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BRAIN AND BEHAVIOUR IN BUMBLEBEES (BOMBUS IMPATIENS): SERIAL REVERSAL LEARNING AND ADULT MUSHROOM BODY DEVELOPMENT

Caroline G. Strang

BRAIN AND BEHAVIOUR IN BUMBLEBEES (*BOMBUS IMPATIENS*): SERIAL REVERSAL LEARNING AND ADULT MUSHROOM BODY DEVELOPMENT

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Caroline G. Strang

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Supervisor	Examiners
Dr. David Sherry	Dr. William Roberts
Supervisory Committee	Dr. Peter Ossenkopp
Dr. William Roberts	Dr. John Mitchell

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Caroline G. Strang

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Abstract

This thesis explores learning in bumblebees (*Bombus impatiens*) and its relation to foraging and development of the mushroom bodies of the bee brain. The first experiment describes the performance of bees on a serial reversal task. Reversal learning requires animals to change their behavior with changes in reward contingencies and is a measure of behavioural flexibility. Results show that bumblebees are capable of improving in a serial reversal task. The second experiment explores the effects of foraging experience on bumblebee mushroom bodies. Mushroom body volume was compared in bees confined to the colony, bees actively foraging, and bees tested on the serial reversal task. In both experiments, bees were housed in simulated foraging environments differing in complexity, to determine the effect of environmental complexity on learning and brain development. The second experiment found no effect of foraging, learning experience or environmental complexity on mushroom body volume.

Keywords: bumblebees, serial reversal learning, behavioural flexibility, mushroom bodies, environmental enrichment

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Table of Contents

List of Figures

CHAPTER ONE

General Introduction

Introduction

Foraging bumblebees and honeybees perform complex behaviours throughout their lives (Heinrich, 1979; Seeley, 1985). This thesis investigates how bees accomplish these behaviours by examining learning and the associated brain areas in bumblebees (*Bombus impatiens*). A serial reversal task was used to measure behavioural flexibility and the ability to 'learn to learn'. The effect of environmental complexity on serial reversal learning was also explored. In addition, the effects of environmental complexity, foraging experience, and serial reversal learning on mushroom body development were examined.

The field of animal cognition has been criticized in the past for studying a limited number of species, mainly the traditional rat and pigeon of the psychology laboratory (Beach, 1950; Papini, 2002). In a recent examination of the species used in comparative cognition research it was shown that the field has broadened the number of species studied and Beach's (1950) assertion that "comparative" cognition was a misnomer has been convincingly refuted (Shettleworth, 2009). However, there is still a notable absence of insect research in animal cognition. Although the number of articles published on insects has increased, there has been almost no increase in the percentage of articles on insects published in comparative psychology journals between the time of Beach's (1950) survey and an updated survey by Shettleworth (2009). Insects make up an overwhelming proportion of extant animal species (May, 1988) yet less than 10% of research in the field (Beach, 1950; Shettleworth, 2009). The argument that animal cognition research should include mammals other than the rat and birds other than the pigeon, is equally applicable to the inclusion of insects. One reason we need to study multiple species is that not all

animals may have found the same solution to learning problems (Shettleworth, 2009). Certainly it seems probable that if there are differences in fundamental cognitive processes they are most likely to be found through exploration of species as phylogenetically distant from mammals and birds and as diverse as insects. Along with differences it is possible we may find commonalities between distantly related species (Papini, 2002). Although they possess vastly different evolutionary histories, insects do share properties with vertebrates. This is evident nowhere more so than in the eusocial insects. The level of sociality in mammals has been linked to brain size and cognition (Dunbar & Shultz, 2007), and the same has been shown in insects (Strausfeld et al., 1998; Farris, 2008). In addition to comparisons of learning and cognition, insects are also ideal for study of the interaction of brain and behaviour. The simplicity of the insect nervous system and brain compared to that of vertebrates makes it accessible on a level not possible in vertebrates and that accessible nervous system is accompanied by a large suite of behaviours (Heisenberg, 1998). These are the same properties that made *Drosophila* so informative for genetics research and the field of brain and behaviour research can capitalize on them as well.

This thesis explores these issues in bumblebees (*Bombus impatiens*). Two chapters, one on behaviour and the other on the brain, examine the relationship between learning and the brain in an insect. In Chapter 2, the behaviour experiment explores behavioural flexibility using a serial reversal task in a more naturalistic paradigm than has been used in the past for bee research. Chapter 3 explores the interaction between adult brain development, experience, and environmental complexity. The next section of

this chapter reviews relevant learning research with bees and provides a general description of the bee brain.

Learning in Bees

Despite the assumption of many people, it has long been known that bees are not mere 'reflex machines' (Menzel, 1990). When von Frisch showed that bees could see colour he also showed that they could learn to associate a colour with reward, proving that bees do in fact learn (von Frisch, 1966). In the near century since von Frisch's studies on honeybee learning a considerable amount of work has been done on associative learning in bees. One focus has been olfactory learning and the proboscis (tongue) extension reflex (PER). Research on the topic has led to detailed understanding of the neural circuit responsible for olfactory conditioning of the PER (Menzel, 1990).

Research on learning in bees has not been limited to study of classical conditioning. In recent years there has been a proliferation of studies displaying feats of learning few would ever have attributed to bees. Much of this work has relied on the ability of bees to perform 'symbolic matching' (Srinivasan et al., 1998; Cooke et al., 2007; Chittka, 1998). Srinivasan et al. (1998) trained honeybees to select a colour-marked exit in a Y-maze based on a smell presented earlier in the maze. The bees successfully learned the task. The same matching ability has subsequently been demonstrated repeatedly in traditional 'delayed match-to-sample' paradigms in which animals are presented with a colour and then, following a delay, required to select that colour for reward (Giurfa et al., 2001; Cooke et al., 2007). Symbolic matching has also been shown in bumblebees in a T-maze sensorimotor task (Chittka, 1998). The discovery that honeybees could solve a delayed match-to-sample task has led to further

demonstrations of remarkable learning ability. Honeybees have been shown to learn the concepts of 'sameness' and 'difference' and to transfer their learning to novel stimuli (Giurfa et al., 2001). They have also been shown to discriminate between stimuli based on number even when the stimuli change in shape, colour and type (Gross et al., 2009). Many of these spectacular displays of learning were the results of studies using honeybees, but bumblebees are not without their own impressive displays of behaviour. It has been shown that bumblebees can perform complex discriminations (Thivierge et al., 2002; Perreault & Plowright, 2009). They can discriminate between patterns and are even able to discriminate between stimuli when stimuli are only partially presented (Thivierge et al., 2002). In addition to their extensive discrimination skills, bumblebees were the first invertebrate species in which interval timing was demonstrated (Boisvert & Sherry, 2006; Boisvert et al., 2007).

One of the criticisms of animal cognition research has been that too many studies are focused on whether or not an animal can perform a behaviour and there is little discussion about how that behaviour is performed (Shettleworth, 2009). This is certainly a relevant consideration for the bee research. With a number of studies presenting spectacular feats of learning there is a danger of bee research becoming overly engaged in showing how 'smart' bees are and failing to consider how they are accomplishing these remarkable behaviours. There have been a few attempts, however, to explore how bees learn and the factors that can affect their performance on learning tasks. In a study of honeybee learning flights it was shown that the duration of the flights changed when factors such as environment complexity and quality of reward were manipulated (Wei et al., 2002). Another study that is most pertinent to the behavioural flexibility research

conducted here is an examination of bees' ability to improve with training on an olfactory discrimination reversal task (Komischke et al, 2002). Bees trained on one or two reversals showed an improved performance, compared to bees with no reversal experience, on a novel olfactory discrimination reversal (Komischke et al., 2002).

The Bee Brain

A large part of what makes learning by bees impressive is that they learn with such a small brain. The range of behaviours of honeybees in the wild has been quantified as 59 distinct motor outputs, a number comparable to that of many mammals (Chittka & Niven, 2011). As Chittka and Niven (2011) point out, a millionfold increase in brain volume for only a doubling or so in behavioural repertoire in vertebrates raises the question 'what are bigger brains for'. In an extensive review Chittka and Niven (2011) make a case that the increase in brain volume in mammals is due largely to increased sensory input and the increased investment in neuronal tissue that accompanies larger body size. This would indicate that the cognitive consequence of drastic differences in the brains of insects and mammals is the quantity of processing rather than kind. Determining the limits and function of the 'amazing mini brains' (Menzel & Giurfa, 1999) of insects is one driving force behind insect neuroscience. Another reason why the brains and behaviour of insects should be of interest to all neuroscience researchers is that their small size makes mapping the brain possible on a scale not yet possible in mammals (Heisenberg, 1998). With a detailed understanding of the neuronal connections in insects within reach, it may be possible to explore the interaction of brain and behaviour at a mechanistic level not possible in more traditional laboratory animals.

Structure. The insect brain is made up of the protocerebrum, the deuterocerebrum (also called the deutocerebrum), and the tritocerebrum (Fahrbach, 2006). The protocerebrum contains the optic lobes, central body and mushroom bodies; the deuterocerebrum consists of the antennal lobes which send olfactory projections to the mushroom bodies; and the tritocerebrum serves to control the sympathetic systems of the insect (Fahrbach, 2006). Of these brain structures the mushroom bodies have been most closely linked to learning and memory, and consequently are of interest here (Strausfeld et al., 1998; Farris, 2005; Fahrbach, 2006).The intrinsic neurons of the mushroom bodies, called Kenyon cells, appear in bilateral clusters in the dorsal part of the protocerebrum (Fahrbach, 2006). Each Kenyon cell projects a neurite that then splits into an axon and a dendrite (Fahrbach, 2006). The dendrite and its arborizations then form the calyces and the axon projects ventrally and splits to form the lobes of the mushroom body (Fahrbach, 2006). There is considerable variation in the structure of mushroom bodies in insects, but the basic structure of Kenyon cell bodies, calyces, and lobes is highly conserved (Strausfeld et al., 1998; Farris, 2005; Fahrbach, 2006). In the Hymenoptera (ants, wasps, and bees) the calyces are bilaterally doubled and divided into three regions the lip, collar, and basal ring (Fahrbach, 2006). The calyces on each side of the brain are identical and merge at what is called the pedunculus, very loosely described as a bridge between the calyces and lobes (Fahrbach, 2006). The calyces are the primary, but not sole, input region of the mushroom bodies (Fahrbach, 2006). In many insects the sensory input into the calyces is exclusively or almost exclusively olfactory, but this is not the case in Hymenoptera (Fahrbach, 2006). The calyces of the Hymenoptera receive olfactory input to the lip compartment, visual input to the collar, and both olfactory and

visual input to the basal ring. In addition to olfactory and visual input the calyces also receive some mechanosensory and gustatory input to the collar and basal ring of the calyces (Fahrbach, 2006). As a result of the variety of sensory information received by the mushroom bodies in the Hymenoptera they are considered a multimodal integration center (Fahrbach, 2006).

Mushroom body development. The development of the mushroom bodies in honeybees has been characterized from the larval stage to adult eclosion (Farris et al., 1999). The first appearance of mushroom bodies occurs in the first and second larval instars, in which two neuroblast clusters occur bilaterally (Farris et al., 1999). The neuroblast clusters develop the characteristics of mushroom bodies throughout subsequent larval instars, but only the Kenyon cells, pedunculus and lobes are present at prepupal stages (Farris et al., 1999). During the pupal stage the neuroblast clusters swell from 16-45 neuroblasts each to approximately 500 (Farris et al., 1999). Cell proliferation continues throughout the pupal stage and the calyces appear (Farris et al., 1999). In the final days of pupal development there is observable death of the neuroblasts and no neuroblasts remain at pupal day 7 (Farris et al., 1999). The death of the neuroblasts ends cell proliferation in the mushroom bodies. No neurogenesis has been observed in adult bees (Fahrbach et al., 1995). The absence of neurogenesis in adults is by no means universal in insects as a number of insects (e.g. crickets and beetles) show significant numbers of new neurons (Cayre et al., 1996).The absence of neuroblasts in the mushroom bodies of adult honeybees, if this is correct, is important because volume changes in the mushroom bodies of adult bees cannot be attributed to the growth of new neurons.

Mushroom bodies and learning. The distinct and conspicuous appearance of the mushroom bodies in insects led immediately upon their discovery to the assumption that they must be involved with learning, memory, and intelligence (Dujardin, 1850). This intuitive association was confirmed in a landmark study by De Belle and Heisenberg (1994). De Belle and Heisenberg (1994) used chemical ablation of larval mushroom body neuroblasts to selectively eliminate the mushroom bodies from the brains of adult *Drosophila*. The remainder of the *Drosophila* brain developed normally, making it possible to observe the behaviour of the ablated flies and determine more specifically the function of the mushroom bodies. The mushroom body ablated flies appeared to behave normally, with similar levels of activity, mating success, and reproduction as controls, however, when tested on an olfactory association the mushroom body ablated flies were severely impaired compared to intact flies (De Belle & Heisenberg, 1994). A similar procedure of chemical ablation of larval mushroom body neuroblasts has been used in honeybees (Komischke et al., 2005). Honeybees with unilateral mushroom body ablation were unable to acquire an olfactory discrimination applied to the ablated side, but retained their learning abilities for discriminations applied to the intact side (Komischke et al. 2005). In addition to the evidence from ablation work, it has been shown that larger mushroom bodies are correlated with improved performance on a learning task in honeybees. Gronenberg and Couvillon (2010) trained honeybees on an olfactory associative task and compared performance with brain volume. Improved performance was associated with greater mushroom body volume (Gronenberg & Couvillon, 2010). Aside from a relationship between total brain volume and performance, the mushroom

bodies were the only brain component significantly correlated with learning (Gronenberg & Couvillon, 2010).

The appearance of larger mushroom bodies in social insects, such as the Hymenopteran order, suggests that mushroom bodies may be important for more complex behaviour as well. It was recently shown through an extensive comparison of eusocial wasp species that increased social interaction was correlated with species differences in mushroom body size (O'Donnell et al., 2011). Increased foraging demands have also been hypothesized to drive greater investment in mushroom bodies in eusocial insects (Mares et al., 2005). The consistently observed increase in mushroom body volume with foraging experience in within species studies in honeybees (Withers et al., 1993; Withers et al., 1995) and other Hymenoptera (O'Donnell et al., 2004; Withers et al., 2007) provides support for this theory. The second study in this thesis tested for possible effects of learning experience, foraging experience and environmental complexity on mushroom body size in bumblebees.

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CHAPTER TWO

Serial Reversal Learning in Bumblebees

Introduction

Animals are constantly learning about their environment, making discriminations, and forming associations in order to complete foraging and navigation tasks required for survival. The importance of this constant processing of information invites the question, when faced with the same learning problem repeatedly does performance improve with experience (Mackintosh, 1974; Shettleworth, 1998; 2010)? That is, do animals 'learn to learn'? The ability to recognize change in the environment and update associations based on new information is adaptive. It is possible to address this issue in animal learning using a serial reversal. Serial reversal learning consists of initial discrimination learning, in which one stimulus is rewarded and another not, and then repeated reversal of the reward contingencies. This requires an animal to reverse its pattern of responding in response to the changing environment to obtain reward. With repeated presentation of similar problems, as in a serial reversal, a measure of improvement with experience can be obtained. The requirement that behaviour constantly change to meet the task demands makes serial reversal learning a measure of behavioural flexibility as well.

The serial reversal task has been used extensively in animal learning research to compare improvement over the course of a number of discrimination reversals in many different species (Davey, 1989; Shettleworth, 1998; 2010). At one point serial reversal learning was widely used as a general measure of intelligence (Mackintosh, 1969; Bitterman, 1969; Davey, 1989). At the time, explanations of inter-reversal improvements were largely attributed to attentional processes and proactive interference (Mackintosh, 1974). In more recent research the serial reversal task is used to explore the ability of animals to 'learn to learn' and their acquisition of abstract strategies. Optimal

performance on a serial reversal task consists of only a single error following each reversal and results from adopting a 'win-stay lose-shift' strategy. Examination of strategy learning using serial reversal learning has provided some interesting results. For example it has been shown that primate species differ in their ability to adopt a strategy in the reversal task (Rumbaugh et al., 1996). This demonstrates the utility of serial reversal for exploring the similarities and differences in learning among closely related species.

As a measure of behavioural flexibility the serial reversal task is enjoying a resurgence in animal learning research. It has recently been used to explore the differences in learning flexibility in animals whose ecology differs in ways that suggest differences in the adaptive benefits of behavioural flexibility (Bond et al., 2007). Bond et al. (2007) compared a number species of corvids on both visual and spatial discrimination reversals. Their results point to serial reversal learning as an indicator of general behavioural flexibility because species that possessed a more complex social structure, presumably increasing the demand for flexible association learning, performed better than other corvid species on reversals in both visual and spatial modalities, even when their initial acquisition of the discrimination was comparatively delayed. Thus, the serial reversal task has become an established method for studying behavioural flexibility in addition to its long standing use in studying 'learn to learn' abilities.

Foraging bumble bees make numerous discriminations. They acquire flower and place preferences and navigate to and from their colony. It is intuitively clear why flexibility would be important for nectar feeders such as bees: the same flowers will not always be the best providers of nectar. Some flowers bloom in the morning and others in the evening. A preferred foraging patch may have been depleted by a competing forager.

Being able to change flower preferences in response to feedback may be adaptive for bees. In addition to the hypothesis that behavioural flexibility may be beneficial to bees studying the flexibility of learning in bees is intrinsically interesting. Bees have a small brain compared to that of mammals and yet they can learn tasks that many mammals find challenging and can acquire a simple learning task (e.g. a colour discrimination) with fewer rewards delivered than some mammals and even human infants (Pearce, 2008). Also, bees perform a suite of at least 59 distinct behaviours in the wild (Chittka & Niven, 2009). One cannot help but wonder at what point the capacity of the bee brain reaches its limit. Is it possible that in prioritizing rapid learning of a large number of ecologically relevant behaviours bees have sacrificed flexibility of those behaviours once established? If this were the case, bees may readily acquire discriminations, displaying their remarkable learning abilities, but struggle with reversal tasks that assess learning flexibility.

Early attempts to explore serial reversal learning in bees provided contradictory results. Menzel (1969) trained honeybees on a two colour discrimination and then repeatedly reversed the reward contingencies. Rather than showing improved learning rate in later reversals, bees began to respond to both stimuli equally, failing to discriminate between the two stimuli. A similar result was found by Couvillon and Bitterman (1986). Couvillon and Bitterman (1986) found that four reversals consisting of eight trials each were readily acquired by bees. However, when bees were required to learn eight reversals consisting of four trials each, their learning pattern resembled that of Menzel's study, with accuracy approaching chance. These initial investigations suggest that bees are sensitive to changes in reward contingencies and do show some flexibility,

in that they can reverse their learned response pattern. Honeybees, however, failed to show inter-reversal improvement - 'learning to learn' - and in fact showed the opposite pattern of a decline in performance.

In an attempt to clarify the contradictory findings of previous work Mota and Giurfa (2010) revisited serial reversal learning in honeybees. They employed the widely used proboscis extension reflex (PER) protocol developed by Bitterman et al. (1983). PER requires that bees be harnessed so that only their proboscis (tongue) and antennae are free to move. Their reflexive proboscis extension to sucrose is then conditioned to odour stimuli administered to their antennae, the bees' olfactory sensory organ. Mota and Giurfa (2010) conditioned honeybees to differentially respond to two odour stimuli. Once the bees had completed five trials with each odour the reward contingencies were reversed. Bees experienced a total of four reversals. Bees significantly changed their response patterns after each reversal, but in the final reversal bees' ability to discriminate between the two stimuli declined and their responses approached equal responding to both stimuli. As was found in the early reversal work, bees' began to generalize their responding to both stimuli after repeated reversals. Although this study, like previous studies, showed a generalization of responding in bees following reversal, there is some reason for skepticism. The PER protocol, although useful in bee learning research of some kinds, limits the number of trials that bees can be given in any experiment. While harnessed in the apparatus bees are unable to return to their colony to deposit nectar and become sated when their honeycrop is filled. Therefore the number of trials and reversals is severely limited by this testing paradigm. Although not done using PER, the early

studies of serial reversal learning in bees were also limited to small trial and reversal numbers.

There has been at least one demonstration of improvement by a bee on a serial reversal task. Chittka (1998) trained a bumblebee (*Bombus impatiens*) to enter a T-maze and choose the right arm to receive a sucrose reward. The bee was trained on this task for 100 trials and then required to switch repeatedly from the right arm to the left arm after 100 trials on each. The initial reversal resulted in a significant increase in the number of errors. The bee's high error rate at the start of each session continued until the fifth reversal. Importantly, however, the bee's performance did eventually improve and after eight reversals the bee performed the reversal with only three errors. This suggests that under certain conditions, such as a vastly increased trial number, bees are capable of showing improvement, as measured by reduction in errors. As with previous serial reversal work in bees, however, there is cause to interpret these results with caution. The study was performed on a single bee. In addition, the bee was performing spatial reversals, not visual or olfactory reversals. There is much evidence to suggest that serial reversal learning can vary depending on the modality being tested. Improvement in spatial reversal cannot therefore be taken as evidence that a similar pattern will occur in another modality. A further consideration is that Chittka's study was done on a bumblebee, not honeybees as in previous work. Because no comparable spatial task has been used with honeybees this cannot be taken as evidence of a species difference in reversal learning. It is certainly worthy of note, however, that the only clear demonstration of inter-reversal improvement in a serial reversal task occurred in a bumblebee.

In addition to looking at patterns of serial reversal learning another point of interest is factors that influence bee reversal learning. It has been shown that bee learning is influenced by the sensory stimulation a bee receives throughout its life. Honeybees' ability to acquire a conditioned PER develops over the first nine days of adult life (Ichikawa & Sasaki, 2003). Bees' learning continues to improve throughout adult development, with foragers acquiring conditioned responses more readily than nurse bees who do not have foraging experience (Ichikawa & Sasaki, 2003). It has also been shown that bees confined and sensorily deprived after nine days of age lose the ability to acquire a conditioned response (Ichikawa & Sasaki, 2003). Learning thus appears inseparably linked to sensory input in bees. It is also known that reversal learning is specifically influenced by development and sensory input in bees. Ben-Shahar et al. (2000) collected nurse and forager honeybees and tested them on a single olfactory reversal using PER. It was found that the nurse bees and foragers acquired a response to a previously unrewarded odour at equal rates. However, nurse bees learned faster than foragers to inhibit responding to a previously rewarded odour that was no longer being rewarded. This suggests that sensory stimulation and foraging experience acquired by bees throughout development influences cognition and reversal learning. One thing that is not clear about the role of foraging in cognitive development of bees is what it is about foraging experience that affects development. Is it the mechanosensory stimulation of flight, the learning demands of locating and retrieving nectar and pollen, or is it the increased sensory input from the environment outside the hive?

I explored serial reversal learning and the factors that influence it in bumblebees. Serial reversal learning was tested by training bees on a simultaneous colour

discrimination followed by repeated reversals. Bees tested in a simulated natural foraging environment were engaged in collecting nectar and carrying it back to their colony throughout testing. The pivotal differences between this study and previous investigations were, 1) a considerably increased number of trials and reversals and 2) free flight testing. In order to address the influence of foraging on reversal learning bees were housed in either an enriched environment or a simple environment, differing in the sensory input available outside the hive.

Methods

Subjects

Subjects were 14 bumblebee workers (*Bombus impatiens*) from four colonies obtained from a commercial supplier (Biobest Canada Ltd., Leamington, ON). Colonies were kept on a 12h light/dark cycle (light onset 6am) in a 3.0 X 8.5 m room. Hive containers were kept in cardboard boxes provided by the supplier, which sheltered the colonies from light. During tagging, pollen feeding, and testing the boxes were opened and the colonies were exposed to light. Bees could leave the colony to forage and had unrestricted access to all parts of the room. Bees foraged *ad libitum* at artificial flower patches for 15-17% sugar solution. Pollen was given directly to the colony through a small opening in the lid of the colony container. Artificial flowers were provided in patches consisting of either five or ten plastic centrifuge tubes secured in polystyrene foam. These patches were placed on tables or mounted on walls. Colonies were given at least 5 days after arrival to begin foraging and habituate to their housing conditions before bees were tested.

Prior to testing, bees were collected while foraging or taken directly from the colony, restrained in a marking tube, and tagged for individual identification with either plastic number tags (Betterbee Inc., Greenwich, NY) affixed with cyanoacrylate glue or Posca paint markers (Mitsubishi Pencil Co.). During testing bees were identified by their colour and number.

Foraging Environments

Two bumblebee colonies were housed in a 'simple' environment and two colonies were housed in a 'complex' foraging environment (Figure 2.1). The simple environment contained eight artificial foraging patches, four consisting of ten artificial flowers mounted on tables and four consisting of five artificial flowers mounted on the walls. The complex environment contained identical foraging patches and additional stimuli: artificial plants placed on the tables; artificial vines hung from the ceiling and placed around wall-mounted foraging patches; carpet; brightly-coloured felt and foam panels placed on the tables and walls. All materials differed from stimuli used during testing (see below). The additional stimuli increased the visual complexity of the environment, and probably tactile and olfactory complexity, too, because bees landed on the stimuli and the stimuli had a variety of distinctive odors detectable by human observers, including floral-like odors from the craft store source of these materials.

Apparatus

The apparatus consisted of two cardboard boxes (20.3 X 20.3 X 10.2 cm) each with a (20.3 X 20.3 cm) clear plastic lid (Figure 2.2). One 20.3 X 10.2 cm side was removed from each box to provide an entrance. Boxes were placed together side-by-side during testing and their relative left-right position could be changed during testing. Each

Figure 2.1. Foraging environments. Images show one half of the room setup for each condition. The yellow box is the hive.

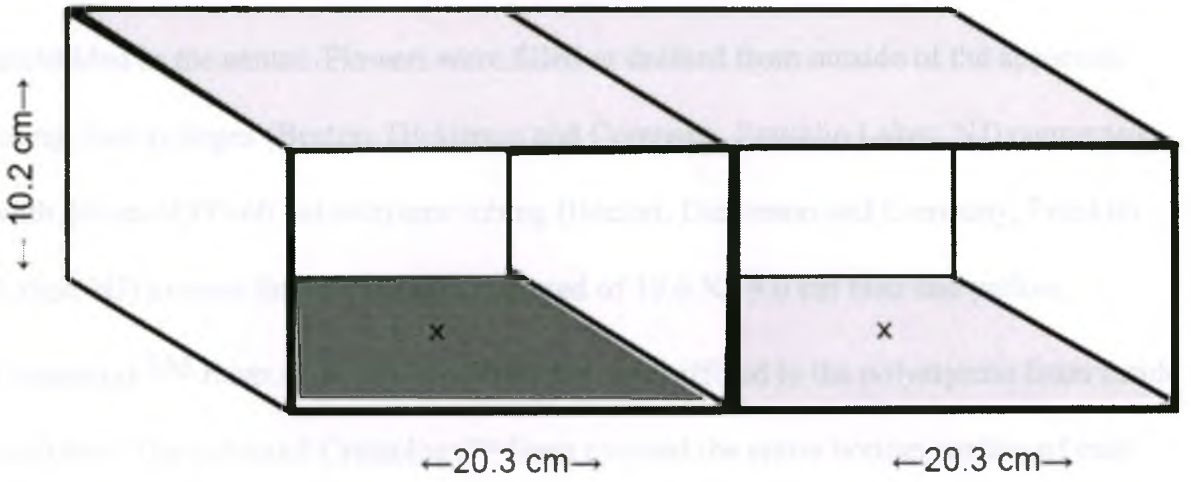

Figure 2.2. Apparatus. The apparatus consisted of two adjacent boxes with a front entrance. The stimuli were presented on the floor of the boxes. The artificial flower locations are designated with 'x's.

box contained a 19.0 X 19.0 cm piece of polystyrene foam with a single artificial flower embedded in the centre. Flowers were filled or drained from outside of the apparatus using 3ml syringes (Becton, Dickinson and Company, Franklin Lakes, NJ) connected with 20 cm of PE-60 polyethylene tubing (Becton, Dickinson and Company, Franklin Lakes, NJ) to each flower. Stimuli consisted of 19.0 X 19.0 cm blue and yellow Creatology^{TM} foam sheets (Michaels Stores Inc.) affixed to the polystyrene foam inside each box. The coloured Creatology™ foam covered the entire bottom surface of each box. The single artificial flower contained in each box was located in the center of the foam. Following each testing session the apparatus was removed from the room and the Creatology™ foam was wiped with 70% isopropyl alcohol after each testing session to remove any odours left by bees during testing.

Behavioural task

Bees were tested individually for five consecutive days. Bees were tested in two sessions per day for a total of ten sessions. Sessions 1 and 2 in a day were separated by 1.5-8 hours.

Pre-training

Prior to training on the simultaneous colour discrimination, bees were shaped to enter the apparatus with only one box and no colour stimuli present. Bees were collected either while foraging or taken from the colony and placed on a portable artificial flower filled with 35-40% sugar solution. While taking sugar solution from this flower, bees were moved to the apparatus entrance. This procedure was repeated until the bees made foraging trips to the apparatus entrance by themselves. The flower was then gradually moved into the apparatus until the bees were making foraging trips into the apparatus,

taking sugar solution to repletion, and then returning to the colony, at which point testing began. Pre-training trials only occurred in the first testing session unless absolutely necessary to initiate foraging trips by a reluctant bee.

Testing Procedure

During each testing session the apparatus contained two colours, blue and yellow, one in each box. Only one colour was rewarded in each session. The rewarded colour in the first session (start colour) was counterbalanced across bees. Each session consisted of 40 trials. Position of colours on the left or right was pseudorandomized with an equal number of right and left positions for each colour in a block of ten trials and no more than three trials in a row of one position. A single choice was recorded for each trial and complete entrance into a box was considered a choice. If bees made a correct choice and entered the chamber of the rewarded colour the artificial flower was immediately filled and the bee was allowed to fill to repletion. If bees made an incorrect choice they were allowed to exit the apparatus and make a second choice. A barrier existed between the two boxes of the apparatus forcing bees to exit the apparatus completely before making a second choice. If bees left the area of the apparatus without making a second choice the trial was considered finished and the bee's next appearance was the start of a new trial. To ensure bees could not use olfactory cues from the sugar solution there was no reward present in the apparatus until after bees had made a choice.

Criterion performance for the initial discrimination in the first session was set at 8 out of 10 correct choices in a sliding block of 10 trials and only bees meeting this criterion in the first session were tested on the serial reversal task. In the serial reversal task reward contingencies were reversed at the start of each session starting with the first

reversal in session two. Bees completed a total of nine reversals over the course of ten sessions.

Olfactory Control Condition

Because it was impossible to clean the apparatus between trials it could not be ruled out that bees in the serial reversal task used odour cues from previous trials to guide their choices within a session. To determine if the simultaneous discrimination could be learned using odour cues, seven bees were tested on the first session initial discrimination under identical conditions to those for bees in the serial reversal task but with the colour stimuli removed.

Data Recording and Analysis

Each bee's first choice in each trial was recorded. The number of errors made in each session was used as a measure of learning for comparisons across sessions (intersession). Learning within sessions (intrasession) was measured by dividing each 40 trial session into four consecutive blocks of 10 trials each and calculating the number of errors per block for each bee. Differences in acquisition of the initial discrimination in the first session by bees in the simple and complex environments were tested with an independent samples t-test on errors in the first session. The effect of start colour on acquisition of the initial discrimination was measured by comparing the first session performance of bees trained with blue rewarded in the first session to bees trained with yellow rewarded using an independent samples t-test. Data from bees in the olfactory control condition (uncued discrimination) was divided into four blocks of 10 trials and analyzed using repeated measures ANOVA to assess intrasession learning. Learning on the serial reversal task was analyzed using the errors in each trial block for each bee and a

repeated measures ANOVA design with trial block and reversal as within subject factors and start colour and foraging environment as between subject factors. Trends across reversals were explored using regression analysis. Chi-square tests were used to compare bees' choices on the first trial in each session to chance.

Seven of the fourteen bees tested did not complete all nine reversals. Complete data were collected and analyzed from fourteen bees for the first four reversals and additional separate analyses were done for the seven bees that completed all nine reversals.

Results

Initial discrimination in the first session

Bees readily acquired the initial simultaneous colour discrimination in the first testing session and met the 8 out of 10 correct criterion within the 40 trial session. No differences were found between the simple and complex foraging environments in acquisition of the initial colour discrimination ($t(12) = -.583$, $p = .579$). A difference was found in acquisition between those bees for whom blue was the rewarded stimulus compared to those for whom yellow was rewarded (start colour), with a response to blue acquired more rapidly ($t(12) = -2.389$, $p = .03$). Consequently, start colour was included in subsequent analyses as a between subjects factor.

Uncued Discrimination

Bees in the simultaneous discrimination task with no colour cues showed no evidence of learning (Figure 2.3). Performance was analysed by dividing the 40 trial session into blocks of ten trials and using a repeated measures ANOVA to determine if performance improved over the course of the session. Performance did not change across

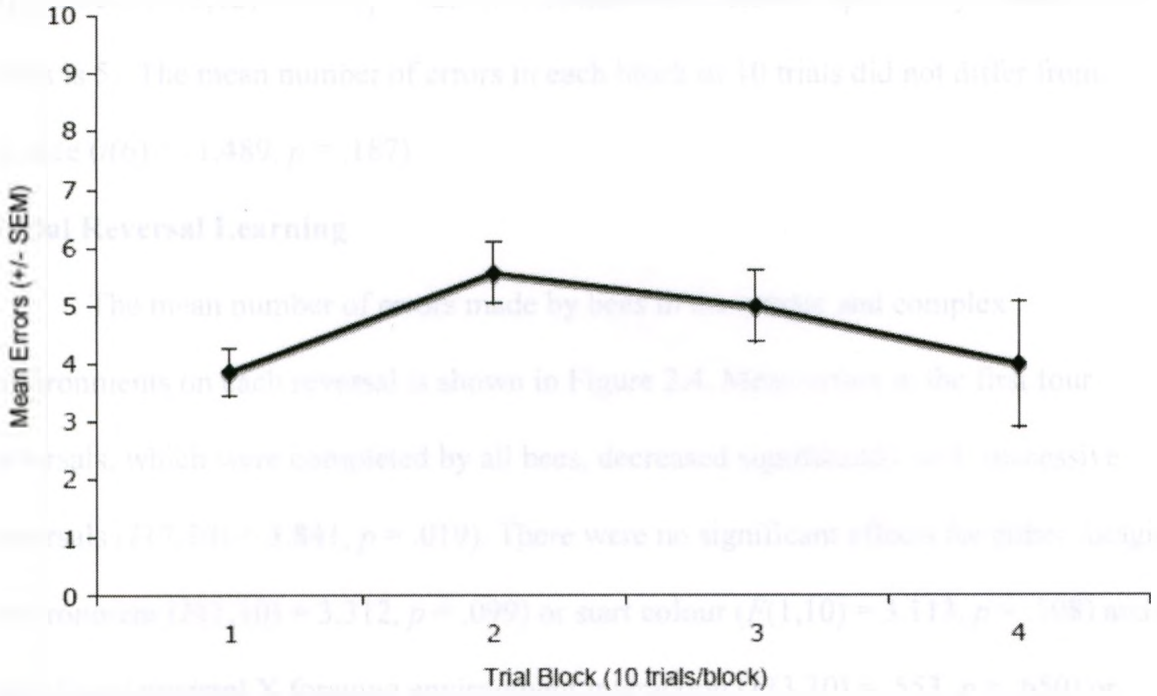

Figure 2.3. Simultaneous discrimination without colour cues. The mean number of errors (out of 10) made by bees in a single session of uncued discrimination does not differ from 5, the number expected by chance.

trial blocks ($F(3,12) = 1.55$, $p = .251$). The number of errors expected by chance in 10 trials is 5. The mean number of errors in each block of 10 trials did not differ from chance ($t(6) = -1.489$, $p = .187$).

Serial Reversal Learning

The mean number of errors made by bees in the simple and complex environments on each reversal is shown in Figure 2.4. Mean errors in the first four reversals, which were completed by all bees, decreased significantly with successive reversals ($F(3,30) = 3.841$, $p = .019$). There were no significant effects for either foraging environment ($F(1,10) = 3.312$, $p = .099$) or start colour ($F(1,10) = 3.113$, $p = .108$) and no significant reversal X foraging environment interaction ($F(3,30) = .553$, $p = .650$) or reversal X start colour interaction ($F(3,30) = 1.452$, $p = .247$). A separate analysis using the 7 bees who completed all nine reversals also found a main effect of reversal ($F(8,24) = 3.444$, $p = .009$), and no significant effect of foraging environment ($F(1,3) = .542$, $p = .515$) or start colour ($F(1,3) = 1.275$, $p = .341$). The mean number of errors per session had a significant quadratic relation with reversal in both the simple ($r = .84$, $F(2,8) = 7.06$, $p = .03$, $r^2 = .7$) and complex ($r = .81$, $F(2,8) = 5.79$, $p = .04$, $r^2 = .66$) foraging environments with errors in each session declining with repeated reversals (Figure 2.5). Learning within sessions was analyzed by dividing the 40 trials per session into four blocks of ten trials each. The mean number errors for each trial block is shown in Figure 2.6. Analysis of the block data from the 14 bees that completed the first four reversals confirmed the main effect of reversal found in the previous analysis along with a significant main effect of block ($F(3,30) = 105.439$, $p < .001$) and a reversal X block interaction ($F(9,90) = 5.885$, $p < .001$). No significant effect of foraging environment was

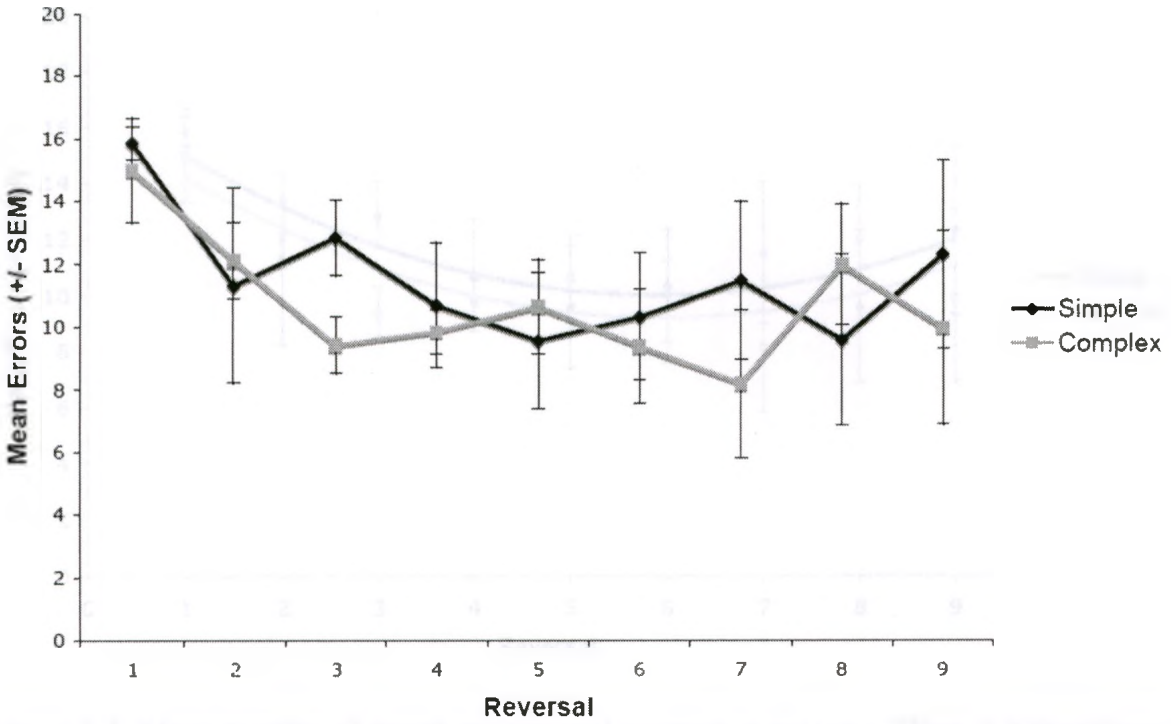

Figure 2.4. Mean number of errors per reversal. The mean number of errors changed significantly across the nine reversals but bees housed in simple and complex foraging environments did not differ significantly.

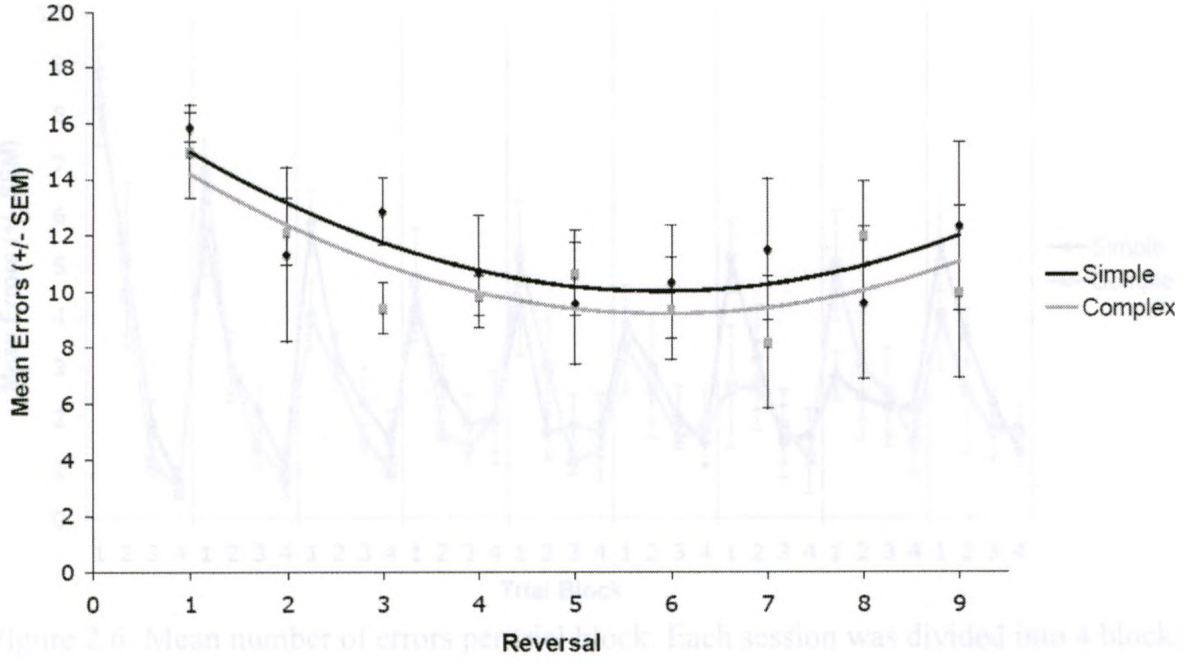

Figure 2.5. Mean number of errors per reversal regression analysis. There is a significant quadratic relation between mean errors and reversal for bees in both the simple and complex foraging environments.

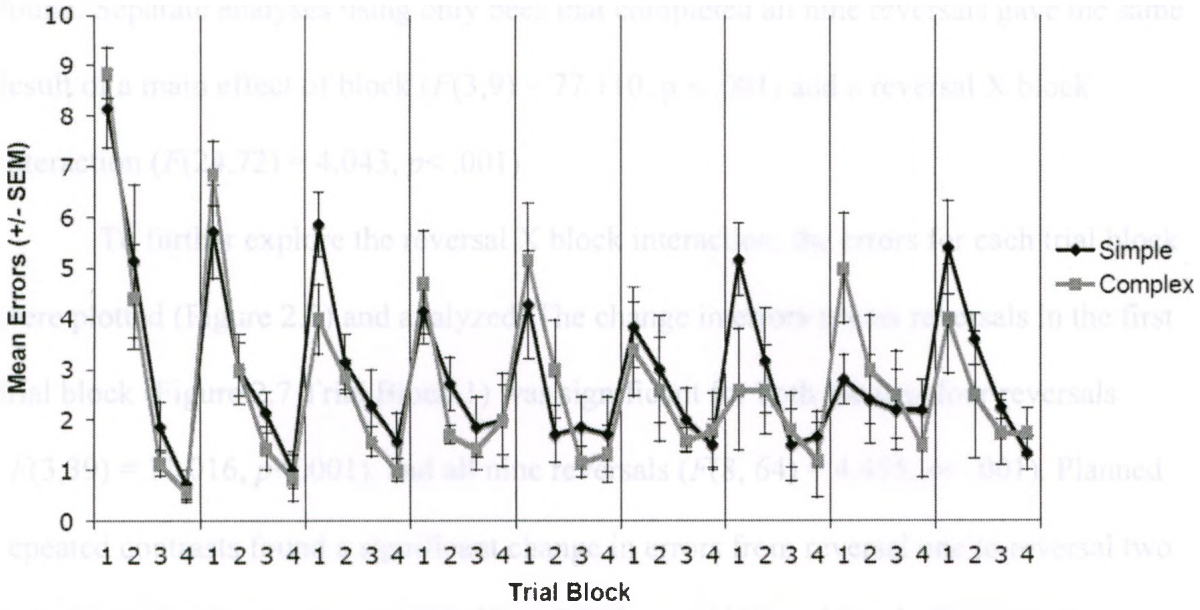

Figure 2.6. Mean number of errors per trial block. Each session was divided into 4 blocks (numbered 1 to 4) of ten trials each. Reward contingencies were reversed on the first trail of each four-block session.

found. Separate analyses using only bees that completed all nine reversals gave the same result of a main effect of block ($F(3,9) = 77.110$, $p < .001$) and a reversal X block interaction ($F(24,72) = 4.043$, $p < .001$).

To further explore the reversal X block interaction, the errors for each trial block were plotted (Figure 2.7) and analyzed. The change in errors across reversals in the first trial block (Figure 2.7 Trial Block 1) was significant for both the first four reversals ($F(3,39) = 11.016$, $p < .001$), and all nine reversals ($F(8, 64) = 4.455$, $p < .001$). Planned repeated contrasts found a significant change in errors from reversal one to reversal two in both the first four reversals ($F(1,13) = 7.803$, $p = .015$), and for the bees who completed all nine reversals ($F(1,8) = 11.571$, $p = .009$). No other significant contrasts were found. Analysis of the second trial block (Figure 2.7 Trial Block 2) for the first four reversals found a significant main effect of reversal ($F(3,39) = 4.065$, $p = .013$), but no effect for the bees who completed nine reversals ($F(8,64) = 1.571$, $p = .151$). The analyses for the third trial block (Figure 2.7 Trial Block 3) found no significant effects of reversal in either the first four reversals ($F(3,39) = .317$, $p = .813$), or all nine reversals ($F(8, 56) = .851$, $p = .563$). The effects of reversal on the fourth trial block (Figure 2.7 Trial Block 4) were also found to be non-significant for the first four reversals ($F(3,36) = 1.807$, $p = .163$), and all nine reversals ($F(8,48) = 1.067$, $p = .401$).

For the first and second trial blocks, quadratic regression of errors across reversals was significant (Figure 2.8; Trial Block 1 $r = .97$, $F(2,8) = 43.51$, $p = .0003$; $r^2 = .94$; Trial Block 2 $r = .86$, $F(2,8) = 8.6$, $p = .017$, $r^2 = .74$).

A comparison of bees' choices on the first trial of each session found that bees' choices were not different from chance in the first trial of the initial discrimination

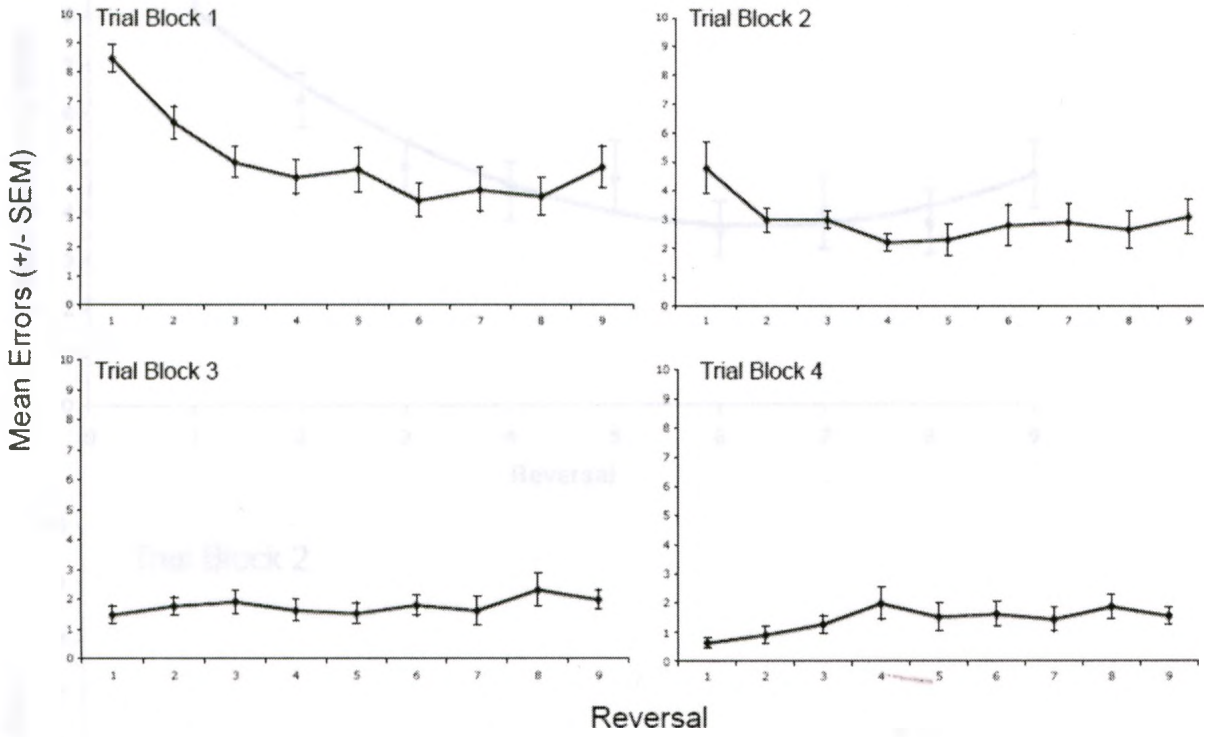

Figure 2.7. Trial block errors per reversal. The mean number of errors in each trial block is shown separately. Each panel represents the mean errors for each block (1-4) for reversals 1-9.

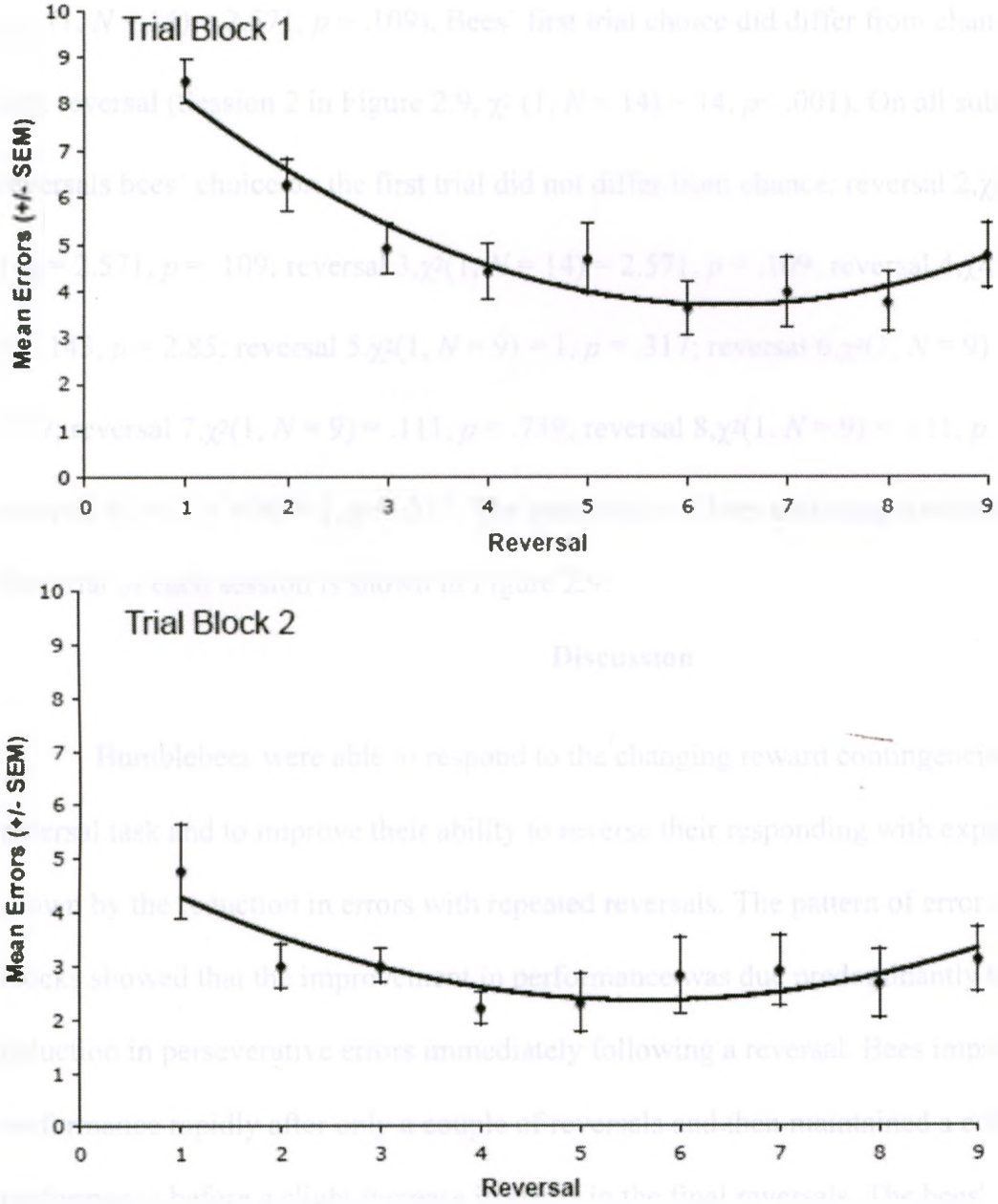

Figure 2.8. Trial block regression analysis. The figure shows regression lines representing the quadratic relationship between mean errors for the first trial block in each reversal (Trial Block 1) and the second trial block in each reversal (Trial Block 2).

(χ^2 (1, N = 14) = 2.571, p = .109). Bees' first trial choice did differ from chance in the first reversal (Session 2 in Figure 2.9, χ^2 (1, N = 14) = 14, p< .001). On all subsequent reversals bees' choice on the first trial did not differ from chance: reversal 2,χ^2 (1, N = 14) = 2.571, p = .109; reversal 3,χ^2(1, N = 14) = 2.571, p = .109; reversal 4,χ^2(1, N = 14) = 1.143, p = 2.85; reversal 5,χ^2(1, N = 9) = 1, p = .317; reversal 6,χ^2(1, N = 9) = .111, p = .739; reversal 7,χ^2(1, N = 9) = .111, p = .739; reversal 8,χ^2(1, N = 9) = .111, p = .739; and reversal 9.χ^2(1, N = 9) = 1, p = .317. The percentage of bees choosing correctly on the first trial in each session is shown in Figure 2.9.

Discussion

Bumblebees were able to respond to the changing reward contingencies in a serial reversal task and to improve their ability to reverse their responding with experience, as shown by the reduction in errors with repeated reversals. The pattern of errors within trial blocks showed that the improvement in performance was due predominantly to a reduction in perseverative errors immediately following a reversal. Bees improved their performance rapidly after only a couple of reversals and then maintained a consistent performance before a slight increase in errors in the final reversals. The bees' performance on the serial reversal task did not improve to the point of one trial reversal that has been shown in a number of species (reviewed in Davey, 1989), but significant improvement over the course of the task is clear evidence that learning occurred across successive 40-trial sessions. This is evidence that bees are capable not only of learning on a trial by trial basis, but can also extract general information about task demands. Thus bees show the ability to 'learn to learn'. These results also show that bumblebees have

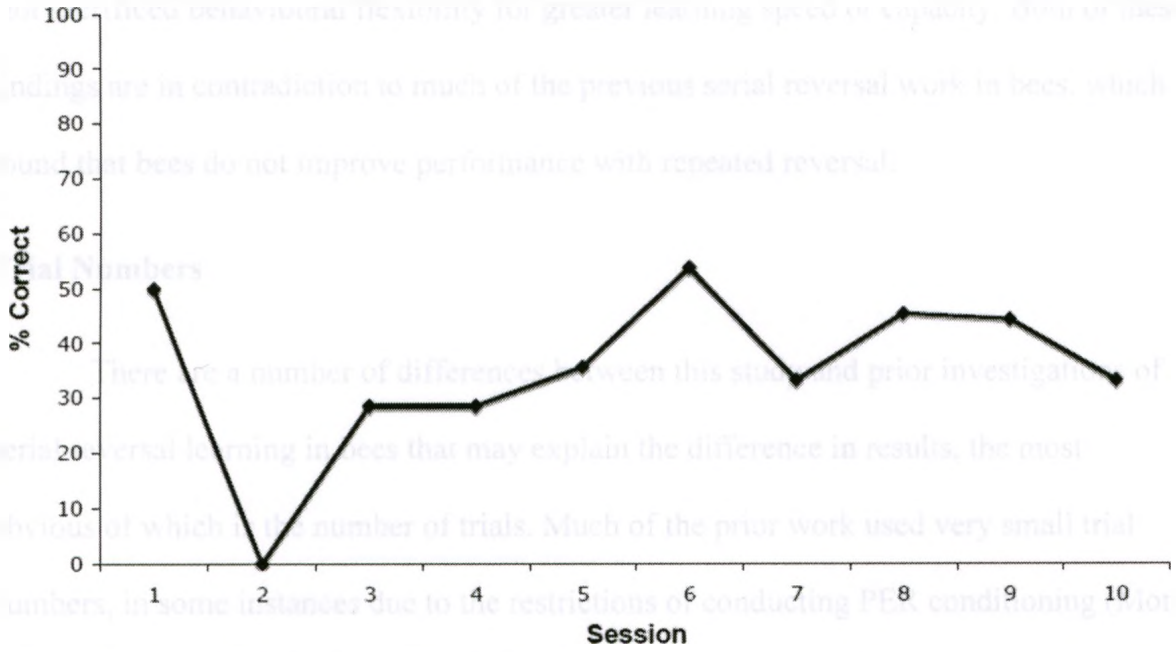

Figure 2.9. First trial choice. The figure shows the percentage of bees that correctly chose the rewarded colour on the first trial in each testing session. Initial discrimination learning occurred in session 1 and sessions 2-9 are reversals.

not sacrificed behavioural flexibility for greater learning speed or capacity. Both of these findings are in contradiction to much of the previous serial reversal work in bees, which found that bees do not improve performance with repeated reversal.

Trial Numbers

There are a number of differences between this study and prior investigations of serial reversal learning in bees that may explain the difference in results, the most obvious of which is the number of trials. Much of the prior work used very small trial numbers, in some instances due to the restrictions of conducting PER conditioning (Mota & Giurfa, 2010). The trial number in this study was much greater than in previous studies and may explain the differences. Also, as mentioned in the introduction, the only previous evidence of improvement in serial reversal learning in bees also used large trial numbers (Chittka, 1998).

There are a number of reasons why large trial numbers may facilitate serial reversal learning. A phenomenon that has long been observed in reversal learning is the overtraining reversal effect (ORE) (Mackintosh, 1974). It was found that, in some situations, animals who received training trials beyond criterion were facilitated when subsequently tested on a reversal compared to controls trained only to criterion (Mackintosh, 1974). These results are counterintuitive because increasing pairings with a reward should strengthen an animal's association between a response and a stimulus, reducing the likelihood of reversal. Nevertheless, overtraining reversal effects have been replicated and the effect appears frequently, depending on testing conditions (Mackintosh, 1974). The bees tested here were not specifically overtrained, but rather

each bee completed an identical number of trials. In a number of sessions, however, bees reached criterion prior to the end of the session and received overtraining trials. This study cannot be considered a demonstration of the ORE because bees were not specifically overtrained, and there was no comparison group without overtraining. Having said that, the connection between overtraining and performance on reversal task does provide a possible explanation for the bees' performance in this study.

Another reason the trial number may make a difference to the performance of bees on the serial reversal task is that trial numbers may have been so low in previous studies that bees were unable to fully acquire the discrimination prior to its reversal. Previous studies were based, like this one, on number of trials for each reversal rather than criterion performance and used considerably fewer trials. It can be seen in previous work that bees did change their behaviour following each reversal (Couvillon & Bitterman, 1986; Mota &Giurfa, 2010) and some authors were careful to confirm that this change in behaviour was significant (Mota & Giurfa, 2010), but because bees did not return to their pre-reversal level of discrimination it cannot be said with certainty that they learned the reversals. If bees were not successfully learning each reversal they may have treated the task as two stimuli on variable interval reinforcement schedules rather than a discrimination. If this were the case it would be an explanation for the averaging performance seen in a number of previous studies. In order to explore a connection between averaging performance and insufficient learning following each reversal, as well as the possibility that improvements are due to the ORE, a series of studies would have to be conducted with a variety of trial numbers and criteria.

First Trial Choice

Sessions 1 and 2 each day occurred consistently in the morning and afternoon respectively, although the actual start time of the sessions was highly variable. As the reward contingencies were reversed at the start of each session it is possible that bees associated one response with the AM session and the other with the PM session. Such an association would facilitate their learning and contribute to their reduction in errors. If bees were associating a colour with the time of the session we would expect them to select the correct response on the first trial. However, bees chose randomly on the first trial in all sessions except the first reversal in which they were worse than chance. This pattern of randomly responding on the first trial with repeated reversals has been found before in rats and occurred when rats were predictably reversed midday (Mackintosh et al., 1968). Although it cannot be ruled out that bees were aided by circadian cues, it seems unlikely given their performance.

Sensory Modality

Although a number of reversal studies in bees have used colour stimuli (Menzel, 1969; Chittka, 1998), much of the work has been done using olfaction (Couvillon & Bitterman, 1986; Mota & Giurfa, 2010). Because this study was done with colour it is possible that the difference in performance is due to the difference in modality. It has been shown in the past that testing conditions can have a significant impact on serial reversal performance, with some species showing improvement in one modality and not in others (Bitterman, 1965). The research on serial reversal learning in bees also gives cause to consider the existence of modality differences. Prior to the present study, the

work by Chittka (1998) was the only demonstration of successful serial reversal learning in bees and it was conducted using spatial learning, not the more commonly used olfactory discrimination. Direct comparisons of performance on matched olfactory, visual, and spatial tasks would be required to test the hypothesis that modality is an important factor but the available results are consistent with the idea that bees are more constant for olfactory learning than other modalities.

Support for potential modality differences in serial reversal learning also comes from observations that bees use colour and odour cues differently while foraging, which could potentially lead to differences in flexibility in learning tasks. Some species of flowers pollinated by bees produce morphs of different colours (Waser & Price, 1983; Wolfe, 1993). In these instances odour cues may be constant across both morphs, but colour cues differ. If flowers were morphologically similar aside from colour, flexibility for colour cues may be beneficial when making foraging decisions (Waser & Price, 1983). Bees could then capitalize on the nectar resources of both morphs without additional costs of learning to handle other types of flower. Research has shown that bees do differ in preference and handling time for colour morphs (Waser & Price, 183), but there are at least some flowers for which multiple morphs are visited with equal preference (Wolfe, 1993). Field observations of Heinrich (1976) provide further evidence that colour and odour cues are treated differently by foraging bees. Bees were observed approaching flowers that were the same colour as their preferred species, landing, but then leaving the flower without sampling or collecting nectar (Heinrich, 1976). It was proposed by Heinrich (1976) that the bees use colour as a distant cue, but confirm flower identity with proximate odour cues upon landing. When the bees left the flowers before

taking nectar, Heinrich (1976) proposed that they had been deceived by colour cues and corrected themselves upon arrival using the proximate odour cues. These two differences in the importance of colour and odour in bee foraging behaviour do not conclusively show that greater constancy for odour cues is adaptive for bees, but they do provide grounds for some hypothesis driven research on modality differences.

Species Differences Between Bumblebees and Honeybees

Thus far previous work on serial reversal learning in honeybees and bumblebees has been considered as a whole, but there may be important species differences. Because of their phylogenetic closeness it is easy to assume that honeybees and bumblebees would share many commonalities in learning. However, research on subtle differences in ecology and their impact on behavioural flexibility give cause to be cautious when generalizing across even closely related species. Bond et al. (2007) compared closely related corvid species and found significant differences in performance on a serial reversal task linked to differences in ecology. It is possible that similar differences also exist between bumblebees and honeybees.

Serial reversal learning by bumblebees was shown to occur in this study as well as in previous work (Chittka, 1998). In contrast, attempts to show serial reversal learning in honeybees resulted in a decline in bees' performance (Menzel, 1969; Couvillon & Bitterman, 1986; Mota & Giurfa, 2010). A direct comparative test would be required to show a species difference in serial reversal learning between honeybees and bumblebees, but there are differences in the natural foraging strategies of honeybees and bumblebees that may lead to species differences in behavioural flexibility. Bees forage for both

nectar and pollen. Analysis of pollen samples taken from foragers shows that individual honeybees forage almost entirely on one pollen species (Free, 1963). In contrast, pollen samples from individual bumblebees were much more likely to contain pollen from more than one species of plant (Free, 1970). This is consistent with behavioral observation that bumblebees use a majoring and minoring foraging strategy in the wild, foraging primarily on a highly preferred species of flower, but regularly visiting a less preferred flower (Heinrich, 1976). It has also been shown that when honeybees' preferred pollen species is no longer available they will either halt foraging completely or switch to foraging only for nectar (Free, 1963). Bumblebees differ in that they will change the flowers that they are foraging on when one is no longer available (Free, 1970).

Why would individual bumblebees and honeybees have different foraging strategies? One proposed explanation is that honeybees recruit fellow bees to nectar sources and they do not need to engage in sampling behavior, as bumblebees do, to find nectar and pollen sources (Heinrich et al., 1977). Without help from their nestmates bumblebees must determine good nectar and pollen sources by sampling a number of flower species in the wild before settling on major and minor flowers (Heinrich, 1976). Bumblebees' need to determine the best sources of nectar and pollen without the help of their nest mates may have resulted in species differences in behavioural flexibility. Serial reversal learning has been used successfully in hypothesis driven comparative work on closely related species to explore the impact of differences in ecology on cognition (Bond et al., 2007). There is considerable justification and scope for using this approach with bees.

Simple and Complex Environment

The manipulation of foraging environment had no significant effect on the performance of the bees on the serial reversal task. This suggests that the increase in visual and olfactory stimulation that comes with foraging may not be the cause of the differences in reversal performance previously observed between nurse and forager honeybees (Ben-Shahar et al., 2000). Honeybees may respond differently to environmental enrichment, and it is possible that greater enrichment, or enrichment of other kinds, might have affected serial reversal learning in bumblebees in the present study. Nevertheless, there was no indication in the present results that enrichment affected serial reversal performance in bumblebees. There are other factors, apart from environmental enrichment, that may lead to differences in serial reversal performance between nurse and forager bees. The bees in this study were matched on the mechanosensory demands of foraging with identically placed foraging patches. If the effects of foraging on reversal learning are connected to changes in mechanosensory experience, no differences between groups would have been found in this study. Another possibility is that the differences in learning between nurses and foragers are a consequence of developmental changes that occur at the time foraging begins, but are not directly caused by foraging. If this were the case the actual experience of foraging would have little impact on the bees' development and the environment manipulations would not be expected to have an effect on their behaviour. There could also be species differences in the development of learning between bumblebees and honeybees. The transition from colony duties to foraging duties is different in honeybees and bumblebees, with the transition to forager determined predominately by age in honeybees (reviewed in

Seeley, 1985) and predominately by size and colony demands in bumblebees (reviewed in Heinrich, 1979). It could be that the differences in nurses and foragers found by Ben-Shahar et al. (2000) on the reversal task may not occur in bumblebees, or may develop differently.

Errors in Successive Reversals

Despite differences between these results and prior studies on serial reversal learning in bees, there is one consistent finding. In this study the bees showed two patterns of performance. One was a reduction in perseverative errors and the other was a slight increase in errors in the final reversals. An increase in errors with continued reversals and a loss of the ability to discriminate between stimuli has also been shown repeatedly in previous work (Menzel, 1969; Couvillon & Bitterman, 1986; Mota & Giurfa, 2010). The pattern has been much more pronounced in other studies, with bees completely losing the ability to discriminate after a number of trials, whereas bees in the present study maintained criterion performance and only showed a small increase in errors. Nonetheless, an increase in overall errors with repeated reversals in serial reversal learning is consistent across almost all studies in bees. A proposed explanation for the bees' behaviour is adoption of an averaging strategy (Mota & Giurfa, 2010). An averaging strategy would occur if bees failed to learn the pattern of reward contingency changes and merely made decisions based on the number of reinforced and non-reinforced experiences with each stimulus (Mota & Giurfa, 2010). If the bees were using an averaging strategy then a chance rate of responding to both stimuli would make most sense. This has been found in previous work (Mota & Giurfa, 2010). In the present

results there is only a moderate increase in errors, not random responding. Thus it seems unlikely that bees are averaging and proactive interference is a more likely explanation.

Colour Preference

Another significant result in this study is faster acquisition of the initial discrimination in the first session when blue was the rewarded colour compared to when yellow was rewarded. That bees have a preference for blue has been demonstrated repeatedly in previous work (Keasar et al., 1997; Heinrich et al., 1977). Here the preference for blue did not persist throughout the study, as there were no differences in performance on the reversals between those bees who started on blue and those bees who started on yellow. This is notable because differences in reversal of foraging preferences in bumblebees trained to go to blue and those trained to go to yellow have been shown before (Heinrich et al., 1977). Heinrich et al. (1977) found that bees who were trained to be constant to blue flowers did not switch to foraging on white flowers when reward contingencies changed. In contrast, bees who were trained to be constant to white flowers did learn to switch when reward contingencies changed (Heinrich et al., 1977). There are a number of differences in procedure between that study and this one, the most important of which is that the bees here were foraging on a single flower for each colour, whereas in Heinrich et al. (1977) bees foraged on patches with multiple flowers of each colour. The reward contingencies also differed. Bees in this study were given all or nothing rewards, whereas in Heinrich et al. (1977) flowers varied in their reinforcement with one colour being more rewarding on average than the other. That both studies found differences in learning for blue over another colour - white or yellow - when artificial flowers were identical suggests that the bee preference for blue 'morphs' cannot be

explained solely by nectar guides or other foraging aids on natural flowers. The bees' behaviour indicates that they can overcome their innate preferences when the task demands require them to do so, but their preference for blue and its influence on learning is certainly intriguing.

Conclusions

This study shows that bumblebees can improve performance on a serial reversal task with experience. In doing so, bees exhibit the capacity to be flexible in learning and to learn task demands that extend beyond trial by trial learning. Differences between these results and previous work require the inclusion of the proviso 'under certain conditions' in the conclusion. The next step is to determine what those conditions are specifically. There are a number of differences between this study and prior work that requires further research. The effect of trial number is the most intuitively obvious difference between this study and others. A comprehensive exploration of trial number and its impact on reversal learning, comparing groups meeting criterion and overtrained, would be necessary to explore differences between the present results and those obtained in previous research. Another promising topic is comparative work on honeybees and bumblebees. The published literature on both species is much less extensive than that on more traditional species used in learning research. Comparative work may reveal substantial differences between bumblebees and honeybees in serial reversal learning, and indicate that research with bees on this relatively straightforward learning paradigm is a promising approach for studying the cognitive consequences of ecological idiosyncrasies of closely-related species.

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CHAPTER THREE

Adult Mushroom Body Development in Bumblebees (*Bombus impatiens*) in Two Foraging Environments

Introduction

Although the intricacies of the relationship between brain size and learning are debated (Roth & Dicke, 2005), the correlation between larger brains and greater cognitive abilities is well established (Lefebvre, 2008). Delving even deeper into the connection between brain and cognition, it has been asserted that selection for a particular behaviour will be reflected in the relative size of the brain regions that support it (Lefebvre, 2008). This relationship holds not only for the vertebrates that dominate research on the topic, but also finds support in work on invertebrates. The mushroom bodies in the invertebrate brain are a multi-modal sensory integration centre which are integral to learning and memory in insects (Strausfeld, 1998). This brain region is larger in eusocial insects, particularly some bee species and other Hymenoptera (Strausfeld, 1998). The increase in mushroom body size has been attributed directly to the increased social interaction (O'Donnell, 2011) as well as the increased behavioural repertoire and demands of foragers (Withers et al., 1993). Thus, the learning and memory demands of social interaction as well as foraging and nurse duties appear to have driven a size increase in the mushroom bodies of bees.

The increased investment in the mushroom bodies is clearly demonstrated in the evolution of the bee brain, but the relationship between a brain region and behaviour can also be explored through adult brain development. It was discovered some time ago that experience throughout an animal's lifetime can influence brain development (reviewed in Rosenzweig & Bennett, 1996; van Pragg et al., 2000). Housing rats in enriched or impoverished environments as well as training them on a certain task can cause a myriad of changes in the brain, from the level of enzyme activity to volume changes in large

cortical regions (Rosenzweig, &Bennett, 1996; van Pragg et al., 2000). One of the most important findings from the extensive study of enrichment induced brain changes in rats was that enrichment does not result in general brain growth, but rather expansion of particular cortical regions with the rest of the brain unchanged (Rosenzwieg & Bennett, 1996; van Pragg et al., 2000). This pattern of brain plasticity during an animal's lifetime is clear evidence that the relationship between brain region volume and behaviour is not restricted to evolutionary differences, but also appears in individual development.

Plasticity in the brain can be categorized into two distinct types of development, experience-expectant and experience-dependent (Greenough et al, 1987). Experience-expectant development encapsulates development that follows a set course throughout an animal's development, often in an age dependent manner with critical periods, and is relatively unaffected by the experiences of each individual (Greenough et al, 1987). Brain changes of this kind are proposed to occur in preparation for learning and behaviour that is common to all members of a species, such as language learning in humans or development of sensory systems (Greenough et al., 1987). The benefit of experience-expectant development is that it efficiently prepares individuals for experiences they are almost certain to have (Greenough et al., 1987). There are, of course, experiences that all individuals of a species do not share, and the brain accommodates these through experience-dependent development. Experience-dependent development reflects an individual's unique learning experiences and results in different patterns of development between individuals (Greenough et al., 1987). The example presented earlier of changes to the rat cerebral cortex in response to environmental enrichment is a prototypical example of experience-dependent brain plasticity.

Bees undergo a number of behavioural changes in adulthood with neural correlates. A eusocial bee colony consists of a queen and a large number of sterile female workers (Seeley, 1985; Heinrich, 1979). The workers are responsible for nurse duties, such as feeding larva, and foraging (Seeley, 1985; Heinrich, 1979). In bumblebees the assignment of jobs within the colony is determined predominantly by colony demand and worker body size (Heinrich, 1979). Some smaller bees will not forage at all, but all bees will spend at least the first few days after eclosion doing jobs inside the colony (Heinrich, 1979). The pattern is considerably different in honeybees. Honeybee workers progress through the different jobs in an age dependent manner referred to as age polyethism. Bees will spend approximately two weeks as a nurse and then transition to a forager (Seeley, 1985). Given the interaction between brain and behaviour described above, one would expect that the considerable changes in a bee's behaviour throughout its lifetime would be reflected in changes in the brain.

Withers et al. (1993) investigated the brain changes related to division of labour in honeybees and did find changes in the brain as the bees progressed through the various stages of adult development. Although changes were found in the olfactory glomeruli as bees transitioned from newly eclosed to nurse bees, the most interesting results were the changes in the mushroom bodies. Withers et al. (1993) showed that as bees aged the volume of their mushroom body neuropil increased, with nurse bees showing greater mushroom body neuropil than one day old bees and foragers showing the largest increase in mushroom body neuropil volume. The increase in neuropil volume coincided with a reduction in volume of the area occupied by the Kenyon cell bodies (Withers et al., 1993). In the initial findings age was confounded with behavioural changes, so the

authors manipulated a colony to produce precocious workers who were matched to nurse bees in age, but had foraging experience: precocious foragers. It was found that precocious foragers had a pattern of brain development similar to normal aged foragers and significantly different from normal aged nurse bees (Withers et al., 1993). Thus it seems that bees' brains reorganize throughout adult development in a manner correlated with behavioural changes.

After the initial discovery that bee brains continue to develop during adulthood a number of studies were conducted to determine the driving force behind the changes and to specifically address the experience-expectant and experience-dependent components. Through manipulating the age of foragers, Withers et al. (1993) had shown that the increase in mushroom body neuropil was not entirely experience-expectant. However a number of subsequent studies have found that there is a large experience-expectant component. Withers et al. (1995) artificially prolonged the duration of nurse behaviour in honeybees and compared workers with normal aged nurse bees. The mushroom bodies of over-aged nurse bees continued to increase in volume despite the delayed behavioural development. Thus, the increase in mushroom body neuropil was shown to occur, at least in part, independent of behavioural experience. In another study that supports the existence of a large experience-expectant aspect of bee adult brain development, bees were completely deprived of sensory or social stimulation at one day old (Fahrbach et al., 1998). Bees were housed in isolation in a dark chamber for 7 days and compared to one day old bees. It was found that even in complete sensory deprivation changes in mushroom body neuropil still occurred (Fahrbach et al., 1998). Unfortunately the dark reared bees were not compared to normally housed 7-day old bees to see if the brain

development was comparable. However, that the mushroom bodies continued to develop without sensory stimulation confirms that development is largely experience-expectant.

One may be tempted to attribute all changes in the bee brain during adulthood to experience-expectant growth after the demonstration that changes occur in complete sensory deprivation, but there is evidence to the contrary. The original study by Withers et al. (1993) showed that precocious foragers had brain changes similar to those of normal aged foragers, clearly indicating that the developmental changes are susceptible to environmental and behavioural influence. Ismail et al. (2006) provided even more convincing evidence of the correlation between increased mushroom body neuropil and foraging experience. Bees were allowed to forage for one week and then were confined to a dark chamber and compared to bees given two weeks of normal foraging. The bees who were confined to the dark chamber following foraging showed smaller mushroom bodies than those who were given two weeks to forage (Ismail et al., 2006). Prior work had shown that the transition from nurse to forager induced reorganization of the mushroom bodies and this work demonstrates that the amount of foraging experience also correlates with greater mushroom body neuropil.

Research on the continued development of mushroom bodies in adult bees has demonstrated convincingly that there is both an experience-expectant component and an experience-dependent component. The pressing question now is the relative contribution of each. It is possible that the experience-dependent component is very small and merely relies on the physiological changes that accompany the onset of foraging. It is also possible that the development of the mushroom bodies is initiated by the same mechanism as foraging behaviour itself and is consequently correlated with foraging, but

not influenced by the environment or experience except when it initiates or halts foraging behaviour. Alternatively, the experience-dependent component could be similar to cortical development in rats and influenced by the complexity of the environment and training. Foraging could serve as an enriching experience, providing increased mechanosensory, visual, and olfactory stimulation as well as increasing the learning demands. The increase in light stimulation alone when bees transition from nurse to foragers is considerable and has been proposed as a possible environmental influence on mushroom body expansion (Fahrbach et al., 1998). Withers et al. (1995) found that bees who spent an artificially prolonged period inside the colony showed comparable mushroom body neuropil to some foraging bees, but also found that those bees spent considerable time at the colony entrance, exposed to both increased light and other sensory stimulation. Consequently, what is proposed to be experience independent increase in mushroom body neuropil may be the result of increased sensory experience.

In this study I explored the influence of environment and sensory stimulation on the development of bumblebee mushroom bodies. The specific aim was to determine if the experience-dependent component of the increase in mushroom body neuropil will respond plastically to environment enrichment in the same manner as has been repeatedly found in vertebrates such as the rat. The onset of foraging, with which the expansion of the mushroom bodies correlates, differs from life inside the colony in many ways, including sensory stimulation, mechanosensory stimulation, increased navigation demands, and increased associative learning demands. Here I test the influence of two of these major differences, increased sensory stimulation and increased associative learning demands. Bees were housed in either enriched, 'complex', environments or

impoverished, 'simple', environments to determine the effects of sensory stimulation. A group of bees from each environment were then tested on a serial reversal task (Chapter 2) to address the influence of increased learning demands on brain plasticity. All of the bees were matched on navigation and mechanosensory components of foraging, which allowed us to isolate the effects of the two variables of interest.

An additional component of this study is the exploration of mushroom body development in bumblebees. The work on bee brain development has been dominated by work on honeybees and the pattern of mushroom body development in bumblebees, although assumed to be similar, is largely unstudied. Mushroom body increases with the onset of foraging have been found in other species of eusocial Hymenoptera (O'Donnell et al., 2004; Withers et al. 2007) providing considerable foundation for the prediction that the phenomenon will occur in bumblebees. Work done in eusocial wasps has shown that as wasps transition from duties inside the colony to foraging, there is an accompanying increase in mushroom body neuropil (O'Donnell et al, 2004). Withers et al. (2007) explored mushroom body development in the solitary orchard bee. The testing conditions were different from those for eusocial bees because each individual solitary bee must forage for food. Instead of confining bees to a colony one group of bees was housed in an impoverished environment, with restrict space and foraging limited to nectar, and another was housed in a natural environment. With 3 weeks of foraging experience, the bees foraging in a natural environment had larger mushroom bodies than bees housed in the restricted environment (both inexperienced and experienced foragers), and larger mushroom bodies than inexperienced bees housed in a natural environment (Withers et al., 2007). This demonstrates that the extent and type of foraging experience influences

the development of mushroom bodies in a bee species other than honeybees. This study examines the pattern of mushroom body expansion in bumblebees in a manner similar to Withers et al. (2007) study of solitary bees. Foraging bumblebees housed in simple and complex environments were compared to bees confined to the colony. This provides a measure of the influence of foraging itself on mushroom body expansion in bumblebees in addition to environment enrichment.

In addition to the growing body of work correlating mushroom body development with foraging there is evidence that directly correlates mushroom body volume and performance on learning tasks (Gronenberg & Couvillon, 2010). Gronenberg and Couvillon (2010) trained honeybees on a PER olfactory discrimination and then compared individual bees' mushroom body volume to their discrimination acquisition. They found that learning performance was positively correlated with mushroom body volume, most significantly with mushroom body calyces volume. The findings of Gronenberg and Couvillon (2010) suggest that a similar correlation between mushroom body volume and performance may be found in the bees that were tested on the serial reversal task in Chapter 2. However, there is research to support the prediction that a relationship between reversal performance and mushroom body volume may be a negative correlation rather than the positive correlation between learning and mushroom body volume found by Gronenberg and Couvillon (2010). Ben-Shahar et al. (2000) found that in honeybees nurse bees performed better on a PER reversal task than foragers. If one assumes that foragers have a greater mushroom body volume than the nurse bees, as has been shown (Withers et al., 1993), the results of Ben-Shahar et al. (2000) suggest that greater mushroom body volume may correlate with greater errors on a reversal task. To

test this prediction I did a direct comparison of mushroom body volume and performance on a serial reversal task.

Methods

Subjects

Subjects were bumblebee workers (*Bombus impatiens*) from four commercial colonies (Biobest Canada Ltd., Leamington, ON). The same colonies were used in the experiment described in Chapter 2. Six of the individual bees from the experiment in Chapter 2 comprised the 'foraging +' condition of the present study. Housing and feeding conditions were as in Chapter 2. All bees were tagged for individual identification using the procedure described in Chapter 2.

Foraging Environments

Bumblebee colonies were housed in either a 'simple or 'complex' environment as described in Chapter 2 (Figure 2.1).

Conditions

Individual bees were raised under three different conditions: colony confined, forage, and forage+. 'Colony confined' bees were collected within 24h of eclosion, tagged, and rendered flightless by a small drop of cyanoacrylate glue applied to their wings. Newly eclosed bees are unable to fly and easily identifiable by their distinct silver colour for the first 24-48h of adult life. Colony confined bees were therefore prevented from gaining any flying experience prior to sacrifice. 'Forage' bees were tagged either within the first 24h of life or while foraging. Bees were released after tagging and

allowed to forage freely to sustain the colony. All bees in the forage condition were monitored to ensure that they continued to forage following tagging and were collected for sacrifice while foraging. 'Forage+' bees were tagged either as newly eclosed bees (24-48h old) or while foraging. Bees were then trained and tested on a learning task (described in Chapter 2). When not being tested forage+ bees had unrestricted access to the room and continued to forage on the foraging patches provided to sustain the colony. Bees were tested for 3-5 days and sacrificed on completion of the final testing session.

Histology and Volume Analysis

Bees were collected either directly from the colony or while foraging, cold anesthetized, and decapitated. Following sacrifice, head capsule size measurements were taken. Width measurements were taken using a magnifying comparator (Edmund Scientific co., Barrington, New Jersey) at the widest point of the head from compound eye to compound eye. Head capsules were then stored in 4% paraformaldehyde and refrigerated for a minimum of 48h prior to dissection. For dissection, head capsules were secured in heated Parafilm (Pechiney Plastic Packaging, Menasha, WI). Brains were removed from the head capsule in bee saline, a mixture of salts (NaCl, KCl, $CaCl_2$, $MgCl_2$), sugars (dextrose, fructose, sucrose), and distilled water mixed to match the osmolarity of bee hemolymph. At the time of dissection the anterior surface of the brain was marked with mercurochrome, to aid in orientation during paraffin embedding. Brains were then immediately placed in 4% paraformaldehyde and stored in a fridge at 5 °C. Brains were kept in fixative for a minimum of 48hrs before further processing. One of the brains used in analysis was dissected out of the head capsule immediately following sacrifice and placed in fixative. All other preparation for this brain was identical.

Brains were embedded in paraffin wax and sliced on a microtome. Brains were sliced anterior to posterior in 5μm sections and serial sections of the whole brain were collected. Tissue was dried at 60°C and stained with HARLECO® hematoxylin (EMD Chemicals Inc., Gibbstown, NJ) and eosin (Sigma-Aldrich, St. Louis, MO). The hematoxylin and eosin stain allowed for easy identification of both mushroom body neuropil and optic lobula (Figure 3.1). The mushroom bodies neuropil (Kenyon cell bodies were not traced) and lobula were traced and area measurements were calculated. Area measurements were taken from every fifth section resulting in a section interval of 20μm on average. The section interval is considered an average because some sections are known to have been lost during processing. It is assumed that the tissue loss occurred randomly and to an equal extent in all treatment groups. Volume was calculated from the area measurement with a formula for the volume of a truncated cone. Mushroom body and lobula volume was calculated separately for left and right structures and combined for a total mushroom body neuropil volume measure and combined lobula volume measure. Area measurements were obtained from digital images captured using a Leica DM5500 B microscope by tracing structure outlines manually with a cursor using Leica Application Suite (Leica Microsystems, Mannheim, Germany). All images were taken using a 10X objective.

Data Analysis

Analyses of the relationship between different brain structures were first done using all bees. The relations between lobula volume and head capsule width, between mushroom body volume and lobula volume, and mushroom body volume and head capsule width were graphed and quantified using linear regression.

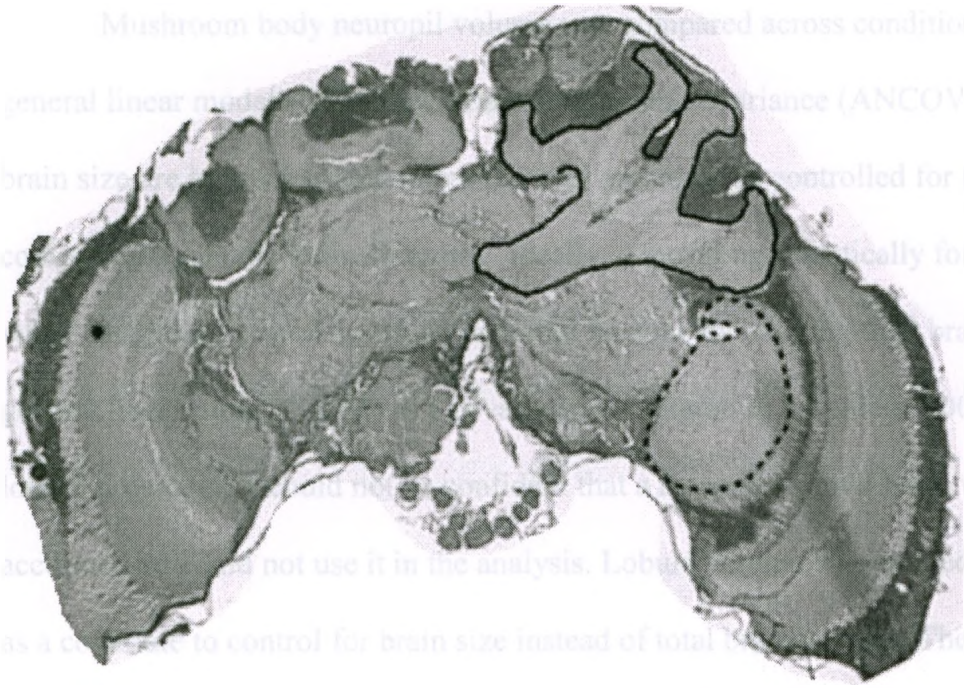

Figure 3.1. Bumblebee brain section. The figure shows mushroom body neuropil (solid line) and the optic lobula (dashed line). Hematoxylin stain (blue) shows cell nuclei and eosin stain (purple) shows fibres and cytoplasm.

Mushroom body neuropil volume was compared across conditions using the general linear model (GLM) univariate analysis of covariance (ANCOVA). Body and brain size are highly variable in bumblebees and must be controlled for statistically when comparing a specific brain structure. Ideally, controlling statistically for total brain size when comparing a specific structure would be done by entering total brain size into a general linear model (GLM) as a covariate (Darlington & Smulders, 2001). Due to tissue loss in processing I could not be confident that a measure of total brain volume would be accurate and could not use it in the analysis. Lobula volume was entered into the model as a covariate to control for brain size instead of total brain volume. The lobula is an easily identifiable structure that was consistently retained during tissue preparation and has been shown to have a consistent relative volume to total brain volume with variation in brain size (Mares et al., 2005).

Differences between colony, forage, and forage+ conditions were analyzed using GLM univariate analysis of covariance (ANCOVA) with lobula as a covariate. In a separate ANCOVA forage and forage+ conditions were combined and compared to the colony condition to analyze the effects of foraging experience. Simple and complex conditions were analyzed using ANCOVA to determine the effects of foraging environment. Colony confined bees were excluded from the foraging environment analysis because their exposure to the additional stimuli is assumed to have been limited.

For the six bees in the forage+ condition performance on the serial reversal task was compared to mushroom body neuropil volume. Mean number of errors per session on the serial reversal task was calculated for each bee and used as a measure of

performance. The relationship between performance and mushroom body neuropil was then graphed and quantified with linear regression.

Results

Only brains that had complete left and right mushroom bodies and lobula were used in analyses. Mushroom body neuropil volume and lobula volume measurements were gathered from 11 bees, and head capsule width measurements were taken from 10 bees.

Total mushroom body neuropil volume ranged from 4.34×10^7 to 7.22×10^7 μm^3. Total lobula volume ranged from $0.94 \times 10^7 \mu m^3$ to $2.29 \times 10^7 \mu m^3$. Head capsule width ranged from 3.5mm to 4.2mm. The relationship between lobula volume and head capsule width was explored by plotting the measurements and performing a linear regression (Figure 3.2). No significant relationship between lobula volume and head capsule width was found ($r = .35$, $F(1,8) = 1.14$, $p = .32$, $r^2 = .12$). Mushroom body neuropil volume was plotted against head capsule width and a linear regression was done (Figure 3.3). No significant relationship was found between mushroom body neuropil volume and head capsule width ($r = .2$, $F(1,8) = .35$, $p = .57$, $r^2 = .04$). Finally, mushroom body neuropil volume was compared to lobula volume (Figure 3.4). Regression analysis revealed a significant relationship between mushroom body neuropil volume and lobula volume ($r = .87$, $F(1,9) = 26.81$, $p < .001$, $r^2 = .75$).

There was no significant effect of condition (colony, forage, and forage+) on mushroom body neuropil volume ($F(2,7) = 2.05$, $p = .20$). The covariate, lobula volume, was significantly related to mushroom body volume ($F(1,7) = 29.02$, $p = .001$). The bees

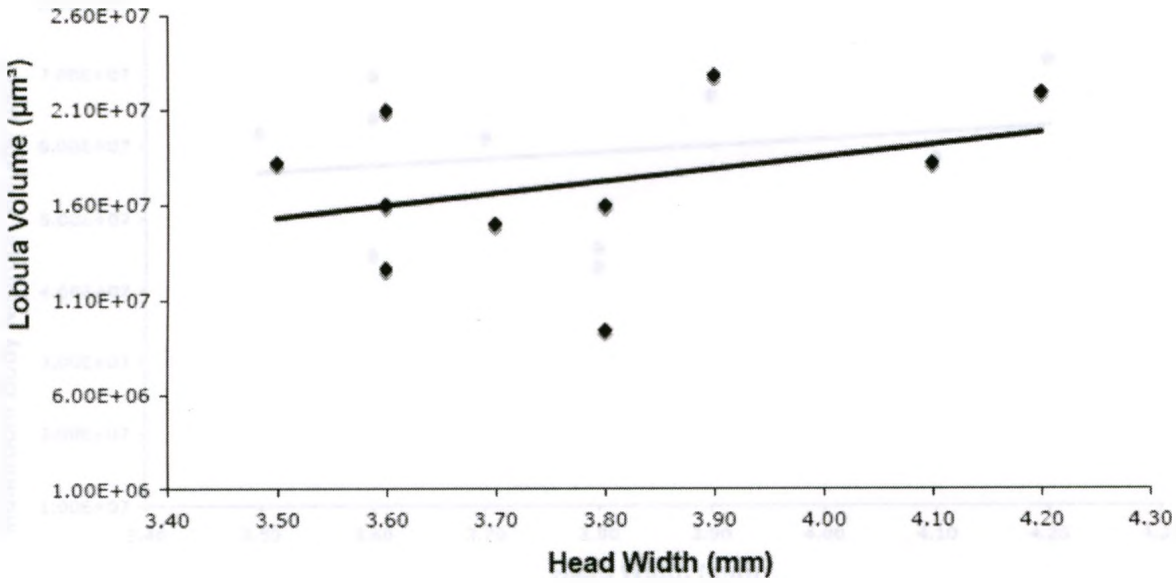

Figure 3.2. Lobula volume and head capsule width. Each point represents the total lobula volume (μm^3) and head capsule width (mm) for each bee (n=10. There is one bee for which head width was not measured). The linear relationship between the two variables is non-significant.

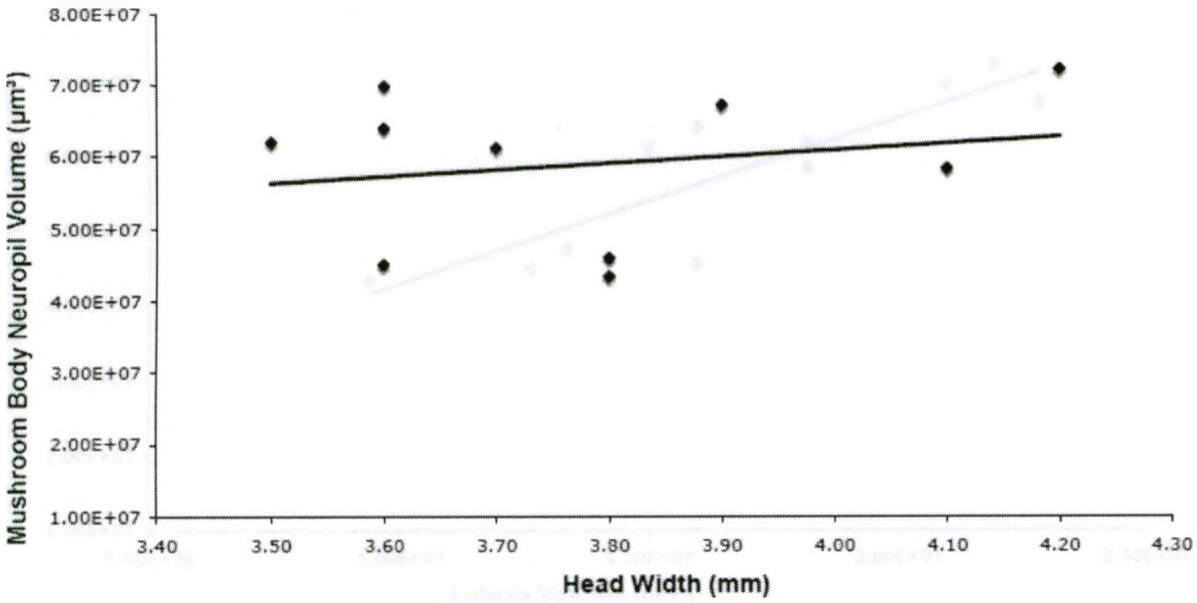

Figure 3.3. Mushroom body neuropil volume and head capsule width. The figure shows the total mushroom body neuropil volume (μm^3) plotted against head capsule width (mm) for each bee (n=10. There is one bee for which head width was not measured). No significant relationship was found between the two variables.

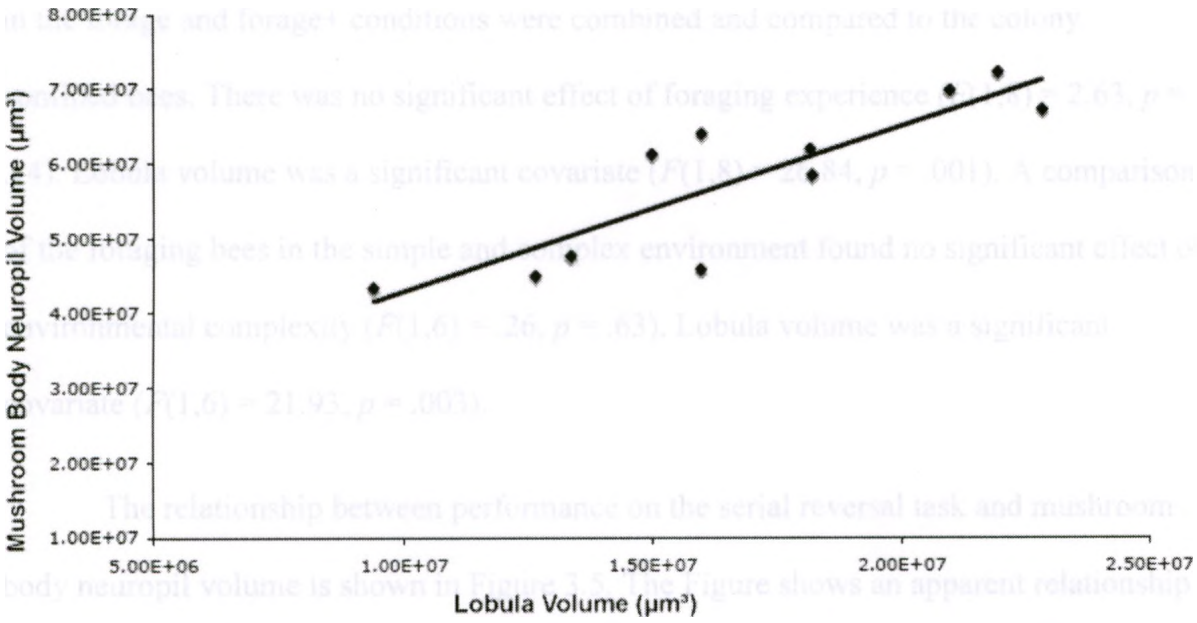

Figure 3.4. Mushroom body neuropil volume compared to lobula volume. The figure shows the total mushroom body neuropil volume (μm^3) plotted against the total lobula volume (μm^3). Each point represents the values for an individual bee (n=11). Linear regression is significant , r = .87, $F(1,9) = 26.81$, $p < .001$, $r^2 = .75$.

in the forage and forage+ conditions were combined and compared to the colony confined bees. There was no significant effect of foraging experience ($F(1,8) = 2.63$, $p = .14$). Lobula volume was a significant covariate ($F(1,8) = 26.84$, $p = .001$). A comparison of the foraging bees in the simple and complex environment found no significant effect of environmental complexity ($F(1,6) = .26$, $p = .63$). Lobula volume was a significant covariate ($F(1,6) = 21.93$, $p = .003$).

The relationship between performance on the serial reversal task and mushroom body neuropil volume is shown in Figure 3.5. The Figure shows an apparent relationship between serial reversal learning and mushroom body neuropil volume, such that greater mushroom body neuropil corresponds to greater number of errors on the reversal task but the linear regression revealed the relationship to be non-significant ($r = .79$, $F(1,5) = 6.55$, $p = .063$, $r^2 = .62$).

Discussion

Mushroom Body Morphology

Knowledge of the bumblebee mushroom bodies and their development is limited when compared to the extensive research that has been done on the honeybee mushroom bodies. One of the goals of this study was to expand current knowledge of bumblebee mushroom bodies. At the level of total mushroom body volume, our bumblebee mushroom body sizes are smaller than those found in previous studies (Mares et al., 2005). Mares et al. (2005) used a different commercial supplier than was used here, so it is possible that this difference in volume is due to differences in the source of the bees. However, it is more likely that the volume measurements here are underestimates of mushroom body volume due to the loss of tissue during processing. The tissue loss was

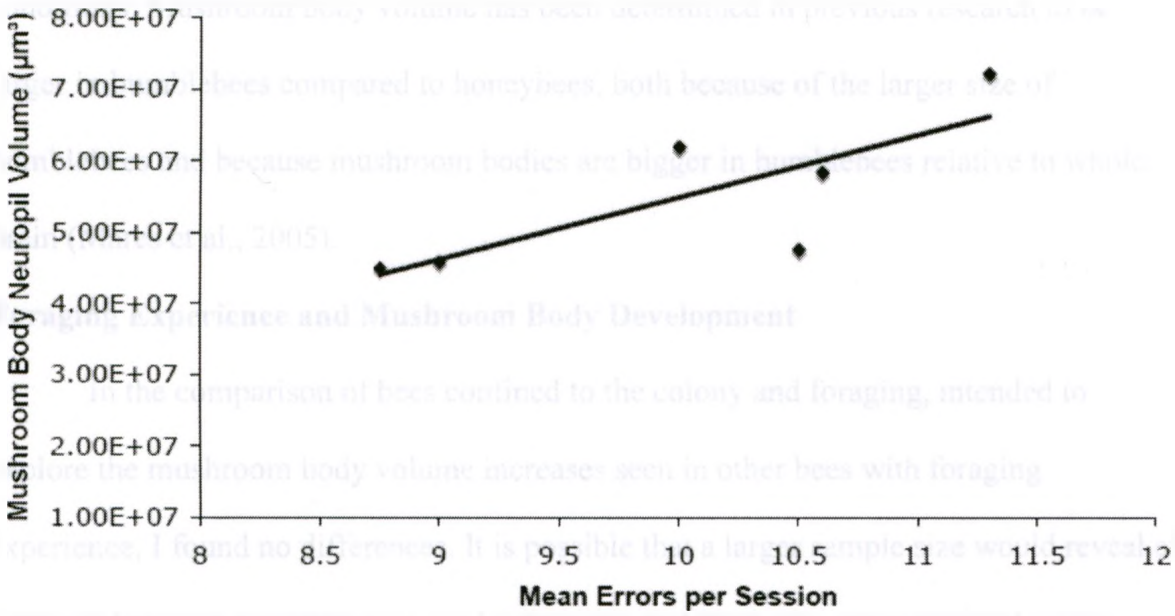

Figure 3.5. Mushroom body neuropil volume and reversal performance. Each point in the figure represents the total mushroom body neuropil volume (μm^3) plotted against mean number of errors per session for an individual bee (n=6).Linear regression was not significant, r = .79, $F(1,4) = 6.55$, $p = .063$, $r^2 = .62$.

random and occurred across all groups, so is assumed not to affect comparisons between conditions. Mushroom body volume has been determined in previous research to be larger in bumblebees compared to honeybees, both because of the larger size of bumblebees and because mushroom bodies are bigger in bumblebees relative to whole brain (Mares et al., 2005).

Foraging Experience and Mushroom Body Development

In the comparison of bees confined to the colony and foraging, intended to explore the mushroom body volume increases seen in other bees with foraging experience, I found no differences. It is possible that a larger sample size would reveal an effect of foraging experience. It is also possible that bees who were confined to the colony were foraging, though not flying. Bees had their wings glued to prevent flying, but a foraging patch was placed on the same table as the colony making walking foraging possible. The bees were also exposed to light during tagging, feeding, and testing. Working with the colony under a red light would make it possible to avoid this unintended exposure to light. Withers et al. (1993) found that the difference between nurse and forager honeybee mushroom body neuropil was not significant, but the ratio of mushroom body neuropil to Kenyon cell volume was significant. I could not be confident that all of the Kenyon cell bodies were preserved through dissection and preparation so no volume measures for that component of the mushroom bodies were calculated. It is possible that there was a change in the ratio comparable to that of Withers et al. (1993) that was undetected. The absence of differences between the two groups could also be related to age. The foragers and the bees confined to the colony used in this study were matched in age. It has been shown that some mushroom body development occurs in

honeybees even when restricted from foraging (Fahrbach et al., 1998). The data here may accurately reflect a lack of differences in the volume of mushroom body foragers and age matched flight restricted bumblebees. In order to determine if this is the case a group of bees confined to the colony (i.e. light restricted), not just restricted from flight would have to be used. The addition of a group of one day old bees would also be useful in determining if the mushroom bodies of colony confined/ flight restricted bees and foragers undergo any adult development at all. Given the pattern of increased mushroom body neuropil with foraging experience in a number of Hymenoptera (Withers et al., 1993; O'Donnell, 2003; Withers et al., 2007), it seems unlikely that it would not be found in bumblebees.

Mushroom Bodies in Simple and Complex Environments

There were no differences between bees in the 'simple' and 'complex' conditions. This suggests that sensory stimulation beyond that of basic foraging had no added influence on mushroom body development. Also I found no differences between the foraging and foraging + conditions. This again suggests that experience beyond that of just foraging has no influence on development of the mushroom bodies in bumblebees. This does not mean that development of the mushroom bodies in bumblebees does not have an experience-dependent component, but rather it shows only that mushroom body development is unaffected by variations in the quantity of visual and olfactory sensory information and training on an associative task. Foraging bees in both environments were still engaging in a number of behaviours that differed from in nurse duties and could influence mushroom body development. Bees were still required to navigate to and from foraging patches and to form associations between the foraging patches and flowers and

sucrose. Also, bees were flying and engaging in foraging related motor behaviours, which provide different mechanosensory input than within colony duties. Differences between enriched and impoverished environments were shown to produce different mushroom body development in solitary orchard bees (Withers et al., 2007). There were a number of very important differences between the environment manipulations in this study and the Withers et al. study. First the bees here were matched in the size of their foraging area. Bees in Withers et al. (2007) were housed in a restricted space inside in the impoverished environment, or outdoors in the enriched environment. The difference in motor output between the two conditions could be responsible for the differences in brain development. Second, the bees in the Withers et al. (2007) study differed in the foraging task in which they were engaged. The impoverished bees were only foraging for sucrose, but the enriched bees were foraging for both sucrose and pollen. Here the bees were matched on both of these factors. The room size was identical for all bees and all bees were foraging only for nectar. Visual and olfactory input were isolated here instead of confounded as they were in Withers et al. (2007). One additional explanation is that the environment enrichment did not actually increase the visual and olfactory input for the bees. The environments appeared different to human observers, but as human and bee vision and other senses are very different, it could be that the complex environment did not constitute a complex environment for the bees.

Another consideration is that changes in response to enrichment that occur in the bumblebee mushroom bodies are isolated to a particular part of the mushroom body neuropil and not apparent when the mushroom body neuropil is analyzed as a whole. Durst et al. (1994) followed up on the Withers et al. (1993) findings with a study that

looked at volume changes in the subcompartments (lip, collar, basal ring, peduncle, and lobes) of the mushroom bodies. They hypothesized that failure of Withers et al. (1993) to find division of labour associated differences in mushroom body neuropil alone was because the different subcompartments may develop differently. Durst et al. (1994) compared the volume of the mushroom body subcompartments of aged matched nurses and foragers and one day old bees. They found that the peduncle and lobes were larger in older bees (nurses and foragers), but there were no differences correlated with foraging experience. The same was true of the basal ring. The most interesting finding is that the lip, which receives olfactory input, increased in volume in nurses compared to one day old bees, and then also increased significantly with foraging experience (Durst et al., 1994). This means that the lip region of the calyces was significantly larger in foragers compared to all other groups. Another very revealing finding is that the collar region, which receives visual input, was no different in nurse bees compared to one day old bees, but was significantly larger in foragers than in either one day old bees or nurse bees (Durst et al., 1994). That volume changes vary across subcompartments has been confirmed in other studies (Withers et al., 1995). Withers et al. (1995) found differences in volume between foragers and one day old bees in the basal ring and collar region. The Withers et al. (1995) findings differ in the patterns of mushroom body volume changes found, but their study included treatments with juvenile hormone that may have differentially affected the subcompartments. These findings suggest that the different sensory worlds of bees doing different jobs for the colony are reflected in the changes in their mushroom bodies. It also means that brain differences between the 'simple' and 'complex' environment in sensory stimulation may not be represented in such a gross

measure as total mushroom body neuropil volume. The hematoxylin and eosin stain used here does not consistently allow the lip, collar, and basal ring to be distinguished. Durst et al. (1994) used the Azan method, staining with a mixture of azocarmine and anilinblue-orange, to visualize these structures. Repeating this study using the Azan method and including analysis of the subcompartments of the mushroom bodies, specifically the lip and collar regions, might reveal differences not found here.

It is also possible that the environment manipulation caused changes in the brain, but the changes are not detectable at the level of volume changes. Changes in dendrites correlated with division of labour and foraging experience have been shown in honeybees (Farris et al., 2001). Farris et al. (2001) looked at the dendritic branching patterns using Golgi impregnation in the collar subcomparment of the mushroom bodies in honeybees with varying degrees of foraging experience. An increased number of dendritic segments were found in the Kenyon cells of bees with foraging experience compared to nurse bees (Farris et al., 2001). In addition to the discovery that complexity of dendritic branching correlates with foraging experience, the Farris et al. (2001) study included another important finding. The groups in which Kenyon cell dendrites were longer or more branched did not necessarily have greater neuropil volume (Farris, 2001). This means that changes in the dendritic branching associated with foraging experience are not necessarily reflected in changes at the more gross volume level. A comparison of total mushroom body neuropil volume between nurse and forager honeybees in Farris et al. (2001) did not show any significant differences, but these groups did differ at the level of dendritic branching in the collar region of the mushroom bodies. It is therefore possible that the differences in the 'simple' and 'complex' foraging environments were reflected

in changes in the brain, but those changes are at the level of dendritic branching or other structural changes not identifiable using volume analysis.

Serial Reversal Learning and the Mushroom Bodies

The relationship between mushroom body volume and reversal learning was not significant, but a trend towards a negative correlation is evident in Figure 3.5. Gronenberg and Couvillon (2010) found the largest correlation between learning and mushroom body neuropil when they analyzed only the calyces. It is possible that here, as in the comparisons previously discussed, looking at the subcompartments of the mushroom bodies would show differences not found at the level of the whole mushroom body neuropil volume comparison. If analysis at the level of mushroom body subcompartments were to show a negative correlation between reversal performance and mushroom body volume it would raise some interesting questions, both about the mushroom bodies' relationship to learning and the way in which the bees are solving the reversal task. If larger mushroom bodies are beneficial for discrimination learning (Gronenberg & Couvillon, 2010) and detrimental for reversal learning it suggests that the two tasks differ in their learning demands and that the mushroom bodies are not universally beneficial for learning, but rather only for specific types of learning.

Conclusions

The results here suggest that no differences exist in the mushroom bodies of bumblebees confined to the colony compared to those allowed to forage. This is surprising given the fairly consistent finding that the mushroom bodies of Hymenopterans increase in volume with experience. Thus, a first priority in future research should be a comprehensive examination of mushroom body development in

bumblebees similar to what has been done repeatedly in honeybees. Bumblebees are unlikely to show an entirely different developmental pattern than that observed in the Hymenoptera studied thus far, but the differences in division of labour in bumblebees and honeybees may result in different patterns of development. Differences in physiological development of bumblebees and honeybees and onset of flight capabilities have been observed (Skandalis et al., 2011), so differences in brain development are not inconceivable.

When more is known about the development of the mushroom bodies in bumblebees it will be possible to draw more confident conclusions regarding the effects of an enriched environment on the experience dependent component of mushroom body development. These results do suggest, however, that increasing the visual and olfactory stimulation for bumblebee foragers does not affect the reorganization of the mushroom bodies in foragers compared to those of bees in an impoverished environment. If it is not visual and olfactory information driving the experience-dependent changes in bees, then what are the precipitating factors? There is some evidence to suggest that restricting foraging to a smaller space and to nectar only has an impact (Withers et al., 2007). Applying similar manipulations to bumblebees would be a logical follow up to this study.

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CHAPTER 4

General Discussion

Summary

The research described here explored behavioural flexibility and brain development in bumblebees and the effect of environmental complexity on both. The behavioural experiment in Chapter 2 used serial reversal learning to probe behavioural flexibility. Reversal learning has long been considered a measure of behavioural flexibility and a measure of an animal's ability to respond to changes in its environment (Davey, 1989). In addition to being a measure of flexibility, serial reversal learning has been used to measure animals' ability to learn-to-learn (Shettleworth, 1998; 2010), that is, their ability to extract general information about a task and improve performance with experience. The previous work on serial reversal learning in bees had produced ambiguous results, with single reversals successfully solved, but declining performance with repeated reversals. The work here showed that bees do improve performance in serial reversal task under certain conditions. Bees made fewer errors on later reversals than on initial reversals, demonstrating intersession learning.

Bees were also tested on the behavioural task in two different environments, 'simple' and 'complex'. The environments differed in the quantity of visual and olfactory stimuli that they contained. It was found that bees in the two environments did not differ in performance, suggesting that enriching the foraging environment does not affect reversal learning in bumblebees.

The brain analyses in Chapter 3 explored the development of mushroom bodies in the adult bumblebee brain. Previous work in honeybees and other Hymenoptera has shown an increase in the volume of mushroom bodies with the onset of foraging (Withers et al., 1993; O'Donnell et al., 2004; Withers et al., 2007). The effect of foraging on

mushroom body development in bumblebees was explored by comparing bees confined to the colony to foraging bees. There were no significant differences in mushroom body neuropil volume between the two groups, suggesting that foraging does not have a detectable effect on mushroom body development in bumblebees. It is possible that differences between colony confined bees and foragers do exist, but were not observable at the level of volume analysis. In order to determine if complexity of the foraging environment influenced mushroom body volume, bumblebees were housed in either a simple or complex environment and the volume of their mushroom bodies was compared. No differences were found between the brains of the bees housed in the simple and complex environments. This suggests that changes in the brains of foraging bees are not significantly, if at all, affected by the quantity of sensory stimulation received during foraging. Alternative explanations were proposed, such as structural changes in the mushroom bodies that might not be evident at the level of volume changes. However, the absence of any differences between bees foraging in environments differing in sensory stimulation suggests that changes in the mushroom bodies of adult bumblebees occur independently of sensory stimulation or are influenced by factors not manipulated here such as light exposure or mechanosensory stimulation.

Mushroom body neuropil volume and performance on a serial reversal task was compared. Although a negative relation appeared to occur between serial reversal performance and mushroom body volume, this relation was non-significant.

Discussion

The success of bumblebees on the serial reversal task demonstrates that they are able to respond flexibly to changes in reward contingencies and improve performance

with experience. This work and other studies (Wei et al., 2002; Komischke et al, 2002) go beyond showing the remarkable learning abilities of bees and begin to examine the nature of their learning and the factors that influence learning. This demonstration of learning flexibility is an important contribution to invertebrate research as all vertebrate species that have been tested display at least slight improvement in a serial reversal task (Davey, 1989). A difference between vertebrates and invertebrates on behavioural flexibility would suggest that flexibility may be something invertebrates have to sacrifice, because of their small brain, to accommodate a rate of learning comparable to mammals on some tasks. However, this study suggests that learning flexibility has not been sacrificed in bumblebees (although the results may be different for honeybees) and continued study of the intricacies and mechanisms of bee learning are necessary to elucidate how they do what they do.

A second contribution of the behavioural work here is an emphasis on the need to explore bee behaviour using procedures that consider bees' ecology and behaviour in the wild. Studies that provide a naturalistic testing situation - this study and that of Chittka (1998) - found bees did demonstrate behavioural flexibility, whereas bees tested using the PER protocol failed to show improvement in the serial reversal task. I mention this not to dispute the usefulness of the PER protocol, as it has been essential to research on the brain and learning in bees (Menzel, 1990) and allows researchers to test learning by bees who are not actively foraging (Ben-Shahar et al., 2000).The PER protocol does have limitations, however, as method of examining complex cognition. There may be a tradeoff between the ease of testing with the PER and external validity.

The brain research described here examines the relative contributions of experience-expectant and experience dependent development to the structure of the mushroom bodies in adult bees. It has been shown that volume increases in the mushroom bodies of honeybees occur in the absence of foraging experience (Fahrbach et al., 1998), but it has also been shown that foraging experience, when age is controlled for, affects mushroom body development as well (Withers et al., 1993). Prior to this work foraging experience had been manipulated as a whole, without further examination of what components of foraging experience might be responsible for the experience-dependent changes in the mushroom bodies. The absence of any differences in the mushroom bodies of bumblebees foraging in environments differing in sensory stimulation suggests that increased visual and olfactory input is not the driving force behind foraging related brain changes in bees. This implies either that the experience-dependent component of mushroom body development is relatively small and inflexible, or that a component of foraging experience other than increased visual and olfactory input, such as light exposure or changes in motor output, account for experience-dependent changes. Finding that increased sensory input is not correlated with larger mushroom bodies serves to direct future research towards other factors that may contribute more to adult brain development in bees.

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