstimulus interval could have different effects on habituation depending on the interval used during testing or training (Davis, 1970). These results indicated that there may be both short-term habituation which dissipates quickly if interstimulus intervals are short, and long-term habituation which is more likely to occur if the interval between stimulus presentations is longer. A theory was introduced by Wagner (1976) which attempted to explain these two habituation processes. He developed his theory from the information-processing models used to explain human memory (Atkinson and Shiffrin, 1968). Wagner's theory distinguishes between short-term memory (STM) and long-term memory (LTM) and suggests that they play different but interacting roles in generating an habituated response. According to Wagner, when a stimulus is presented it elicits rehearsal until the formation of a representation of the stimulus in short term memory (STM) occurs. When the

stimulus is presented again it primes the representation in short-term memory and a response is generated (in the case of habituation, decreased response to the stimulus occurs). If the stimulus is presented repeatedly, a representation of the stimulus and the associated environmental cues are stored in long term memory (LTM). So stimuli can be represented in STM in two ways: (1) recent presentation of a stimulus (self-generated priming), or (2) associated environmental cues that provoke retrieval from LTM (retrieval-generated priming).

The debate between the dual process theory and Wagner's theory continues (MacIntosh, 1987), and Davis and File (1984) state that "in general, investigators who work with relatively simple organisms adhere to the first, but those who work with complex organisms adhere to the second" (p. 193). Recently, evidence has been growing which challenges their view (Jordan, 2000; Pedreira et al., 1998; Rankin, 2000). For example, studies in habituation with organisms such as the *Caenorhabditis elegans* nematode (302 neurons) show that associative processes may be at work even in these simple creatures (Rankin, 2000). While short-term habituation was examined in Experiments 1 and 2, the next questions addressed in this thesis focus on long-term habituation.

Before any theoretical explanations can be pondered, the behaviour of bees must be observed to determine if habituation is retained and under what circumstances. Rose and Rankin (2001) suggest that there is a "need to understand the dynamics of a behavior before attempting to determine its underlying mechanism" (p. 63). Two variables have been chosen to test long term habituation: time and context. Once the bee has stopped responding to the attractive flower does the passage of time cause the bee to "forget" that the flower is

unrewarding, and are environmental cues such as background foliage used to "remember" the unrewarding flower. To avoid undue repetition the reader is referred to the introductions of each experiment in Manuscript #2, pp. 57 and 63 for the literature review and subsequent rationale for time and context. Experiments 3 and 4 built in replications of the habituation design in Experiments 1B and 2B. Once habituation was established, Experiment 3 then tested the retention of the habituated response over a 2 hour time delay and a 24 hour time delay. Based on the literature review for Experiment 3, it was expected that bees would retain habituation for the 2 hour time delay but not the 24 hour delay.

After habituation was observed, Experiment 4 tested the degree to which context was used as an associative cue. Half of the bees were exposed to the context (maze) without any stimuli present and the other half remained in the colony for the same time period. Based on the Wagner model (1976, 1979), it was expected that the group exposed to the context alone would experience a disruption in the association between the context and the stimuli and would demonstrate increased responding to the previously habituated stimuli. In contrast, it was predicted that the group that remained in the colony between sessions would retain habituation.

Significance

In summary, the primary contribution of this thesis is the development of a method using no reward that enables more accurate assessment of unlearned responses in bumble bees. It goes on to test the viability of this method by examining simple colour and pattern choices by bumble bees on their first visits outside the colony. This begins to address the challenge by Tinbergen (1951) and other bee researchers (e.g., Manning, 1954) to build

foundational knowledge about unlearned processes in order to better understand what gets the bee to the flower in the first place and what is truly learned once experience with the flower occurs. The second contribution of the thesis is examination of short and long term habituation in bumble bees. Habituation is considered to be learning not to respond to stimuli that tend to have no significance in the life of the animal (Thorpe, 1963). This is an area of learning in bees that has been previously ignored and, if the existence of habituation is supported in the data, it will illustrate bees' ability to learn even in the absence of reward.

Manuscript #1

Paper submitted for publication to Animal Behaviour

How do bumble bees first find flowers?:

Unlearned approach responses and habituation

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Abstract

In the absence of previous experience with flowers, how do bees distinguish between possible food sources and non-rewarding objects? Unrewarding stimuli (colours in Experiment 1 and patterns in Experiment 2) were presented in a radial arm maze. The unlearned approach responses of bumble bees (*Bombus impatiens*) were recorded and significant preferences were obtained: the bees chose yellow and blue over other colours, and radial patterns over concentric patterns or unpatterned discs. Habituation was demonstrated when choices proportions for the same pattern by the same bees decreased over two test sessions. When an attractive novel pattern was presented in the third session the trend was reversed. This study confirms both that truly naive bees have unlearned colour and pattern preferences and that learning not to approach these stimuli occurs in the absence of reward.

Introduction

Worker bees provide for themselves and their colony by foraging for nectar and pollen. Much of the literature on bee foraging behaviour has focussed on foraging efficiency and how bees might achieve it. But before a worker bee can bring food back to the colony and learn how to find more, she faces the problem of identifying possible food sources: How do bees find flowers in the first place? In other words, how is it that they tend to approach flowers rather than cars, mailboxes, cows, rocks etc.. This question has been raised frequently since at least Manning (1956), but it has received little attention in the literature until recently–some research on the topic is described below. The behaviour of a worker on her very first foraging trip, before she has obtained any rewards from flowers, might be termed exploratory behaviour. We use the term here not to signify exploration in the teleological sense that bees have any conscious goal of information seeking, but rather in the sense that their behaviour serves to expose them to the conditions which are most likely to yield reinforcement and lead to subsequent learning of what is important for them (Shettleworth, 1998).

In this paper we examine two aspects of exploratory behaviours in bumble bees (*Bombus* spp. Hymenoptera: Apidae). Because these behaviours occur prior to reward from flowers they can be described as unlearned behaviours [a seldom used alternative expression is "prefunctional" (Hogan, 1988)]. The first aspect of exploratory behaviour that we examined is approach by the bees to particular colours and patterns. Some of the impetus for this part of the research stemmed from our own anecdotal observations of bees landing on illustrated packets of flower seeds, blue and white patterned soccer balls, and clothing with

floral prints. In our lab a photograph of a flower elicited the strongest possible response from a naive bee: approach, landing and proboscis extension (Plate I). These unrewarding objects seem to have been "mistaken" for flowers, and so we began to investigate whether they had "fooled" the bees by virtue of some particular characteristics such as their colour or pattern. The second aspect of exploratory behaviour that we examined was possible habituation to stimuli with these characteristics. Even if bees have unlearned preferences for some floral characteristics, these preferences would be expected to wane after a few unrewarded visits. Unless the preferences were modifiable, the bees might persist indefinitely in their unrewarding behaviours and never find food.

While the overriding interest in bee behaviour has often been on learned behaviour, recently the question of unlearned behaviour has received attention. This research has suggested, for example, that flower-naive honeybees have unlearned colour preferences for blue and green (Giurfa , Núñez, Chittka & Menzel. 1995) and pattern preferences for radial flower-like patterns (Lehrer , Horridge, Zhang & Gadagkar 1995). These studies and others before them, however, have used methods which potentially interfere with an accurate assessment of unlearned responses because they typically involve rewarding the bees in the experimental apparatus, such as a Y-maze or radial arm maze, before testing them. For example, bees might be rewarded for approaching an ostensibly neutral stimulus such as black and white checkerboard or a grey disk, and then tested for preferences on new colours or patterns where all the new stimuli are thought to be equally similar or dissimilar to the neutral ones. Using this approach, it is not possible to rule out for certain the possibility that the rewarded experience on the neutral stimuli may have affected the behaviour on the new

stimuli–indeed, in explicit tests of stimulus generalization, some colour choices by bumble bees have been found to be controlled by generalization from learned colours (Gumbert 2000). One way to obviate this difficulty is not to use reward at all. In this way the unlearned ("innate") preferences of bumble bees have been investigated (Lunau 1990, 1991, 1992; Lunau & Maier 1995; Lunau, Wacht & Chittka 1996) by testing their reactions to artificial flowers in a flight cage. The approach flight from 1 metre and antennal touching were found, for example, to be elicited by optical flower signals such as gradient of spectral purity (Lunau 1990). Although this method rules out any associative learning from training stimuli, the dependent measure of approach flight can itself be intractable. At a distance of 1 metre, it may be difficult to discern whether a bee is truly approaching the test stimuli or whether it is simply in free flight.

The present series of experiments combines the approaches described above to testing unlearned behaviour: bees were tested in a radial arm maze, and so a clear-cut and easily measurable dependent variable of entrances into a corridor could be used. Unlike previous maze studies, however, no reward in the maze was given prior to testing for preferences. The specific questions addressed by the experiments presented here are: (1) Do naive bumble bees have preferences for pattern and colour? (2) If so, are these preferences altered by repeated exposure?

General Methods

Subjects

Five colonies of bumble bees, *Bombus impatiens* Cresson, were used for the full series of experiments. The colonies were raised in the laboratory from captured queens according to the procedure developed by Plowright & Jay (1966). All bees were identified by gluing numbered labels of various colours onto the thorax. In the colony the bees were provided with an ad libitum supply of pollen and honey solution (honey and water 2:1 by volume).

Materials and Apparatus

Each colony was housed in a wooden domicile 30 cm x 15 cm x 15 cm. The domicile was attached to a screened flight cage (1.83m x 1.83m x 1.83m) by a wooden walkway (33 cm). Glass plates were placed on the top of the walkway with two vertical gates to allow easy identification and corralling of bees leaving and entering the colony. Three panels of fluorescent light fixtures illuminated the flight cage.

A radial arm maze, modelled on that of Lehrer et al. (1995), was constructed with twelve corridors (15 cm long) which opened onto a central area (22 cm across). The entrance to each corridor was 6 cm wide. The floor and ceiling of the maze were made with 1/8" clear plexiglass and the external walls and corridors were made with 1/8" opaque grey plexiglass. All pieces were fixed with a plexiglass epoxy except for the ceiling which was removable. Testing stimuli (round - 12 cm diameter; rectangle - 14 cm x 15 cm) were positioned vertically at the end of each corridor which was 14 cm high x 14 cm wide.

Procedure

Training to forage in the flight cage: The colony was deprived of honey solution for two days prior to training. On the first day of training, bees leaving the colony for the first time were allowed to forage freely from eight pots (1.5 cm diameter, 0.5 cm deep) filled with honey solution. The pots were placed on a wooden tray approximately 1 m from the floor in the far right corner (relative to the colony entrance) of the flight cage. This foraging session lasted approximately 2 h and was implemented to select foragers for further training the next day. On the second day, bees that were observed foraging on the previous day were again allowed to forage and the identifying numbers of these bees were recorded. After one hour of foraging, the bees were returned to the colony. Only the bees that were observed on both training days were selected for testing.

Testing for preferences. The tray of pots was removed from the cage and the 12-arm radial maze was put in its place, on top of a 1 m high stool in the centre of the flight cage. The stimuli were placed at the end of the corridors in the maze. As each of the selected bees exited the walkway, it was captured in a small vial and immediately placed into the centre of the maze. Bees were tested at this point in the procedure to ensure that they were still in "foraging mode", having just made several trips to the tray of pots filled with honey solution. Once the bee began to fly in the maze, the experimenter then recorded the first twenty or thirty choices. A choice was determined to be made when the bee flew forward across the entrance to the corridor toward the stimulus. Between Test sessions the maze was rotated 90° to control for position of stimuli, shadows, and other inconsistencies in lighting. Also,

the experimenter's position in relation to the maze was changed. This procedure was repeated in its entirety with two more colonies.

Statistical analysis

For all experiments in this series, the data were counts and a log-linear analysis was carried out using GLIM version 4.0 statistical software (Francis, Green, & Payne, 1993).

EXPERIMENT 1A: TESTING FOR COLOUR PREFERENCES

Part A of the first experiment tested naive bumble bees' responses to blue, yellow, white, and red stimuli where no reward was offered in association with the stimuli. Although the colours referred to in this paper are named as they appeared to the experimenters, colour will be defined at the physical level (i.e., dominant wavelength, spectral purity, and intensity) as we cannot draw conclusions about the perceptual experience of colour for bees from understanding human perception of colour (i.e., hue, saturation, and brightness). The quotation at the head of this paper illustrates the commonly held view that blue is the colour that bees "love best". That bees have a preference for blue and yellow has been confirmed in the literature (e.g., Lunau & Maier 1995). Testing for these preferences here is necessary to validate our method. It is also a prerequisite for subsequent testing of the effects of repeated exposure (Experiment 1B). The objective of this research was not, however, to investigate the details of colour perception and so if colour preferences for blue and yellow did emerge, we could not conclude whether they were due to differences in hue, saturation or brightness.

Methods

The methods were described under General Methods. Forty-five naive bumble bees from three colonies were used. Three replicates of four differently coloured discs made from construction paper were used: Yellow, blue, white, and red. The colours were assessed by eye on three dimensions according to Munsell's (1967) specifications (Hue=Wavelength, Chroma=Spectral Purity, Value=Intensity): Yellow = Hue-5Y, Chroma-8, Value-8.5; Blue = Hue-5PB, Chroma-8, Value-5; Red = Hue-2.5R, Chroma-10, Value-5. A within-subjects design was used where choices of four different colours out of n = 20 choices were recorded for each bee.

Results and Discussion

Thirteen bees from Colony 1, 10 bees from Colony 2, and 10 bees from Colony 3 were observed making a total of 660 colour choices. The mean number of choices for each colour (blue, yellow, white and red) out of 20 choices is displayed in the pie chart in Fig.1. A significant main effect for colour was found ($\chi_3^2 = 205.90$, $P < 0.001$). Fig. 1 shows that bees chose blue and yellow stimuli more frequently than white or red. The effect of colour interacted with colony ($\chi_6^2 = 23.08$, $P < 0.001$): in Colony 2 the bees favoured yellow over blue more so than in the other colonies, but both blue and yellow were still more attractive than either red or white.

EXPERIMENT 1B: TESTING FOR HABITUATION TO COLOUR

Habituation consists of a response decrement resulting from repeated stimulation (Harris 1943): "Behaviour changes in such a way that time and energy are not wasted in

unnecessary, inappropriate, or ineffective behaviours" (Shettleworth 1998, p. 142). Habituation has been well-documented in a wide variety of invertebrates and vertebrates (e.g., Harris 1943). It is a form of learning, and as such, it does not include response decrements due to fatigue, trauma, aging, etc. (Thompson & Spencer 1966). Such explanations can be ruled out by presenting the animal with a novel stimulus: if an increase in responding is observed then factors such as fatigue can not have been responsible for the waning in behaviour.

In a review of the literature on habituation in 1943, Harris concluded that it had not yet been systematically studied in Hymenoptera. In the ensuing years the study of habituation in this Order has remained all but untouched. Experiment 1B tested whether habituation takes place when stimuli are repeatedly presented without reward. Bees were repeatedly exposed to the same stimulus to see if responding would decrease given that no reward was offered. Then, after 60 presentations of the original stimulus, a novel stimulus was presented to assess whether the decreased responding was due to fatigue or other confounding variables. Evidence in favour of habituation would be obtained if the bees responded to the novel stimulus more frequently than to the habituated stimulus. Three conditions were planned to test these hypotheses: a Control condition in which the stimulus array (yellow vs grey stimuli at the end of the corridors in the maze) was unchanged over 3 Test Sessions, and two "Array Change" conditions in which the yellow stimuli were replaced with a different colour on the third Test Session.

Methods

Subjects, Materials and Procedure

The methods were as described in the General Methods above, with the following exceptions: Twenty seven naive bumble bees from three colonies were used (nine bees per condition). A binary choice between six yellow rectangular stimuli (14 x15 cm) and six corridors with no stimuli (just the grey end of the corridor) was presented for each condition. Blue stimuli were introduced in the Yellow to Blue Array Change condition and red stimuli in the Yellow to Red Array Change (see below). The number of choices allowed was increased from 20 to 30 to increase the opportunity for habituation. If the bee stopped choosing before thirty choices were recorded, no data points were used (4 bees). Each day 3 bees were tested and returned to the colony after testing. When Test Session 1 was completed, the tested bees were allowed to forage from the pots of honey solution for at least 15 min. Once the bees returned to the colony after foraging and re-entered the walkway, they were tested a second time using the same procedure as above. Again, after Test Session 2 when the tested bees re-emerged from the colony, they were allowed to forage for at least 15 min. Once they returned to the colony and came out again, Test Session 3 commenced using the same procedure as above. Testing began at approximately the same time each day and terminated on the same day when each of the three bees had completed three test sessions.

Control: All Yellow Array: Bees were presented with 6 yellow rectangles and 6 corridors were left empty (grey plexiglass). The yellow stimuli were not placed in alternating corridors: the assignment of colour to corridor was random.

Yellow to Blue Array Change: The procedure was identical to the All Yellow Array condition with the exception of the stimuli used during Test Session 3 where the 6 yellow rectangles were replaced with 6 blue rectangles. The empty grey corridors remained as the alternative choice.

Yellow to Red Array Change: The procedure continued as in the Yellow to Blue Array Change but the 6 yellow stimuli used in the first two Test Sessions were replaced in Test Session 3 with red instead of blue rectangles.

Design

A 2 (Yellow/Blue/Red vs Grey) x 3 (Test Sessions) within-subjects design was used. Number of choices out of n=30 for each bee was examined for each condition, where condition was a between-subjects factor.

Results and Discussion

For each condition, 3 bees each from Colonies 1, 2 and 3 were observed making 90 choices over 3 Test Sessions for a total of 2,430 choices. The mean number of choices out of 30 for each stimulus and Test Session is displayed for all 3 Conditions in Fig. 2.

All Yellow Array: The bees showed a significant preference for yellow over grey ($\chi_1^2 = 42.16$, $P < 0.001$). Bees' choices, however, depended on Test Session ($\chi_2^2 = 36.10$, $P < 0.001$). This interaction was not dependent on colony ($\chi_{10}^2 = 8.94$, NS): Figure 2a illustrates that preference for yellow over grey was most pronounced in Test Session 1, whereas no preference for yellow was found for Test Sessions 2 and 3. The number of

choices for yellow decreased significantly from Test Sessions 1 to 2 ($\chi_1^2 = 30.39$, $P < 0.001$).

Yellow to Blue Array Change Again, a main effect of yellow vs grey was found ($\chi_1^2 = 46.92$, $P < 0.001$) and choice varied according to test session ($\chi_2^2 = 25.95$, $P < 0.001$). This interaction was not dependent on colony ($\chi_{10}^2 = 6.87$, NS). The means in Fig. 2b indicate that bees preferred yellow over grey in Test Session 1 but not in Test Session 2 ($\chi_1^2 = 19.71$, $P < 0.001$). When the blue stimuli were introduced in Test Session 3, number of choices for grey did not decrease when compared to Test Session 2, i.e. there was no increase in "interest" when the new stimulus was introduced.

Yellow to Red Array Change: As in the first two conditions the effect of yellow vs grey was significant ($\chi_1^2 = 36.80$, $P < 0.001$). Choice depended on Test Session ($\chi_2^2 = 23.66$, $P < 0.001$). This interaction was not dependent on colony ($\chi_{10}^2 = 2.873$, NS). The means in Fig. 2c indicate that bees preferred yellow over grey in Test Session 1. This preference waned in Test Session 2 ($\chi_1^2 = 6.01$, $P < 0.025$). When the red stimuli were introduced in Test Session 3, number of choices for grey did not decrease–again, the new stimulus did not elicit renewed approach behaviour. In Test Session 1 the most frequent choice for all of the bees was yellow except for one bee which showed no preference for yellow.

In summary, bees chose the yellow over the grey stimuli in Test Session 1 in all 3 conditions. The response decrement between Test Sessions 1 and 2 suggests that habituation may have occurred. Unfortunately, neither the introduction of a previously attractive (Experiment 1A) blue stimulus nor a previously unattractive red stimulus produced any

renewed responding in Test Session 3, and so the evidence for habituation is not at hand. Fatigue may possibly account for the response decrement though this is unlikely given how frequently and actively bees forage in a natural environment. Alternatively, a novel colour stimulus may not be sufficiently different to provoke responses, that is, one colour may generalize to another. Perhaps in order to trigger an increase in responding, the novel stimulus must differ along other dimensions besides colour, or perhaps along more salient dimensions, e.g., pattern, shape, size.

EXPERIMENT 2A: TESTING FOR PATTERN PREFERENCES

This experiment tested for preferences by bumble bees of radial over circular patterns. The patterns used were based on the stimuli used by Lehrer et al. (1995) in a series of tests for pattern preferences in honey bees. We chose one pattern which produced marked preference (a radial pattern), and one which produced marked avoidance (a concentric pattern). Rather than present the patterns in black and white, blue and yellow stimuli were used since these colours were shown to be attractive to bees in Experiment 1A.

Methods

Groups of 10 to 13 naive bumble bees from 3 colonies were used for a total of 33 bees. Four replicates of 3 stimuli made from construction paper were used: a radial pattern with 6 alternating wedges of blue and yellow; a blue and yellow concentric circular pattern with 4 bands; and a plain blue disc. A within-subjects design was used where choices of three different stimulus types out of n=20 choices were recorded.

Results and Discussion

Nine bees from Colony 1, 19 bees from Colony 2, and 5 bees from Colony 3 were observed making a total of 660 pattern choices. The mean number of choices for each stimulus type (radial, blue, and concentric) is displayed in Fig. 3. Figure 3 reveals preferences for the radial pattern and accordingly, the choices for the blue disc and the concentric pattern were combined in analysis and compared with the radial pattern. Congruent with observations obtained by Lehrer and colleagues (1995), bees showed a significantly higher choice proportion for the radial pattern ($\chi_1^2 = 29.07$, $P< 0.001$) compared to the concentric and plain discs combined. The effect of pattern did not interact with colony ($\chi_2^2 = 2.06$, NS).

EXPERIMENT 2B: TESTING FOR HABITUATION TO PATTERNS

Although a response decrement over time occurred in Experiment 1B, the failure of a novel stimulus to provoke responses left in question whether bees' responses were indeed habituated. Thus, some changes were applied to Experiment 2B: The number of choices observed in Test Sessions 1 and 2 were lowered from 30 to 20 to control for possible effects of fatigue and the Test stimuli included the addition of pattern to colour.

Methods

Subjects, Materials and Procedure

Forty-five naive bumble bees from three colonies were used. A binary choice between patterned stimuli vs blue discs (shown to be attractive in Experiment 1A) made from construction paper were used for each condition. The patterned stimuli varied for each

condition (Fig. 4). In the two control conditions, the same stimuli were used for all three Test Sessions. For the other three conditions ("Array Changes"), the patterned stimuli were changed between Test Sessions 2 and 3.

Controls: All Radial Array and All Floral Array: For the All Radial Array condition the bees were presented with 6 radial discs of alternating blue and yellow and 6 plain blue discs for all 3 Test Sessions. Assignment of discs to corridors was random. Two bees stopped choosing before 20 choices were recorded and so none of their data points were used . In the All Floral Array condition, 6 floral stimuli and 6 blue discs were presented for all 3 Test Sessions. The floral stimuli were copies of the photograph of wild flax (Plate I) that we had previously seen a bee probing.

Array Changes: Radial to Floral, Floral to Radial and Radial to Concentric: The procedures for these conditions were identical to those described above with the exception that the stimuli used during the third Test Session were different from those in the first two. In the Radial to Floral Array Change, the 6 radial discs were replaced with 6 floral stimuli. The Floral to Radial Array Change was administered in reverse: the floral stimuli were presented for the first 2 Test sessions followed by the radial pattern in the Test Session 3. The Radial to Concentric Array Change was similar to the Radial to Floral Array Change, but during Test Session 3 the unattractive concentric pattern was presented.

Design

A 2 (pattern vs blue disc) x 3 (Test Sessions) within-subjects design was used. Number of choices for each stimulus type out of n = 20 was examined for each bee for each of five conditions, where condition was a between-subjects factor.

Results and Discussion

For each of the five conditions, 9 bees from 3 colonies were observed making 60 choices over 3 Test Sessions for a total of 2,700 choices (9 bees x 60 choices x 5 conditions). The mean number of choices for each stimulus and for each Test Session are displayed for all five conditions in Fig. 4.

Controls

All Radial Condition: There was a significant main effect of pattern vs blue disc ($\chi_1^2 = 30.63$, $P < 0.001$). Bees' choices depended on test session ($\chi_2^2 = 37.37$, $P < 0.001$; interaction with colony $\chi_{10}^2 = 2.81$, NS). Figure 4a illustrates that preference for the radial pattern was stronger in Test Session 1 than Test Session 2 ($\chi_1^2 = 29.15$, $P<0.001$), whereas no preference was shown for the radial pattern over the blue disc for Test Sessions 2 and 3 . In other words, in Test Sessions 2 and 3 the bees chose the radial pattern and the blue disc in approximately equal proportions.

All Floral Array: The effect of pattern vs blue disc was significant ($\chi_1^2 = 4.64$, $P < 0.05$) and choice depended on Test Session ($\chi_2^2 = 46.21$, $P < 0.001$), interaction with colony $\chi_{10}^2 = 4.54$, NS). Figure 4b shows that the floral pattern was preferred over the blue disc in Test

Session 1 but this preference waned by Test Session 2 ($\chi_1^2 = 26.28$, $P < 0.001$). As in Test session 2, bees showed no preference for the floral pattern over the blue disc in Test Session 3.

Array Changes

Radial to Floral Array Change: Again, a significant main effect of pattern vs blue disc was shown ($\chi_1^2 = 103.0$, $P < 0.001$) and choice varied according to Test Session ($\chi_2^2 = 23.55$, $P < 0.001$; interaction with colony $\chi_{10}^2 = 5.95$, NS). Figure 4c shows that bees preferred the radial pattern over the blue disc in Test Session 1 but not in Test Session 2 ($\chi_1^2 = 21.31$, $P < 0.001$). When the floral pattern was introduced in Test Session 3 an increase in responding occurred: the number of choices for the blue disc decreased significantly when compared to Test Session 2 ($\chi_1^2 = 12.17$, $P < 0.005$).

Floral to Radial Array Change: Figure 4d shows effects similar to those described for the Radial to Floral Array Change Condition. The effect of pattern vs blue disc was significant ($\chi_1^2 = 23.40$, $P < 0.001$) and again choice depended on Test Session ($\chi_2^2 = 29.20$, $P < 0.001$; interaction with colony $\chi_{10}^2 = 9.56$, NS). As in the All Floral Array, bees' preference for the floral pattern over the blue disc decreased significantly from Test Session 1 to 2 ($\chi_1^2 = 17.23$, $P < 0.001$). When the radial pattern was introduced in Test Session 3, number of choices for the blue disc decreased significantly compared to Test Session 2 ($\chi_1^2 = 25.44$, $P < 0.001$): the new pattern triggered an increase in responding.

Radial to Concentric Array Change: As in the other conditions the effect of pattern vs blue disc was significant ($\chi_1^2 = 21.75$, $P < 0.001$) and choice depended on Test Session ($\chi_2^2 = 20.65$, $P < 0.001$; interaction with colony $\chi_{10}^2 = 6.32$, NS). Figure 4e indicates that bees

preferred the radial pattern over the blue disc in Test Session 1. This preference waned in Test Session 2 ($\chi_1^2 = 5.47$, $P < 0.025$). When the concentric pattern was introduced in Test Session 3, number of choices for the blue disc did not decrease.

In summary, in all five conditions bees showed a preference for the patterns (either radial or floral) over the blue discs in Test Session 1 and this preference diminished by Test Session 2. When a previously attractive stimulus was introduced in Test Session 3, number of choices for the blue disc decreased (both in the Floral to Radial and the Radial to Floral Array Changes). Not any new stimulus will elicit renewed responding, however: when the unattractive stimulus (concentric pattern) was introduced in Test Session 3, there was no increase in approach behaviour

General Discussion

How do bumble bees first find flowers? Our results elucidate two components of exploratory behaviour. The first component (Experiments 1a and 2a) is an unlearned preference for certain floral characteristics. Certain colours and patterns seem inherently attractive to bumble bees. In our radial arm maze in which the bees never once obtained reward, blue and yellow was chosen over red or white (Fig. 1), and radial patterns were approached more frequently than concentric patterns and unpatterned stimuli (Fig. 3). Moreover, no colony differences in these preferences were obtained. These unlearned preferences are important for three reasons: (1) they show that bumble bees, like honey bees, seem to have an "innate preference for flower-like patterns" (Lehrer et al. 1995), though this

in itself is not surprising, (2) they rule out the possibility that these preferences might in any way be attributable to reward obtained in the maze and (3) they are a prerequisite for studying the effect of repeated exposure discussed below. The method described here could be used in future research to address other issues such as the roles of spectral purity (Lunau 1992) vs dominant wavelength (Gumbert 2000) in colour preferences. One methodological improvement for the future might be to measure time to choose in addition to choice.

Colour and pattern preferences are advantageous to bees insofar as they facilitate landing on flowers rather than other objects, and obtaining food. If the preferences persisted for long in the absence of reward, however, they would soon become a liability instead of an asset. This second aspect of exploratory behaviour has received little if any attention in the literature. A waning with time in approach behaviour to yellow was observed in Experiment 1B. Unfortunately we could not conclude that this decrease was an instance of habituation (as opposed to fatigue or some other explanation other than learning), because renewed responding was not obtained when a new stimulus (either blue or red) was presented. Such evidence was obtained, however, in Experiment 2B: a decrease in responding to floral patterns (relative to blue discs) over three Test Sessions was obtained (Fig. 4b), but the introduction of a radial pattern in Test Session 3 triggered approach behaviour (Fig. 4d). Similarly, a decrease over time in responding to radial patterns was obtained (Fig 4a), but the introduction of a floral pattern sparked exploration once more (Fig. 4c). Most interesting from an ecological point of view was that there seemed to be a limit on what would trigger the renewal in approach behaviour. Even after habituation to a radial stimulus occurred, the bees did not then approach a concentric stimulus (Fig. 4e). Apparently, after a few

unrewarding experiences with potential flowers, bees will move on to other potential flowers and not just anything.

The results described above fill a void in the psychological literature on habituation, which has been neglected in Hymenoptera. A variety of theories have been advanced to account for habituation, and these fall broadly into two groups. The first group suggests that repeated presentation of a stimulus results in a degeneration in the transmission in a stimulus-response pathway (habituation), and that sensitization can be generated by the introduction of a strong novel stimulus which increases transmitter release causing disruption of habituation (e.g., Castellucci & Kandel 1976; Groves & Thompson 1970; Thompson & Spencer 1966). The second group, originally conceived by Sokolov (1963) and developed by Wagner (1976, 1979), states that when the stimulus is received in the sensory register a representation is then formed in memory. When the stimulus is presented again, a response is generated based on the extent to which it is a familiar or an unfamiliar stimulus (e.g., Sokolov 1963; Wagner 1976; Wagner 1979). Mackintosh (1987) argues that to date habituation studies can be explained by either group of theories. Distinguishing between these two sorts of theories as an account for the habituation documented here is a challenge for future research. One possible avenue for further work is to explore the role of context which acts as an associative cue used to prime the memory according to the Wagner model (1976, 1979).

A few examples in the literature have studied the behaviour of naive bees. One such example is that of *Bombus consobrinus* which specializes on *Aconitum* (monkshood) and shows unlearned behavioural adaptions to handling these flowers (Laverty & Plowright 1988). Compared to naive workers of generalist species (*B. fervidus*, *B. pennsylvanicus*)

which usually initiate probing in the wrong places and often fail to locate the nectary, naive workers of *B. consobrinus* are more likely to probe in the vicinity of the nectary and to quickly obtain reward. Another example traces the developmental sequence of how unlearned behaviours in bees are fine tuned by experience: blue is not only a colour that is preferred by naive honey bees, but it is also learned faster than other colours (Menzel 1985). The interplay between rewarded experience and unlearned behaviour is relevant to the current interest in the "cognitive ecology of pollination" (Chittka & Thomson 2001). Our research underscores the further point that absence of reward is also effective in shaping unlearned behaviours into efficient foraging behaviours: bees can learn quickly not to approach that to which they were first drawn.

Acknowledgments

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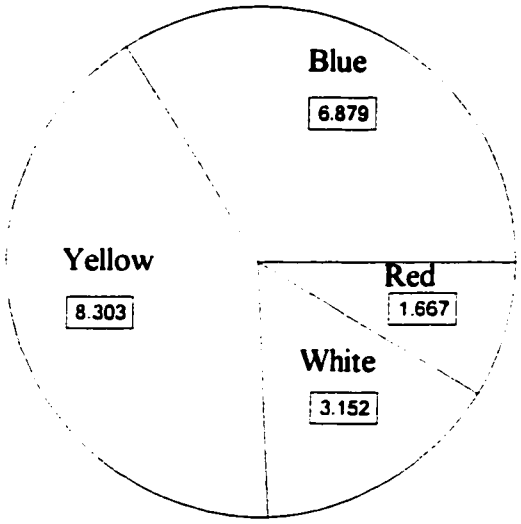
Figure Captions

Figure 1 Mean number of choices for each colour out of 20 choices (n=660 observations) in Experiment 1A.

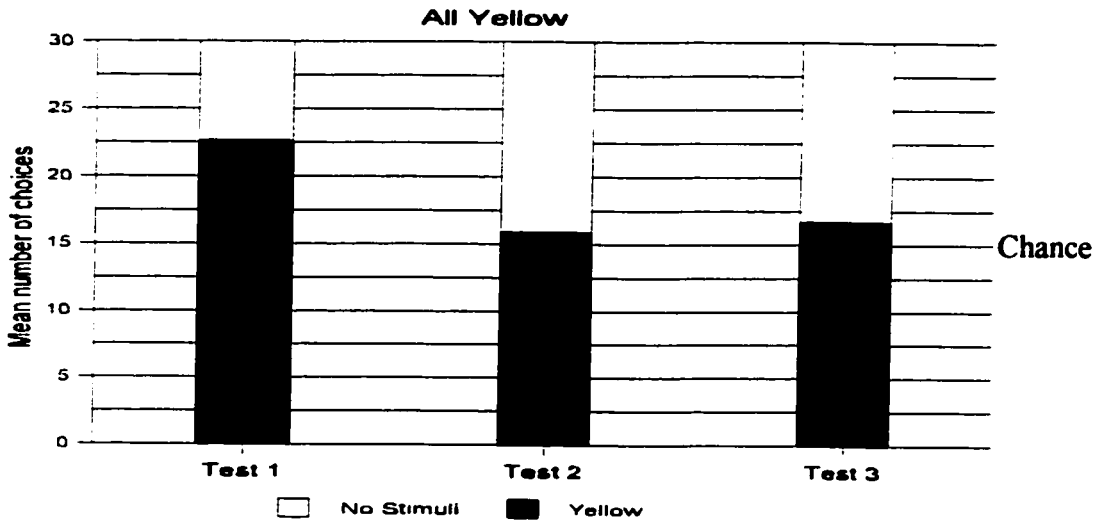
Figure 2 Mean number of choices out of 30 over three Test Sessions separated by 15 minutes for 3 conditions in Experiment 1B (a) Control condition - Yellow stimuli used for 3 Test Sessions; (b) On the 3rd Test Session the yellow stimuli are replaced with blue; (c) On the 3rd Test Session yellow stimuli are replaced with red.

Figure 3 Mean number of choices for each stimulus type out of 20 choices (n=660 observations) in Experiment 2A. The choice proportion for the radial pattern was significantly greater than the choice proportion for the other two stimulus types combined ($P < .001$).

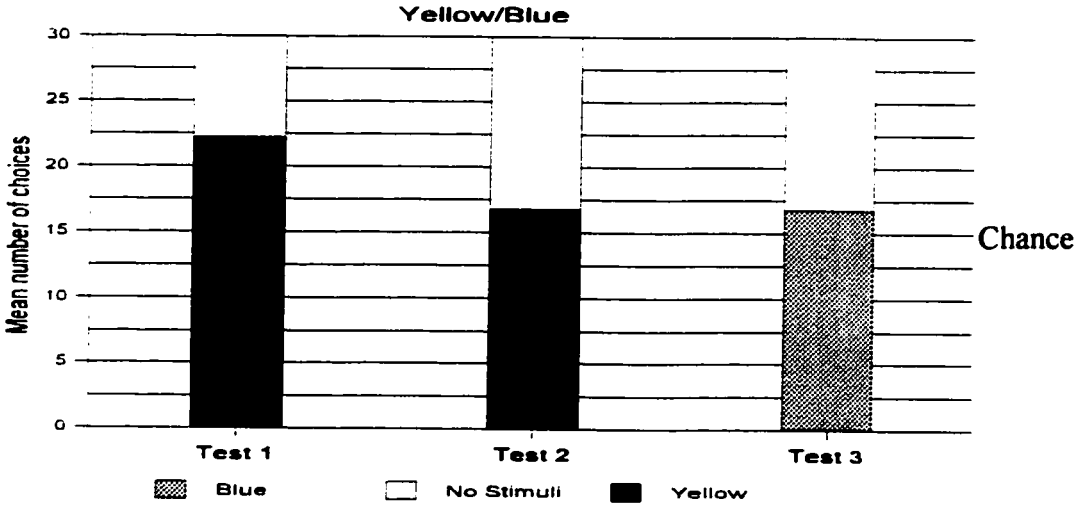
Figure 4 Mean number of choices out of 20 over 3 Test Sessions separated by 15 minutes for five conditions in Experiment 2B. Two control conditions: (a) Radial stimuli vs blue discs used for 3 tests; (b) Floral stimuli vs blue discs used for 3 tests; Three conditions in which a stimulus change occurred on the 3rd Test Session: (c) the radial stimuli are replaced with floral; (d) floral stimuli are replaced with radial; (e) Radial stimuli are replaced with concentric.


Blue
6.879
Yellow
8.303
Red
1.667
White
3.152

(a)


All Yellow
Mean number of choices
30
25
20
15
10
5
0
Chance
Test 1
Test 2
Test 3
No Stimuli
Yellow

(b)


Yellow/Blue
Mean number of choices
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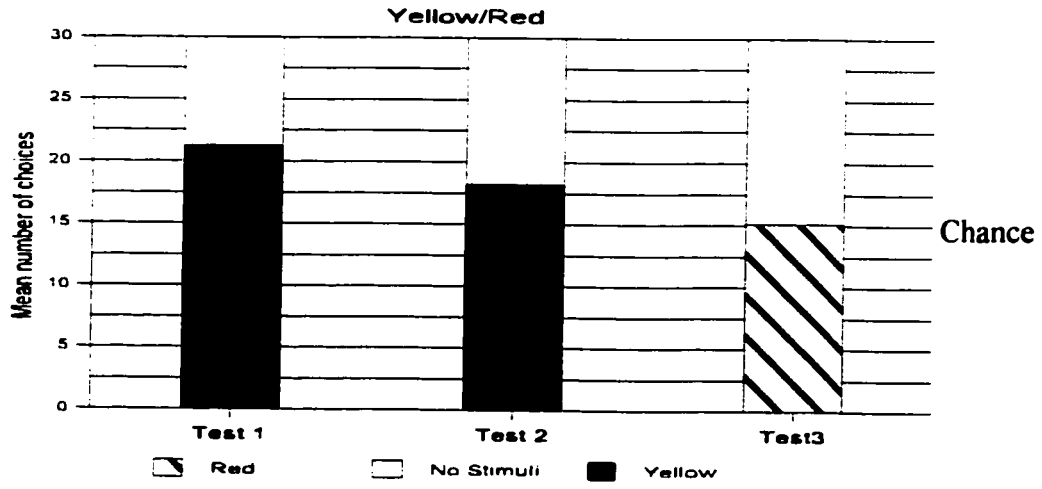
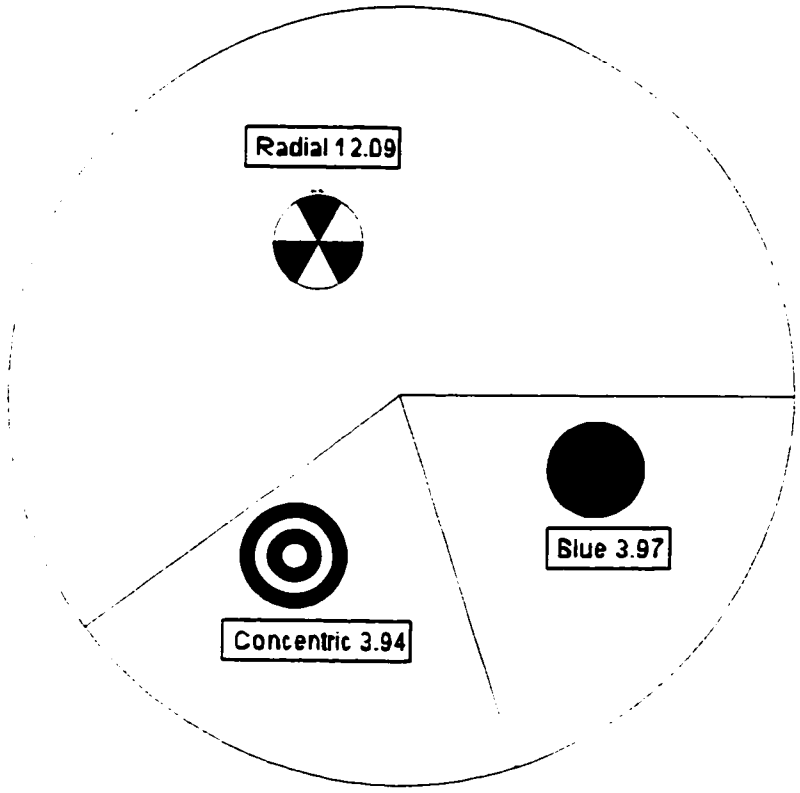
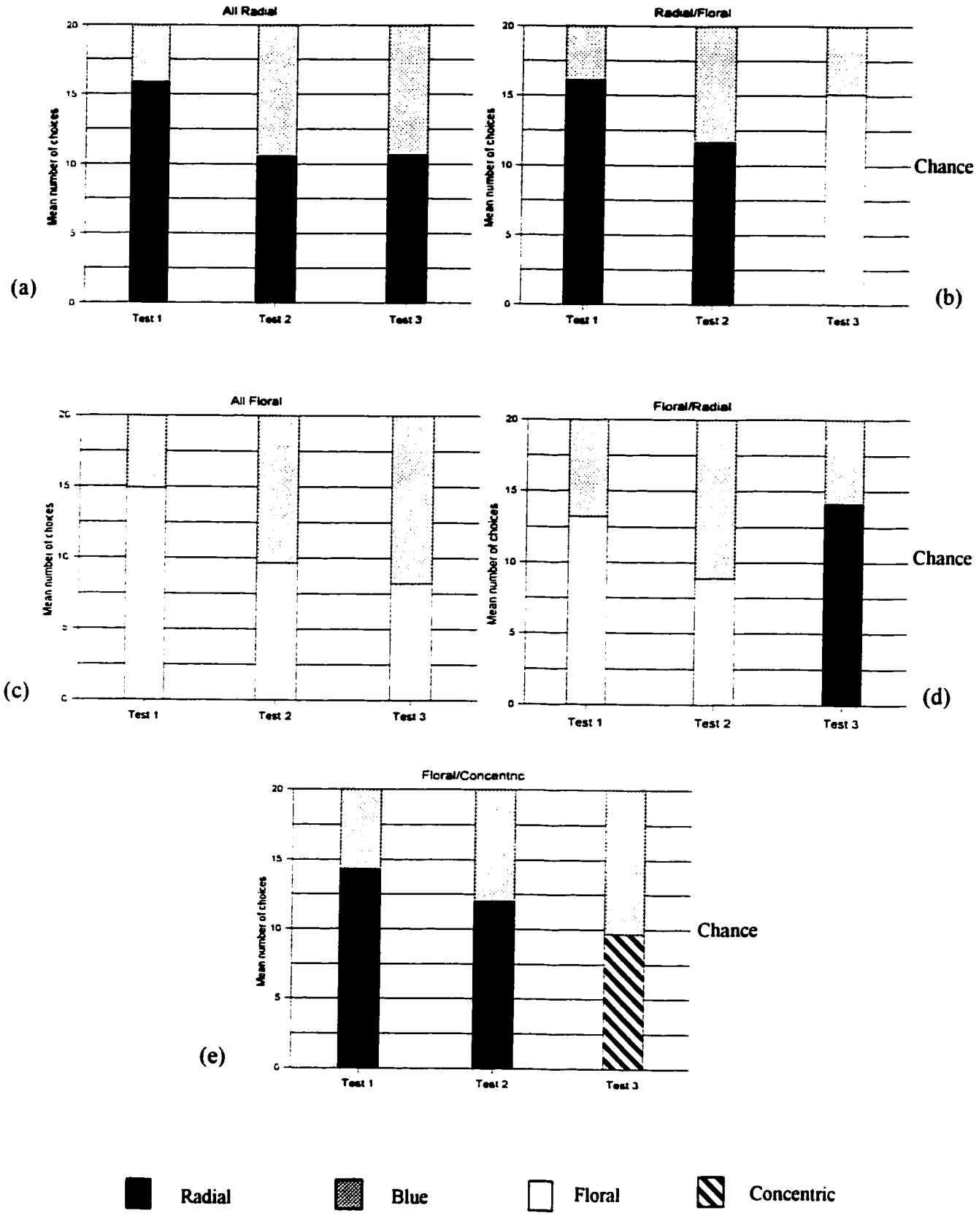
25
20
15
10
5
0
Chance
Test 1
Test 2
Test 3
Blue
No Stimuli
Yellow

(c)


Yellow/Red
Mean number of choices
30
25
20
15
10
5
0
Chance
Test 1
Test 2
Test3
Red
No Stimuli
Yellow


Radial 12.09
Blue 3.97
Concentric 3.94


All Radial
Radial/Floral
All Floral
Floral/Radial
Floral/Concentric
Mean number of choices
Test 1
Test 2
Test 3
Chance
(a)
(b)
(c)
(d)
(e)
Radial
Blue
Floral
Concentric

Manuscript #2

The effects of time and context on habituation to visual stimuli by bumble bees

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Abstract

Two experiments tested the length of time that habituation is maintained by bumble bees and the degree to which contextual cues contribute to habituation. In Experiment 1 once bees habituated to a radial pattern, response decrement was maintained after a stimulus presentation delay of two hours. After a 24 hour delay, however, the preference was restored. In Experiment 2 exposure to the context without stimulus presentation did not affect the retention of the habituated response. These findings confirm bumble bees' ability to habituate to a stimulus and delineate its time course as lasting in the range of minutes and hours but not days. They call into question the associative role of contextual cues in habituation for bumble bees.

Keywords: Habituation, bumble bees, long-term retention, context, memory.

Introduction

Habituation as a form of learning has been well-established in the literature across species (Harris, 1943; Peeke & Herz, 1973). It was first demonstrated in bumble bees in two recent experiments by Simonds & Plowright (submitted manuscript, 2002). Using a method designed to allow responses to stimuli even in the absence of reward, a series of experiments was conducted to determine whether bees habituate to an initially attractive stimulus. Bees were observed making choices in a radial arm maze between a blue and yellow radial stimulus and a blue disc over three successive test sessions. Reward was never obtained in the maze. An initial preference for the radial stimulus in the first test session disappeared in the second and third test sessions. When a previously attractive novel stimulus was presented in the third test session, however, choice proportions increased significantly for the novel stimulus, thereby ruling out other explanations for the lowered responding such as fatigue. The habituation observed in these experiments was interpreted in terms of Wagner's (1976/78/79) model which states that when a stimulus is presented it elicits rehearsal until the formation of a representation of the stimulus in short term memory (STM) occurs. When the stimulus is presented again it primes and compares the representation in short-term memory and results, in this case, in decreased responding to the radial stimulus. This type of rehearsal is referred to by Wagner as "self-generated priming" of STM.

As these experiments represent the first documentation of habituation in bumble bees, replication is necessary. In addition, exploration of some of the conditions for this type of learning would contribute to understanding the maintenance of the habituated response. Replication of habituation was incorporated into the current experiments and two potential controlling variables were chosen for investigation: time in Experiment 1 and context in Experiment 2. The rationales for these choices are presented below. Findings from these

experiments will replicate support for habituation by bumble bees in the absence of reward and, furthermore, it holds the potential to move beyond just the existence of habituation by bees but may begin to isolate the mechanisms and controlling stimuli implicated.

Experiment 1: How long do bees retain an habituated response?

Retention of habituation has been demonstrated in vertebrates with durations ranging from 24 hours to up to 42 days (e.g., Davis, 1972; Leaton, 1974,1976). Even invertebrates (e.g., crabs) show retention of habituation after 24 hours (Lozada, Romano & Maldonado, 1990; Pedreira, Dimant, Tomsic, Quesada-Allue, & Maldonado, 1995). Based on these results habituation is widely thought to be "a relatively permanent change in behaviour" (p. 248, Leaton, 1976). There is good evidence that a number of factors contribute to the retention of the habituated response, for example, the number of stimulus exposures, the length of the inter-stimulus interval, and the salience of the environmental cues associated with the stimulus (Bonardi, Guthrie, & Hall, 1991; Davis, 1970; Pedreira, Romano, Tomsic, Lozada, & Maldonado, 1998; Rankin, 2000; Staddon & Higa, 1996).

Although the study of habituation in bumble bees is recent, the existence of long-term retention of responses in bees has been demonstrated (Gerber, Wüstenberg, Schültz & Menzel, 1998; Gould, 1987). In an experiment designed to assess long-term retention in honeybee classical conditioning, propionic acid was used for the conditioned stimulus and the unconditioned stimulus was sucrose solution administered to an antenna (Gerber et al., 1998). The measured response was proboscis extension. Intervals between trials were 30 seconds, 1 minute, 3 minutes, or 20 minutes. Bees were able to retain the conditioned response across all trials.

These data tell us that bees have the ability to retain a learned response over time. Given that habituation is considered to be a learned response, the assumption should be that bees will retain an habituated response over time. The question remains how long? The foraging behaviour of bumble bees observed while responding to unequally rewarding artificial flowers may provide some guidance (Keasar, Motro, Shur & Shmida, 1996). The experiment was designed to examine memory retention of foraging skills and results showed that the bees' ability to forage efficiently, determined by probing times and inter-visit intervals, increased steadily over the period of the observation day but decreased significantly overnight. The authors suggest that this decrease may make sense in the face of overnight changes in nectar and pollen availability. Although this study says nothing about habituation retention, the findings suggest that there is some forgetting of learned information overnight.

The current experiment attempts to replicate earlier findings of habituation by bumble bees to a radial stimulus. Once habituation is demonstrated, repeated presentations of the stimuli will occur after a 2 hour delay and a 24 hour delay to examine the retention length of the habituated response. Based on the evidence presented above, it is expected that bumble bees will retain the habituated response after the shorter delay but responding will increase significantly after the 24 hour delay showing forgetting of the habituated stimuli.

Method

Subjects

Thirty naive bumble bees, *Bombus impatiens*, from three colonies were used. The colonies were raised in the laboratory from captured queens according to the procedure developed by Plowright and Jay (1966). All bees were identified by gluing numbered labels of various colours onto the thorax. In the colony the bees were provided with an ad lib

supply of pollen. They were also given access to 67% honey solution except for periods preceding and during the experiment (see below).

Materials and Apparatus

Each colony was housed in a wooden domicile 30 cm x 15 cm x 15 cm. The box was attached to a screened flight cage (1.83m x 1.83m x 1.83m) by a wooden walkway (33 cm). Glass plates were placed on the top of the walkway with two vertical gates to allow easy identification and corralling of bees leaving and entering the colony. Three panels of fluorescent light fixtures illuminated the flight cage.

A radial arm maze, modelled on that of Lehrer et al. (1995), was constructed with twelve corridors (15 cm long) which opened onto a central area (22 cm across). The entrance to each corridor was 6 cm wide. The floor and ceiling of the maze were made with 1/8" clear plexiglass and the external walls and corridors were made with 1/8" opaque grey plexiglass. All pieces were fixed with a plexiglass epoxy except for the ceiling which was removable. Testing stimuli were positioned vertically at the end of each corridor which was 14 cm high x 14 cm wide. Six blue and yellow radial patterns (each with 6 wedges) and six plain blue disks were used as stimuli (12 cm diameter).

Procedure

Training to forage in the flight cage: The colony was deprived of honey solution for two days prior to training. On the first day, bees leaving the colony for the first time were allowed to forage freely from eight pots (1.5 cm diameter, 0.5 cm deep) filled with honey solution. The pots were placed on a wooden tray approximately 1 m from the floor in the far right corner (relative to the colony entrance) of the flight cage. This foraging session lasted approximately 2 h and was implemented to select foragers for further training the following day. On the second day, bees that were observed foraging on the previous day were again

allowed to forage and the identifying numbers of these bees were recorded. After one hour of foraging, the bees were returned to the colony. Only the bees that were observed on both training days were selected for testing.

Testing for preferences. The tray of pots was removed from the cage and the 12-arm radial maze was put in its place, on top of a 1 m high stool in the centre of the flight cage. The stimuli were placed at the end of the corridors in the maze. As each of the selected bees exited the walkway to forage, it was captured in a small vial and immediately placed into the centre of the maze. Bees were tested at this point in the procedure to ensure that they were still in "foraging mode", having just made several trips to the tray of pots filled with honey solution. Once the bee began to fly in the maze, the experimenter then recorded the first twenty choices. A choice was determined to be made when the bee flew forward across the entrance to the corridor toward the stimulus.

Testing for retention of habituation over time. Immediately following this procedure, the bees that were tested in the first test session were retested. Before the second test session, the test bees were allowed to forage from the honey pots placed in the flight cage for at least 15 minutes. This part of the procedure was included to maintain foraging behaviour. The second test session proceeded exactly as the first and was implemented to increase the opportunity for habituation to the stimulus. Once the bees were returned to the colony after this test session they were confined there for a delay period of two hours calculated separately for each bee. When the test bees re-emerged into the corridor after the two hour delay, they were tested one at a time for a third test session using the same procedure and stimuli as above. The following day (after a 24 hour delay calculated separately for each bee from the end of the second test) the same bees were tested a fourth time using the procedure above.

Between Test sessions the maze was rotated 90 ° to control for position of stimuli, shadows, and other inconsistencies in lighting. Also, the experimenter's position in relation to the maze was changed. This procedure was repeated in its entirety with two more colonies.

Design and analysis

Thirty bees made 80 choices each over four test sessions for a total of 2,400 choices. A 2 (pattern) x 4 (test session) within-subjects design was used where binary choices of the radial pattern versus blue discs were recorded (out of 20). A log-linear analysis was carried out for all analyses using GLIM statistical software Version 4.0 (Generalized Linear Interactive Modelling; Francis, Green, & Payne, 1993). The test statistic used in tests of significance for counts was a χ^2 value (as in Plowright, Cohen-Salmon, Landry & Simonds, 1999).

Results

Ten bees from Colony #1, seven bees from Colony #2, and thirteen bees from Colony #3 were observed making 80 choices over four test sessions for a total of 2,400 choices. The mean number of choices was calculated for each stimulus type (radial and blue) and test session. Figure 1 shows that, in Test Session 1, bees chose the radial pattern more frequently than the blue disc ($\chi_1^2 = 79.53$, $p < 0.001$). When Test Sessions 1 and 2 were compared, bees' choices for the radial pattern depended on Test Session ($\chi_1^2 = 40.44$, $p < 0.001$): the preference for the radial pattern waned. This effect did not interact with colony ($\chi_2^2 = 2.65$). These results suggest that habituation to the radial pattern occurred by Test Session 2. Test Session 3 was compared to Test Session 2 to investigate whether there was evidence of

recovery after a two-hour time delay. Bees' choices did not depend on Test Session (χ_1^2 = 1.92) suggesting that habituation was maintained after a two-hour delay. After a 24-hour delay (compared to Test Session 2), bees' choices did depend on Test Session (χ_1^2 = 10.80, $p < .005$) demonstrating that bees did not retain the habituated response overnight. Choice proportion at Test Session 4 was comparable to that at Test Session 1. This effect did not interact with colony ($\chi_2^2 = 3.54$). Figure 2 shows the choice pattern for radial for all bees across 20 trials for four test sessions.

Discussion

Results confirmed habituation in bumble bees originally illustrated in Simonds and Plowright (submitted manuscript, 2002). Habituation was retained over a 2 hour time delay but not over 24 hours. From an ecological point of view, it may be disadvantageous for a bee to retain an habituated response overnight, at least to a floral pattern. Observation of patterns of floral pollen and nectar resources indicates that some flowers visited on one day may again be rewarding on the next (Real & Rathcke, 1988; Zimmerman & Pyke, 1986). Therefore, if bees' period of retention was 24 hours, their visitation patterns may miss potentially profitable flowers.

The lack of retention over 24 hours suggests the period of retention is relatively short when compared to studies with other species. Some possible explanations for this discrepancy are procedural. Rate specificity is known to have an effect on how quickly habituation occurs and how rapidly it dissipates (Davis, 1970; Pedreira et al., 1998; Staddon & Higa, 1996). That is, shorter inter-stimulus intervals result in rapid acquisition and dissolution of the habituated response. A design which allows for more stimuli presentations with larger inter-stimulus intervals might possibly result in longer retention.

Experiment 2: Role of contextual cues in maintenance of habituation

The previous experiment illustrates that habituation is maintained over time. For bumble bees it appears to be a relatively short period of time, but if we refer to Wagner's (1979) model, encoding of the habituated stimuli has been sufficient to form a representation in LTM that can be retrieved into STM (retrieval-generated priming). Experiment 2 was designed to investigate the role of context, which is thought to be a key variable in long-term habituation (Wagner, 1976, 1979). Wagner contends that associations are developed between the stimulus and its context so that the "contextual cues can lead to a retrieval-generated priming of STM and thus lead to a relatively permanent decrement in the measured response to the stimulus" (p. 203). In other words, a context-stimulus association is formed which maintains the habituated response. This contention should mean that separating the stimulus from its context would extinguish this association and result in the context losing its ability to retrieve the appropriate representation. Consequently, the habituated response should be restored.

Evidence was garnered for the associative model in an experiment which used groups of rabbits (Whitlow & Pfautz as cited in Wagner 1976). A tone was presented 32 times over two hours until habituation was apparent. The following day the Control Group was left in their cages but the Extinguished Group was placed in the experimental chamber with the recording apparatus attached but no tones were administered. The third day both groups were given 32 presentations of the same tone as in the first test session. The Control Group showed only minimal response to the tone in the last test session, whereas the Extinguished Group showed a response magnitude almost as large as the initial presentations in the first session. Similarly, Jordan et al. (2000) tested rats who had been habituated to a tone administered in a startle chamber (measured by lick suppression and head-orienting). After

habituation of lick suppression and orienting had occurred, rats who were exposed to the chamber without tone presentations between test sessions showed increased licking and orienting when compared to rats who either remained in their cages or were exposed to an alternate context between test sessions. In both studies, unpairing the context with the stimulus is effective in restoring responding.

Most studies investigating the context-stimuli association have used vertebrates, but more recently Rankin (2000) and Tomsic et al. (1998) have tested invertebrates (nematodes and crabs, respectively). After demonstrating that nematodes, organisms with only 302 neurons, were capable of habituating to a stimulus, Rankin used sodium acetate as a salient context cue. Both groups were habituated to a tap (measured by withdrawal response). The context change group was first tested in the presence of sodium acetate but then rested on a plain plate for 1 hour before being tested for retention of habituation again in the presence of sodium acetate. The extinction group was subjected to the same procedure but during the resting stage they were exposed to sodium acetate (unpaired with the tap). Habituation to the tap was maintained by the context change group but not by the extinction group. Tomsic et al. (1998) tested crabs who had habituated (decreased running response) to an opaque screen passing overhead and found that exposure to the testing container without the presence of the screen restored the crabs' running response. These studies support Wagner's model according to which the association between context and stimulus serves as a controlling mechanism for maintaining habituation.

Nevertheless, despite the growing evidence that context-stimuli associations develop and contribute to responding, Marlin and Miller (1981) observed no evidence of this association using a similar basic design to those experiments cited above. Rats were habituated to a tone (measured by startle response). The experimental group was exposed to

the testing apparatus without the tone presentation and the control group was placed in home cages. The experimental group showed no greater startle response during the final test session than the control group. Other evidence at odds with Wagner's (1976) associative model did not use an extinction design but instead tested whether the habituated response was retained in a new context (Hall & Channell, 1985; Hall & Honey, 1989; Leaton, 1974). The question of whether these contradictory results occur due to a lack of context-stimuli association or other variables such as salience of context remains unanswered. Relatively few experiments have been conducted to form robust support on either side of this question, and so Experiment 2 will continue the investigation of whether contextual cues play a role in maintaining the habituated response using a similar design to the studies cited above (Jordan, 2000; Rankin, 2000; Whitlow & Pfautz in Wagner 1976), in this case to the radial stimulus for bumble bees. After habituation has occurred, one group of bees will be placed in the radial maze without any stimuli present while the control group will remain in the colony. Then both groups will be retested with the original stimuli. Based on the predictions of Wagner's (1976) model, it is hypothesized that the group exposed to the contextual cues (unpaired with the stimuli) between test sessions (the "Maze" group) will show an increased response to the originally habituated stimulus in the last test session, but the context change group (the "Home" group) will not.

Method

Subjects and Procedure

Forty two naive bumble bees, *Bombus impatiens*, from three colonies were used. The colonies were raised, labelled, and fed as described in Experiment 1. The materials and apparatus were as in Experiment 1.

The procedure for test sessions followed as per Experiment 1 except that bees were tested a total of three times within one day. The first and second test sessions were again designed to establish an habituated response in bees. During the period between the second and third test sessions half of the total number of foragers tested, chosen randomly, remained in the colony (Home Group) and the other half were placed in the maze without stimuli presented for a period of 180 seconds (Maze Group).

Design and analysis

Forty two bees made 60 choices each for a total of 2,520 choices. The same analysis was conducted in the first part of Experiment 2 as in Experiment 1. A 2 (pattern) x 3 (test session) within-subjects design was used where binary choices of radial hexagons versus blue discs were recorded (out of 20). The second part of the analysis for the second and third test sessions used a 2 (pattern) x 2 (test session) x 2 (group) mixed design. The effect of interest was whether the effect of test session on pattern choice depends on group (i.e., a 3 way interaction).

Results

Fourteen bees each from three colonies were observed making 60 choices over three test sessions for a total of 2,520 choices. The mean number of choices was calculated for each stimulus type (radial and blue) and test session. Results are displayed in Figure 3. In Test Session 1, bees chose the radial pattern more frequently than the blue disc ($\chi_1^2 = 99.35$, $p < 0.001$). This effect was not dependent on group ($\chi_1^2 = .13$, NS) and was consistent across colonies ($\chi_2^2 = 1.58$, NS). When Test Sessions 1 and 2 were compared, bees' choices

for the radial stimuli decreased over test sessions ($\chi_1^2 = 30.78$, $p < 0.001$). This interaction was neither dependent on group ($\chi_1^2 = 0.64$, NS) nor on colony ($\chi_2^2 = 2.65$, NS).

Test Session 3 was compared to Test Session 2 to investigate whether there was a difference between groups in response to the radial stimuli after the Maze Group had spent time in the test maze without stimuli presentation. Choices for the radial pattern did not depend on test session ($\chi_1^2 = 3.31$, NS) but did depend on group ($\chi_1^2 = 12.19$, $p < .025$). Figure 3 shows this latter interaction to be a greater choice proportion for the radial stimuli for both Test Sessions 2 and 3 by the Maze Group compared to the Home Group. No increase in preference for the radial pattern was obtained between Test Sessions 2 and 3 for the Home Group, and although Figure 3 shows a slight increase for the Maze Group, the predicted three-way interaction between pattern, test session, and group was not significant ($\chi_1^2 = 1.88$, NS). Figure 4 shows the choice pattern for radial for all bees in the Maze Group (top graph) and all bees in the Home Group (bottom graph) across 20 trials for three test sessions.

Discussion

As in all our previous habituation experiments the bees in both groups showed decreased responding to the stimulus from Test Session 1 to Test Session 2. Although there was a small increase in responding to the radial stimulus by the Maze Group in Test Session 3, the difference was not significant and so these data fail to support Wagner's associative model of habituation for bumble bees. The usual cautionary remarks regarding a failure to reject the null hypothesis apply here. Jordan et al. (2000) found that no effect of context was apparent until the interval of exposure to the context without the stimuli was increased. The 180 second interval may not have been sufficient to disrupt the context-stimuli association, in

fact, it may be that limited exposure actually maintained the strength of the association. Also, the salience of the context may not have been sufficient to provoke an association in the first place. The maze is constructed of grey plexiglass and when the stimuli are placed at the ends of the corridors only a small amount of grey area is exposed and, it is difficult to determine to what degree the bees perceive this surface. A larger and more natural environment may serve to improve the design of the experiment such as a facsimile of green foliage. This change would fit with Rankin's (2000) and Evans and Hammond's (1983) suggestion that the more biologically relevant the contextual stimulus, the more likely it is to be used in associative learning. Lastly, the time necessary to distinguish short from long term habituation can be arbitrary and it might be that the interval between test session 2 and 3 was inadequate to illustrate that long term habituation had occurred. If this is the case, the presentation of the stimuli without its associative cues (i.e., context) would provoke self-generated priming rather than retrieval-generated priming.

Despite these potential limitations of the experiment, Baker and Mercier (1982) warn against the pitfalls of suggesting that further manipulations of the context-stimuli pairing will serve to strengthen the associations rather than accepting that the associative model was simply not supported. Instead, limitations of the Wagner model need to be considered, at least as it might apply to bumble bees. It remains that this experiment used the same manipulation as other studies testing for context-stimuli association and found no association (Jordan et al., 2000; Rankin, 2000). Our results are consistent with many others reporting no association (Hall & Channell, 1985; Hall & Honey, 1989; Leaton, 1974; Marlin & Miller, 1981). Bees' ability to learn by association is not in question as they do associate specific floral characteristics such as colour and nectary guides with food sources (Ney-Nifle et al., 2001). Therefore, the following functional explanations should be considered: 1) the

immediate environmental context for most flowers is very similar (i.e., green foliage) and would render the use of this level of context to aid learning for bees irrelevant; 2) bees have greater mobility than other invertebrates used in the study of context (i.e., crabs and nematodes) and so either remote context cues may be more useful than immediate context cues or, again, context is not practical as an associative cue for retention of habituation in bees.

In summary, the merit of context as an associative cue for maintaining long-term habituation in bumble bees remains in question. Further research extending the time in the empty maze and the time interval between test sessions to ensure long-term habituation has occurred may help to clarify the results. In addition, using more salient, biologically relevant contextual cues and distal rather than proximal cues would possibly provoke stronger support for a context-stimuli association.

General Discussion

The existence of long-term habituation in bumble bees was supported by the evidence presented in Experiment 1. The use of contextual cues to retain the habituated response, however, was put in question in Experiment 2. Several studies cite the necessity of a context-stimulus association in maintaining long-term habituation (Jordan et al., 2000; Tomsic et al., 1998). When these findings are considered together, they may support a more ecologically-based explanation first raised in Experiment 1. Habituation has been shown to exist across all species but it makes sense that the properties of habituation would vary according to their environment. Petrinovich (1984) agrees and states "the relative strengths of the different components [of habituation] should vary with different species according to the dictates of the ecological pressures to which they have had to adapt" (p. 51). If

contextual cues are used to facilitate long-term habituation as Wagner's model suggests, then perhaps the use of these cues is not as important for bees given the need to respond according to floral productivity which can fluctuate overnight. Further research is implicated in two areas. First, to determine whether contextual cues are used in some circumstances, the observation of habituation to stimuli that are not associated with foraging behaviour but instead with behaviour associated with danger or threat would be beneficial (e.g., hissing in response to nest disturbances; Kirchner & Röschard, 1999). Many other studies finding a context-stimulus association have tested threat responses rather than orienting responses (e.g., Rankin, 2000; Tomsic et al., 1998). Once habituation is established, then rate and stimulus specificity can be tested to determine whether the duration of retention can be lengthened and whether contextual cues are used to facilitate the retention. Second, to examine the merit of an ecological explanation an experimental design should be developed that incorporates multiple time delays to better determine the length of retention and then to manipulate and map patterns of floral productivity onto this information. In bee research the dominant view is that general learning principles can account for the details of behaviour (Bitterman, 1996), and the present research in no way contradicts this view. An ecological approach to learning (Kamil & Yoerg, 1982; Shettleworth, 1993) would suggest, however, that the details of behaviour might be more easily uncovered with the bee's foraging ecology in view.

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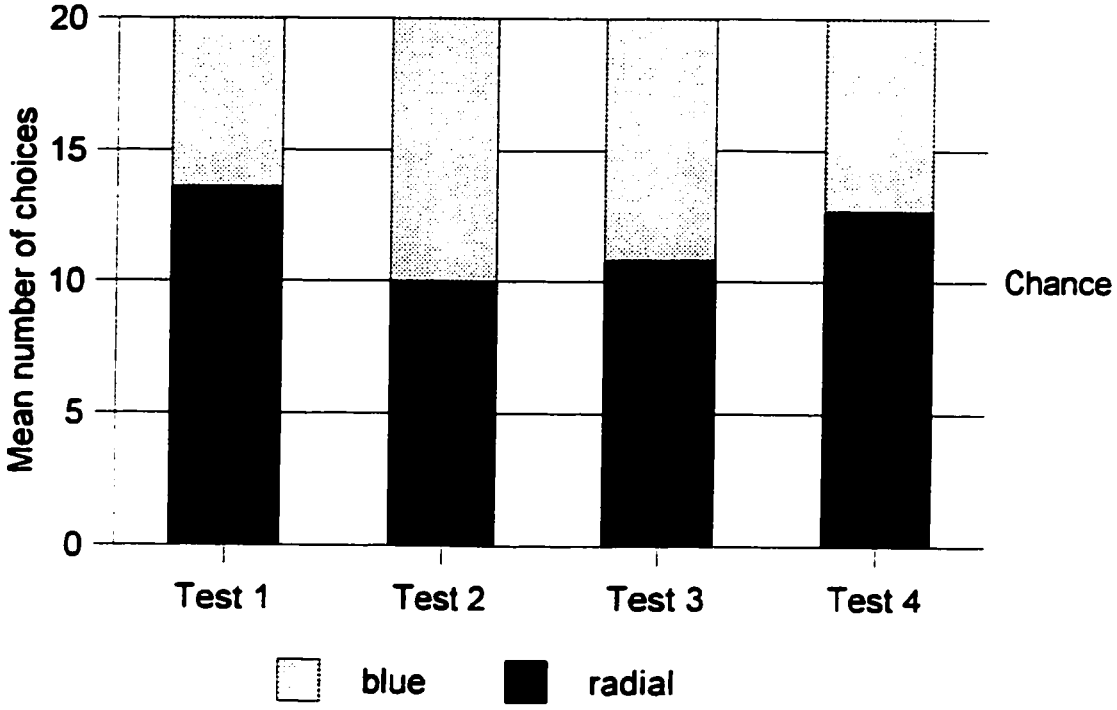
Figure Captions

Figure 1. Experiment 1. Mean number of choices out of 20 for radial vs. blue over four test sessions (n=2,400) separated by 15 min foraging sessions. The 1st session shows a preference for the radial stimuli, the 2nd session and 3rd session show habituation, and the 4th session shows a restored preference for radial over blue after a 24 hour delay.

Figure 2. Experiment 1. Total number of choices for radial for all bees (30) for each trial across 4 test sessions.

Figure 3. Experiment 2. Mean number of choices out of 20 for radial vs. blue over three test sessions (n = 2,520) separated by 15 min foraging sessions. Between tests 2 and 3, (a) Home Group remained in the colony and (b) Maze Group was placed in the maze without stimuli for 3 minutes. The decrease in responding to the radial pattern was significant for both groups. The apparent restoration in responding on Test 3 for the Maze Group was not significant.

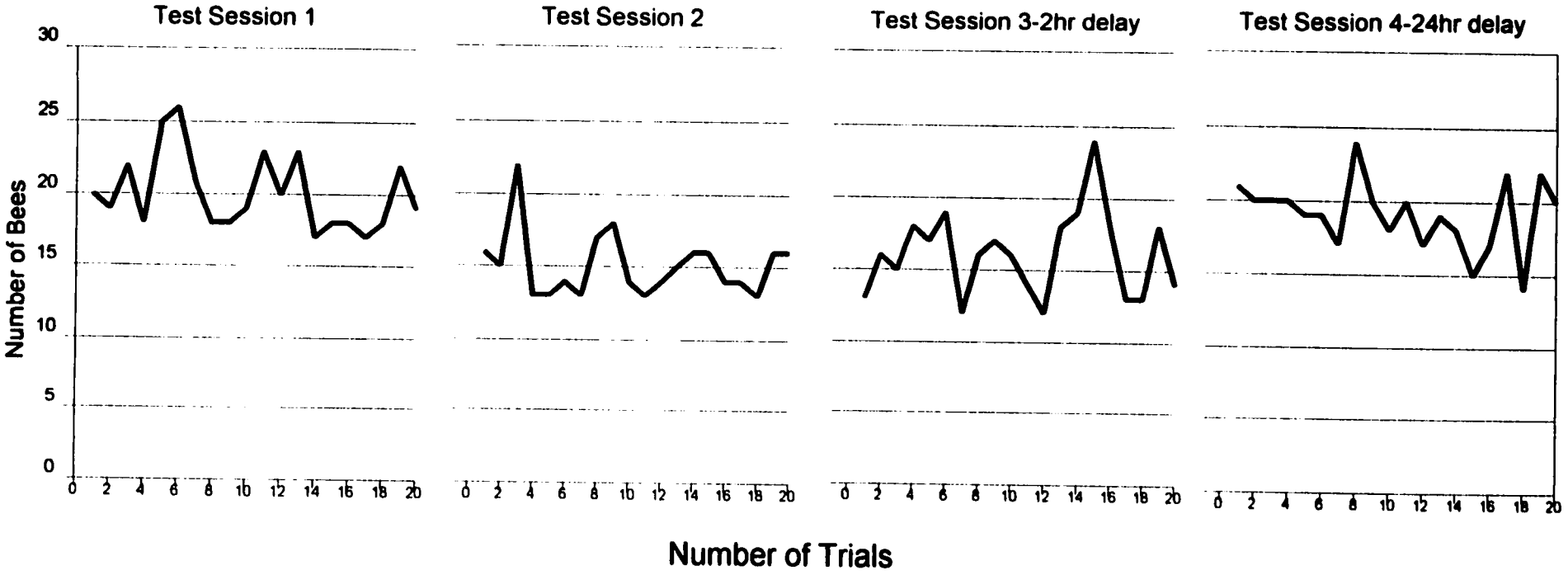
Figure 4. Experiment 2. Total number of choices for radial for bees (22) in Home Group for each trial across 3 test sessions. Total number of choices for radial for bees (22) in Maze Group for each trial across 3 test sessions.

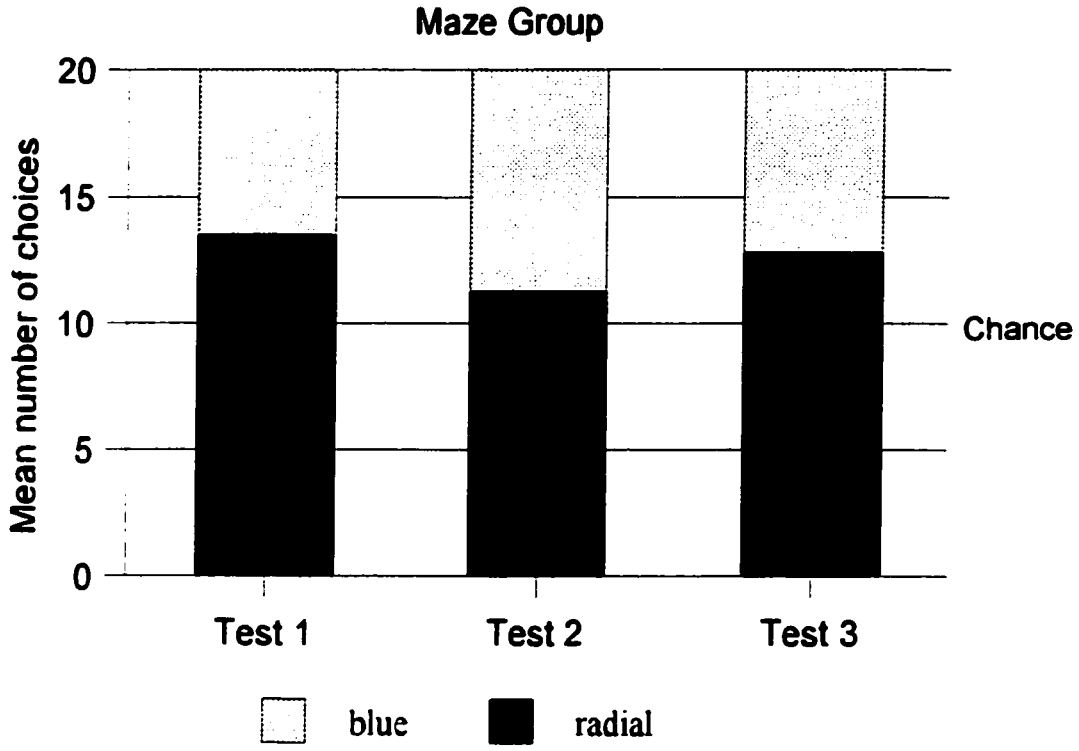


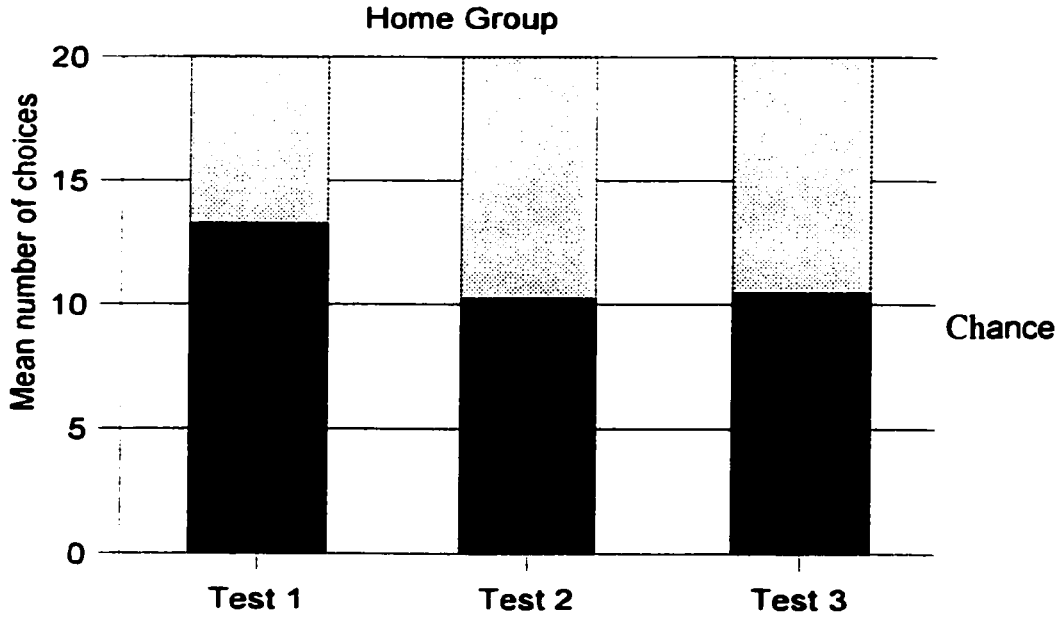
Test 2: 15 min after Test 1

Test 3: 2 hours after Test 2

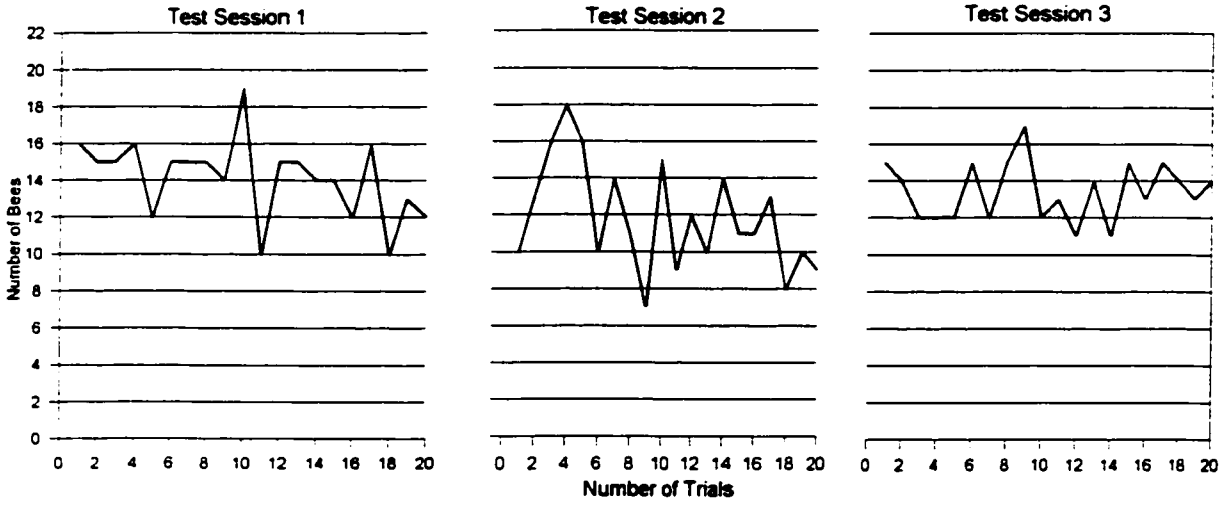
Test 4: 24 hours after Test 3


Test Session 1
Test Session 2
Test Session 3-2hr delay
Test Session 4-24hr delay
Number of Bees
30
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20
Number of Trials

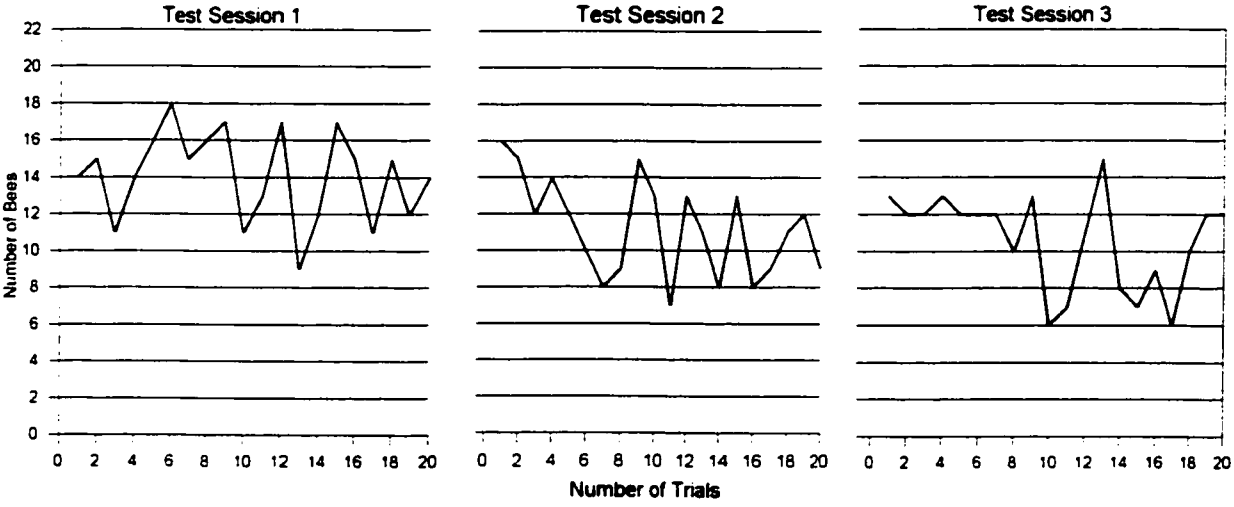

Maze Group
Mean number of choices
20
15
10
5
0
Chance
Test 1
Test 2
Test 3
blue
radial


Home Group
Mean number of choices
20
15
10
5
0
Chance
Test 1
Test 2
Test 3

Maze Group



Home Group



General Discussion

The first question 'How do bumble bees first find flowers?' evolved into other questions about the effect of repeated exposure to floral stimuli in the absence of reward. The results of the first experiments found that bees were attracted to certain colours and patterns (i.e., blue and yellow; radial) prior to experience with them, and these results concur with earlier studies (e.g., Lehrer et al., 1995). In the second half of these same two experiments bees' preference for the stimuli waned showing evidence for habituation in bumble bees, an area previously ignored in the literature. Results from the third experiment showed that the habituated response was retained for at least two hours but disrupted after 24 hours indicating that long-term habituation exists for bumble bees. Although bees illustrate the retention of habituation, the results do not correspond with other studies testing invertebrates which have found retention periods of greater than 24 hours (e.g., Lozada, Romano & Maldonado, 1990; Pedreira, Dimant, Tomsic, Quesada-Allue, & Maldonado, 1995). The final experiment failed to support a context-stimulus association in maintaining long-term habituation. There has been mixed evidence regarding the associative role of context, but the most recent studies have been positive (e.g., Rankin, 2000). Explanations for the discrepancies with other studies in the last two experiments are discussed in the second paper.

Although the method worked well for these experiments, in order to gather more information and expand the breadth of possibilities for its use certain improvements are noted. Methodological improvements would include: (1) an additional dependent variable such as time that would better measure lack of responding; (2) an increase in the size of the apparatus to allow for a more natural flying environment and greater distance from the stimuli; (3) presentation of the habituated stimulus without the binary choice; and, (4) the

development of a sophisticated method of projecting the stimulus image at the ends of the corridors to allow for more variation in presentation of stimuli, e.g., massed vs distributed trials; variable interstimulus intervals. Certain design changes may render more and/or clearer information about the properties of habituation for bees. For example, in Experiments 1 and 2 a fourth test session could be included where the habituated stimulus is presented again to see whether the novel stimulus disrupted habituation. A simpler context design could be implemented where some bees are tested in the same context as the original habituation test situation and others are tested in a different context. This design change would further and more clearly test the merit of Wagner's associative model for habituation in bumble bees.

Results from Parts A of Experiments 1 and 2 primarily showed that the method did allow collection of informative data on unlearned responses to floral stimuli. The possibilities for further work on variables affecting attraction to visual stimuli are endless. For example, manipulations of the size or dimension of the stimuli, the shape of nectary guides and their contrast with the corolla, degrees of wavelength, spectral purity, or intensity have the potential to provide information that would establish a foundation of knowledge and begin to address Tinbergen's (1951) challenge to psychologists, at least in the study of this species, that states "because learning and other higher processes are modifications of innate mechanisms, . . . the study of learning processes has to be preceded by a study of the innate foundations of behaviour" (p. 6).

Future directions for habituation research are also limitless. First, given that this is the first formally reported research on habituation for bumble bees, we would benefit from experiments designed to discover more about what kinds of stimuli are most effectively habituated, what types of novel stimuli disrupt it, do interstimulus intervals modify the strength of the habituated response and, if so, how, what are the effects of massed versus

distributed trials, and so on. In other words, how does habituation in bumble bees line up with the research already conducted with other species? It is perhaps too soon in the study of habituation with bumble bees to design experiments for the strict purpose of testing the existing theories of habituation. The foundational behavioural and physiological experiments need to be conducted to get a sense of the limits and controlling mechanisms of habituation. There is a need to simply gather information about the basic processes of the phenomenon in bumble bees first. That said, in collecting these data we need to bear in mind the ultimate goal of explaining at least three levels of processes involved in the phenomenon of habituation in bumble bees: behavioural processes, neuronal processes, and evolutionary processes.

Theories such as Wagner's (1976) attempt to explain at an abstract level consistencies in the phenomenon of habituation that occur despite the differences in neuronal processes of the individual species (Whitlow & Wagner, 1984). The results in Experiment 1B were not conclusive regarding the existence of habituation in bumble bees as no increased responding occurred when a novel stimulus was presented, however, the results in Experiment 2B did support habituation in bees. According to Wagner, the initial response to the radial stimulus occurred because the stimulus was novel, i.e., there was no representation of the stimulus in STM. Once the stimulus was presented it elicited rehearsal until a representation of the stimulus was formed in STM. On further presentations this representation primed the STM and inhibited responding. Results of Experiment 3 indicate that the representation was stored in LTM. When the stimulus was repeated over three test sessions a delay of 2 hours did not disrupt the habituated response in the fourth test session. Wagner explains this by suggesting that when the habituated stimulus is presented repeatedly the representation of the stimulus and its associated environmental cues are stored in LTM. A presentation of the radial

stimulus then primed a retrieval from LTM into STM and resulted in inhibited responding. As explained above, evidence did not support the use of environmental cues to aid this retrieval-generated priming in bumble bees. Although the theory presents an interesting model of habituation it is difficult to test and tells us little about *why* a particular response is generated and then inhibited on repeated presentations.

The principle theories mentioned earlier that explain habituation have generated a large number of studies dedicated to finding evidence to support or refute one group or the other. Often evidence of association is used to make this distinction but does it? In the introduction to this thesis I expressed the bumble bees' ability to engage in associative learning between a food source and the surrounding features of the flower. The mere existence of association in habituation experiments does not necessarily equate with support for Wagner's model. The model was originally developed 25 years ago and was derived from experiments with humans (Sokolov, 1963) and from an information processing model developed for human memory (Atkinson & Shiffrin, 1968). Perhaps it is unreasonable to expect that this model would fit for an organism with 302 neurons such as the nematode or a bumble bee. We know that these organisms are capable of classical conditioning and that conditioning and its extinction play a role in maintaining and disrupting habituation. The process of understanding habituation is slowed by trying to fit the evidence into the model. Instead, the observations about classical conditioning should drive the next step in research. This is not to say that research energy is not well placed in the development of theories, but if the model does not adequately explain habituation for all species future research time should be spent modifying the existing models or developing new ones based on current behavioural and neurophysiological evidence.

Once a base of information about habituation in bumble bees has gained more depth and breadth, the ultimate aim of this area of research is to explain how exploratory behaviour in the absence of reward contributes to the bees' ability to survive and thrive in its environment. The ecological importance of these results has been discussed in detail in the discussion sections of the individual papers, but overall it makes sense that bees need to have ways of finding food sources and learning when those sources are no longer rewarding. The attraction to floral stimuli over other objects, and the subsequent shaping of the response to floral stimuli when reward is not available means that bees are able to forage more efficiently. Functional queries drive us to understand how an organism adapts to the demands of the environment (Peeke & Petrinovich, 1984). Glaser states that habituation may play an important role in general adaptation to the environment (as cited in Groves & Thompson, 1970).

In summary, future research should include the pursuit of more perceptual information about what guides the bee to the flower in the first place to better distinguish between unlearned and learned processes. Second, it should continue to develop an understanding of the basic processes of habituation in bumble bees given that this research represents the first evidence of habituation in bees. Third, this basis of knowledge can contribute to the development of new theories that more adequately explain habituation across species. Finally, if the primary goal is to understand how organisms adapt to their environment, then experimental design and theory development should also focus on ultimate explanations.

This is by no means the end of the story but rather the beginning. The primary contribution of the thesis remains the development of a method to distinguish between unlearned and learned responses, and to examine exploratory behaviour in the absence of

reward. The results gleaned from these experiments represent contributions in both of these areas. The information extracted from future perceptual and learning experiments using this method holds the promise to build a more accurate picture of exploratory behaviour in bumble bees before more classic forms of learning associated with reward begin to occur.

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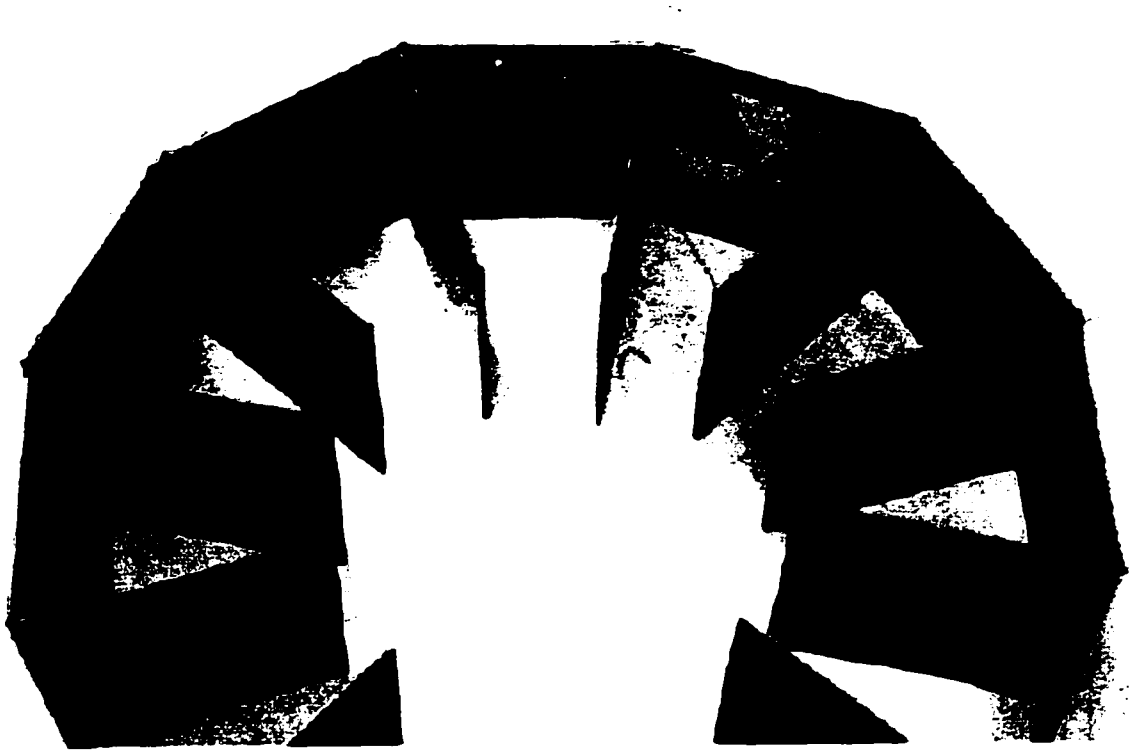
APPENDIX A

12 Arm Radial Maze



APPENDIX B

Tables 1-12 presenting individual data for Experiments 1-4.

Tables 13-16 presenting mean number of choices for Experiments 1-4.

Table 1. Experiment 1A. Choices for colour stimuli.

Bee	Colony	Blue	Yellow	White	Red	Total
G80	5	8	8	3	1	20
P93	5	8	11	1	0	20
P12	5	10	7	1	2	20
G30	5	7	9	3	1	20
P73	5	6	11	2	1	20
W9	5	11	7	2	0	20
P15	5	8	8	3	1	20
G11	5	7	10	3	0	20
Y58	5	5	11	2	2	20
W19	5	8	8	3	1	20
B14	5	9	7	3	1	20
G18	5	8	9	2	1	20
Y48	5	8	10	1	1	20
B39	1	7	11	1	1	20
W36	1	10	6	3	1	20
W39	1	5	11	2	2	20
B45	1	8	9	1	2	20
Y62	1	3	6	7	4	20
B38	1	3	10	4	3	20
B58	1	7	10	3	0	20
Y71	1	3	9	4	4	20
G47	1	6	9	3	2	20
B51	1	6	6	3	5	20
R1	2	6	7	7	0	20
R2	2	8	11	1	0	20
R3	2	7	8	4	1	20
R4	2	3	7	8	2	20
R5	2	8	4	6	2	20
R6	2	9	6	3	2	20
R7	2	6	6	3	5	20
R8	2	6	7	4	3	20
R9	2	5	8	5	2	20
R10	2	8	7	3	2	20
Total		**227**	**274**	**104**	**55**	**660**

Table 2. Experiment 1B - Condition 1. Colour choices over three test sessions for yellow vs grey.

Bee	Colony	Session 1		Session 2		Session 3	
		Yellow	Grey	Yellow	Grey	Yellow	Grey
B28	1	24	6	18	12	20	10
G25	1	26	4	18	12	13	17
G40	1	23	7	11	19	17	13
YD	2	20	10	23	7	22	8
Y21	2	23	7	16	14	16	14
P33	2	23	7	17	13	15	15
P94	3	23	7	17	13	15	15
W61	3	20	10	11	19	16	14
G45	3	22	8	12	18	16	14
Total		**204**	**66**	**143**	**127**	**150**	**120**

Table 3. Experiment 1B - Condition 2. Colour choices for yellow vs grey over two sessions and blue vs grey for third session.

Bee	Colony	Session 1		Session 2		Session 3	
		Yellow	Grey	Yellow	Grey	Blue	Grey
Y87	1	22	8	18	12	17	13
P19	1	25	5	15	15	19	11
G78	1	21	9	17	13	22	8
W44	2	22	8	20	10	17	13
W45	2	23	7	17	13	15	15
Y34	2	24	6	17	13	12	18
G66	3	16	14	16	14	18	12
G56	3	25	5	16	14	15	15
W62	3	22	8	15	15	16	14
Total		**200**	**70**	**151**	**119**	**151**	**119**

Table 4. Experiment 1B - Condition 3. Individual colour choices to a maximum of 30 for each session..

Bee	Colony	Session 1		Session 2		Session 3	
		Yellow	Grey	Yellow	Grey	Red	Grey
Y78	1	25	5	21	9	18	12
W50	1	15	15	15	15	16	14
G55	1	22	8	18	12	14	16
YD	2	21	9	20	10	15	15
W48	2	18	12	15	15	13	17
G4	2	22	8	20	10	13	17
P74	3	23	7	17	13	16	14
W73	3	21	9	16	14	16	14
YB	3	24	6	22	8	15	15
Total		**191**	**79**	**164**	**106**	**136**	**134**

Table 5. Experiment 2A - Choices made by individual bees for radial, blue, and concentric patterns.

Bee	Colony	Radial	Blue	Concentric	Total
Y86	1	15	3	2	20
G28	1	10	6	4	20
G35	1	13	3	4	20
P1	1	13	4	3	20
G90	1	7	8	5	20
P2	1	14	3	3	20
G93	1	11	4	5	20
B61	1	12	3	5	20
B55	1	15	2	3	20
R1	2	12	6	2	20
R2	2	12	4	4	20
R3	2	15	4	1	20
R4	2	13	5	2	20
R5	2	13	3	4	20
R6	2	14	3	3	20
R7	2	13	5	2	20
R8	2	9	4	7	20
R9	2	12	4	4	20
R10	2	13	2	5	20
R11	2	11	6	3	20
R12	2	15	3	2	20
R13	2	13	4	3	20
R14	2	12	1	7	20
R15	2	14	1	5	20
R16	2	9	5	6	20
R17	2	12	3	5	20
R18	2	10	3	7	20
R19	2	13	5	2	20
W67	3	10	3	7	20
G52	3	13	4	3	20
G61	3	10	6	4	20
P91	3	11	6	3	20
UN	3	10	5	5	20
Total		**399**	**131**	**130**	**660**

Table 6. Experiment 2B - Condition 1. Pattern choices over 3 test sessions presenting radial vs. blue disc.

Bee	Colony	Session 1		Session 2		Session 3	
		Radial	Blue	Radial	Blue	Radial	Blue
P73	4	16	4	9	11	11	9
G45	4	13	7	11	9	9	11
P33	4	16	4	13	7	14	6
W52	1	16	4	8	12	7	13
W45	1	19	1	10	10	13	7
W47	1	16	4	12	8	12	8
W14	5	14	6	9	11	8	12
P18	5	18	2	12	8	12	8
W57	5	15	5	11	9	10	10
Total		**143**	**37**	**95**	**85**	**96**	**84**

Table 7. Experiment 2B - Condition 2. Choices for radial vs. blue over two sessions and floral pattern vs blue for third session.

Bee	Colony	Session 1		Session 2		Session 3	
		Radial	Blue	Radial	Blue	Floral	Blue
G70	4	16	4	18	2	18	2
G48	4	15	5	11	9	14	6
B85	4	15	5	10	10	15	5
B52	1	17	3	11	9	15	5
B37	1	18	2	12	8	17	3
B21	1	18	2	9	11	13	7
RD	5	15	5	10	10	15	5
G99	5	17	3	11	9	13	7
B10	5	14	6	13	7	16	4
Total		**145**	**35**	**105**	**75**	**136**	**44**

Table 8. Experiment 2B - Condition 3. Choices for floral pattern vs. blue disc over three sessions.

Bee	Colony	Session 1		Session 2		Session 3	
		Floral	Blue	Floral	Blue	Floral	Blue
Y67	4	15	5	2	18	5	15
Y15	4	15	5	10	10	8	12
Y10	4	18	2	12	8	10	10
B38	1	13	7	9	11	6	14
P23	1	15	5	13	7	12	8
B54	1	16	4	9	11	8	12
Y45	5	13	7	7	13	7	13
G10	5	16	4	11	9	9	11
Y42	5	13	7	14	6	9	11
Total		**134**	**46**	**87**	**93**	**74**	**106**

Table 9. Experiment 2B - Condition 4. Choices for floral vs. blue over two sessions and radial vs blue for third session.

Bee	Colony	Session 1		Session 2		Session 3	
		Floral	Blue	Floral	Blue	Radial	Blue
G86	4	11	9	9	11	14	6
Y60	4	13	7	11	9	12	8
P50	4	8	12	9	11	13	7
B96	1	16	4	8	12	16	4
B57	1	16	4	10	10	14	6
P38	1	14	6	8	12	15	5
Y33	5	12	8	9	11	11	9
G12	5	15	5	6	14	15	5
B7	5	14	6	10	10	17	3
Total		**119**	**61**	**80**	**100**	**127**	**53**

Table 10. Experiment 2B - Condition 5. Choices for radial vs blue for two sessions and concentric vs blue for third session.

Bee	Colony	Session 1		Session 2		Session 3	
		Radial	Blue	Radial	Blue	Concentric	Blue
W50	5	18	2	15	5	10	10
Y38	5	12	8	13	7	8	12
Y23	2	16	4	9	11	11	9
RD	2	13	7	12	8	10	10
W60	2	13	7	10	10	13	7
RD1	1	15	5	14	6	10	10
RD2	1	12	8	11	9	8	12
B26	1	14	6	11	9	8	12
B27	1	16	4	13	7	9	11
Total		**129**	**51**	**108**	**61**	**87**	**93**

Table 11. Experiment 3 - Pattern choices for individual bees over four test sessions (2 immediate, 2 hour delay, and 24 hour delay) presented with radial vs. blue stimuli.

		Time 1		Time 2		Time 3		Time 4	
Bee	**Colony**	**Radial**	**Blue**	**Radial**	**Blue**	**Radial**	**Blue**	**Radial**	**Blue**
W48	1	16	4	11	9	12	8	15	5
R	1	13	7	8	12	12	8	12	8
BY	1	16	4	12	8	15	5	11	9
GY	1	9	11	7	13	9	11	5	15
RY	1	16	4	11	9	9	11	13	7
W	1	14	5	9	11	9	11	13	7
G	1	11	9	8	12	8	12	14	6
B	1	16	4	10	10	15	5	12	8
BW	1	8	12	9	11	16	4	10	10
GW	1	15	5	10	10	8	12	14	6
R	2	15	5	8	12	9	11	13	7
Y	2	15	5	12	8	13	7	15	5
W	2	13	7	11	9	9	11	15	5
R2	2	16	4	11	9	15	5	8	12
B	2	7	13	12	8	13	7	16	4
G	2	12	8	7	13	13	7	12	8
GW	2	14	6	6	14	6	14	14	6
GY	3	14	6	11	9	11	9	14	6
YW	3	16	4	8	12	11	9	13	7
RB	3	16	4	11	9	11	9	9	11
BW	3	16	4	11	9	9	11	13	7

Table 11 Continued

Table 11 (Cont'd). Experiment 3 - Pattern choices for individual bees over four test sessions (two immediate, 2 hour delay, and 24 hour delay) presented with radial vs. blue stimuli.

		Time 1		Time 2		Time 3		Time 4	
Bee	**Colony**	**Radial**	**Blue**	**Radial**	**Blue**	**Radial**	**Blue**	**Radial**	**Blue**
G	3	13	7	13	7	10	10	13	7
W	3	15	5	8	12	7	13	16	4
Y	3	16	4	8	12	7	13	14	6
RW	3	13	7	11	9	8	12	16	4
YR	3	14	6	9	11	12	8	13	7
RB2	3	12	8	14	6	13	7	9	11
B	3	14	6	14	6	15	5	13	7
OR	3	9	11	9	11	10	10	12	8
GB	3	14	6	11	9	9	11	13	7

Table 12. Experiment 4 - Pattern choices for individual bees in the Control Group and the Experimental Group over 3 test sessions presenting radial vs. blue disc.

Bee	Colony	Group	Test 1		Test 2		Test 3	
			Radial	Blue	Radial	Blue	Radial	Blue
B57	1	1	15	5	11	9	10	10
Y100	1	1	16	4	11	9	9	11
B22	1	1	15	5	9	11	9	11
G12	1	1	13	7	11	9	11	9
W31	1	1	5	15	10	10	10	10
P25	1	1	15	5	8	12	11	9
G2	1	1	14	6	10	10	12	8
W1	2	1	10	10	12	8	10	10
G	2	1	17	3	11	9	15	5
Y	2	1	11	9	15	5	10	10
W2	2	1	17	3	13	7	16	4
R62	2	1	12	8	10	10	7	13
BY	2	1	15	5	9	11	7	13
LY	2	1	13	7	11	9	9	11
BW	3	1	16	4	8	12	11	9
GY	3	1	13	7	9	11	13	7
W	3	1	15	5	8	12	8	12
Y	3	1	16	4	7	13	8	12
RW	3	1	13	7	11	9	11	9
YR	3	1	6	14	11	9	9	11

Table 12 Continued

Table 12 (Cont'd). Experiment 4 - Pattern choices for individual bees in the Control Group and the Experimental Group over 3 test sessions presenting radial vs. blue disc.

Bee	Colony	Group	Test 1		Test 2		Test 3	
			Radial	Blue	Radial	Blue	Radial	Blue
RB	3	1	12	8	10	10	14	6
B58	1	2	15	5	9	11	15	5
G60	1	2	14	6	11	9	6	14
P18	1	2	16	4	9	11	14	6
P46	1	2	15	5	11	9	12	8
G100	1	2	15	5	11	9	13	7
R82	1	2	6	14	10	10	15	5
G17	1	2	14	6	15	5	14	6
LY	2	2	15	5	15	5	16	4
BY	2	2	14	6	9	11	16	4
Y2	2	2	12	8	15	5	13	7
R	2	2	13	7	10	10	8	12
R78	2	2	15	5	9	11	14	6
G	2	2	13	7	13	7	15	5
B	2	2	17	3	17	3	17	3
Y	3	2	16	4	13	7	16	4
GB	3	2	7	13	13	7	11	9
G	3	2	13	7	10	10	14	6
Y2	3	2	14	6	8	12	12	8
BW	3	2	12	8	13	7	13	7
B	3	2	14	6	9	11	5	15
W	3	2	14	6	7	13	12	8

Table 13.

Mean number of choices for Experiment 1B - Conditions 1-3.

Condition	Colour	Session 1	Session 2	Session 3
1	Yellow	22.667	15.889	16.667
	Grey	7.333	14.111	13.333
2	Yellow/Blue	22.222	16.778	16.778
	Grey	7.778	13.222	13.222
3	Yellow/Red	21.222	18.222	15.111
	Grey	8.778	11.778	14.889

Table 14.

Mean number of choices for Experiment 2B - Conditions 1-5.

Condition	Pattern	Session 1	Session 2	Session 3
1	Radial	15.889	10.556	10.667
	Blue	4.111	9.444	9.333
2	Radial/Floral	16.111	11.667	15.111
	Blue	3.889	8.333	4.889
3	Floral	14.889	9.667	8.222
	Blue	5.111	10.333	11.778
4	Floral/Radial	13.222	8.889	14.111
	Blue	6.778	11.111	5.889
5	Radial/Conc	14.333	12.000	9.667
		5.667	8.000	10.333

Table 15.

Mean number of choices for radial vs blue stimuli in Experiment 3 (Time).

Pattern	Test 1	Test 2	Test 3	Test 4
Radial	13.600	10.0	10.8	12.667
Blue	6.400	10.0	9.2	7.333

Table 16.

Mean number of choices for radial vs blue stimuli in Experiment 4 (Context).

	Test 1		Test 2		Test 3	
Pattern	**Group 1**	**Group 2**	**Group 1**	**Group 2**	**Group 1**	**Group 2**
Radial	13.286	13.524	10.238	11.286	10.476	12.810
Blue	6.714	6.476	9.762	8.714	9.524	7.190