

**Causes and consequences of variation in
learning performance in the bumblebee
(*Bombus terrestris*)**

Karen Elizabeth Smith

School of Biological Sciences, Royal Holloway University of London

Thesis submitted for the degree of Doctor of Philosophy

January 2016

Declaration of Authorship

I hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Karen E. Smith, January 2016

Abstract

Animal cognition has been studied for decades, yet there are still many unanswered questions about how and why such variation in cognitive abilities exists, within and among species. The reasons for variation in cognitive abilities may have been ignored as just ‘noise’ in the past. However, there has been recent interest in quantifying the costs and benefits associated with variation in cognitive ability, to make inferences about its adaptive significance. In this thesis I add to this area of research, by examining a number of potential explanations for variation in learning ability, observed both within and among colonies, and what the ultimate consequences of such variation might be for the bumblebee *Bombus terrestris*.

I start by comparing variation in individual performance in an olfactory and visual learning task (chapter 2), finding that there is neither a trade-off nor correlation in learning ability across the two tasks. I further explored individual variation within colonies in chapter 3, by assessing whether there is an association between foraging preferences and olfactory learning. In chapter 4, I investigated the fitness consequences of variation in visual and olfactory learning performance in a field setting, finding that better learning ability was not adaptive in the environment tested, indicating it may come with costs. Finally in chapter 5, I extended the scope of my thesis, by exploring the impacts of a negative anthropogenic factor (neonicotinoid pesticide use) found in natural environments, on learning performance and memory formation.

My work shows the clear utility of proboscis extension reflex conditioning as a paradigm for learning and memory studies using bumblebees. Taken together, my findings give insight into the potential adaptive significance of variation in learning performance, the costs it may come with and how stress (via pesticide exposure) can affect the allocation of resources in cognitive abilities.

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Chapter 1

General Introduction

1. General Introduction

1.1 What are learning and cognition?

Learning is one of a number of processes, including memory and decision making, that make up animal cognition, defined as the mechanisms by which animals acquire, process, store and act on information from their environment (Shettleworth 2010). Learning will be the focus of this thesis, and this process has now been studied in a wide range of taxa from insects to humans, using various learning tasks. It may have been assumed that animals with larger brains have a greater cognitive capacity; however comparatively simple animals, like insects, use only a small number of neurons to solve complex cognitive tasks (Chittka & Niven 2009). Even relatively simple organisms, such as nematode worms (*Caenorhabditis elegans*), have been shown to be able to learn and remember about stimuli, environments and their physiological state (Rankin, Beck & Chiba 1990; Rankin 2004).

There are a number of proposed definitions for learning from different perspectives. For example, Shettleworth (1998) defined learning as ‘a relatively permanent change in behaviour as a result of experience’, whereas Dukas (2008) defined learning as ‘the acquisition of neuronal representations of new information’. Neuronal changes within an animal cannot be seen; therefore in many cases (and in this thesis) learning is assessed indirectly, by measuring changes in behaviour towards an exposed stimulus (Dukas 2008). One issue where care needs to be taken when testing learning performance is that the animal’s physical state is not causing the change in behaviour. For example, an animal that has not eaten for a longer of period of time compared to another may be more likely to find food because of hunger, not because they have learnt where the food is located (Shettleworth 1998). Therefore, cognitive tasks need to be designed to test ecologically relevant stimuli for the animal in question, and all individuals need to be tested under the same conditions. There are three levels of learning that we can investigate: can an animal learn the task or not, how fast can it learn and what is its final (asymptotic) level of task performance (Dukas 2008)? In this thesis, I take all these approaches into account.

Learning is very important for insects as, although some insects may be relatively short lived, they live in environments that can be extremely changeable, in terms of the amount and type of resources available. Learning can enable them to respond to these changes in the most efficient way. Learning in insects has been studied for a long time and they have become a model species, they can learn tasks quickly, there is considerable variation within species and can be studied in a lab environment easily. Research on behavioural genetics in the fruit fly

Drosophila Melanogaster has provided valuable information on the cellular, molecular and evolutionary bases of behaviour (Sokolowski 2001). Fruit flies have a heritable foraging gene (*for*) which comes in two variants (*for^R*) and (*for^s*), Mery *et al* (2007) found that flies with the *for^R* allele have better short term learning and flies with the *for^s* allele show better long term memory. The foraging behaviour of these flies appeared to favour the polymorphism they had, indicating that there may be selection for a particular allele depending on the environment they inhabit. Learning ability has also been shown to be heritable in honeybees (*Apis mellifera*) and good and poor olfactory learning can be selected for (Brandes 1985). Even though learning ability was selected for using olfactory PER conditioning performance, it was shown to have a genetic basis independent of the sensory stimulus (visual or olfactory) and task situation (i.e. lab or field based) (Menzel & Brandes 1980). It is interesting that we find this level of variation within species and poses the question that it must be maintained for a reason, this is the main focus of this thesis and a question I will address.

1.2 Causes of variation in learning performance

I have split this discussion of the causes of variation in learning performance into four sections, the first discussing how innate behaviours may make learning more or less useful, the second how different situations and scenarios can affect the learning ability of individuals living in these and whether there is experimental evidence for predicted theories. The third section discusses how differences within individuals such as specialisations, rank and sex may determine learning ability. The final sections discusses how the costs associated with learning may determine when individuals invest in learning. Within these sections I discuss the both the potential mechanisms that cause variation of learning in individuals, for example physiology, genes, heritability or development and also the effect of different environmental conditions.

In this section I use examples of both within and between species comparisons in my discussions. Clearly, the best way to determine causes of variation within animals is to use within species comparisons, in this case we can be surer that the cause that we are testing is the reason for the differences in learning performance as they do not vary in any other way. Interspecific comparisons, even between closely related species, can be problematic, as there is potential for a third factor to be causing the observed differences in behaviour, such as differences in ecological requirements. However, between species comparisons are also provide us with valuable information and in many cases, this is the only way to test a hypothesis, for example the differences in learning ability between hoarding and non – hoarding birds which is discussed in more detail later in this section.

1.2.1 The use of innate behaviours

Depending on the situation or the environment an animal inhabits, it is possible that innate (or unconditioned) behaviours could be valuable and used instead of learning. By innate, I mean behaviours that are not learnt. The individual is born with these pre-programmed behaviours, probably because selection has favoured them in the environment that the individual inhabits. For example, naïve insects have been shown to have preferences for certain colours (Raine & Chittka 2007a), that are thought to help naïve insects locate rewarding flowers (food). Giurfa and colleagues (1995) showed that such preferences indeed matched floral reward levels. Additionally, when colonies from wild-caught bumblebee queens (*B. terrestris*) were reared in the lab, Raine & Chittka (2007) found a positive correlation between violet preference in the lab and foraging performance in the field. The area that the queens were caught in was used for the field tests, so these findings are consistent with the view that the bee's preferences were adapted to the local flora.

There is also evidence to suggest, that prey that have coexisted with predators in the same habitat for a long period of time, may show innate avoidance behaviour towards these predators, without needing to learn that they are dangerous. It has been suggested that alien predators are more dangerous than native predators to prey, in a wide range of mammals and birds (Salo *et al.* 2007). This is likely to be driven partly by innate responses giving the prey an advantage against the native predators. Naïve tadpoles of the western spadefoot toad (*Pelobates cultripes*) show reduced activity levels to dragonfly (*Anax imperator*) chemical cues (native predator), compared to red swamp crayfish (*Procambarus clarkii*) chemical cues (alien predator) (Polo-Cavia & Gomez-Mestre 2014). These two examples show evidence of how innate behaviours can be valuable and adaptive. However, the changeability of the environment is thought to be important in determining the use of both innate behaviours and learning ability. For example, when innate preferences are not useful or reliable predictors of rewards they can be quickly erased or suppressed by learning (e.g. Giurfa *et al.* (1995), Gumbert (2000)). The implications and costs of using learning as opposed to innate behaviours is discussed further in section 1.2.4.

1.2.2 Influence of different situations

There are a number of factors that are anticipated to be important in determining the costs and benefits of learning ability for an individual, the first being the environment in which the individual lives. It is assumed that learning will only be selected for, when the benefits outweigh the cost (Stephens 1991; Dunlap & Stephens 2012), therefore it may not always make sense to invest in cognitive abilities. Theory predicts that in unpredictable and changeable environments learning is more important, than in more stable environments (Stephens 1991), where innate behaviours may be more useful. Although, if environments are too changeable, what an animal has learnt would become incorrect too quickly to be useful, and again it is more likely innate responses will be more valuable. This highlights that there is probably a small window of unpredictability when learning is the optimal strategy. There is some evidence from between species studies to support this. For example, Roudez, Glover & Weis (2008) compared the ability to find hidden food in invasive green (*Carcinus maenas*) and native blue crabs (*Callinectes sapidus*). Over multiple trials, the invasive crabs learnt to locate the food associated with the visual cue more quickly, indicating that faster learning might be important in their successful invasion of multiple environments. In addition, vole species that occupy larger home ranges, that will be likely to contain more diverse environments, have better spatial memory than those with smaller home ranges (Sherry & Healy 1998).

There is limited evidence of within-species differences in cognitive abilities between environments. Black-capped chickadees (*Poecile atricapillus*) that live in harsher environments have better learning ability (these individuals are both faster problem solvers and show reduced neophobia), compared to birds living in less harsh environments (Roth, LaDage & Pravosudov 2010). Therefore, this supports cognitive ability being heritable and being selected for in environments that require learning. However, it is likely to take time for such differences in performance to be selected over generations. For example, even though learning would have been more useful in the complex environment, fruit flies (*Drosophila melanogaster*) that were either exposed to a 'complex' or 'simple' environment during their development showed no difference in their learning abilities, as larvae or adults (Durisko & Dukas 2013). Living in different environments is also likely to influence the use of different cues. Odling-Smee & Braithwaite (2003) showed that when spatial learning was tested in populations of three-spined sticklebacks (*Gasterosteus aculeatus*) from different environments, they used different cues to solve the task. Populations from the stable habitats (ponds) used local landmarks; whereas populations from unstable habitats (rivers) did not, as in their environment they are less likely to be reliable.

The lifespan of an animal could also be potentially important in determining the benefits of learning to an individual, although evidence to support this is currently lacking. This is potentially due to the difficulty of testing the learning performance of two animal species with different lifespans, using the same cognitive task. Additionally, animals may vary in other ways apart from lifespan which may influence how well they perform. For example, a longer living animal may be more likely to experience a greater diversity of situations in which they need to learn. Alternatively, a long-lived animal may live in a very consistent environment in which learning is no more important than to a shorter-lived animal.

A third example of when cognitive ability may be selected for is when animals perform certain behaviours (e.g. caching) on a regular basis. There is evidence of particular behaviours being linked with specific areas of the brain being enlarged. For example, caching animals will hoard food in a number of locations, for varying lengths of time (depending on the perishability of the food), and then at a later time will need to relocate these caches. Correspondingly, hoarding species have a larger hippocampus (a region of the brain implicated in spatial learning and memory (Sherry *et al.* 1989; Hampton & Shettleworth 1996)) compared to non-hoarding species (Krebs *et al.* 1989). Following on from this, there have been numerous studies that have assessed whether spatial memory is better in species that rely on caching food, with some studies finding differences and other not (e.g. Shettleworth & Krebs 1986; Shettleworth *et al.* 1990; Clayton & Krebs 1994b; Clayton & Krebs 1994a; Healy 1995; Biegler *et al.* 2001). There is again the potential in this situation for differences in ecological requirements, for example, to explain the differences between hoarding and non-hoarding species. However, in this case there are no known species in which only some individuals hoard, to facilitate a comparison among individuals within a species.

1.2.3 Influence of an individual's specialisation, social rank and sex

Within species, there is evidence to suggest that the specific role an individual takes within a group is linked to individual learning ability. In the honeybee (*Apis mellifera*), a social insect, the 'reproductive ground plan' hypothesis (Amdam *et al.* 2004) has been proposed to drive the foraging specialisation of workers, and this is correlated with a suite of traits, including learning performance. Honeybees show age polytheism (Seeley 1982), whereby they go through a predictable set of roles, starting with in-nest tasks and ending with foraging, once they are about 3 weeks old. Contrastingly, bumblebees do not show age polytheism, as some workers will never become foragers (Jandt & Dornhaus 2009). In honey bees it has been shown that the sucrose responsiveness of 5-day old bees correlates with the

resources they collect, when they start to forage 2-3 weeks later (Pankiw & Page 2000), with nectar foragers having a higher response threshold for sucrose than pollen foragers. This means they bring back a higher concentration of nectar, which is beneficial to the colony and a potential ultimate reason for the link. Sucrose responsiveness is further correlated with olfactory and tactile learning ability, with pollen foragers being better learners (Scheiner, Erber & Page 1999; Scheiner, Page & Erber 2001a; Scheiner, Page & Erber 2001b). The larger the difference is between the bee's sucrose response threshold and the sucrose reward concentration used for the learning task, the better the bee will be able to learn (Scheiner, Erber & Page 1999). Therefore, if nectar foragers are given a higher sucrose concentration reward during the learning task, they will learn better, and will even be able to learn comparably to pollen foragers if the difference between response threshold and reward is the same (Scheiner *et al.* 2005). This suggests that adult behaviour (learning performance) is determined during the bees' development. The ability to learn to ignore an odour that is not predictive of reward (latent inhibition) has additionally been shown to be stronger in pollen foragers, and has been suggested that pollen foragers are generally more sensitive to environmental stimuli (Latshaw & Smith 2005). The same relationship between sucrose responsiveness and learning ability has been shown in ant foragers and nurses, with foragers having a lower sucrose response threshold and having higher learning acquisition rates, than nurses (Perez *et al.* 2013).

Bumblebees differ to honeybees in a number of ways. Firstly, bumblebee colonies are much smaller than honeybee colonies; therefore honeybee colonies have the capacity for individual workers activity to be less important. Whereas, all bumblebee workers need to have a role, and foragers will forage for their whole lives, compared to around 7 days in honeybees (Dukas & Visscher 1994). The waggle dance allows honeybee foragers to alert and recruit other workers to a profitable food source (Frisch 1967), while individual bumblebees must rely on their own individual learning ability to find food rewards from flowers in the landscape. Secondly, not all worker bees are the same size, and there can be up to 10-fold mass difference among workers within a colony (Jandt & Dornhaus 2009). Size difference has been linked to the division of tasks individual bees will perform (Brian 1952; Free 1955; Goulson *et al.* 2002; Jandt & Dornhaus 2009), with smaller bees more likely to perform within-nest tasks, and larger bees more likely to act as foragers. Whether foraging specialisation in bumblebees is driven in the same way as in honeybees, is currently less well understood, and something that I explore further in Chapter 3.

Rather than having a specific role within a group, some gregarious animal groups have a social hierarchy, in which the most aggressive individuals are dominant over others and have the

highest rank (Chase 1980). Whether it is cognitive ability that determines what rank an animal takes, or whether their rank determines individual cognitive ability, has been suggested to vary both between species, and also to depend on the situation in which cognitive ability is tested. As described in the paragraph above there are links between specialisations or tasks performed by insects and learning ability, however to my knowledge whether dominance or rank within insects affects learning ability has not been studied. However, there are examples of this in other animals. Prior to pairing into social groups, individual laboratory mice (*Mus musculus*) showed no difference in cognitive ability, but once paired the individual in the dominant rank performed better in the cognitive task (Barnard & Luo 2002). This suggests that cognitive abilities are to some extent determined by rank, but there are inconsistencies between species. When tested in isolation, low ranked individual long-tailed macaques (*Macaca fascicularis*) learnt better than higher ranking individuals (Bunnell, Gore & Perkins 1980), whereas higher ranking starlings (*Sturnus vulgaris*) are faster learners than lower ranking individuals (Boogert, Reader & Laland 2006). However, the context can change how individuals perform, for example, when testing in a group compared to in isolation. Dominant rhesus macaques (*Macaca mulatta*) performed better than lower ranked individuals, when tested within a group, however when they are tested in isolation, their learning abilities are comparable (Drea & Wallen 1999). Overall, this suggests that context is very important, and that in some species, individuals with a higher rank have better learning ability, although it appears that less dominant individuals may hide their cognitive abilities when in the company of higher ranked individuals.

The sex of the individual could also influence cognitive abilities. The tasks that males and females perform for their different reproductive strategies will in part determine the environments that they will encounter, which could affect the importance of cognitive abilities. Within insect species there are a couple of examples showing females having a higher or better learning rate than males while foraging (Church, Plowright & Loyer 2001; Kandori *et al* 2009). This could be explained by the roles that males and females have within the colony, as female bees are the workers that normally collect the food, so may have more incentive to forage (Church *et al* 2001). Alternatively Kandori *et al* 2009 suggest that it may be due to female butterflies requiring more nectar to produce eggs, therefore they need to be able to forage more efficiently, and hence enhanced learning ability is selected for in females. Males have been shown to outperform females in spatial cognition tasks in a number of species, for example; Cuttlefish *Sepia officinalis* (Jozet-Alves, Moderan & Dickel 2008); Deer mice *Peromyscus maniculatus* (Galea *et al.* 1994) and Eastern water skink *Eulamprus quoyii* (Carazo *et al.* 2014). One proposed reason for this difference in cognitive ability is the size of home range sizes (Gaulin & Fitzgerald 1986). In some species (e.g. Meadow voles *Microtus*

pennsylvanicus), males have larger home ranges, which means that potentially they have more spatial information to process and remember (Madison 1980). There is also evidence of the reproductive state of the individual having an effect on cognition differently between sexes. Female pinyon jays (*Gymnorhinus cyanocephalus*) that had mated were less accurate at retrieving caches after long intervals (4 months) than males, however accuracy was comparable to males in unmated females (Dunlap *et al.* 2006). It is suggested that one reason for this could be due to reallocation of resources, by the female to focus on reproduction, rather than memory formation. This highlights that cognitive performance many change through an individual's life.

1.2.4 Costs of learning

As discussed above, not all animals can learn comparably; even within a single species. This suggests that learning abilities may involve some form of cost. These costs could range from just an investment of time to an energetic cost, or even a life history cost such as reduced reproductive output, all which will affect the animal's fitness.

The pioneering work in this area is by Mery and Kawecki using fruit flies (*Drosophila melanogaster*). These experiments use fly populations that have gone through multiple generations of either a high or low learning selection regime (Mery & Kawecki 2002). In each generation, the high learning line was given a choice of two oviposition substrates, after being exposed to them first in a conditioning period, when one of these contained quinine (substrate containing quinine alternated each generation). Flies that learnt the aversive association, laid eggs on the substrate without quinine, and had their eggs reared for the next generation. Low learning lines were given no conditioning, and the eggs were reared from each flavour substrate in alternating generations. In their first experiment, they show that the low learning selection line larvae had higher competitive ability compared to the high learning line, when the quantity of food was restricted, and hence larval competition was more intense (Mery & Kawecki 2003). This implies that there is a constitutive fitness cost to learning ability, meaning the cost is paid whether learning ability used or not.

In a second experiment, Mery & Kawecki (2004) tested the learning ability of one set of high learning line flies and not another, finding that the flies that needed to use their learning ability showed reduced rates of productivity (egg-laying). This was performed in parallel for low learning line flies; they found no difference in the productivity between these two groups. This study therefore indicates that there may be an operating cost to learning, paid only when individuals show learning. As the low learning line flies exposed to the learning task did not

learn, they paid no operating cost. The proximate basis for this cost is unclear, but the authors suggest that it could reflect the energetic costs involved in collecting and processing the information they have learnt. This produces a trade-off between using energy for learning ability or fitness related traits such as reproduction.

It is very likely that there is some sort of energetic cost to learning an association, as it involves the development and maintenance of cellular mechanisms (Laughlin, van Steveninck & Anderson 1998; Dukas 2008). It has been shown that when fruit flies form long term memory, which requires protein synthesis, the lifespan of individuals is shortened (Mery & Kawecki 2005). Correspondingly, recent work in honeybees (*A. mellifera*) showed that bees that learnt an associative task, lived for a shorter time afterwards, than bees that did not learn during the same task (Jaumann, Scudelari & Naug 2013). In an additional second experiment, the same authors showed that bees that have been starved, compared to being satiated, had reduced learning ability. However, these experiments used methods that could have affected the conclusions, as bees that showed a PER response later or did not show a response at all were grouped together as non-learners. This is a factor that in the experiments in this thesis is controlled for, by categorising these bees differently and excluding those that do not show a PER response over a specified number of times. This is very important as bees that are not responding with a proboscis extension being classed as non-learners, could be explained by the bees not being motivated to take part in the task not that they cannot learn the task. Both of Jaumann, Scudelari & Naug's experiments suggest that there is an energetic cost to learning and memory retention in terms of reduced longevity, and when energetically challenged learning ability is poorer, as the individual may need to allocate the energy elsewhere. However, it is possible that learning variation and this affect reported on longevity, could be explained by part of a wider differences in behaviour that are more directly involved in a reduction of life span. For instance, bees that are good at learning could forage more actively and therefore the increased activity and energy levels used is decreasing the lifespan.

The honeybee immune system has the potential to be challenged by the invasion of host parasites (Schmid-Hempel 1998). When immune responses are triggered, this has negative impacts on olfactory learning performance (Mallon, Brockmann & Schmid-Hempel 2003). This suggests that there may be a trade-off between use of resources in the immune and nervous system. Nutritional stress has also been shown to reduce learning ability in a number of vertebrate studies, e.g. (Bush & Leathwood 1975; Tonkiss & Galler 1990; Nowicki, Peters & Podos 1998; Gil *et al.* 2006). However, the same was not found when the learning ability of individual honeybees was tested from a whole colony of honeybees, that were either nutritionally stressed or not (Mattila & Smith 2008). The learning ability of honeybees from

the nutritionally stressed colonies was no different to honeybees from control colonies. As honeybees live as a single (colony) unit, they were able to reallocate resources, so that learning was not affected at the cost of reduced brood rearing. Therefore, an individual may have the choice in some circumstances of what to invest their energy in. All the above studies were performed in controlled lab setting, but whether these same costs would be found in a more natural setting is more unclear and some field examples are discussed in section 1.4.2.

1.3 Fitness consequences of variation in cognitive ability

The fact that we see variation in learning ability within individuals of the same species means that there is the potential for selection to act up on this. Whether differences in cognitive ability actually have fitness consequences, are discussed in these next two sub-sections, first looking at laboratory studies and then field studies. It is important to measure fitness consequences and this gives us a tangible measure of benefits or costs to an individual. The number and quality of offspring produced (i.e., reproductive output) are an accepted and good proxy for direct fitness. In reality, fitness is quite hard to assess in a meaningful way, the individual's offspring need to survive and to be of sufficient quality to mate themselves to increase the individual's fitness. Therefore, this would involve following the individual and it's offspring for a long period of time. The bowerbird study that I go onto discuss in further detail in section 1.4.2 is a good example of the problems that can arise. These birds do not mate until they are around 7 years old and the learning task is built around the mating display they produce for females. Therefore, prior experience of the birds is impossible monitor for this period of time. Foraging choices, mate choices and nest choices will be expected to affect the numbers and quality individuals are able to produce.

1.3.1. Laboratory studies

The majority of laboratory studies assessing the fitness consequences of cognitive ability, have been performed on animals that are fairly short lived. These animals are easier to keep in the lab, and their reproductive output can be assessed over a shorter period of time. For example, in a study that compared the number of offspring produced by parasitoid wasps (*Biosteres arisanus*), they found that wasps that were allowed to learn about host substrates, produced significantly more offspring, than wasps that were not allowed to learn (Dukas & Duan 2000). In addition, grasshoppers (*Schistocerca americana*) that were given reliable cues to learn to associate with a balanced diet, learnt to visit and feed from this diet, more often than grasshoppers in a random treatment group (Dukas & Bernays 2000). Subsequently,

grasshoppers in the learning treatment achieved higher growth rates than those in the random group, which may translate to a fitness advantage, if such individuals produce more and larger eggs. In a predation context, tadpoles of the western spadefoot toad (*P. cultripipes*) that were able to learn the association between predator cues and conspecific alarm cues, have increased survival when exposed to predators, compared to naïve individuals, giving them an adaptive advantage (Polo-Cavia & Gomez-Mestre 2014). However, lab studies can only give us so much information, and we cannot assume that the same outcomes would be found in the wild and I discuss some of the key studies in the next section.

1.3.2. Field studies

Whether differences in learning ability actually impact the fitness of individual animals in a natural setting, is a question that has only really started to receive attention relatively recently. There is now evidence that fitness related traits, such as foraging performance (Raine & Chittka 2008), mating success (Keagy, Savard & Borgia 2009) and reproductive effort (Cole *et al.* 2012), are influenced by cognitive ability in the wild. I now discuss these studies in more detail, highlighting some of the weaknesses and strengths of each study, and identifying fruitful areas for further research.

A pioneering study in this area assessed whether learning ability in a lab based visual learning task of bumblebee colonies (*B. terrestris*), was related to colony foraging performance in a natural setting (Raine & Chittka 2008). These authors found a positive correlation between average learning ability of individuals within colonies in the laboratory and their colony nectar collection rate from real flowers in the field. As food supply is closely linked to colony reproductive output in bumblebees (Schmid-Hempel & Schmid-Hempel 1998; Pelletier & McNeil 2003), this makes collection rate a good proxy measure for colony fitness. Therefore, this study suggests that faster learning ability may give colonies an advantage at collecting nectar from flowers efficiently, and likely also more resources to invest into reproduction. The strengths of this study are that the bees that had their learning ability tested were naïve to foraging on flowers in wild, as they had been reared in the lab, with no prior experience that could affect their performance. Secondly, the learning task used is a well-established task that is an accepted paradigm to assess inter-individual and inter-colony variation, in the dynamics of learning, rather than just whether or not the individual can perform the task (e.g. Raine *et al.* 2006b). However, this study also has weaknesses. Firstly, the authors make the assumption that the learning ability of 15 bees from each colony represented the learning performance of the whole colony. Secondly, the authors cannot entirely rule out whether the observed

correlation was not caused by a third variable, such as colony condition or parasite load, linked causatively to learning and foraging performance.

A second study used mating success of the male satin bowerbird (*Ptilonorhynchus violaceus*) as a measure of fitness (Keagy, Savard & Borgia 2009). Male bowerbirds build bowers, a structure in which courtship occurs, in order to impress potential mates. Males have a strong aversion to red objects and will try to remove them from their bower site. The cognitive task in this study involved a problem that birds had to solve in order to remove the red objects. They found a positive correlation between problem solving ability (time to solve the task and remove red objects) and male mating success. This study gives us evidence of cognitive ability influencing the fitness of individuals in another species. They were also able to test cognitive ability without taking the birds into captivity, which avoided the potential problem that birds may not perform naturally under laboratory conditions. However, there are again some weaknesses of this study. For example, the male bowerbirds tested were not naïve, and therefore, differences in experience between individuals, may have influenced how well they performed when solving the task. With longer-lived animals this is a harder factor to control. Secondly, the use of a problem-solving task to test cognition has been discussed in recent reviews (Rowe & Healy 2014; Thornton, Isden & Madden 2014), as potentially not a good measure of cognition. A problem solving task involves presenting individuals with one new task to solve in a set period of time, which in some cases, may have no relevance to a real life situation. This could mean that the individual solves the task by chance or due to a factor other than cognition, such as their strength, allowing them to better be able to solve the task (Thornton, Isden & Madden 2014). Another example could be motivation of individuals, however, cognitive tasks should be designed to mitigate issues like this, and it is unlikely that this is a reason we see variation in cognitive ability in well-designed experiments. Additionally, it is currently unclear what cognitive abilities are needed during problem solving tasks, and it is likely to involve the use of more than one, which is important to know if we want to understand how they have evolved or what they may be selected for (Rowe & Healy 2014). It has been suggested that testing problem solving ability would be improved by presenting problems multiple times, to gain a rate of change in ability (Thornton, Isden & Madden 2014). In addition, using a wider range of tasks would minimise the chance effects of an individual doing well in one particular test, when they are not a good learner.

The most recent study to tackle this question used wild-caught Great tits (*Parus major*) as a study species (Cole *et al.* 2012). Cognitive ability was tested, by briefly taking the birds into captivity in individual cages, using a problem-solving task, before reproductive performance in the wild was assessed. They found that females that could solve the task had larger clutch

sizes (higher fecundity) in the wild, but were more likely to desert their nests. Therefore, they found contrasting positive and negative effects associated with cognitive ability, unlike the above studies showing only positive effects. One of the real strengths of this study was that they measured multiple fitness traits, which is important to get an overall picture of why cognition may be adaptive and is the only study to date to do this. However, the study has the same two weaknesses as discussed for the Keagy, Savard & Borgia (2009) study- the prior experience of test birds could not be controlled and problem solving was used as a measure of cognition.

These three studies have greatly increased what we know about the consequences of differences in cognitive ability for animals in the field. However, there is still much we do not know, in chapter 4 I build on this area of work, by addressing some of the weaknesses of these previous studies.

1.4 Bumblebees

1.4.1. Study species

Bumblebees (*B. terrestris*) are the study species I used in all my thesis experiments. Bumblebees typically have an annual life cycle which starts from a single mated queen emerging from hibernation in early spring. The queen must choose a nest site, provision with pollen and then lay her first batch of eggs, which take 4 – 5 weeks to complete development and emerge as workers (Alford 1975). During this period, the queen must incubate the brood, forage and feed them. This is one of the most vulnerable stages of colony growth, as the queen is responsible for all tasks. Once the first workers emerge, the queen will gradually stop foraging and the workers will take on this role. Colonies grow up to 200–500 workers in size before they produce sexuals (males and new queens, also called gynes) and then the colony will then decline (14–24 weeks following founding; Goodwin 1995). Bumblebees are a eusocial Hymenoptera species, and workers cannot mate (but can lay unfertilised eggs that develop into males). Reproduction is therefore measured at the colony level: the sexual offspring produced by the founding queen. Colonies that produce a greater number of sexuals are likely to have higher fitness, and this has been shown to be proportional to the amount of food brought back to the colony (Pelletier & McNeil 2003). However, fitness will only be higher if males go on to mate, additionally that gynes mate, survive hibernation and initiate a colony of their own. Biomass is also important, as gynes that weigh less are less likely to survive hibernation (Beekman, van Stratum & Lingeman 1998). Larger males have been shown to be more successful at mating, although age was also important with younger males outperforming older males (Amin, Bussiere & Goulson 2012).

Bumblebee workers show variation in size and there can be up to a 10 fold difference in size within a single colony (Alford 1975), although the difference in size is typically less than this. There is evidence to suggest that pupae at the edges of the brood are fed less, and therefore, become smaller adult workers (Couvillon & Dornhaus 2009). However, it is unclear whether this brood is just 'forgotten' about or for an adaptive reason, so that colonies have workers varying in size. Pyke (1978) suggested that bumblebee body size could be explained by the maximization of net rate of energy intake. Although different tasks within the colony have been seen to be performed by workers of all sizes (Jandt & Dornhaus 2009), it has been observed that larger workers tend to forage, whereas smaller workers perform in nest tasks (Brian 1952; Free 1955; Goulson *et al.* 2002; Jandt & Dornhaus 2009). Larger workers have been shown to be more efficient at foraging (Goulson *et al.* 2002; Spaethe & Weidenmüller 2002), and this likely to be driven in part by their better visual acuity (Spaethe & Chittka 2003). There is no evidence to suggest that smaller workers are better nurse bees than larger bees. However, smaller *Bombus impatiens* workers live longer than larger workers under starvation conditions (Couvillon & Dornhaus 2010), and therefore could help colony survival under difficult conditions. This could be one adaptive reason for size differences in workers within colonies; however it is unclear whether there are other reasons.

1.4.2 Experimental use

Bumblebee colonies can be obtained from commercial suppliers, which make it possible to control the age and prior experience of the bees for experiments. Because of their relatively small colony size, they can be kept in the lab easily, are amenable to the conditions and will perform relatively naturally i.e. will perform the tasks they do in the wild, such as caring for brood and foraging for nectar and pollen. Bumblebee colonies can also be used for field-based experiments, because foraging bees will reliably return to the colony after each foraging bout. Therefore, colonies can be placed in any environment an experiment requires, as long as there is food in the surrounding area that bees can collect, which would not be possible for most animals. Many studies have used paint marks or coloured/numbered tags to identify individuals. However, new technology is opening up more opportunities for researchers, for example, radio-frequency identification (RFID) tagging technology allows the foraging activity of individuals to be recorded automatically (Molet *et al.* 2008; Gill, Ramos-Rodriguez & Raine 2012).

1.4.3 Cognitive ability

Bees forage in complex environments, where they are faced with flowers varying in multiple ways (e.g. colour, scent, shape), and they need to learn to identify which flowers provide the highest reward, and remember both the locations of flowers and their colony. Bumblebee foragers have been shown to have foraging preferences in flower type, and after initially sampling multiple flowers they will typically specialise on certain type that they will forage from the majority of the time (Heinrich 1976). However, they have been shown to minor on other species which is thought to be an adaptive behaviour to track other resources (Heinrich 1979). Species which are specialists of flowers have been shown to be more efficient at handling these flowers than generalist bumblebee species (Lavery and Plowright 1988).

Bees have been shown to have surprisingly high level of cognitive abilities for their body, and brain, size (Chittka & Niven 2009). The default expectation would typically be that smaller brain size would mean they are less capable of more complex behaviours and processes. Shettleworth (2010) outlines that a change in view that complex cognitive abilities, found in bees for example, could be explained by complex processes such as insight rather than simple mechanisms would further our understanding. Honeybee cognition has been studied more widely than bumblebee cognition (Sherry & Strang 2014). One of the most impressive cognitive abilities of honeybees to date being the learning of sameness-difference rules, whereby they can learn a rule, to either pick the same as matched sample or the different one, and apply it to different situations (e.g. cues in a different sensory modality) (Giurfa *et al.* 2001). In addition, honeybees have been shown to be able to learn many other processes. For example, responding to a stimulus only in certain contexts (Menzel & Giurfa 2001) and responding to the number of cues not the features of them (Dacke & Srinivasan 2008). Sequence learning of landmarks (Collett, Fry & Wehner 1993; Saleh & Chittka 2007) and reversal learning (Menzel 1993; Chittka 1998) are a couple of examples of learning processes shown in both bumblebees and honeybees.

To test olfactory learning in bumblebees, there has been growing use and success with the proboscis extension reflex, and this is discussed further in section 1.5.5. The majority of studies to test bumblebee learning have used visual cues, for example, there is evidence to support that they are able to associate colour (Raine *et al.* 2006b), patterns (Fauria *et al.* 2002) and shapes (Muller & Chittka 2012) with reward in free flight tasks. Bumblebees have also been shown to be able to optimise both, the distance they travel to flowers (by selecting the shortest route), and visiting the most rewarding flowers first (Lihoreau, Chittka & Raine

2010). Recently iridescence (Whitney *et al.* 2009) and electric fields (Clarke *et al.* 2013) of flowers, have also been identified as cues bumblebees can use to discriminate between flowers. Therefore, bees have the potential to use multiple features of flowers to associate with reward, and the plant is likely to present all these cues to increase the likelihood of efficient pollination. This is supported by the finding that when more than one cue (colour and odour) is presented, bumblebees have been shown to make more accurate decisions compared to one cue alone (Kulahci, Dornhaus & Papaj 2008). Whether there is a trade off in learning across different sensory modalities is a topic I investigate in chapter 2. As well as learning for themselves, there is additional evidence that bumblebees can use social information, to learn more quickly, which flowers contain nectar (Leadbeater & Chittka 2007) and which flowers to visit, to avoid predation (Dawson & Chittka 2014). However, it has been suggested that social information is not more important than a bee's individual learning experience (Leadbeater & Florent 2014).

1.4.4. Effects of anthropogenic stressors

In the modern world, bees, along with many other animals, are faced with numerous potential anthropogenic stressors, in the changing environment in which they live, that include habitat loss, parasites and disease, invasive species, pesticide exposure and potential interactions between these factors (Didham *et al.* 2007). Variation in learning ability could be one mechanism that helps bumblebee colonies deal with these types of stressors within the environments they inhabit. The intensification of farming has meant that habitats in and around farmland have changed in recent years, through hedgerows being removed to increase field sizes, which has increased fragmentation of the landscape. This may mean that nesting sites for certain animals have been lost, or that to find suitable food they will have to look further away and search for longer to find suitable food. For example, in environments where resources are sparse, bees performed longer foraging bouts and the colony's weight increase was smaller (Westphal, Steffan-Dewenter & Tschardtke 2006). This could reduce the number of workers or reproductive output the colony is able to produce, and therefore their contribution to crop pollination.

Bee populations have been shown to be declining on a global scale (Biesmeijer *et al.* 2006; Potts *et al.* 2010). Bumblebees are an important pollinator of flowers and crops (Garratt *et al.* 2014), and declines in bumblebees are of great concern as they are essential in food production and maintaining wild flower biodiversity (Vanbergen *et al.* 2013). Habitat loss is one potential driver of decline, increasing landscape fragmentation and reducing species richness (Williams 1988). Introduced parasites and disease can also cause stress to insects. Honeybees infected

with *Nosema ceranae* were shown to be energetically stressed, and subsequently, had a shorter lifespan (Mayack & Naug 2009). Finally, exposure to pesticides can cause stress to insects and has been shown to impair foraging performance (Gill, Ramos-Rodriguez & Raine 2012; Feltham, Park & Goulson 2014; Gill & Raine 2014), decrease reproduction output (Whitehorn *et al.* 2012) and navigation ability (Henry *et al.* 2012; Fischer *et al.* 2014).

The consequences of these environmental stressors on learning performance is less well known. In honeybees, nutritional stress (Jaumann, Scudelari & Naug 2013), parasite infection (Kralj *et al.* 2007) and exposure to pesticides (Decourtye *et al.* 2004a; Decourtye *et al.* 2004b; Williamson & Wright 2013) have all been shown to reduce learning ability in honeybees, but also see (Williamson, Baker & Wright 2013). This indicates that there could be energetic costs of exposure to these stressors, and that the resources available to invest in learning and memory may decrease, therefore this may not represent a choice of the individual but a consequence of pesticide exposure. To date, experiments investigating the effect of pesticide exposure on learning and memory have almost exclusively been performed under laboratory conditions. Additionally, in many cases, the level of exposure has not been comparable to what individuals would experience in the field. Therefore, it is important that future studies use field realistic profiles of exposure. Furthermore, all the studies on learning and memory have used honeybees. However, the biology of honeybees and bumblebees differs substantially, and therefore we cannot necessarily assume that the impacts will be the same. For example bumblebees have been shown to be less able to continually metabolise pesticide bodily residues than honeybees, this may be due to honeybees being better pre-adapted to deal with these (Cresswell *et al.* 2012, 2014). Therefore, bumblebee colonies may be more sensitive to pesticide exposure and its effect on behaviour. In chapter 5 I address some of these knowledge gaps, by assessing the effects of field realistic doses on learning and memory in bumblebees for the first time.

1.4.5 Proboscis extension reflex (PER) conditioning in bumblebees

The vast majority of learning studies on bumblebees to date have used free flight tasks (e.g. (Gumbert 2000; Kulahci, Dornhaus & Papaj 2008; Raine & Chittka 2008; Leonard, Dornhaus & Papaj 2011) as discussed in section 1.5.3. However, when testing olfactory learning there are potential problems that need to be controlled. In an enclosed space, like a flight arena, it is possible that odours may mix if there is not the correct ventilation. It is also important that the bees do not return to the colony with a strong smell of the odour, as this could affect the responses of other bees (Dornhaus & Chittka 1999). For example when odours have been presented with food within the hive, this has been shown to later influence the foraging floral

choice of the same bees outside the hive (Gil & De Marco 2006; Arenas, Fernández & Farina 2007).

Conditioning the proboscis extension reflex is one of the most robust and reliable tools available to study learning and memory in invertebrates, and has been used with great success in honeybees (*Apis mellifera*) for over 5 decades (Giurfa & Sandoz 2012). This paradigm is particularly effective for olfactory conditioning, as it gives precise control over the timing and duration of odour presentation, and avoids unintended odour mixing. To date, early attempts to use condition proboscis extension using visual cues, has shown very low response rates at the end of the trials in intact honeybees (e.g. 30% of bees responding: Dobrin & Fahrbach 2012), and those studies reporting higher response rates involve removal of at least one antenna prior to conditioning (Niggebruegge *et al.* 2009). The latter technique is clearly problematic if you intend to use bees in subsequent behavioural tests, involving olfactory/gustatory sensation. However, a more recent study using Africanized honeybees, found that the bees could learn the visual PER task better when their antenna were not removed, reaching learning rates of 50% (Jernigan *et al.* 2014). Although their learning ability in the visual PER task was still lower than the olfactory PER paradigm.

Conditioning the proboscis extension reflex, involves bees learning the association of a conditioned stimulus, typically an odour, with a sucrose reward (the unconditioned stimulus) (Bitterman *et al.* 1983). These paired presentations of the conditioned (odour) and unconditioned stimulus (food) are presented to the bees several times in a series of trials, separated in time by an inter trial interval (ITI). Once the subject has learnt this association (between odour and reward), they will respond to the odour with a proboscis extension prior to the reward being offered.

The paradigm has not been used as widely in bumblebees, and early studies have shown lower success rates than honeybees. The first attempt at olfactory conditioning of bumblebees (*B. terrestris*) using PER conditioning was by Laloi *et al.* (1999), who found that only 30-40% of the bumblebees learnt the association by the end of the training trials. In comparison, 70-80% of bees responding is commonly seen in honeybees at the end of conditioning (Wright, Carlton & Smith 2009). The bumblebees were also seen to struggle in the harnesses, and persistently try to escape during conditioning. More recent studies using PER paradigm to study learning in bumblebees have had higher success rates (Riveros & Gronenberg 2009; Toda, Song & Nieh 2009), probably because of modifications to the methods used to restrain the bumblebees. Toda, Song & Nieh (2009) compared the standard harnesses that have been used in honeybee studies with a capsule to restrain the bumblebees (*Bombus impatiens*), and found a much

higher response rate of learning with the capsule, reaching a final level of performance, with around 51% of bees extending their proboscis to the conditioned odour. Riveros & Gronenberg (2009) further improved on this by harnessing bumblebees (*Bombus occidentalis*) in a plastic tube, using 2 metal pins which formed a yoke around the bee's neck preventing them from escape. They found that by the end of the trials, 71% of the bees had learnt the association – i.e. comparable to the performance of honeybees in this paradigm.

However, the work on bumblebees to date appears to show that bumblebees are slower learners in this paradigm than honeybees, with bumblebees typically taking 5-7 trials (Riveros & Gronenberg 2009; Toda, Song & Nieh 2009), to reach the same performance levels that honeybees manage in 2-3 trials (Bitterman *et al.* 1983). Therefore, the number of training trials given to bumblebees tends to be higher; up to 20 compared to 6-12 in honeybees. There are also other differences in the conditioning of bumblebees compared to honeybees, in honeybees in odour stimulus is typically presented for 4–6 seconds, whereas it was observed by Laloi *et al* (1999) that in bumblebees, this odour presentation was not long enough to elicit proboscis extension. The experiments above have typically used an odour presentation of 8-12 seconds, and this is typically preceded by clean airflow (not containing the odour) for 10-12s (which is not used in honeybee conditioning).

These experiments suggested strong potential for PER conditioning to become a valuable method of assessing olfactory learning ability in bumblebees. When I started my PhD this technique had only recently been applied successfully to the North American species *B. impatiens* (Riveros & Gronenberg 2009), and an initial goal of my thesis was to develop and optimise a PER conditioning protocol for *B. terrestris* to employ in all experiments for my thesis work. Since I began using the technique it has also been shown to be successful in *B. terrestris* by Sommerlandt, Rossler & Spaethe (2014). The potential for using colour cues has also been shown to be successful in *B. impatiens* (Riveros & Gronenberg 2012).

1.5. Thesis outline

The aim of this thesis is to investigate potential causes and adaptive consequences of variation in learning performance in the bumblebee (*B. terrestris*). I start by exploring potential causes of variation in learning performance in chapters 2 and 3, followed by examining the consequences of variation in learning performance in the field in chapter 4. Finally in chapter 5, I investigate the impacts of an environmental stressor (pesticide exposure) on learning and memory. More detail about each chapter is given below:

Chapter 2: Comparison of individual visual and olfactory learning

Bees use a variety of sensory cues to locate floral rewards, but there has been little investigation of how the same individual bees learn cues in different sensory modalities. In this chapter, I compared the learning performance of individual bees in an olfactory and visual learning task. The olfactory task used proboscis extension reflex (PER) conditioning and the visual task used a free flight discrimination task. I hypothesised that there would either be a trade-off in performance across the two tasks (i.e. learning in one came at a cost to learning in the other modality), or that performance would be correlated (i.e. they are either all round good or bad learners).

Chapter 3: The association between foraging preferences, sucrose responsiveness and olfactory learning performance

In chapter 2, I identified considerable variation in olfactory learning performance within colonies, and here, I followed up by investigating a potential cause of this variation. Bumblebee colonies divide tasks among individual workers within the colony, but why certain individuals choose to perform different tasks is relatively poorly understood (particularly in comparison to task allocation in honeybees). In this chapter, I investigated whether sucrose responsiveness and olfactory learning performance (previous variables used in honeybee work) correlates with the nectar or pollen foraging preferences of individual bumblebees.

Chapter 4: The relationship between individual learning ability and field foraging performance

Foraging in the wild, compared to in the laboratory, bees are faced with an increased diversity and complexity of stimuli, longer distances to travel and the potential predators. Therefore, I wanted to investigate whether findings from lab based studies on the fitness consequences and costs of learning, are also applicable in the field. In this chapter, I explored whether variation in individual learning performance, has consequences for foraging performance in a natural setting. The learning performance of individuals was tested in the lab in either a visual or olfactory task, and then their foraging performance in the field was measured. I hypothesised that better learning bees would perform more foraging, and do this more efficiently, due to the advantage their cognitive abilities would give them in the field.

Chapter 5: The impacts of an anthropogenic stressor on olfactory learning and memory

When foraging in the field, bumblebees face a number of potential stressors that may impact behavioural traits, including cognitive performance. In this chapter, I investigated what effect either acute or chronic exposure to varying levels of a neonicotinoid pesticide, had on the odour learning and memory capabilities of individual bumblebees. I hypothesised that pesticide exposure would also have negative effects on bumblebee learning and memory, as has previously been shown in honeybees.

Chapter 6: General discussion

In the final chapter, I summarise the results from the four research chapters, draw together and discuss the wider significance of my finding and outline avenues for future research arising from my thesis. As well as discussing the strengths and weaknesses of the proboscis extension reflex with bumblebees.

Chapter 2

A comparison of visual and olfactory learning performance

Published as: Smith K. E, Raine N. E (2014). A comparison of visual and olfactory learning performance in the bumblebee *Bombus terrestris*. Behavioral Ecology and Sociobiology 68 (9):1549-1559.

2. A comparison of visual and olfactory learning performance

2.1 Abstract

Animals use cues from a range of sensory modalities to discriminate stimuli as predictors of reward. While there is appreciable variation in the cognitive performance of animals, we know surprisingly little about the extent to which learning varies among individuals, across different sensory modalities. Do individuals that are good at learning in one sensory modality also perform well in another (performance is correlated between modalities), or do individuals demonstrate specialisation in learning performance in one modality (trading-off performance between modalities)? I tested these hypotheses by examining the performance of 76 *B. terrestris* workers, from four colonies, in both an odour and visual learning task. Olfactory learning was assessed using proboscis extension reflex (PER) conditioning and visual (colour) learning was examined using a well-established free flying paradigm. My results showed neither a correlation, nor a trade-off, in individual performance for learning tasks using different sensory modalities. However, there was considerable variation among workers within each colony in their performance in both learning tasks. This extent of inter-individual variation in learning ability across sensory modalities could be adaptive for colonies dealing with changeable foraging conditions. There was also significant inter-colony variation in final task performance level in the olfactory learning task, and both the strength and persistence of blue preference in the colour learning task. In this chapter, I demonstrate variation in olfactory learning performance across multiple bumblebee colonies using PER conditioning, suggesting this is an effective paradigm for assessing associative olfactory learning performance both within and among colonies.

2.2 Introduction

Learning, or the adaptive modification of behaviour based on experience, affects almost every aspect of animal behaviour. The fact that all animals are able to learn, in at least a rudimentary way, suggests this trait allows them to be able to survive and adapt in their environment. However, there is appreciable variation in the learning ability of animals both among (Biegler *et al.* 2001; Healy, de Kort & Clayton 2005) and within species: e.g. birds (Katsnelson *et al.* 2011; Cole *et al.* 2012), butterflies (Snell-Rood & Papaj 2009) and bumblebees (Raine *et al.* 2006b; Raine & Chittka 2007b; Alghamdi *et al.* 2009). This variation is likely to be due to the costs and benefits associated with learning (Dukas 2008). If there were no costs associated with enhanced learning performance, and better learners had higher fitness, then we might expect all individuals to be good at learning. However, this is not what has been observed, and

the considerable (inter- and intraspecific) variation in learning performance indicates that potential fitness costs, such as those associated with enhanced learning and/or long-term memory performance (Mery & Kawecki 2003; Mery & Kawecki 2004; Mery & Kawecki 2005), are likely to be significant constraints. As such, animals operating in ecological conditions that do not demand high levels of cognitive performance, are likely to benefit (in terms of enhanced fitness) if they do not invest valuable resources into learning.

Variation in learning ability may also be associated with the type of environment in which an animal lives: in rapidly changing environments fast learning could be more important than in environments which are constant (or less changeable). For example, if a bee forages in an environment in which the rewards provided by each flower species are consistent at all times, then it would not need to learn which are the most rewarding flower species (and when) but could perform equally well using innate behaviours (such as colour preferences) which are adapted to that specific environment (Raine & Chittka 2007b). Especially in eusocial species, where individuals are not just foraging for themselves but for the colony as a whole, differences in learning ability may be beneficial for the colony and help it to adapt to changing conditions. There is evidence to suggest that individual bee decision strategies fall along a continuum of a speed-accuracy trade-off, with some workers making faster but more inaccurate, or slower but more accurate, foraging decisions (Chittka *et al.* 2003; Burns & Dyer 2008; Muller & Chittka 2008), and that these alternatives could be more effective in different environments. Unexpectedly, the fast, inaccurate bees bring back more nectar due to higher collection efficiency. Burns and Dyer (2008) suggest that a colony with mixed foraging strategies will reduce the variation in nectar collection rate, thereby increasing colony fitness and the likelihood of colony success compared to a colony with a single foraging strategy. Therefore, having a mixture of bees with varying learning performance within a colony, could be adaptive as different individuals forage more efficiently across a range of conditions.

In their environment animals are typically faced with signals comprised of cues from multiple sensory modalities (e.g. visual, olfactory, tactile and auditory), and they make important decisions based on this information: for example females choosing a mate based on a male's mating display or a bee choosing to collect nectar and pollen from a plant based on its floral display. Multimodal signals are potentially beneficial both to the signaller and receiver, as they can be used to enhance the chances of the signal being received and/or increasing the speed with which a receiver responds. Animals show enhanced learning of signals comprising cues from more than one sensory modality, compared to single modality cues, increasing the speed or accuracy of decisions, the longevity of memory and overall foraging success (Kunze & Gumbert 2001; Reinhard, Srinivasan & Zhang 2006; Kulahci, Dornhaus & Papaj 2008;

Leonard, Dornhaus & Papaj 2011). A combination of cues in different sensory modalities can be important in some situations, for example in the solitary bee *Hoplitis adunca* which forages solely for the pollen of its host plant *Echium vulgare*. *H. adunca* has been shown to use a visual cue (blue colour) to initially locate potential host flowers and then uses olfactory cues to recognise the correct host flower when it gets closer to the target (Burger, Doetterl & Ayasse 2010). Without the multimodal combination of these two cues, *H. adunca* are unable to successfully locate their host flower.

However, we know little about how well animals can learn to use multiple sensory components of signals when they are presented separately, whether individuals are consistent in their learning across modalities (Muller & Chittka 2012) or whether they show enhanced learning ability in specific sensory modalities, at the cost of reduced learning performance in others. Such trade-offs in learning performance could be driven by preferential allocation of limited resources, generating differences in individual brain architecture to support learning and memory in one or other sensory modality, as suggested by differential investment between paperwasp castes (e.g. (O'Donnell *et al.* 2014)). In this chapter, I examined whether the level of performance shown by individual bumblebees (*B. terrestris*) in an odour and a visual associative task are correlated. There are three predicted outcomes: (1) individual bees that are good at learning in one sensory modality and will also perform well in another (i.e. a positive correlation in learning performance between sensory modalities), or (2) that individuals will demonstrate specialisation in learning performance in one sensory modality (i.e. a trade-off in learning performance between modalities), or (3) that the learning performance of individuals is unpredictable across sensory modalities (i.e. no correlation in learning performance between sensory modalities).

2.3 Methods

2.3.1 Experimental setup

I obtained four bumblebee (*B. terrestris*) colonies, each containing a queen and 24-52 workers on arrival, from Syngenta Bioline Bees (Weert, The Netherlands). Prior to experiments, colonies were transferred to a bipartite wooden nest-box (with a transparent Perspex lid) and fed pollen and unscented sucrose solution *ad libitum*, without exposure to colour or odour stimuli associated with food. All workers were uniquely marked on the thorax with numbered, coloured tags (Opalith tags; Christian Graze KG, Germany) upon eclosion so that individuals could be identified and potential age effects could be assessed with an accuracy of ± 1 day.

During the experiment the nest-box was connected to a flight arena (see below). Pollen was still provided *ad libitum* directly into the nest-box, but workers now had to collect sucrose solution from a gravity feeder in the flight arena. All workers that took part in learning tasks were foragers that collected sucrose solution from this feeder. The performance of 76 workers from the four colonies was tested (two colonies May-June 2012; two colonies October-November 2012) using both an olfactory and a colour (visual) learning paradigm. A further 19 and 13 workers completed either the olfactory or the visual task respectively, but not both, and these bees were included in separate analyses of odour learning (n = 95) and colour learning (n = 89 bees). The order in which learning performance were tested (i.e. odour then colour or colour then odour) was allocated for each worker at random.

2.3.2 Odour learning

Olfactory learning was assessed using the proboscis extension reflex (PER) conditioning paradigm. Foragers were removed from a feeder containing sucrose solution in the foraging arena using forceps and kept on ice until they became quiescent (*ca.* 5 min). Subsequently, they were put into harnesses that secure the bee's head using a yoke fastened to the top of the harness using tape (following a method similar to Riveros and Gronenberg 2009: see Figure 2.1). Bees were harnessed in the early afternoon (*ca.* 1300-1400) and fed to satiety (with sucrose solution) two hours later. Bees were trained 18 hours after harnessing (*ca.* 0800-0900 the next morning). Prior to training I assessed the responsiveness of each bee by touching its antenna with a droplet of 50% (v/v) sucrose solution. Bees that extended their proboscis were considered motivated to participate (Giurfa & Sandoz 2012). On average, 79% of bees extended their proboscis, and these individuals were fed a small droplet of sucrose solution to maintain motivation to the start of the experiment (15 minutes later).

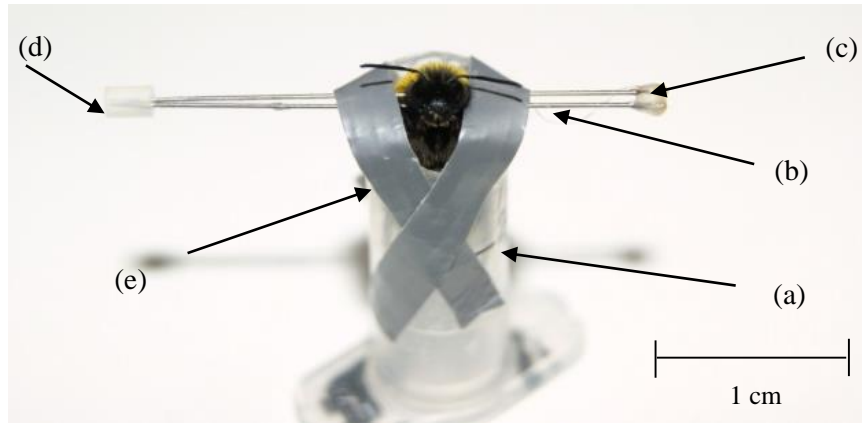


Figure 2.1 Harnessing technique used for the proboscis extension reflex (PER) paradigm (a) Harness made from a cut down 2ml plastic syringe (internal diameter 8.2mm), with a v-shaped groove at the front to allow easy proboscis extension. (b) Yoke to secure the bee's head in place, made from 2 entomology pins (size 0) glued together at one end (c) and secured at the other end with a plastic earring backing (d). (e) Tape used to fasten the yoke to the top of the harness on either side of the bee's head.

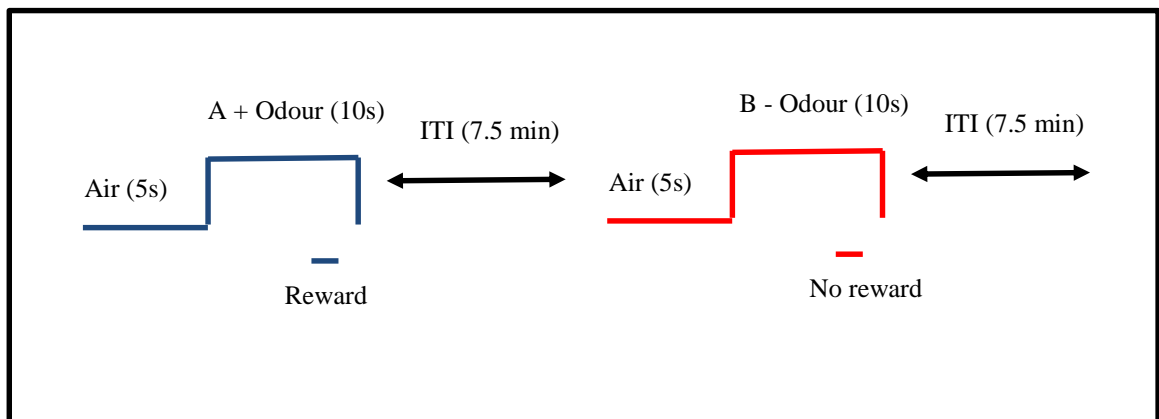


Figure 2.2 Timeline of one rewarding trial (blue) and one unrewarding trial (red)

Bees were trained using differential conditioning, so they had to learn to distinguish between two odours: one odour (A) was paired with a reward (A+), the other (B) was unrewarding (B-). The odours used were lavender and lemon (essential oil, Calmer Solutions Limited). For half of the bees, lavender was the rewarded odour (and lemon was unrewarded), for the other half the reverse. I found no significant difference in learning performance depending on which odour was rewarding (Mann-Whitney, $Z = 0.878$, $p = 0.380$). Each bee was trained individually in an odour extraction hood every time it was exposed to the odour. An odour tube (*ca.* 3cm away) pointed towards the bee (containing 1 μ l of the essential oil odour on a

piece of filter paper) delivered a precise stimulus to the bee. A programmable logic controller (PLC) computer controlled the volume of air, flow rate and duration of stimulus presentation to each bee. Odour tubes were changed every 20 uses to maintain consistency of odour strength.

Bees were presented first with clean air for 5s and then the air containing the odour for 10s. For the rewarding odour, the bee was presented with 0.8µl of 50% sucrose solution (using a Gilmont syringe) 6s into the odour stimulus by touching their antenna to elicit a proboscis extension, and was allowed to consume the droplet (Figure 2.2). For the unrewarding odour, the bee's antennae were touched with an empty syringe tip 6s into the odour stimulus. Both odours were presented to the bee 15 times with an inter trial interval (ITI) of 7.5 minutes in a pseudorandom order (i.e. 30 presentations during a period of 3 hours 45 minutes). Results from the unrewarding odour presentations are not included in analyses as none of the bees responded (with a proboscis extension), indicating that they could clearly discriminate between the odours. Once a bee learns the association between the rewarding odour stimulus and reward, they extend their proboscis as soon as the odour is presented (prior to reward delivery). For each rewarding odour presentation I recorded whether the bee responded prior to reward delivery or not, giving a binary response. If a bee did not extend its proboscis when their antennae were touched on more than 20% of the conditioning trials, they were deemed insufficiently responsive to participate and excluded from analyses. After bees completed the odour conditioning they were taken out of the harnesses and reintroduced to the colony.

2.3.3 Colour learning

Colour learning was assessed using a well-established paradigm in which individual bees learn to associate yellow as a predictor of flower reward, and learn to ignore blue (unrewarding) flowers for which they have an innate preference (Raine *et al.* 2006b; Ings, Raine & Chittka 2009; Raine & Chittka 2012). Each colony nest-box was connected to a flight arena (120 x 100 x 35 cm) by a transparent Perspex tube. Shutters along the tube allowed the flow of bees between the nest-box and the flight arena to be controlled. Bees were pre-trained to forage on 20 bicoloured yellow (Perspex Yellow 260) and blue (Perspex Blue 727) artificial flowers placed in the flight arena. Each flower (24 x 24 mm: half yellow and half blue) was placed on a glass cylinder (40 mm high) to raise them above the floor of the flight arena. During pre-training these flowers were rewarded with 15µl of 50% sucrose placed in the middle of the flower. All bees were allowed to forage freely on the flowers and the rewards were replenished as they were consumed, this gave bees equal opportunity to associate both blue and yellow with reward. To ensure I only assessed the learning performance of motivated foragers, an

individual bee was only tested once it had completed at least 5 consecutive foraging bouts. Subsequently, I recorded the number of flowers (each rewarded with 5µl of 50% sucrose) the bee visited in each of three further foraging bouts, and calculated the mean to estimate the volume of sucrose solution it collected per bout.

Each bee was then tested individually in the flight arena with 10 blue and 10 yellow flowers. Yellow flowers contained reward (50% sucrose solution) and blue flowers were unrewarding (empty). The volume of sucrose solution reward in the yellow flowers was calculated from the mean volume that bee consumed (+10%) during the 3 foraging bouts at the end of the pre-training. For example, if a bee visited all 20 flowers then 110µl (= 20 x 5µl = 100µl + 10 %) would be divided equally among the 10 rewarding flowers (11µl/ flower). All flowers had a recessed well in the middle of the upper surface (depth = 2mm; diameter = 4 mm) and the sucrose solution was pipetted into the wells. This meant that bees were unable to see the reward (or absence of reward) until they landed on flowers. Bees were regarded as choosing a flower when they either approached (oriented towards a flower with their head < 2 cm away) or landed on it. While landing on, and sampling from, a flower gives bees direct feedback (positive from yellow and negative from blue flowers), approaching a flower is also valuable to the learning process. Approaches allow bees to familiarize themselves with the flowers in preparation for landing and/or provide information on whether flowers have already been visited and emptied from scent marks left by previous visits (Saleh *et al.* 2007). The frequency of both approaching and landing on each flower colour changes predictably with individual experience in this assay (Appendix 2.1; Ings, Raine & Chittka 2009; Raine & Chittka 2012), indicating that both these behaviours represent a choice for a particular flower, and including all choices gives us a more sensitive measure of learning. While the frequency of both approaches to and lands on yellow flowers increase as bees gain experience, the number of blue approaches and lands both decrease (Appendix 2.1). Once a bee fed from (probed) a yellow (rewarding) flower a further 99 choices were recorded, so each bee made a sequence of at least 100 choices. Visits to (unrewarding) blue flowers were considered as errors, and visits to yellow flowers as correct choices. The choice sequence was recorded using EthoLog 2.2.5 software (Ottoni 2000), providing detailed data on the timing of each flower choice and the duration of each foraging bout. The choices prior to the first yellow probe gave information on the innate colour preference for individual bees. The strength of blue preference (over yellow) was calculated by the percentage of blue flowers chosen (defined as either approaching or landing on, but not feeding from) before the first yellow probe, and the persistence of blue preference is calculated from the number of flowers visited before the first yellow flower was probed. Flowers were changed, and their positions re-randomized between

foraging bouts, to prevent bees using either scent marks or previous flower positions as predictors of reward.

2.3.4 Curve fitting and analysis

I fitted sigmoidal curves (Boltzman: $y = (A_1 - A_2)/(1 + e^{(x - x_0)/dx}) + A_2$) to assess the choice performance of individual bees in each task using Microcal Origin (see Appendix 2.2 for further details on the curve). The same curve was fitted to behavioural data from both tasks to produce comparable measures of learning performance across modalities. Consistently high r-squared values for fits indicate the curves provided a very good description of the choice data (mean \pm SE: colour $r^2 = 0.895 \pm 0.009$; olfactory $r^2 = 0.957 \pm 0.008$).

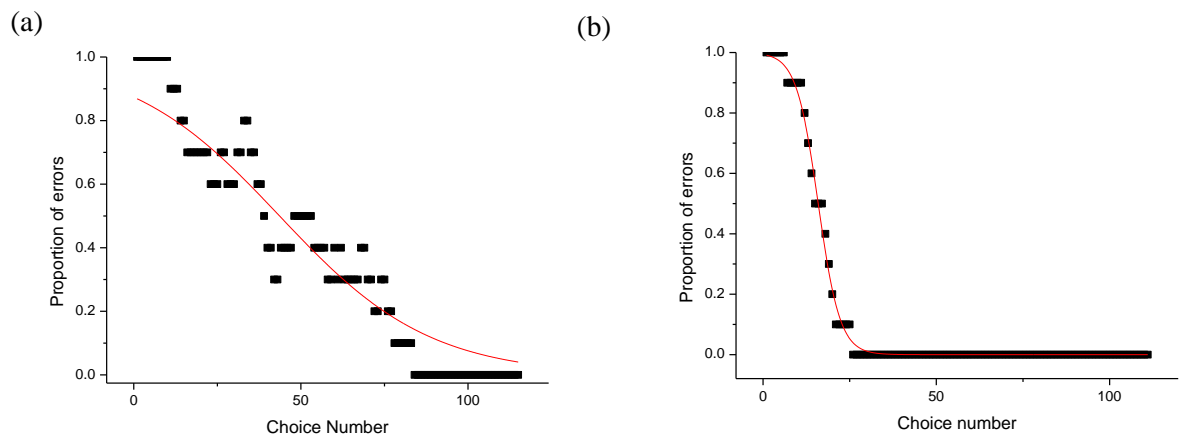


Figure 2.3 Example sigmoidal curve fits to individual choice data in the colour learning task. (a) A slower learning bee (learning score = 2.08, $r^2 = 0.917$) and (b) a faster learning bee (learning score = 0.83, $r^2 = 0.996$). Each data point (black square) represents the proportion of errors made by the bee in the 10 choices up to and including the focal choice. For choices 1-9 the previous choices are all assumed to be errors.

A moving accuracy index was generated for each consecutive flower visit by calculating the number of errors (blue choices) for the sequence of ten choices ending with that focal visit (Leadbeater & Chittka 2007). For choices that were not preceded by 9 visits (i.e. choices 1 to 9) I assumed that all previous visits were to blue flowers (i.e. errors) as all bees initially showed a strong innate preference for blue. If a bee made 10 blue choices it would have a moving accuracy index of 1, and every yellow choice would decrease this value by 0.1. The moving accuracy index (y axis) was plotted against flower choice number (x axis). All flower choices made by each individual bee in the colour learning task were included in their learning

curves (i.e. all choices made prior to the first yellow probe and the subsequent 99 choices). For the olfactory learning each bee made a binary choice at each odour presentation (i.e. responded, or not, prior to the reward associated with the odour) for each of the 15 trials. Again I used a moving accuracy index for each presentation by calculating the number of errors (no response prior to reward) for the 5 responses up to and including the focal response. For choices that were not preceded by 4 responses (i.e. choices 1 to 4) I assumed these would have been errors. Therefore a bee making 5 ‘no responses’ prior to the reward would have a moving accuracy index of 1, and for every consecutive correct choice this would decrease the accuracy index by 0.2.

Given the dynamic nature of the learning process I produced a colour learning score that takes into account both differences in the slope and shape of the fitted curves. For the colour learning task I summed the proportion of errors made by each individual at choice 0 (the first time they probed a yellow flower), 20, 40, 60, 80, 100. I created a comparable odour learning score for the olfactory task by summing the proportion of errors at rewarding presentation 1, 3, 6, 9, 12 and 15. This gives a learning score out of 6 for both the colour and olfactory learning task (see Figure 2.3 for example learning curves with learning scores). Bees that did not learn the olfactory task, so could not have a curve fitted to their performance, were allocated the maximum learning score of 6.

All statistical analyses were performed in IBM SPSS (version 19), apart from the linear mixed effect model comparing the olfactory and colour learning score performed in *R* (Pinheiro *et al.* 2014).

2.4 Results

2.4.1 Odour learning

Bees from all colonies showed improved learning performance over the 15 rewarded olfactory conditioning trials (Figure 2.4), with a higher proportion of bees responding with a proboscis extension prior to reward (showing they had learnt the task) as the trials progressed. I found significant inter-colony variation in the final level of learning performance at the end of the trial (Kruskal-Wallis on proportion of bees responding in trials 14 and 15, $H = 9.211$, $p = 0.027$; Figure 2.4). However, there was no intercolony variation in the proportion of bees that learnt the task, determined to be those bees that responded positively to the conditioned stimulus at least once (Kruskal-Wallis, $H = 6.434$, $p = 0.092$), or the olfactory learning score which we calculated for comparison with the visual learning performance (Kruskal-Wallis: $H = 5.755$, $p = 0.124$). Additionally, I found no significant difference in the total number of presentations to which bees responded positively among colonies (Kruskal-Wallis, $H = 6.519$, $p = 0.089$), however there was appreciable variation in the extent to which individual bees within each colony learnt this task (range = 0-13 learnt responses out of 15; Figure 2.5).

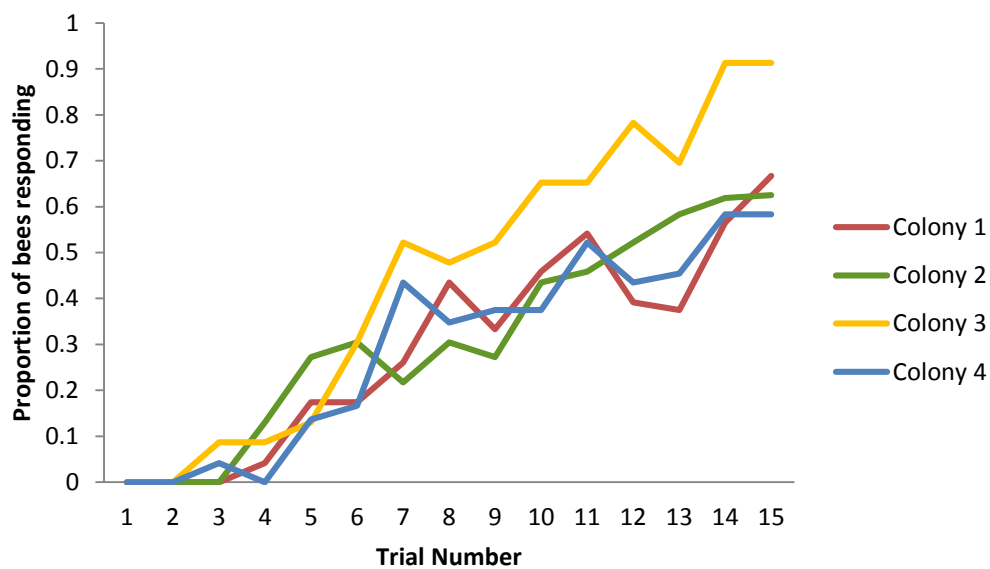


Figure 2.4 Learning curves across the 15 rewarded trials. Curves show the proportion of bees in each colony learning to associate the conditioned odour with reward (determined by a proboscis extension to the rewarding odour prior to reward presentation). Data shown are for all bees tested in this paradigm: colony 1: $n = 24$, colony 2: $n = 24$, colony 3: $n = 23$, colony 4: $n = 24$.

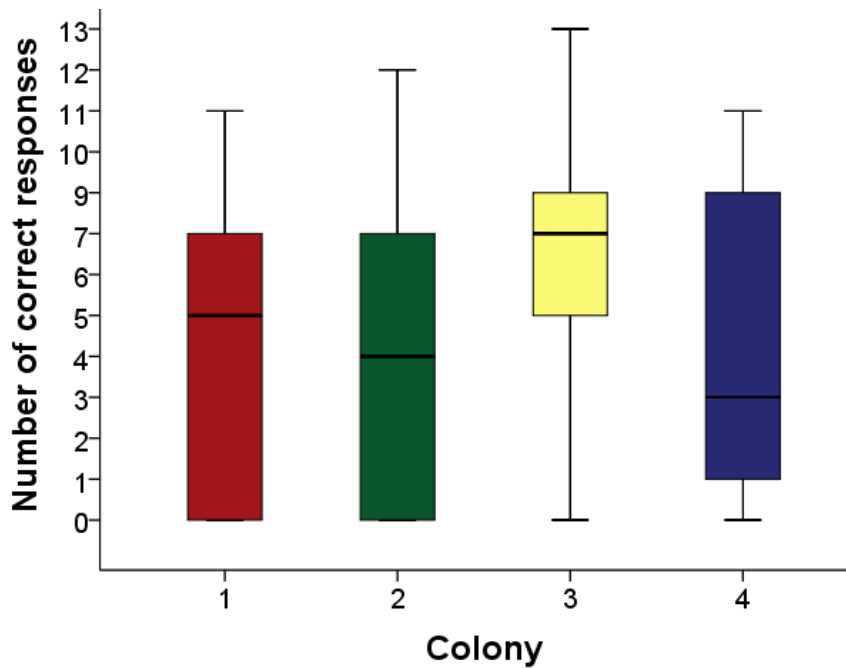


Figure 2.5 Box and whisker plot showing individual variation in the number of correct responses made during the 15 PER conditioning trials within each colony. The thick horizontal bar in each box is the colony median, whilst the lower and upper edges represent the 25% and 75% quartiles respectively. Whiskers indicate maximum and minimum values that are not outliers. Data shown are for all bees tested in this paradigm: colony 1: n = 24, colony 2: n = 24, colony 3: n = 23, colony 4: n = 24.

2.4.2 Colour learning

Bees from all colonies showed an initial preference for blue over yellow before they fed from (probed) a rewarding (yellow) flower for the first time. However, there was significant variation among colonies in both the strength (measured as the percentage of blue flowers chosen before probing a yellow rewarding flower for the first time: Kruskal–Wallis, $H = 14.550$, $p = 0.002$, Table 1A) and persistence of this blue preference (measured as the number of flower choices individual bees made prior to their first yellow probe: Kruskal-Wallis, $H = 14.107$, $p = 0.003$, Table 1B). Interestingly, the strength and persistence of blue preference were not correlated with one another (Spearman’s rank correlation, $\rho = 0.030$, $p = 0.785$), meaning that bees with the strongest blue preference did not always make the most choices before probing a rewarding flower. Similarly, neither strength nor persistence of blue preference were significantly correlated with colour learning score (Spearman’s rank correlation: strength, $\rho = 0.192$, $p = 0.076$; persistence, $\rho = 0.157$, $p = 0.142$).

Table 2.1 Performance of the four colonies in the colour learning task. Data shown are for all bees tested in the visual task: colony 1: n=24, colony 2: n=23, colony 3: n=19, colony 4: n=23.

^aMean (\pm SE) strength of blue preference (the percentage of blue flowers bees chose before probing a yellow, rewarding flower for the first time). ^bMean (\pm SE) persistence of blue preference (the number of choices bees made before they probed their first yellow flower).

^cMean (\pm SE) colour learning score—a measure of learning that takes into account differences in the slope and shape of the curve. ^G Mean (\pm SE) *t* value—a measure of learning performance used in previous studies (see Raine & Chittka 2008 for details).

Colony	Strength of blue preference (%) ^a	Persistence of blue preference ^b	Colour learning score ^c	Median colour learning score	Minimum colour learning score	Maximum colour learning score	<i>t</i> value ^G
1	74.15 \pm 3.23	32.15 \pm 5.19	1.22 \pm 0.07	1.18667	0.764317	2.00871	23.38 \pm 2.75
2	90.67 \pm 2.30	75.26 \pm 14.26	1.33 \pm 0.08	1.31097	0.831307	2.61239	19.03 \pm 3.11
3	86.38 \pm 2.78	41.53 \pm 4.85	1.45 \pm 0.10	1.50868	0.743053	2.16425	22.81 \pm 3.07
4	81.30 \pm 3.16	26.96 \pm 5.82	1.31 \pm 0.07	1.24622	0.885828	2.16441	24.25 \pm 3.55

I found considerable variation in the learning ability of individual bees in the colour learning task, that made between 1 and 24 mistakes (blue choices) after they probed their first yellow flower (Figure 2.3 shows example learning curves for one of the worst (Figure 2.3a) and best (Figure 2.3b) learning bees with learning scores of 2.082 and 0.833 respectively). I found no significant inter-colony variation in colour learning performance using colour learning score as a measure of learning performance (Kruskal–Wallis, $H = 3.247$, $p = 0.355$: Table 2.1). I repeated my analysis of inter-colony variation using individual *t* values calculated for each bee. The *t* value is an established measure of learning performance calculated from the slope of an exponential decay curve fitted to the flower choice data for each individual bee (this analysis has been used in numerous previous studies, for example, see Raine *et al* 2006; Raine & Chittka 2008; Ings, Raine & Chittka. 2009; Raine & Chittka 2012 for additional methodological details). These analyses revealed no significant intercolony variation in colour learning performance using the *t* value as a measure of task performance (Kruskal-Wallis, $H = 3.614$, $p = 0.306$: Table 2.1D). In addition, I found a strong correlation between the colour

learning score and t value for individual bees (Spearman's rank correlation, $\rho = 0.573$, $p < 0.001$) indicating both are robust and comparable measures of learning performance.

2.4.3 Comparison of learning in two modalities

The results showed no correlation between individual performance in the colour and odour learning tasks in any of the four colonies (Figure 2.6: Spearman's rank correlation: colony 1, $\rho = 0.181$, $p = 0.420$; colony 2, $\rho = 0.034$, $p = 0.889$; colony 3, $\rho = 0.004$, $p = 0.989$; colony 4, $\rho = 0.157$, $p = 0.548$), although there was considerable variation in the learning scores among individuals within each colony, especially for the olfactory task (range, olfactory: 1.97–6; colour: 0.74–2.61, Figure 2.6). Considering the performance of all 76 bees tested there was still no relationship between performance in the odour and colour task, taking into account variation in worker body size (thorax width), the order in which bees completed the task (odour or colour learning first) and including colony as a random factor (Linear mixed effects model: $T_{73} = 3.025$ $p = 0.816$)

Whilst there was significant variation in worker body size among colonies (Kruskal–Wallis: $H = 8.073$, $p = 0.045$), I found size had no effect on learning performance in either modality (Partial correlation, colony 1: $\rho = 0.047$, $p = 0.844$; colony 2: $\rho = 0.040$, $p = 0.876$; colony 3: $\rho = 0.096$, $p = 0.734$; colony 4: $\rho = 0.164$, $p = 0.559$). There was no effect of the order in which bees completed the task on their learning performance in either modality (comparison of learning performance between bees that completed the test first or second; olfactory: 36 bees completed first, ANOVA, $F_{35} = 0.599$, $p = 0.442$; visual: 40 bees completed first, ANOVA, $F_{39} = 0.503$, $p = 0.480$). Age effects could be assessed for 50 (of the 76) tested bees, the others eclosed before colonies arrived in the laboratory. I found no significant difference in the age when bees were tested among colonies in either the colour or odour test respectively (Kruskal–Wallis: $H = 6.092$, $p = 0.107$; $H = 3.186$, $p = 0.364$), and there was no significant relationship between age and learning performance in either modality (Spearman's rank correlation, olfactory: $\rho = 0.031$, $p = 0.831$; visual: $\rho = 0.107$, $p = 0.460$).

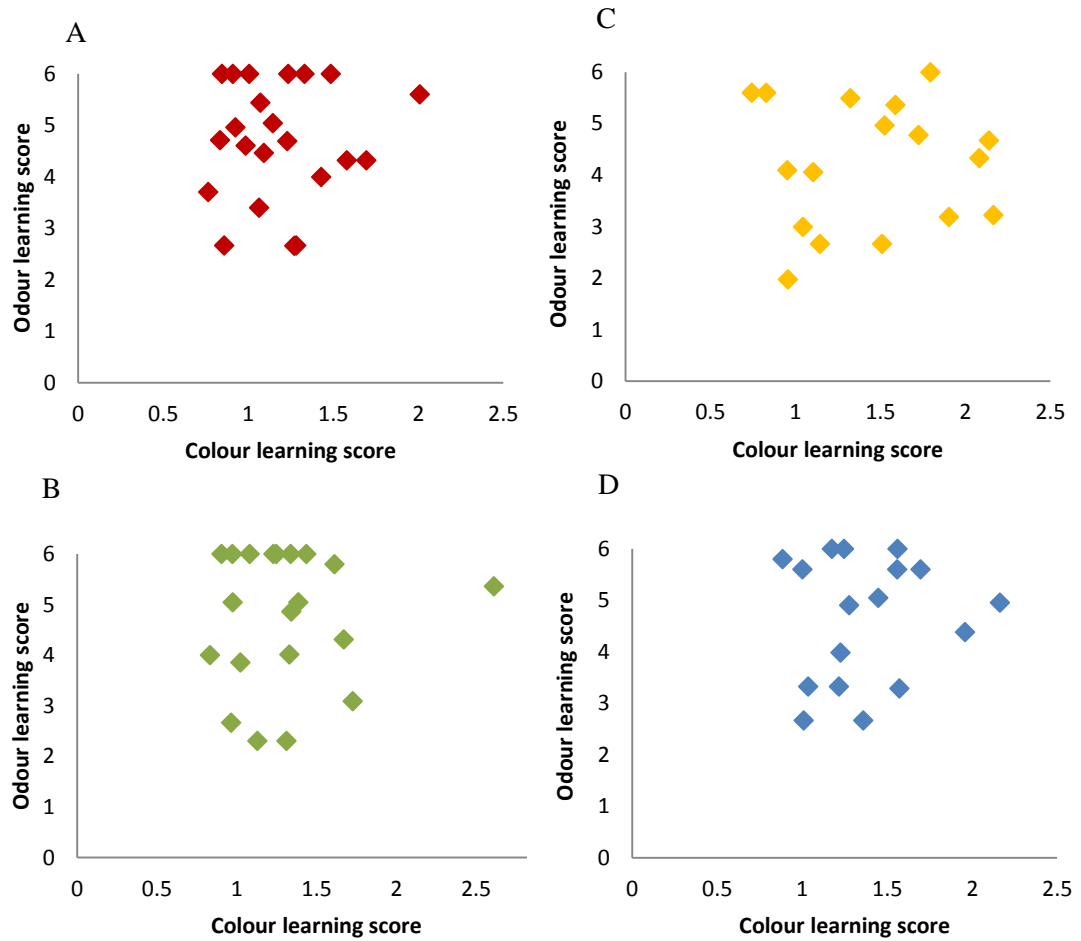


Figure 2.6 Scatter graphs showing the colour and odour learning scores for all workers tested (A-D = colonies 1-4 respectively). Data shown are for all bees that completed both the olfactory and visual task: colony 1: n = 22, colony 2: n = 20, colony 3: n = 17, colony 4: n = 17.

2.5 Discussion

The results from this chapter show no evidence to suggest either a correlation, or a trade-off, in individual performance for learning tasks using different sensory modalities. However, I found there was considerable variation among workers in each colony in their performance in both the colour and odour learning task. Additionally, I found significant variation among colonies in some traits: such as final level of performance in the olfactory learning task, and both strength and persistence of blue preference in the colour task. In this chapter, I demonstrate for the first time variation in olfactory learning performance across multiple bumblebee colonies using the PER paradigm. The response levels and learning rates in this study are the best reported to date for *B. terrestris* and demonstrate that PER conditioning is

a robust and effective paradigm for assessing associative olfactory learning performance both within and among colonies for this bumblebee species.

Results from previous studies using PER with bumblebees have indicated that there may be interspecific differences in learning performance, with *B. occidentalis* achieving higher levels of task performance (85.6% of individuals showing at least one learnt response and an asymptote of 71% at end of learning task: (Riveros & Gronenberg 2009)) than *B. terrestris* (64.8% of individuals showing at least one learnt response and an asymptote performance of ca. 30-60% at end of learning task: (Laloi *et al.* 1999)). However, I found that learning and acquisition rates were considerably higher than previous studies using *B. terrestris* and more comparable to those of *B. occidentalis*. Performing 15 conditioning trials, rather than 10 used by Laloi *et al.* (1999), may have been a contributing factor for the improved success, potentially indicating the need for a greater number of conditioning trials for *B. terrestris* to learn effectively. However, a more recent study using *B. terrestris* reported acquisition rates of ca. 60% after 10 conditioning trials (Sommerlandt, Rossler & Spaethe 2014), although they report a much higher dropout rate of unresponsive bees (70% vs. 21% in this study). Results from this chapter and Sommerlandt, Rossler & Spaethe (2014) indicate that using a harnessing procedure modified from Riveros & Gronenberg (2009), appears to be a key factor in improving the response rates for *B. terrestris*. Although levels of acquisition achieved in odour learning trials have improved significantly since this revised harnessing technique was first reported in bumblebees (Riveros & Gronenberg 2009), they are still lower than in honeybee studies (which regularly report ca. 90% of individuals learning the task). While I found that average colony performance (mean \pm SE = 69.69 \pm 7.40%) was broadly comparable to that found in earlier studies investigating associative odour learning in bumblebees, one of the colonies reached performance levels comparable to honeybees (colony 3 = 91.30%: Figure 2.4). However, it is noteworthy that honeybees are usually trained with shorter odour presentations and will typically learn after fewer odour-reward exposures (e.g. Giurfa & Sandoz 2012).

In the colour learning task individual bees varied appreciably in their learning performance and I found significant intercolony variation in both the strength and persistence of the innate preference for blue prior to the first time bees probed a rewarding (yellow) flower. These results are consistent with previous studies that assessed variation across at least 6 *B. terrestris* colonies (Raine *et al.* 2006b; Raine & Chittka 2008; Ings, Raine & Chittka 2009; Raine & Chittka 2012). However, I found no significant intercolony differences in learning performance using the learning scores generated from sigmoidal curves fitted to the data. This contrasts with previous studies that report significant intercolony variation in colour learning

performance when comparing the decay constant (t value) from exponential decay curve fits to learning data from individual bees (Raine *et al.* 2006b; Raine & Chittka 2008; Ings, Raine & Chittka 2009; Raine & Chittka 2012). When comparing both methods of curve fitting to the learning data, I found a strong correlation in learning performance measures between the sigmoidal curve (colour learning score) and the exponential decay curve (t value) fits to the choice data for individual bees. This consistency between curve fitting methods indicates that the lack of intercolony variation in learning performance observed in this study is a genuine result, which could be the result of comparing across fewer colonies than earlier studies (that all report comparisons for at least 6 colonies). Sigmoidal curves fits were chosen for this study as they could be fitted in a comparable manner to both colour and odour learning data from the same individual bees.

When I compared the learning performance of individuals between modalities I found no clear relationship between performance in the olfactory and visual learning tasks. Bees that were good at learning to associate cues with reward in one modality were not consistently good at learning in the other modality. Furthermore, there was not a consistent trade off in learning performance between modalities. These results are intriguing but I do not believe the patterns observed are because we tested learning tasks in each modality using a different paradigm. Whilst being harnessed for PER conditioning may have been more stressful for participating bees, I found no evidence that individuals tested in this paradigm first performed any differently when faced with the colour learning task (when compared to bees that experienced the tasks in the reverse order). Furthermore, I ensured that all bees were motivated to participate, shown either through repeated and consistent foraging on bicoloured flowers in the colour learning task or their responsiveness to sucrose solution prior to testing in the odour learning task. While the presentation of colour cues can be precisely controlled for bees flying freely in a flight arena, this is not the case for odour cues as they begin to diffuse and mix immediately following application. In contrast, PER conditioning provides precise control over the timing and duration of odour presentation and avoids unintended odour mixing. Results published since I completed the experimental work for this chapter, indicate it is now possible to study visual learning using the PER protocol for intact harnessed bumblebees (Riveros & Gronenberg 2012), which opens up interesting avenues for future investigation.

Clearly some bees were good at learning irrespective of modality, others were poor learners in both modalities, and others were good at one and poor at the other. The data are more consistent with the view that individual bees within a colony use different learning strategies when making foraging decisions. Some individuals may be more sensitive to differences in olfactory cues when assessing and learning the reward value of flowers, while other bees are

more attuned to variation in colour cues. A mix of individual behavioural strategies may actually be beneficial for the performance of the colony in environments with reward distributions that vary appreciably in either space, time or both (Burns & Dyer 2008; Jandt *et al.* 2014). This appears to be case for variation in decision speed and accuracy among individual foragers in honeybee colonies (Burns & Dyer 2008), so similar pressures could also generate and maintain variation in learning ability in a range of sensory modalities in bumblebee colonies. Variation in individual learning phenotypes (across sensory modalities) could allow a colony to exploit a heterogeneous environment, such as the range of flower species in bloom in different parts of the colony flight range or at different times during the season, more effectively. For some of these flower species odour cues may be more reliable predictors of reward, whilst in other species visual cues may provide better indicators, so a mix of learning phenotypes could ensure minimum variation in foraging performance over time (Burns & Dyer 2008; Chittka, Skorupski & Raine 2009). Currently very little is known about how the foraging performance of individual bees is linked to their learning ability and is something I investigate in chapter 4, or whether bees pay more attention to the floral cue modality in which they learn best. These would be interesting areas for future research.

Another potential explanation for the high levels of variation I saw among individuals within each colony could be differences in the resources they collect (i.e. pollen, nectar or both). As colonies were provided with *ad libitum* pollen into the nest, I do not know whether individual foragers would have collected pollen, nectar or both if they had a choice of resources in the flight arena. Pollen foraging honeybees show lower response thresholds to sucrose and also perform better in olfactory learning tasks than nectar foragers (Scheiner, Page & Erber 2001a; Scheiner, Page & Erber 2001b), suggesting that foraging specialisation could influence learning performance. Although the resources (pollen or nectar) collected by bumblebees (*B. impatiens*) on their first day of foraging significantly predicts their lifetime preference, individuals can also demonstrate flexibility and are able to switch from nectar to pollen collection when pollen foragers are removed (Hagbery & Nieh 2012). In chapter 3, I investigate whether learning performance may predict task specialisation in bumblebees and these data could help explain the variation in learning ability observed in this chapter.

In a recent paper, Muller & Chittka (2012) investigated the discrimination performance of individuals bumblebees from two colonies faced with a colour, a shape and an odour learning task in a flight arena. Intriguingly their results indicate consistent individual discrimination performance in these three tasks, i.e. bees that were good at discriminating colours, were also good at discriminating shapes and odours. There are several differences in experimental design between their study and the work presented in this chapter, which could explain the

contrasting results. Firstly, Muller and Chittka (2012) trained their bees using floral arrays in which the unrewarding flowers were penalised with quinine hemisulphate (a stimulus bumblebees find highly aversive: Chittka *et al.* 2003) that will influence the dynamics of the learning process. Secondly, presenting odours in a flight arena gives limited control over the quantity or duration of odour presentations, and cannot prevent odour mixing, compared to computer controlled PER conditioning. Thirdly, testing bees with two visual learning tasks (colour and shape), and the order in which each task was presented, could affect learning dynamics. More than half the bees Muller and Chittka (2012) tested completed at least one visual discrimination task before undertaking the odour discrimination, although they report no effect of task order. Fourthly, the contrasting patterns of results could be explained by different approaches to assessing learning performance in the two experiments: while Muller and Chittka (2012) used individual saturation performance as an index of learning, our learning score took into account flower choice information across the entire learning process. Another intriguing possibility is that differences between Muller and Chittka's (2012) results and those in this chapter could indicate that some colonies have individual foragers with consistent learning performance while others do not. If this is true then testing four colonies, rather than two, makes it less likely all would contain such consistent individuals. However, it would be interesting to conduct future experiments to resolve whether variation in experimental designs explains these differences.

Overall, the results in this chapter indicate that individual bees show no consistency in their ability to perform tasks testing learning in different sensory modalities. However, the appreciable variation in learning phenotypes observed in all colonies we tested could be beneficial to colonies foraging in changeable environments. Examining how variation in learning ability in different sensory modalities may link to individual foraging success and/or task specialisation within the colony will help us gain a better understanding of the adaptive value of individual and colony level variation in learning performance in a range of environmental conditions.

Chapter 3

The association between foraging preferences, sucrose responsiveness and olfactory learning performance

3. The association between foraging preferences, sucrose responsiveness and olfactory learning performance

3.1 Abstract

Animals living in eusocial groups show division of labour. Bumblebee colonies need to forage for both pollen and nectar to survive. Evidence for why some bumblebees show foraging preferences, to either collect pollen or nectar, is relatively unknown in comparison to honeybees. There is considerable variation in individual learning performance within colonies; the tasks they choose to perform could be associated with this variation. In this chapter, I first observed foraging bumblebees (*Bombus terrestris*) preferences for pollen and nectar. This was done under changeable provisions of pollen and sucrose, that colonies would be likely to experience in the wild, to assess differences in individual foraging flexibility. Bees then had their sucrose responsiveness threshold (SRT) and olfactory learning ability tested, using the proboscis extension reflex (PER) paradigm. I found that there was considerable variation in foraging preferences of individual bumblebees; 23% foraged only for pollen; 30% only for sucrose and the remaining bees showed a flexible preference, to forage for both pollen and sucrose depending on demand. In contrast to findings from honeybees, there was no difference in the SRT between the nectar and pollen foragers. This difference could be due to bumblebees being more sensitive to sucrose rewards when foraging. However, the flexible bees had a higher sucrose response threshold, which could be explained by them being more sensitive to changes in resource levels of pollen and sucrose in the colony, rather than the absolute levels of reward. Both pollen and flexible foragers showed better olfactory learning performance than nectar foragers, which might have an adaptive function, assuming learning to collect pollen is more challenging than learning to collect sucrose.

3.2 Introduction

Eusocial bees live as a single colony unit and divide the tasks among individuals within the colony. There are a number of tasks that need to be completed for their successful survival, ranging from within-nest tasks, such as feeding and caring for brood or nest thermoregulation, to foraging for nectar and pollen to provision the colony. Honeybee division of labour has been studied in detail, and is thought to be driven by the ‘reproductive groundplan’ (Page *et al.* 2006). When honeybees begin foraging, at around 3 weeks old, their ovary development determines whether they forage for pollen or nectar; bees with more developed ovaries forage

for pollen. Furthermore, this is correlated with learning performance; honeybee pollen foragers learn quicker, and their final level of performance is higher in both tactile and olfactory tasks (Scheiner, Page & Erber 2001a; Scheiner, Page & Erber 2001b), compared to nectar foragers. In addition, there is also a positive correlation between sucrose responsiveness threshold (SRT) and learning ability, where pollen foragers have a lower threshold for sucrose compared to nectar foragers (Scheiner, Erber & Page 1999). The same correlation has been shown in ants, with foragers having a lower SRT and better learning ability (i.e. higher accuracy), compared to nest workers (Perez *et al.* 2013). Whether bumblebees show the same link between division of labour and learning performance has yet to be investigated.

Bumblebees differ considerably from other bee species, and therefore we cannot assume that the same process would drive variation in learning performance. Both honeybees and stingless bees show age polytheism, whereby as they age they complete a predictable set of tasks, starting with within-nest roles, and finally becoming foragers (Seeley 1982). Bumblebees do not show age polytheism, as some workers will never become foragers (Jandt & Dornhaus 2009). However, unlike honeybees and stingless bees, not all worker bees are the same size, and there can be up to 10-fold mass difference among workers within a colony (Jandt & Dornhaus 2009). Size difference has been linked to the division of tasks individual bees will perform (Brian 1952; Free 1955; Goulson *et al.* 2002; Jandt & Dornhaus 2009), with smaller bees more likely to perform within-nest tasks, and larger bees more likely to act as foragers. Larger bees have been shown to collect nectar at a higher rate (Goulson *et al.* 2002; Spaethe & Weidenmüller 2002), and the visual detection and resolution of larger bees (4.7mm thorax width) is significantly better than smaller bees (3.5mm thorax width) (Spaethe & Chittka 2003). Honeybee colonies are also much larger than bumblebee colonies; therefore honeybee colonies have the capacity for individual workers activity to be less important. Whereas, all bumblebee workers need to have a role, and foragers will forage for their whole lives, compared to around 7 days in honeybees (Dukas & Visscher 1994). The waggle dance allows honeybee foragers to alert and recruit other workers to a profitable food source (Frisch 1967), while individual bumblebees must rely on their own individual learning ability to find food rewards from flowers in the landscape.

The handful of studies that have investigated foraging specialisations in bumblebees, report dramatically different degrees of specialisation by individuals, in terms of nectar and pollen collection (O'Donnell, Reichardt & Foster 2000; Hagbery & Nieh 2012; Konzmann & Lunau 2014). The longest of these studies (over 100 days) found that what an individual (*B. impatiens*) collected on their first day of foraging, broadly predicted their lifetime foraging preference (Hagbery & Nieh 2012). In the two colonies observed, 16% and 36% of individuals

specialised on pollen or nectar only collection. In comparison, three *B. bifarius* colonies, observed over a 42 day period (4 – 5 days per week), had between 27 and 42% of individuals specialising on pollen or nectar collection (O’Donnell 2000). Finally, no specialists were found in one *B. terrestris* colony, where individuals were observed for 21 - 41 days (Konzmann & Lunau 2014). These studies were observed on different bumblebee species, over different timescales and developmental stages, which could explain some of the variation in results. In addition, they used different criteria to classify bees as ‘specialists’. None of these studies have investigated why certain individuals show these specialisations, apart from their differences in size, which show inconsistent results.

The above studies did not manipulate the provision of sucrose and pollen, and in the field bumblebee colonies will experience considerable changes in supply and demand for resources. Firstly, their annual lifecycle means they experience an initial period of growth, followed by a population decline once the queen dies, meaning that pollen demand initially increases and then declines. Hagbery & Nieh (2012) found evidence for this with a decrease in pollen foraging during their experiment. Secondly, there could be adverse weather conditions, which may mean foragers are lost or resources may be more or less available at certain times, and the colony needs to adapt to this. There is evidence for bumblebee colonies being flexible when resource levels are depleted, by increasing or decreasing the number of foraging bees (Cartar 1992), the number of foraging bouts per bee (Cartar 1992; Pelletier & McNeil 2004) or the levels of pollen collection per bout (Plowright *et al.* 1993). Colonies have additionally been shown to send out more foragers when the colony is under stressful conditions (e.g. pesticide exposure), as more foragers are needed because individuals are returning with less pollen after each foraging bout (Gill, Ramos-Rodriguez & Raine 2012). It has also been shown that when pollen foragers are removed, the remaining individuals will switch task to pollen collection (Hagbery & Nieh 2012).

There is considerable variation in learning performance between individual bumblebees (Raine *et al.* 2006b; Raine & Chittka 2008; Raine & Chittka 2012), and there is evidence to suggest enhanced learning performance comes with costs (Mery & Kawecki 2003; Mery & Kawecki 2004; Mery & Kawecki 2005). It therefore might not make adaptive sense for resources to be allocated to learning ability in all individuals. Foraging role could be a potential explanation for this, and recent work on *Bombus huntii* has indicated that bees that perform within-nest tasks (nurse bees) are better at olfactory learning (i.e. learning associations between odour as a predictor of food reward), than foragers (Hannaford *et al.* 2013). Whether there are differences in learning performance, depending on individual foraging preferences in bumblebee foragers, has not been studied. In this chapter, I observed

the foraging preferences of individual bumblebees, during changeable conditions of pollen and sucrose availability inside the nest, as would be experienced in the wild. Therefore, the foraging preference I observed for each bee, was representative of what their overall foraging preference over time would be. After the foraging period, I tested the sucrose responsiveness and olfactory learning performance of all foraging bees. I was interested to understand whether individual variation in foraging preferences, would be associated with sucrose responsiveness or olfactory learning performance. Additionally, if pollen collection efficiency was associated with pollen foraging preference. I hypothesised that if I found clear foraging preferences for pollen and nectar, as is found in honeybees that similarly learning and sucrose responsiveness would be correlated and would be determined by the bees' foraging role.

3.3 Methods

Two bumblebee (*B. terrestris audax*) colonies were obtained from Biobest (Westerlo, Belgium), each containing a queen and either 54 (colony 1) or 34 (colony 2) workers on arrival. Colonies were each transferred to bipartite wooden nest-boxes with transparent plastic lids. All workers present were uniquely marked on the thorax, with numbered tags (Opalith tags; Christian Graze KG, Germany), so that individuals could be unambiguously identified. All subsequent bees were marked upon eclosion, so that potential age effects could be assessed. Over the 28 days of the experiment, colony 1 grew from 79 to 123 workers and colony 2 from 51 to 101 workers. After the experiment ended, all foragers had their thorax widths measured as a proxy for body size.

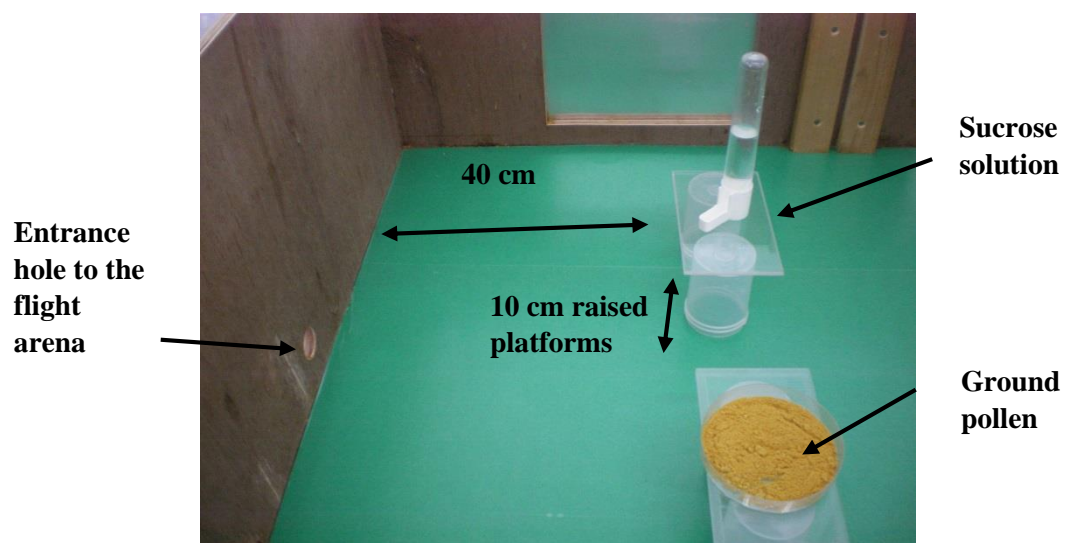


Figure 3.1 Photograph of the experimental set up in the flight arena.

3.3.1 Assessing foraging preference

The colony nest-boxes were attached to flight arenas (120 x 100 x 35 cm) by a transparent Perspex tubes. Pollen and sucrose were presented on separate raised platforms in the flight arena (Figure 3.1), to ensure that bees had to fly and make a definite choice to collect each resource. Frozen honeybee-collected pollen (Koppert Biological systems: Weert, The Netherlands) was ground using a coffee grinder (Wahl mini grinder, ZX595), until it was a fine powder, and approximately 5 grams was presented in a 89mm petri dish for bees to collect. During pilot experiments, I found that the pollen became unattractive and difficult for the bees to collect after around 10 minutes. Therefore, pollen was changed every 10 minutes. Pollen could not be weighed afterwards to estimate amount collected, as pollen was scattered out of the dish during collection, and so measurements would have been inaccurate. Sucrose (40% v/v) was presented in a gravity feeder, and was weighed before and after each session to give the mass collected. On the first day of the experiment, the colonies were allowed access to the arena for the whole day, where they could collect pollen and sucrose *ad libitum*. After this initial day, foragers from the colonies were allowed into the flight arena for 3 hours per day, in two 1.5 hour sessions over an 8-day period. As both colonies could not be observed simultaneously, they were each observed in a morning session (either 8.30am – 10.00am or 10.15am – 11.45am) and an afternoon session (either 12.30pm – 2.00pm or 2.15pm – 3.45pm). Whether colonies were observed in the first or second slot within each session alternated on a daily basis. I recorded the resource type collected in each foraging trip, the size of the pollen loads (visually classified as small, medium or large, see Appendix 3.1 for estimated weights) and the time bees spent in the flight arena on each foraging bout (to the nearest 10 s).

I carried out the experiment under three different food storage scenarios: 1. P - Pollen limited and abundant sucrose stores (sucrose: 0.45g x number of worker in the colony; pollen: none); 2. S - Sucrose limited and pollen in excess (sucrose: 0.05g x number of bees in the colony; pollen 0.45g x bees in the colony); and 3. C - Control amount of sucrose and pollen given (0.15g x number of bees in the colony for both pollen and sucrose). Food stores were provided at the end of the day, and I measured its effect the next day by observing how the bees foraged. The sequence of provision types given is shown in Table 3.1. After the 8 days of observation, all bees that had been observed foraging on at least 2 of the days, were removed from the colonies to test both their sucrose responsiveness and olfactory learning performance. Then another 8 days of observations began, and I repeated this for 3 cohorts of bees. A total of 60 bees had their foraging preference measured (colony 1 = 35, colony 2 = 25), however one bee

died from colony 2 prior to harnessing, and could not have its SRT and learning performance tested, a further 8 bees were excluded due to unresponsiveness (for details see below).

Table 3.1 The provision types were given in pairs, each colony always started with 2 control days (C1) and then two days of either P (pollen limited) or S (sucrose limited), then 2 more control days (C2) and then two days of P (pollen limited) or S (sucrose limited). The number of bees foraging on each day is given in brackets below each provision type.

<i>Colony</i>	<i>Cohort</i>	<i>Day 1</i>	<i>Day 2</i>	<i>Day 3</i>	<i>Day 4</i>	<i>Day 5</i>	<i>Day 6</i>	<i>Day 7</i>	<i>Day 8</i>
<i>1</i>	1	C (n = 4)	C (n = 8)	P (n = 8)	P (n = 8)	C (n = 7)	C (n = 9)	S (n = 7)	S (n = 5)
	2	C (n = 4)	C (n = 9)	S (n = 9)	S (n = 9)	C (n = 9)	C (n = 9)	P (n = 9)	P (n = 10)
	3	C (n = 10)	C (n = 11)	P (n = 15)	P (n = 16)	C (n = 14)	C (n = 13)	S (n = 11)	S (n = 9)
<i>2</i>	1	C (n = 9)	C (n = 9)	S (n = 9)	S (n = 9)	C (n = 9)	C (n = 10)	P (n = 10)	P (n = 9)
	2	C (n = 2)	C (n = 1)	P (n = 5)	P (n = 6)	C (n = 6)	C (n = 6)	S (n = 5)	S (n = 4)
	3	C (n = 3)	C (n = 5)	S (n = 7)	S (n = 6)	C (n = 6)	C (n = 6)	P (n = 8)	P (n = 7)

3.3.2 Assessing learning performance and sucrose responsiveness

I used olfactory PER to test the learning ability of the bees. This allowed me to test bees that foraged for pollen and nectar, as other free flight tasks require bees to forage for nectar. This task also allowed me to take all foragers out of the colony, and test at the same time for each cohort. In addition, this method also allowed me to test the bee's sucrose responsiveness prior to conditioning, as they were in a harness for the learning task, and this is how SRT has been tested previously in honeybees.

Individual bees were harnessed using methods described in section 2.3.2. The morning after harnessing (ca. 08.30), bees had their sucrose responsiveness threshold (SRT) tested. The bee's antenna was touched with increasing concentrations of sucrose to determine their threshold for sucrose, by examining at what concentration they started to respond and extend their proboscis. The concentrations of sucrose used were water, 1%, 3%, 5%, 8%, 10%, 15%,

20%, 25%, 30% and 50%, for each concentration I recorded whether this elicited a proboscis extension, or not. These were presented to the bees with a 2 minute gap between presentations. A small droplet of the final concentration (50%) was fed to the bees to increase motivation for the olfactory task, which started 15 minutes later. This time delay is comparable to honeybee sucrose responsiveness testing, where olfactory conditioning starts directly afterwards (Scheiner et al 2001a & b), and why I chose to do the testing in this way and not do the responsiveness testing at another time (e.g. following harnessing).

The bees were conditioned in the same set up as described in section 2.3.2, with the only difference being that bees were trained using only one odour (absolute conditioning), instead of two (differential conditioning) as used in Chapter 2. The odour used was lemon essential oil (Calmer solutions), which was presented to the bee 15 times, with an inter trial interval (ITI) of 12 minutes. All odour presentations were rewarded with 0.8µl of 50% sucrose solution (v/v). For each odour presentation, I recorded whether the bee responded prior to reward delivery, with a proboscis extension or not, giving a binary response measure. Eight out of the 59 bees did not extend their proboscis when their antenna was touched on more than 3/15 of the conditioning trials, these bees were excluded from the experiment and classed as insufficiently responsive to participate in the task.

3.3.3 Analysis

To analyse changes in the number of foraging bouts performed over the four provision types (C1, S, C2 & P), I used the proportion of sucrose bouts performed as the response variable. The control provision was given to the colonies for two periods in each cohort (Table 3.1; C1 and C2); and I decided to include these as two distinct provision types. If a bee collected both pollen and nectar during the same foraging bout, this was counted as one pollen bout and one nectar bout. A generalised linear mixed model (GLMM) was used to test for differences in the proportion of sucrose bouts over the four provision types, using the *Glmer* function in *lme4* package (Bates *et al.* 2014), and assuming binomial distribution. Colony, cohort, and bee were included as random effects and provision type as a fixed factor. Paired t tests were used to compare individual's pollen collection, which foraged for pollen in both the S and P provision types.

To show how the provision types affected individuals foraging response, I categorised bees in 8 groups of how they could respond to the changes. Due to the sample sizes being too low to

perform statistical analysis, this data is just included for visual comparison. The first three groups are for bees which did not change their foraging in response to provision changes:

Category 1. Only forage on sucrose - always at same rate do not take provision into account.

Category 2. Only forage on pollen - always at same rate do not take provision into account.

Category 3. Forage on both pollen and sucrose – do not take provision into account.

The second three groups are for bees that did change their foraging in response to the provision changes but this did not change what resource they foraged for:

Category 4. Only forage on sucrose - increase/ decrease foraging trips depending on demand.

Category 5. Only forage on pollen – increase/ decrease foraging trips depending on demand.

Category 6. Forage on sucrose and pollen - increase/ decrease foraging trips depending on the resource in demand.

The final two groups are for bees that did change their foraging in response to the provision changes, but unlike the other groups switched to additionally forage for the resource in demand:

Category 7. Forage on pollen in control provisions – additionally foraged for sucrose when in demand.

Category 8. Forage on sucrose in control provisions – additionally foraged for pollen when in demand.

To analyse each bee's performance in the SRT and PER, the number of responses to each in the series of sucrose concentrations (gave a score out of 11) and the number of correct PER responses (gave a score out of 15), were used respectively as the response variables. Bees were split into three groups, based on what they foraged for (pollen only, nectar only or both (flexible)), and this was included as a fixed factor in the model. Pollen foragers were classed as bees that foraged predominantly for pollen and performed no more than 2 sucrose collection bouts. Flexible foragers were classed as bees that foraged for both pollen and sucrose, performing at least 3 bouts of each type of foraging. Nectar foragers were classed as bees that foraged predominantly for sucrose and performed 2 or fewer pollen bouts. To compare provision and forager types within each model pairwise post hoc comparisons were used, to perform multiple comparisons using the `glht` function from the `multcomp` package (Hothorn, Bretz & Westfall 2008).

A pollen collection rate (mg / min) was calculated for each foraging bout. The estimated pollen load size collected (mg; see Appendix 3.1) was divided by the time spent in the arena (min) for that foraging bout. A linear mixed effects model, using the `lme` function from the `nlme` package (Pinheiro *et al.* 2014), was used to analyse the average pollen collection rate response

variable as these are not count data. Colony and cohort were included as random effects. Forager type (pollen only and flexible), learning performance (PER score), number of bouts performed (measure of experience) and bee body size were included as predictors. Paired t tests were used to compare individual's pollen collection rate improvement over bouts. All analyses were performed in R version 3.1.0 (R Core Team 2014).

3.4 Results

3.4.1 Colony response to provision changes

A total of 4704 sucrose foraging bouts and 706 pollen foraging bouts were observed from 60 bees, over the 24 observation days. Bees spent on average nearly 6 times longer in the flight arena when collecting pollen (mean \pm SE: 13.69 \pm 0.54 minutes), compared to sucrose (mean \pm SE: 2.66 \pm 0.03 minutes). Bees responded to the provisioning changes by increasing the proportion of sucrose bouts they performed, in comparison to the pollen in demand provision type (P) (GLMM: S. $Z = 12.57$, $p < 0.001$; C1. $Z = 7.44$, $p < 0.001$; C2. $Z = 8.33$, $p < 0.001$, Figure 3.2).

I found that the average pollen load size collected was larger when pollen was in demand (P), compared to when it provisioned in abundance (S) (Paired t test of bees that foraged in both provision types $t = -2.354$, $p = 0.0317$, $n = 19$, Table 3.2). Therefore, as well as bees increasing the number of bouts performed, bees increased the amount they collected per foraging bout when pollen was in demand. However, the average pollen collection rate was not greater when pollen was in demand (P) (Table 3.2).

Table 3.2 Summary of the pollen foraging during each provision type. C1 = control 1, P = pollen limited and sucrose in abundance C2 = control 2, S = sucrose limited and pollen in abundance. Data presented are mean \pm SE.

	Provision type			
	C1	C2	P	S
Total number of foraging bees	46	56	59	48
Number of bees foraging for pollen	24	33	37	19
Mean pollen load size (mg)	15.10 \pm 1.37	15.17 \pm 0.95	16.83 \pm 1.11	13.66 \pm 1.19
Mean bout time (min)	11.63 \pm 1.12	10.15 \pm 1.19	15.57 \pm 1.10	13.56 \pm 1.56
Mean collection rate	1.50 \pm 0.14	1.56 \pm 0.14	1.33 \pm 0.10	1.49 \pm 0.11

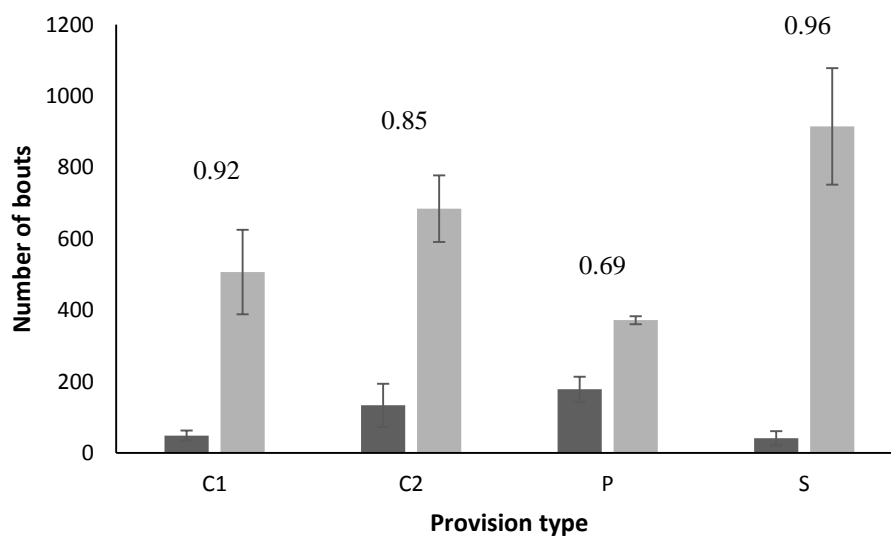


Figure 3.2 Mean (\pm SE) number of bouts performed by the foraging bees from each colony in the four provision types. Number of pollen bouts (dark grey bars) and number of sucrose bouts (light grey bars). C1 = control 1, P = pollen limited and sucrose in abundance C2 = control 2, S = sucrose limited and pollen in abundance. Proportion of sucrose bouts given above each provision type.

3.4.2 Individual responses to provision changes

I found differences in the foraging preferences of individual bees; 23% of bees foraged only for pollen, 30% only for sucrose and the remaining 47% foraged for both pollen and sucrose, across the four provision types. However, I found differences in these percentages between the two colonies (Figure 3.3); colony 1 had more bees that only foraged for pollen only or sucrose only than colony 2 (52% vs 40%). This left colony 2 with a greater percentage of bees with a flexible preference, foraging for both pollen and sucrose (60% vs 48%). Additionally, for both colonies I found that bee size was not significantly different between the 3 foraging preferences (Figure 3.4 a & b, colony 1: Kruskal- Wallis Chi-squared = 1.5596, $p = 0.4585$; colony 2: Kruskal- Wallis Chi-Squared = 0.9476, $p = 0.6226$).

When I categorised bees based on how they responded to the provision manipulations, I found that the majority of bees (colony 1: 71% and colony 2: 72%, Figure 3.5) responded to the manipulations by increasing or decreasing the amount they foraged for the same resource they foraged for in the control provisions (whether that be pollen, sucrose or foraging for both), but did not switch to the other resource (i.e. if they foraged for sucrose in the control period they just increased or decreased the number of sucrose bouts but did not switch to forage for pollen). Colony 1 had a much larger proportion of bees that did switch to forage for the other resource when it was in demand (0.17 compared to 0.04, Figure 3.5). Interestingly, neither colony had any bees that were categorised in category 7 which is bees that forage for pollen in the control provisions and switch to additionally foraging for sucrose in the manipulated provisions (Figure 3.5).

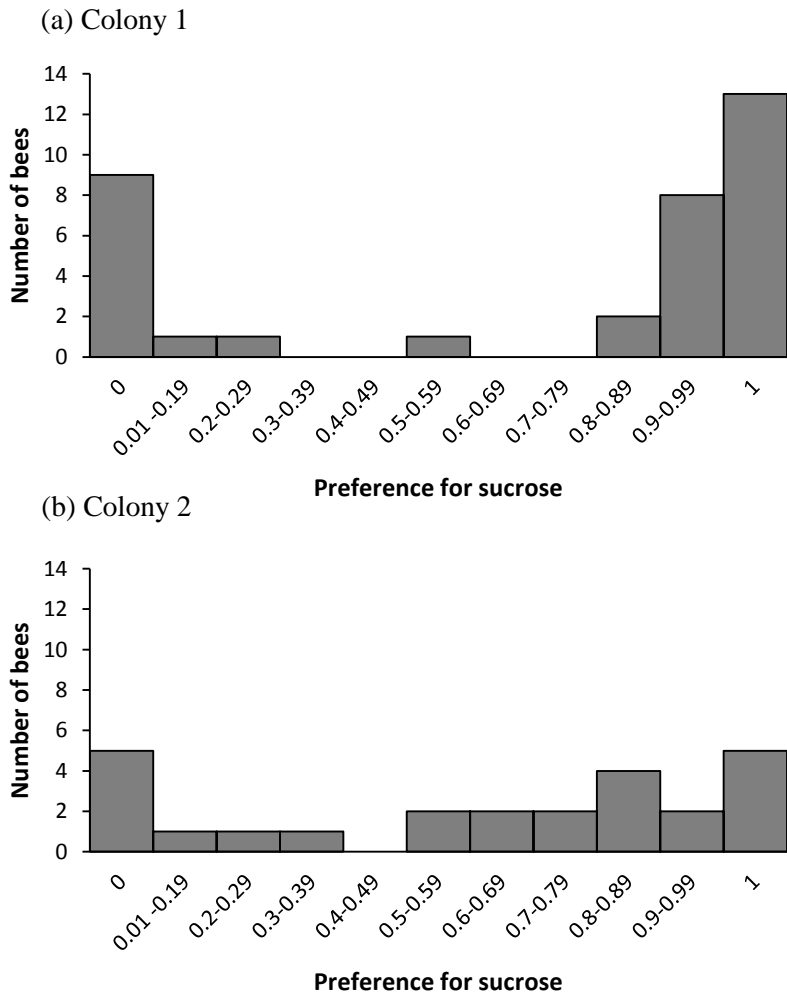


Figure 3.3 Histograms showing the overall foraging preference for sucrose (number of sucrose foraging bouts / total number of foraging bouts) based on foraging across all provision types for (a) colony 1 (n = 35 bees) and (b) colony 2 (n = 25 bees).

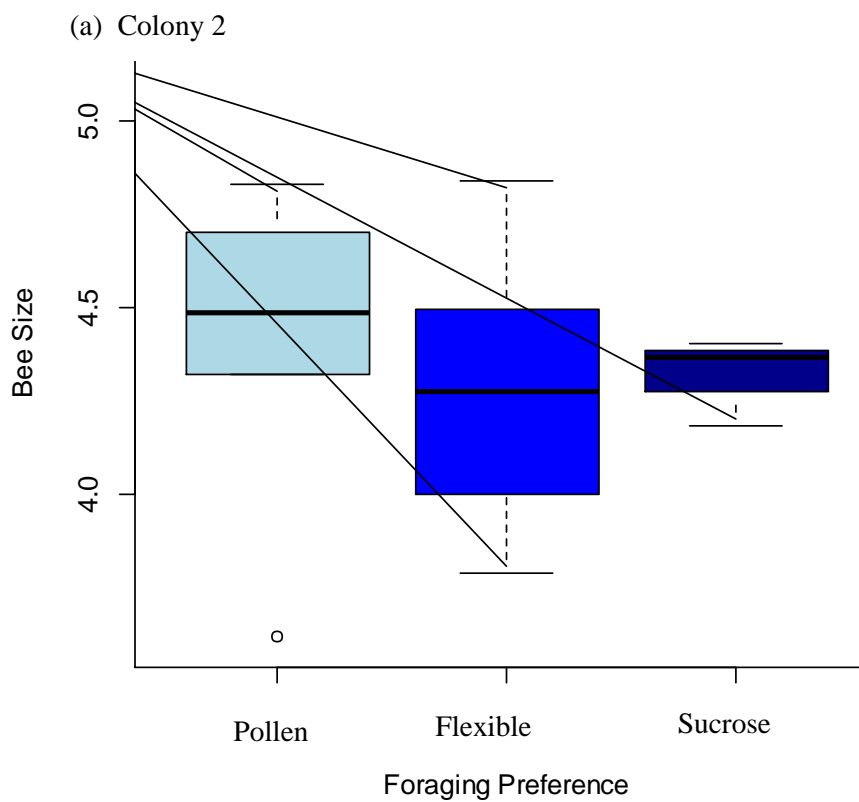
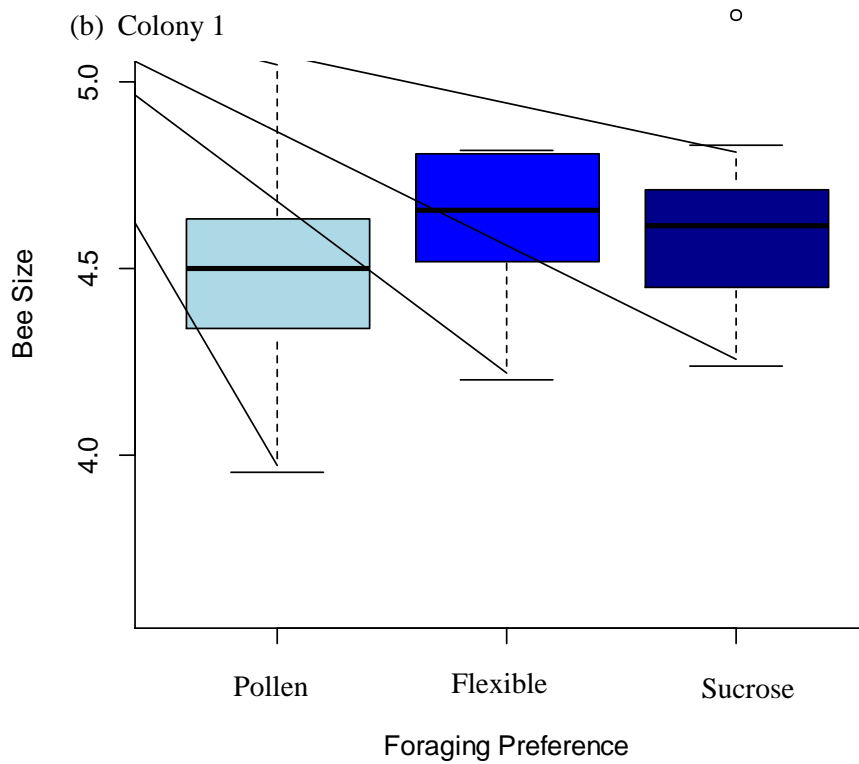


Figure 3.4. a & b. Boxplots showing the bee size (mm) for each foraging preference, pollen (colony 1 n = 10, colony 2 n = 6), flexible (colony 1 n = 5, colony 2 n = 12) and sucrose (colony 1 n = 15, colony 2 n = 3). Further details of the foraging preferences is outlined in section 3.3.3.

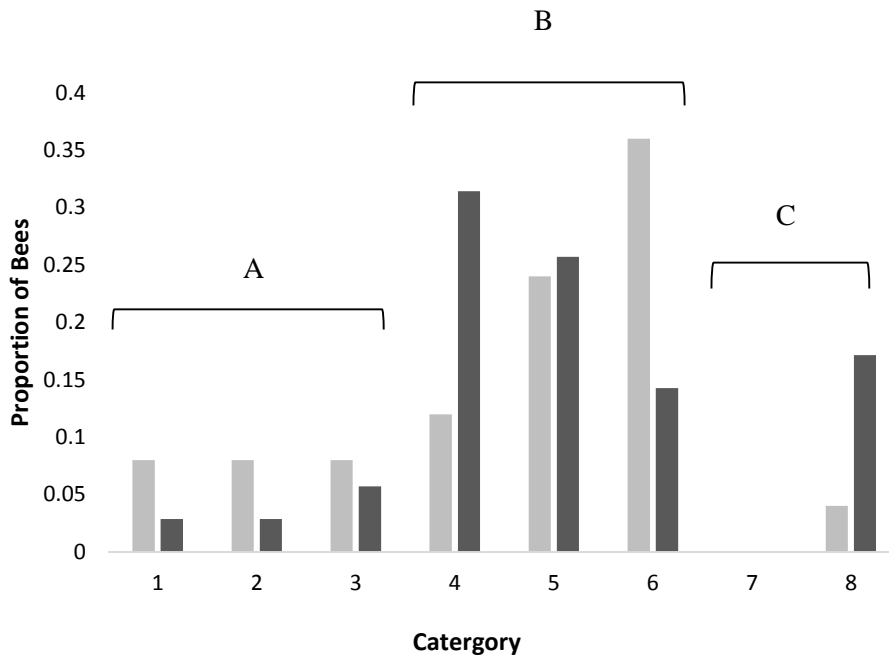


Figure 3.5. The proportion of bees from colony 1 (dark grey bars) and colony 2 (light grey bars) that foraged in the 8 foraging response categories (for details of the categories see section 3.3.3.) Group A – categories of bees that do not respond to changes in resource provision. Group B – categories of bees that do respond to the changes in resource provision but do not change the resource they are foraging for. Group C – categories of bees that do respond to the changes in resource provision by switching to additionally forage for the other resource.

3.4.3 Sucrose responsiveness and olfactory learning performance

I found that the bees with a flexible preference responded to fewer of the sucrose concentrations in the SRT test (i.e. they started to respond when sucrose concentrations were higher), than bees with a preference for sucrose foraging (GLMM, $Z = -2.38$, $p = 0.0173$, Figure 3.6). There was no difference between bees with a pollen and sucrose preference (GLMM, $Z = -0.855$, $p = 0.3925$), or bees with a pollen and flexible preference (Tukey post hoc, $p = 0.281$).

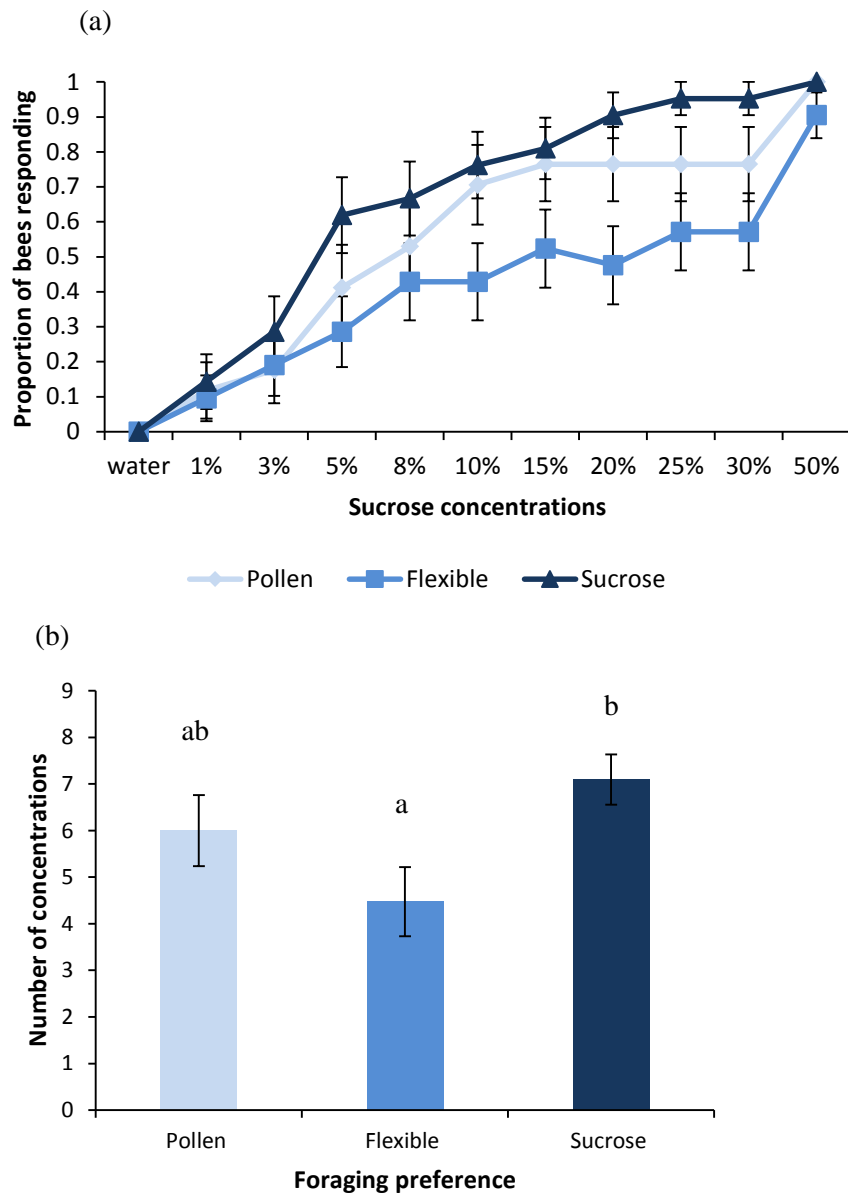


Figure 3.6 (a) Sucrose response curves, showing the proportion of bees responding with a proboscis extension to each sucrose concentration. (b) Mean number of sucrose concentrations bees with each foraging preference responded to with a proboscis extension. Data shown are means \pm SE, pollen foragers: $n = 17$, flexible foragers: $n = 21$, nectar foragers: $n = 21$. Significant differences indicated with letters.

Bees with a preference for sucrose foraging responded correctly fewer times during the PER task, and their final level of task performance was lower, compared to both bees with a pollen and flexible preference (GLMM, $Z = 4.167$, $p < 0.001$ and $Z = 3.381$, $p = 0.0007$, Figure 3.7). Post hoc tests showed no difference in performance between bees with a pollen and flexible

preference ($p = 0.694$). There was no correlation between sucrose responsiveness and olfactory learning ability for bees from either colony (Spearman rank correlation, colony 1: $\rho = -0.1515$, $p = 0.4241$, colony 2: $\rho = 0.3289$, $p = 0.1453$).

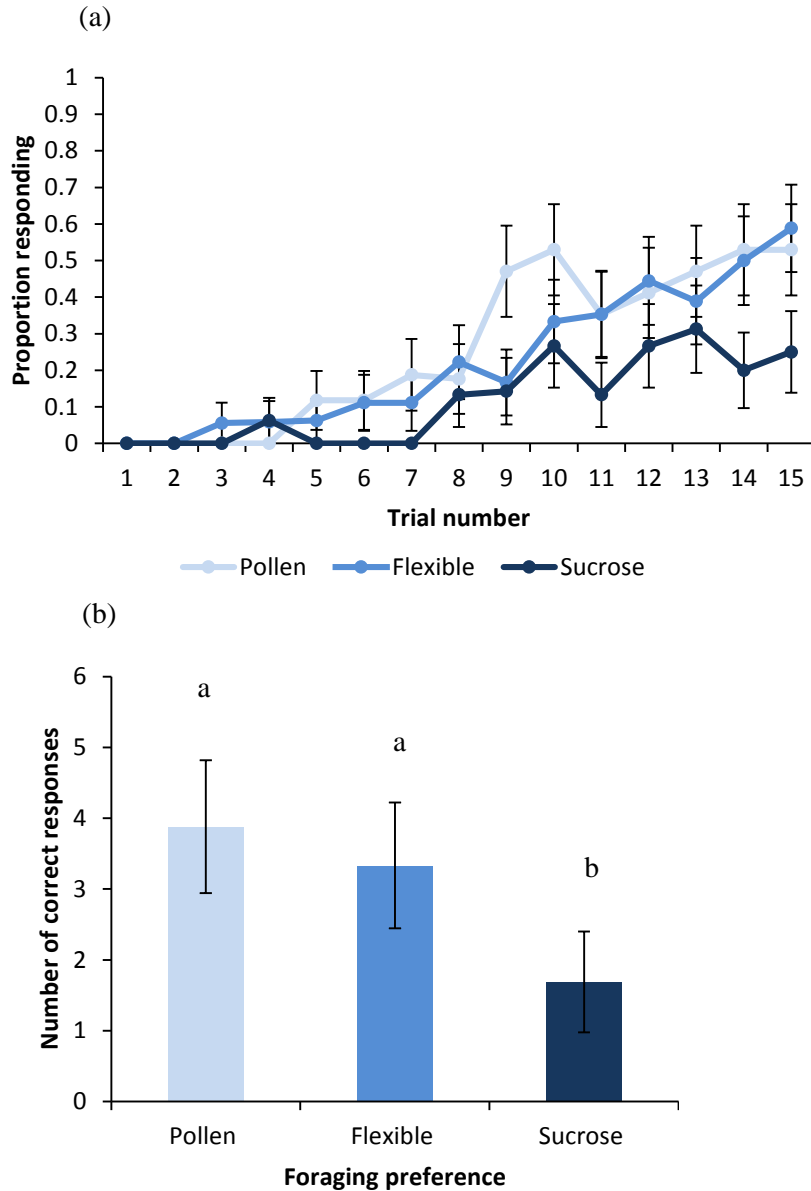


Figure 3.7 (a) Olfactory learning curves, showing the proportion of bees responding with a proboscis extension prior to reward on each trial. (b) Mean number of correct responses during PER conditioning for bees from each foraging preference class. Data shown are means \pm SE, pollen foragers: $n = 17$, flexible foragers: $n = 18$, nectar foragers: $n = 16$. Significant differences indicated with letters.

3.4.4 Pollen collection efficiency

Bees increased their pollen foraging efficiency during their first 4 pollen foraging bouts (the lowest number of pollen bouts an individual bee performed), by increasing their pollen collection rate (Bout 1 vs 4; t test, $t = -4.672$, $p < 0.001$). I found that the average pollen collection rate was not predicted by whether the bee foraged for pollen only or was flexible (LME, $t = -0.776$, $p = 0.444$), or their learning performance (LME, $t = 0.527$, $p = 0.602$). However, collection rate increased with increasing number of bouts bees performed (experience level) (LME, $t = 4.489$, $p = 0.001$), and with increasing bee body size (LME, $t = 4.495$, $p = 0.0001$).

3.5 Discussion

By observing the foraging preferences of individual bumblebees under changeable conditions of demand for sucrose and pollen, as would be experienced under natural conditions, I was able to establish the flexibility of their preferences. I found that 30% of bees only foraged for sucrose, 23% only for pollen and the remaining 47% showed varying flexibility in their collection preferences during the 8 days of observation. The results of categorising the bees responses to the provision changes show that the manipulations were successful in changing the foraging behaviour of bees, as there was only 11% and 24% of bees from colony 1 and 2 respectively that did not change their foraging in response to the changes. The work in this chapter is the first to assess, whether these preferences can be predicted by individual SRT response and/or olfactory learning performance. While I found that bees with flexible foraging preferences responded to the fewest sucrose concentrations during the responsiveness test, meaning they have a higher sucrose response threshold (SRT), I found no difference in the responses between the pollen and nectar foragers. Pollen and flexible foragers learnt better (responded correctly on more occasions) in the olfactory PER task, compared to nectar foragers. Surprisingly, there was no relationship between bee responses in the SRT and PER tasks, as has been shown previously in honeybees. Finally, I found that a bee's average pollen collection rate, was not predicted by whether bees were pollen only or flexible foragers or olfactory learning performance, it was determined by experience and bee size.

Firstly, I show that bumblebee colonies can adapt to changeable provisioning conditions, by increasing foraging for the resource in greater demand (Cartar 1992). This was achieved by the colony increasing the number of bees foraging, the activity level of individual foragers, and I also show that individuals increase their load size they collect when pollen is in demand. The collection rate of pollen was not greater when pollen was in demand. A potential reason

could be that there were more bees foraging for pollen, and there was only limited space in the petri dish to forage, so therefore it took the bees longer. Alternatively, bees were collecting larger loads than they had previously, and this may have required extra handling time. Secondly, I found that there was considerable variation in individual bees preferences for pollen and sucrose (Figure 3.3), the percentage of bees specialising on collection of each resource was greater in colony 1, compared to colony 2 (52% vs 40%). This suggests that there is colony variation in how workers behave, and how worker foraging effort is organised. Colony 1 was slightly bigger than colony 2 (123 vs 101 workers at the end of the experiment), however both colonies were still in their growth phase. Similarly to Hagbery & Nieh (2012) I found that there was not a significant difference in bee size between bees with different foraging preferences. Additionally, as was also found by Hagbery & Nieh (2012) I did not find the same pattern in both colonies, pollen foragers tended to be smaller in colony 1 and larger in colony 2 than bees with the other 2 foraging preferences. This again suggests that further studies are needed in this area to determine the reasons for this inconsistent pattern across colonies.

In comparison to previous studies that have assessed bumblebee foraging specialisation, I found that more bees (53%) specialised on foraging solely for sucrose or solely for pollen, during the experiment. Although these bees might be specialists, they may just have a stronger preference to collect the resource they specialised in across the experiment. There could be a number of reasons why this might not be their overall preference; firstly I only observed foraging for 8 days, other studies observed for more days; 100 days (Hagbery & Nieh 2012), 21 – 41 days (Konzmann & Lunau 2014), 42 day period – 4 -5 times a week (O'Donnell, Reichardt & Foster 2000). Secondly, this could be explained by variation among bumblebee species, as two of the studies were on other species (O'Donnell, Reichardt & Foster 2000; Hagbery & Nieh 2012). Finally, if colonies were tested at different developmental stages, their nutritional needs may have been different. For example, Konzmann & Lunau (2014) highlight that their colonies were at a particularly late developmental stage and therefore needed less pollen. Overall, all these studies including the work in this chapter, indicate that there are differences between colonies, and this may depend on a number of factors.

I found that flexible foragers responded to the fewest sucrose concentrations, and therefore had the highest SRT (significantly higher than sucrose foragers). The higher SRT of the flexible foragers suggests they could be more responsive to the abundance of overall resources in the colony, rather than the absolute or relative abundance of either pollen or nectar. Konzmann & Lunau (2014) showed that bumblebees preferentially forage for the most rewarding sugar concentrations; however they did not find the same in pollen foraging.

However, bumblebees have been shown to be able to associate differences in pollen rewards with colour cues (Nicholls & de Ibarra 2014). It would be interesting to repeat my experiment with a choice of rewards, in sucrose and pollen, to examine whether the flexible (generalist) bees discriminated differently among sugar concentrations, compared to sucrose or pollen foraging specialists.

Honeybee pollen foragers are more sensitive to lower sugar concentrations than nectar foragers (Pankiw & Page 2000). Honeybees will not collect rewards that are lower than their response threshold (Pankiw 2003). A potential reason for nectar foragers having a higher response threshold, could be to ensure they bring back higher (more valuable) rewards to the colony. However, I did not find the same in bumblebees, with nectar and pollen foraging bees having comparable response thresholds. In an experiment measuring free flight sucrose sensitivity in honeybees, they found that there were three groups of nectar foragers that differed in their sensitivity to sucrose (Mujagic & Erber 2009). The largest group were insensitive and collected sucrose only above 10%, but the other two groups collected much lower concentrations. In another study that compared the responses of honeybees and bumblebees to changes in reward at a specific site, they found that bumblebees were more likely to leave a site when reward decreased than honeybees (Townsend-Mehler, Dyer & Maida 2011). Therefore, potentially bumblebee colonies have more sensitive nectar foragers and therefore, have a similar threshold to bumblebee pollen foragers, unlike honeybees where the majority of nectar foragers are less sensitive to sucrose.

Mommaerts, Wackers & Smagghe (2013) assessed the gustatory responses of *B. terrestris* to three sugars (glucose, sucrose and fructose), and found that bees were least responsive to sucrose: half the bees responded to 5.5% fructose and glucose solution, and it took a concentration of 40% sucrose to achieve the same levels of response when harnessed. However, free moving bees given a choice between 30% solution of each sugar showed a preference for sucrose (66% of bees). These different responses between the two experimental set ups could be due to tarsal sugar perception (de Brito Sanchez *et al.* 2008), in addition to antennal input, in the free moving bees. However, sucrose sensitivity in the field and lab in honeybees has been shown to correlate only partially (Mujagic & Erber 2009). Therefore, they may not correlate in bumblebees either, although Mommaerts, Wackers & Smagghe (2013) did not test the sensitivity of different concentrations in free moving bees. However, they used a harnessing method that has found poorer response rates in the past, compared to the method I developed (chapter 2; Smith & Raine 2014), and could be the reason that I found bees in this chapter responding at much lower concentrations (>50% of bees respond to 8% sucrose

solution). By testing the sensitivity of free moving bees, and using the harnessing protocol used in this thesis this issue could be resolved.

Although lower sucrose response thresholds (SRTs) have been linked to enhanced learning performance in both honeybees (Scheiner, Page & Erber 2001a; Scheiner, Page & Erber 2001b) and ants (Perez *et al.* 2013), I did not find this same pattern in bumblebees. Nectar foragers had the lowest SRT, and were the poorest learners in the olfactory PER learning task. Both pollen foragers and flexible foragers responded correctly to significantly more of the trials during the PER task, and were therefore better learners. The only previous work on task specialisation and learning in bumblebees (*B. huntii*) found that nurse bees were better learners than foragers, and they suggest this could be due to an inhibition of learning to increase foraging efficiency (Hannaford *et al.* 2013). I did not test the learning ability of nest workers so it is difficult to compare our work. However, Hannaford *et al.* (2013) found that foragers learning ability is extremely poor and they do not improve over time. In contrast, I found that the foragers in this experiment learn much better than this, and therefore this could be a bumblebee species effect they are finding. A potential reason for pollen and flexible foragers being better learners could be due to the difficulty of pollen foraging. Pollen collection can take more than 3 times longer than nectar collection to learn to collect effectively from simple flowers (Raine & Chittka 2007c). Additionally, I show in this experiment that bee's pollen foraging efficiency improves with experience, and this is further evidence for pollen collection being a skill that requires learning ability. Therefore, it may not be optimal for the colony for slower learning nectar foragers to collect pollen.

Finally, I assessed whether the pollen collection rate between the pollen only and flexible collectors differed. I found that there was no difference between the two groups. Therefore, all bees had to learn how to forage for pollen most efficiently, pollen only foragers were not only collecting pollen because they were most efficient at collecting it. Additionally, olfactory learning ability did not predict pollen foraging, and this could indicate that other types of learning are more important, such as motor learning. I found that worker size and experience were more important in determining how efficient a bee is at pollen foraging, rather than what they have a preference for collecting. Cartar (1992) found a similar outcome, in that the bees that switched from nectar to pollen collection were no less efficient at collecting pollen, than the non-switching bees (i.e. pollen only collectors). However, bees that switched from pollen to nectar foraging were less efficient than the non-switching bees i.e. nectar only foragers. It is generally assumed that the benefit of specialising at a task is that you are more efficient at it than a generalist (Goldsby *et al.* 2012), although this is not always the case (Dornhaus 2008). I did not find this either and there could be a few reasons for this. Firstly, the pollen collection

in this experiment was relatively easy compared to foraging from complex flowers, which the pollen only foragers may be much better at, and this would be an interesting area to explore further. Secondly, bees in the flight arena were all foraging from the same pollen dish, and therefore the bees may have been able to learn socially from one another (Leadbeater & Chittka 2007), which in a natural foraging environment may have been more unlikely to happen, as most flowers can only accommodate one bee collecting pollen from at a time.

Overall, the results from this chapter show that there is variation in the foraging preferences of bumblebees (*B. terrestris*), and some individuals have stronger preferences for pollen or sucrose than others. I show evidence to suggest that these preferences may, in part, be associated with sucrose response threshold and olfactory learning ability, although these two traits do not correlate. Pollen and flexible foragers showed higher accuracy during the olfactory learning task in comparison to nectar foragers, which could be linked to the increased complexity of pollen foraging. I did not find bees that were specialising on pollen collection being more efficient at the task, as I may have expected. It would be interesting to test this in a more environmentally realistic situation, and to assess in what other ways learning performance maybe influencing the activity and decisions made by individual bees.

Chapter 4

The relationship between individual learning ability and field foraging performance

The experiment for this chapter was conceived, designed, and lab work executed, jointly with another PhD student (Lisa Evans), and we both undertook these tasks equally. The writing and analysis are all entirely my own work. An undergraduate student (Emily Parsons) also helped with the weighing of foraging bees during the experiment and inputted part of this, as part of her third year project.

4. The relationship between individual learning ability and field foraging performance

4.1 Abstract

Cognition in animals has been studied for decades, yet there is still much that is unclear about why we see such variation in individual cognitive abilities within species. It may be assumed that learning performance measured in the lab will influence fitness in the field; however there are few studies that actually test this idea, and those that do could be improved upon. Bees forage in complex environments and learning performance is potentially important in a number of situations, including locating the most rewarding flowers and flower handling. Variation in visual learning performance has been correlated with foraging success at the colony level, but there are potential colony level traits that could confound this. I tested the learning performance of individual naïve bumblebees (*Bombus terrestris*), in either a visual or an olfactory task, in controlled laboratory conditions before allowing bees to forage in a realistic field setting. Individual foraging performance was measured using RFID tagging technology to record foraging activity and by the mass of collected food to assess foraging efficiency. I found that poorer visual learners contribute more to colony foraging effort as they forage as efficiently as good learners, but for longer. Whether bees could learn or not during the olfactory task appeared to be important in determining how long bees foraged for, with non-learners foraging for less time. Variation in olfactory learning performance of bees that learnt the association between odour and reward during the task in the lab is suggestive of the same as the visual learning results; poorer learners foraged for longer but not more efficiently than better learners. However, the sample size limits the conclusions I can draw from the olfactory learning data. My results demonstrate that better learning performance may come with costs such as reduced longevity, and will not always benefit the fitness of the individual. The environment that individuals are foraging in will potentially influence the importance of learning, and alternative environments may favour individuals of different learning abilities.

4.2 Introduction

Historically, studies attempting to assess the fitness implications of variation in cognition have focussed on the between species variation (Healy, de Kort & Clayton 2005). This is potentially problematic as different species can vary in their ecological requirements, and therefore it's hard to conclude whether cognitive abilities are the cause of the fitness differences. However, we know there is considerable variation in cognitive performance within species (Raine & Chittka 2008; Snell-Rood & Papaj 2009; Katsnelson *et al.* 2011; Cole *et al.* 2012). Research

has now begun investigating why this variation exists, and how differences in learning performance affect the ability of individuals to perform activities in the wild, such as finding food, reproducing or caring for young (Thornton & Lukas 2012).

There are a number of pioneering studies that have investigated how cognitive performance affects individual fitness, using various life history traits that were discussed in detail in section 1.4.2. For example, Cole *et al.* (2012) found that Great tits (*Parus major*) that could solve a cognitive task had increased fecundity (clutch size), but were also more likely to desert the nest compared to non-solvers. A positive correlation between problem solving ability and mating success also occurs in the male satin bower bird (*Ptilonorhynchus violaceus*) (Keagy, Savard & Borgia 2009), but not in male spotted bower birds (*Ptilonorhynchus maculatus*) (Isden *et al.* 2013). A potential explanation for this difference in relationship could be that the female's mate choice could be based on male social status, rather than cognitive ability. These results suggest that cognitive ability can have quite a profound effect on the individual, from their ability to produce young, attract a mate and find enough food. Such variation might potentially reflect variation between environments, in the importance of both cognitive performance and the other life-history traits in question. However, one factor these studies fail to take into account is the prior experience of the individual, and this is a problem I address in this chapter.

Bumblebees are good model organisms for studying how variation in learning and memory may be adaptive. They can be kept in the lab due to their relatively small colony size, and are amenable to lab-based learning tasks. Field-based foraging performance can also be measured, because workers will reliably return to the colony after each foraging bout. Importantly, as bumblebee colonies can be obtained from commercial suppliers the bees are naïve and have no prior experience of foraging, which other studies investigating the fitness consequences of cognition have been unable to control (Keagy, Savard & Borgia 2009; Cole *et al.* 2012). Bees forage in dynamic and complex environments, in which the most rewarding flower species will change. To forage efficiently bees need to adapt to these changes, and therefore cognitive abilities are thought to be important in their success (Raine *et al.* 2006a). The amount of food brought back to the colony has been shown to be proportional to colony reproductive output (production of males and gynes) (Pelletier & McNeil 2003), and therefore foraging performance can be a good proxy measure of fitness in bumblebees. At a colony level, faster learning bumblebee colonies (*B. terrestris*) have been shown to collect up to 40% more nectar when foraging in the field, than slower learning colonies (Raine & Chittka 2008). However, there can be considerable individual variation in learning performance within colonies (Raine & Chittka 2008; Raine & Chittka 2012; Evans & Raine 2014; Smith & Raine 2014; chapter

2) and assessing colony performance could be confounded by colony level traits, such as parasite infection (Schmid-Hempel & Muller 1991; Schmid-Hempel & Stauffer 1998). There are many factors that are important in successful foraging; these include efficiency of collection, length of foraging career and the quality of reward (nectar and/or pollen) collected. Therefore, it is important as many of these are assessed as possible in an experimental setting.

Flowers signal to pollinators using a range of cues (visual, olfactory and tactile), and it has been suggested that the ability of bees to learn, is improved when they have more than one cue to associate with reward (e.g. a colour and an odour compared to only a colour alone; (Kunze & Gumbert 2001; Reinhard, Srinivasan & Zhang 2006; Kulahci, Dornhaus & Papaj 2008; Leonard, Dornhaus & Papaj 2011). This highlights that bees use both visual and olfactory cues while foraging, and therefore learning tasks using both olfactory and visual cues are ecologically relevant. In addition, we do not know if learning performance in different sensory modalities (e.g. visual, olfactory and tactile) affects foraging performance differently. In this experiment, I tested the learning performance of individual bumblebees (*B. terrestris*), in either a visual free-flying discrimination task or an olfactory restrained absolute conditioning task. I then tested whether performance in the lab-based learning task predicted: (1) foraging efficiency (rate of pollen and nectar collection) and (2) foraging activity (total time spent foraging), in the field. I hypothesised that better learning bees would perform more foraging and do this more efficiently, due to the advantage their cognitive abilities would give them in the field.

4.3 Methods

4.3.1 Experimental setup

Ten bumblebee (*B. terrestris*) colonies were commercially obtained (Biobest, Belgium), each containing a queen and 23-49 workers (mean = 32). Five of the colonies were assigned to the olfactory absolute conditioning learning task and the other five to the visual discrimination learning task. Colony sizes were matched between learning task types as far as possible. The colonies were each transferred to wooden nest-boxes with four chambers, two rear chambers to house the brood, connected to the two front chambers by a small hole; a mesh divider ran down the centre between the two sides of the box (Figure 4.1). All colonies had their brood split equally and half was put in each rear chamber of the colony boxes along with half of the workers. The queen was swapped between sides every 24 hours, the aim being that she would lay eggs equally in each brood chamber (Schmid-Hempel & Schmid-Hempel 1998). The mesh inbetween the chambers allowed the colony to be kept as a unit allowing transmission of

olfactory signals/pheromones, but bees could not move between sides. This design permitted me to retain one half of the bees in each colony (inner side) in the lab, where their learning performance could be tested, and allowed the other half, access via a network of tubing, to the natural environment surrounding our campus (outer side) (Gill, Ramos-Rodriguez & Raine 2012; see Figures 4.2, 4.3a). Above each of the exit holes were unique black and white patterns, to aid bees in navigating back to their colonies (Figure 4.3b). Bees on the outer side of the colony were not provisioned with sucrose or pollen, therefore needed to leave the colony to forage outside.

Colonies were checked daily for newly emerged workers. Bees on the inner side of the colony were tagged on the thorax with uniquely numbered, coloured tags (Opalith tags; Christian Graze KG, Germany) so that individuals could be unambiguously identified and potential age effects could be assessed. Once individual bees had completed the learning task they had an RFID tag (Microsensys GmbH: mic3-Tag 64 bit read only transponder; *carrier frequency*: 13.56 MHz; measuring: 2 x 1.6 x 0.5mm; mass: 4mg) glued on top of their coloured tag, and were transferred to the outer side of the colony box. This allowed me to follow the foraging activity of these individuals. All other bees on the outer side of the colony box had one of their wings clipped. This meant that they could still perform tasks within the colony, but could not go out and forage; therefore the RFID tagged bees were the only bees foraging.

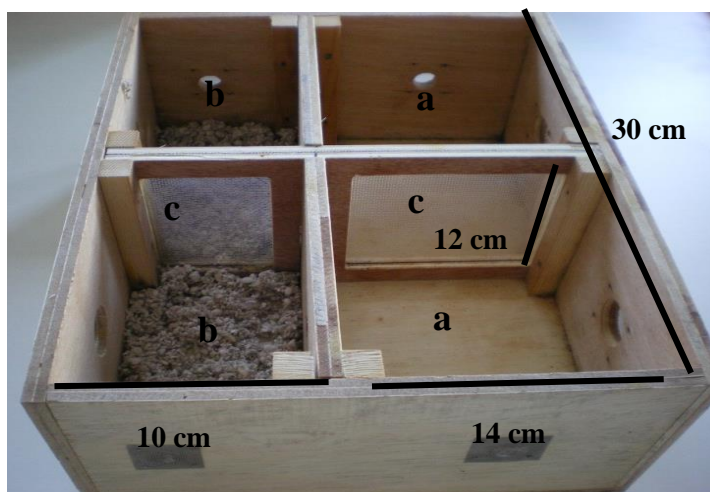


Figure 4.1 Photograph of split colony box. The rear brood chambers are indicated by a, the front chambers by b and the mesh divider is shown by c. Dimensions of the boxes are indicated along the sides.

4.3.2 Foraging activity and efficiency

Once transferred to the outer side of the colony, the two RFID readers (see Figure 4.2) recorded the time of each entrance and exit of each tagged bee. Having two RFID readers

allowed me to know the direction in which the bee was moving. This gave information on the number of days on which a bee foraged, and the number and duration of foraging bouts. The first bees were RFID tagged on the 5th July 2013, and RFID tagged bees were monitored until the 10th August. Each colony was also observed for 3 hours per day in one of the 3 sessions (9am – 12pm, 12pm – 3pm, 3pm – 6pm), 5 days a week (20 days total). During these sessions foraging performance was measured by recording the mass of foragers exiting and returning to the colonies, using the ‘animal weighing’ function, on a balance placed under a Perspex tunnel near the colony entrance (balance accurate to one thousandth of a gram, Figure 4.2 & 4.3c). Three weights were taken and the average of these was used. In addition, I recorded whether bees were carrying pollen and scored the size (very small, small, medium, large or very large) and colour using a pollen colour guide, to estimate the plant groups they had been foraging on (Kirk 2010). The daily order of colony observations was assigned in a pseudo-random order, to account for different activity levels at different times of day. Details of the weather conditions during the experiment are given in the appendices (Appendix 4.1).

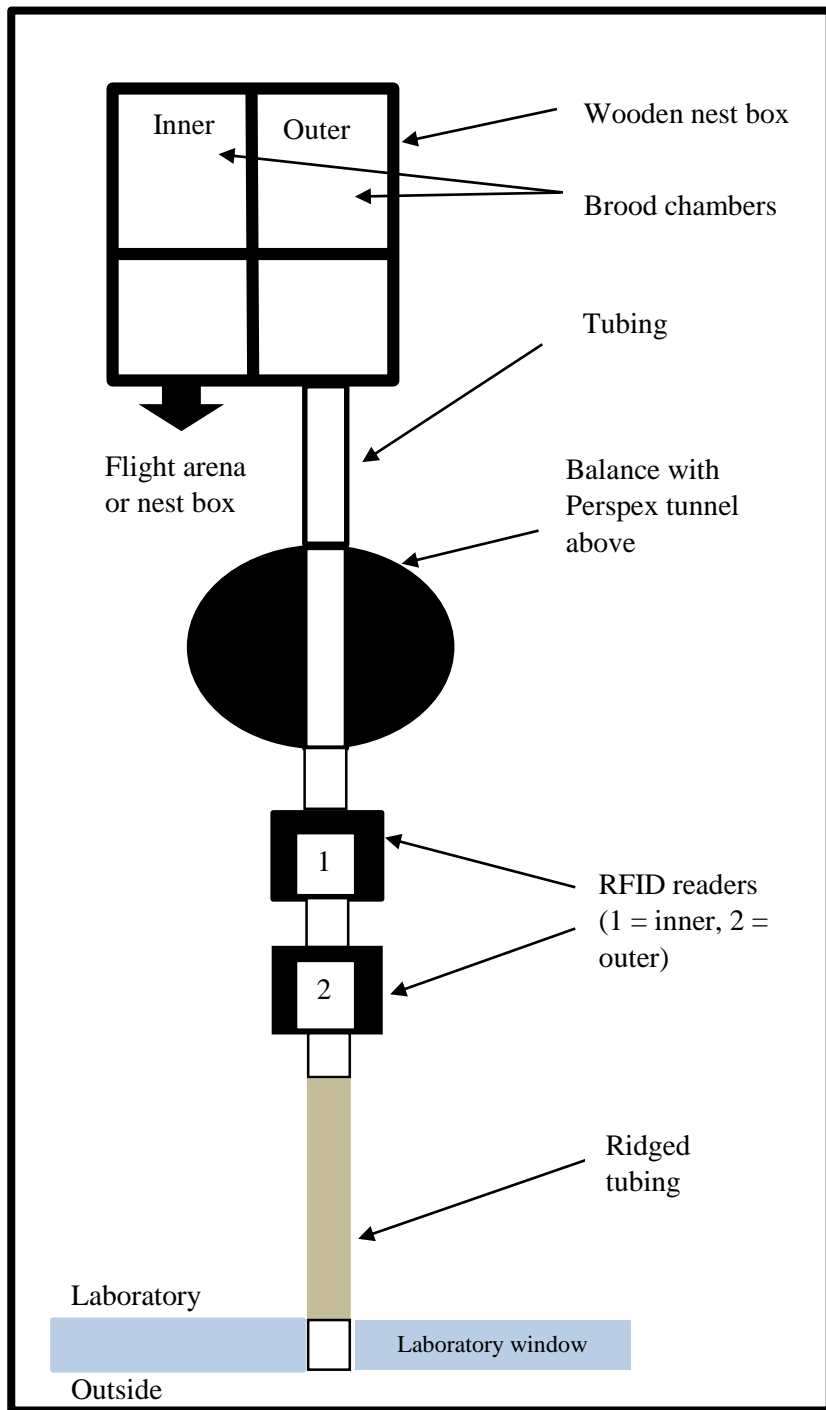


Figure 4.2 Diagram of the experimental set up (not to scale). The inner side of the split colony box was connected to a nest box or flight arena. The outer side of the split colony box was connected to the outside environment by a number of tubes. Firstly, there was a short tube which connected to a Perspex square tunnel, with gates to control the flow of bees and part of the base was cut out so that bees walked directly on top of the balance below. Next a pair of RFID readers with a short piece of tubing between them and this was then connected to another short piece of the square Perspex tube with gates so that during observations bees could be held in this area at

busy times. Finally, this connected to a final piece of ridged tubing to allow bees to climb to the window exits and forage in the outside environment.

(a)

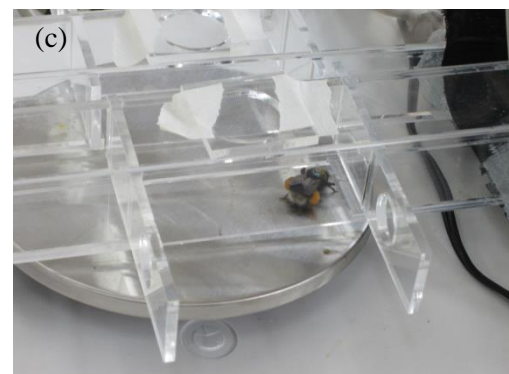


Figure 4.3 Photographs of (a) the lab set up, (b) the window panels on the third floor lab window from the outside and (c) the Perspex tunnel that allowed foraging bees to be weighed on the balance beneath.

4.3.3 Visual learning performance

The inner side of each visual learning colony nest-box was connected to a flight arena (120 x 100 x 35 cm) by a transparent Perspex tube. Bees were given access to *ad libitum* sucrose (50% v/v) in the flight arena except for when the learning task was taking place; pollen was given directly into the brood chamber every other day. Visual learning was tested in the same way as described in section 2.3.3, whereby bees has to learn to associate yellow flowers with reward and ignore blue unrewarding flowers.

To assess learning performance a learning score was created by fitting a first-order exponential decay function curve:

$$(y = y_0 + Ae^{-x/t})$$

to the data for each bee (Microcal Origin pro 8.6). In this equation, 'y' is the number of errors (blue choices) and 'x' is the cumulative number of flower choices since the first yellow probe. 'y₀' is the fitted saturation performance level (the number of mistakes the bee is making when learning performance plateaus). 't' is a fitted decay constant, which represents the rate of change in task performance therefore is a measure of learning speed, and 'A' is the slope amplitude. The curve starts from the proportion of errors (blue choices) the bee made prior to probing the first rewarding yellow flower. Flower choices from this point were grouped in bins of 10 choices and the number of errors made (blue flower choices) in each group was calculated. This gave 11 points (starting point and error value from each group of 10 choices) for each bee and the learning curves were fitted to these. Given the dynamic nature of the learning process I created a single learning score (error rate) from these curves that takes into account both difference in the slope and shape of the fitted curves. To do this I summed the number of errors made at 3 points along the curve (choice number 5, 50 and 100 after probing the first yellow flower). This produced a learning score out of a maximum of 30, the lower the bees score was the fewer errors the bee made during the learning task, therefore they were better at learning the task.

4.3.4 Olfactory learning performance

The inner side of the odour learning colony boxes were connected to a further nest box (14 x 24 x 12 cm), where bees were allowed to forage for 50% (v/v) sucrose solution provided *ad libitum*, and pollen was provided every other day directly into the brood chamber. Observations were made throughout each day, to identify foragers. Bees that were observed foraging on at least two days a total of 3 times, were classed as foragers and selected to be tested in the olfactory task. Olfactory learning was assessed by harnessing bees as described

in section 2.3.2 and using the proboscis extension reflex (PER) paradigm with absolute conditioning (as described in section 3.3.2).

The number of odour presentations (out of the 15), in which bees failed to respond to the odour with a proboscis extension prior to the reward, was used to assess learning performance. This approach was used instead of the number of correct responses, which I used in my other chapters, because this made low scores representative of better learning bees for both the olfactory and visual task presented in this chapter. I additionally tried the curve fitting approach I used in chapter 2 to analyse the olfactory learning data, but this made no difference to the results, so I chose to present the simpler approach of using number of failed responses. The more odour presentations to which bees responded with a proboscis extension prior the reward being offered, the lower score they obtained in the task (i.e. a low score indicates high learning performance). Based on this score, I categorized bees as good learners (score of 2 – 5), average learners (score of 6 – 9), poor learners (score of 10 – 14) and bees that did not learn (score of 15). Unlike the visually tested bees, olfactory learning performance was then analysed as a factor; I decided to take this categorical approach because of the distribution of the data, there was a large proportion of bees scoring 15 (non learners) (see Figure 4.4a & b for histograms of the visual and olfactory learning performance distributions). Five out of 85 bees were excluded from the experiment and classed as insufficiently responsive to participate, as they did not extend their proboscis when their antenna was touched on more than 3/15 of the conditioning trials. Of the remaining 80 bees 12 of these did not respond on either 1 or 2 of the trials, however this was not linked to learning rate (i.e. these bees were not always poor learners, see Appendix 4.2.).

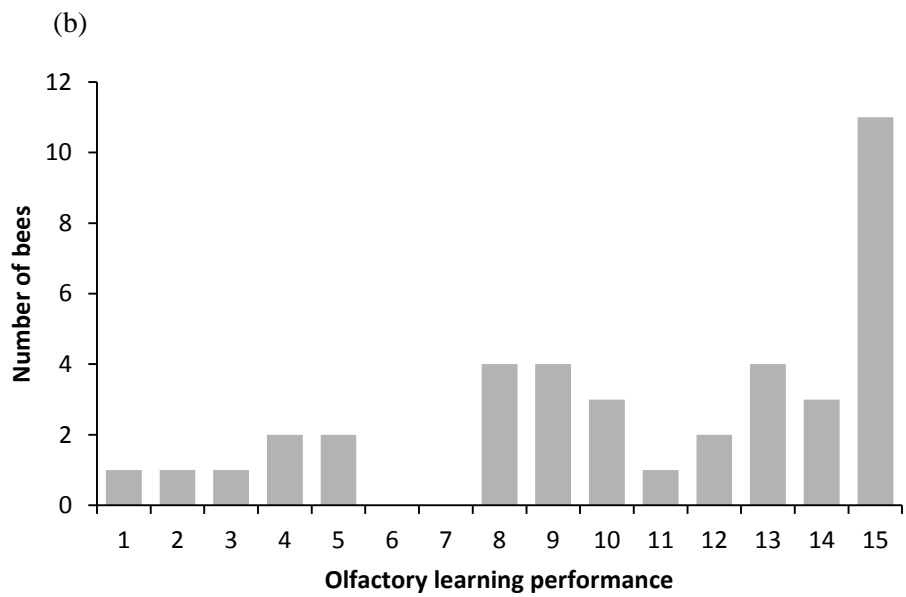
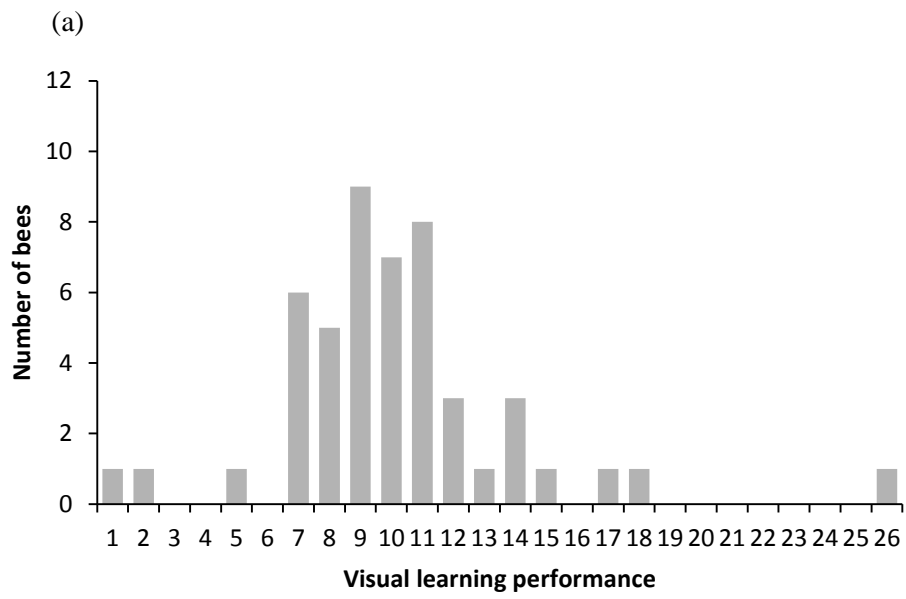


Figure 4.4 Histograms of the learning performance of bees tested in the (a) visual task and in the (b) olfactory task.

4.3.5 Foraging efficiency - analysis

I made over 1300 observations of bees entering and exiting the colonies. I classed bouts in which the bees were carrying pollen as “pollen” bouts and those without pollen as “nectar” bouts. Bouts in which bees had collected a very small amount of pollen were classed as nectar foraging bouts.

I took a conservative approach, by only calculating collection rates for bees that I observed completing at least 3 foraging bouts of the resource in question. I did this as I felt that the collection rates may be skewed if the bout observed was not representative of the majority of bouts they performed. The mean nectar collection rate for each bee was based on bouts in which bees did not collect any pollen. The length of each observation session meant that bees did not always complete a foraging bout (i.e. exit and return from foraging) within the observation period, therefore I used the average in and out weights of bees to work out their foraging efficiency. The negative values seen in Figure 4.5a can be explained by using the average, however this allowed me to increase the sample size I could achieve allowing statistics to be performed. Nectar collection rate was calculated by firstly subtracting the average departure (‘out’) weight from the average arrival (‘in’) weight for each bee, which gave an indirect measure of the mass of nectar collected. This was then divided by the average bout duration for that bee and multiplied by 1000 (to convert from grams to milligrams); this gave me a collection rate per minute for each bee. This was done in a similar way for pollen collection rate, using only bouts in which pollen was collected. The weights were not used for the pollen collection rate, as pollen collecting bees tended to return lighter than they departed. Therefore, I used an average of the pollen load size (scored as 1 – 4: 1 = small, 2 = medium, 3 = large and 4 = very large), divided by the average bout duration to give a rate of pollen collection.

All analyses were performed in *R* (R Core Team 2014). The RFID data showed some foragers were drifting between multiple colonies (mean \pm SE = 4.09 ± 0.17 colonies), which is a similar level to a previous study (Gill, Ramos-Rodriguez & Raine 2012). For each foraging bee I calculated the colony that it foraged for the most, and called it the ‘majority colony’ (Gill, Ramos-Rodriguez & Raine 2012). In 48% of cases, the majority colony was the natal colony. On average, foraging bees performed $61.88 \pm 2.33\%$ of their foraging bouts for their majority colony, compared to $37.36 \pm 3.51\%$ for their natal colony. Therefore, I felt that majority colony was a better measure of colony membership, and used this measure in the models. I used linear mixed effects models to analyse the two response variables, nectar and pollen collection rate, using the *lme* function from the *nlme* package (Pinheiro *et al.* 2014). To

investigate whether learning performance predicted foraging efficiency, I adopted a bottom-up model building approach, which is both more conservative than a stepwise deletion approach and also more appropriate given our limited sample size because it avoids over parameterization of the model (Raihani & Bshary 2012). Firstly, I specified a basic model including only majority colony as a random effect e.g. nectar collection rate $\sim 1 + (1|\text{majority colony})$. I then created four new models in which colony age, worker age, worker mass or experience were added to the basic model. I calculated the AICc value (Akaike Information Criterion – corrected version for small sample sizes) for each model (selMod function from the pgirmess package (Giraudoux 2014)) and the best model of this subset was identified as the model with the lowest AICc value. I then added learning performance to the best model to identify the resultant effect on AICc value. If the AICc was significantly lowered (i.e. $\Delta\text{AICc} > 2$) I concluded that learning was important in predicting that response variable. The fit of the best model was checked by plotting the fitted vs residual values for the model.

The predictor variables were calculated as follows: colony age was the age of the colony when each bee was RFID tagged and began foraging. Worker age was the age of the bee from when it eclosed (NB: bees present in the colonies upon arrival were assumed to have eclosed 5 days prior to colony arrival: 15/06/13). Worker mass gives an indication of the bee size based on the average of the bees out weights that were measured during the foraging efficiency observations. Bees for which I did not have a weight were allocated the average mass based on the other bee's weights from their natal colony. Experience was included to take into account where within the bee's foraging career bouts were observed. It might be expected that a bee's performance may be poorer if only observed on its first few bouts. This was calculated by averaging the bout numbers I observed the bees foraging on. For example a bee observed on its 2nd, 10th and 30th bout would be given an experience value of 14 (i.e. $2 + 10 + 30 / 3$).

4.3.6 Foraging activity data – analysis

When analysing the RFID data I classified a foraging bout as when a bee spent at least 8 minutes out of the nest (0.8% of bouts excluded). Whilst some previous studies have used a higher threshold of 10 minutes (Capaldi & Dyer 1999; Peat & Goulson 2005), I chose 8 minutes because some bees ($n = 7$) were consistently making short foraging trips of around this time, this was further supported by the foraging efficiency data, showing they were returning and offloading pollen each time. Bouts when the bee spent the night outside were excluded (3% of bouts).

I generated three response variables from the foraging effort data: number of days on which an individual was observed to forage, average bout duration and average number of bouts per day. Generalized linear mixed models (GLMMs glmer function in lme4 package (Bates *et al.* 2014)) were used to analyse the count data response variable (number of days foraged), assuming a Poisson error distribution. Linear mixed effects models (lme function from the nlme package (Pinheiro *et al.* 2014)) were used to analyse the average bout duration and average number of bouts completed per day response variables. Average bout duration was square root transformed to improve model fit based on inspection of model residuals.

I investigated whether learning predicted foraging performance in a similar way to the foraging efficiency data, as described above. I ran five models in total. The first a basic model including only majority colony as a random effect: i.e. number of days foraged $\sim 1 + (1|majority\ colony)$. I then created four new models in which colony age, worker age, worker mass or experience were added to the basic model. Learning performance was then added to the best model based on AICc values, as explained above. Results of a Grubbs test on the worker mass data from the olfactory learning colonies detected an individual outlier, this bee was subsequently excluded from analyses.

4.4 Results

4.4.1 Visual learning bees

I tested 86 bees from 5 colonies and found no significant inter-colony variation in visual learning performance (Kruskal–Wallis, $H_4 = 6.370$, $p = 0.173$). All of these bees were RFID tagged and allowed to forage outside; 49 of the 86 bees foraged, while the other 37 bees never returned to the colony once they left. I found no significant difference in learning performance between bees that foraged and bees that left and never returned (Mann Whitney U, $Z = 1.227$, $p = 0.220$).

4.4.1.1 Foraging efficiency of visual learners

I observed 44 of the 49 visually tested bees that foraged outside while RFID tagged, exiting or returning from foraging bouts, on at least one occasion. I observed on average 11% (range 3 – 30%) of the foraging bouts completed by each bee, which was correlated with the total number of bouts they performed (Pearson’s correlation, $t = 0.22$, $p < 0.001$). I found that the nectar and pollen collection rates were not predicted best by the models that included learning

performance (Table 4.1 and Figure 4.5); therefore learning performance was not a good predictor of foraging efficiency.

Table 4.1 Candidate models to predict the nectar collection rate and pollen collection rate response variables for the bees tested in the visual paradigm that we observed at more than 2 foraging bouts for (n = 22 and 31 respectively). The AICc value of each model and the Δ AICc from the best model are presented. The model with the lowest AIC value from the top four models (indicated with an asterisk) had learning ability added to it to assess whether this significantly decreased the AICc value. The best model (based on the lowest AICc value) is highlighted in bold.

Model name	Nectar collection rate		Pollen collection rate	
	AICc	Δ AICc	AICc	Δ AICc
Basic	70.51	0.88	-168.87*	0.00
Colony age	69.63*	0.00	-166.53	2.34
Worker age	72.46	2.84	-167.91	0.96
Worker weight	73.03	3.40	-166.82	2.04
Experience	70.22	0.60	-166.58	2.29
Best model + learning	71.09	1.47	-167.25	1.61

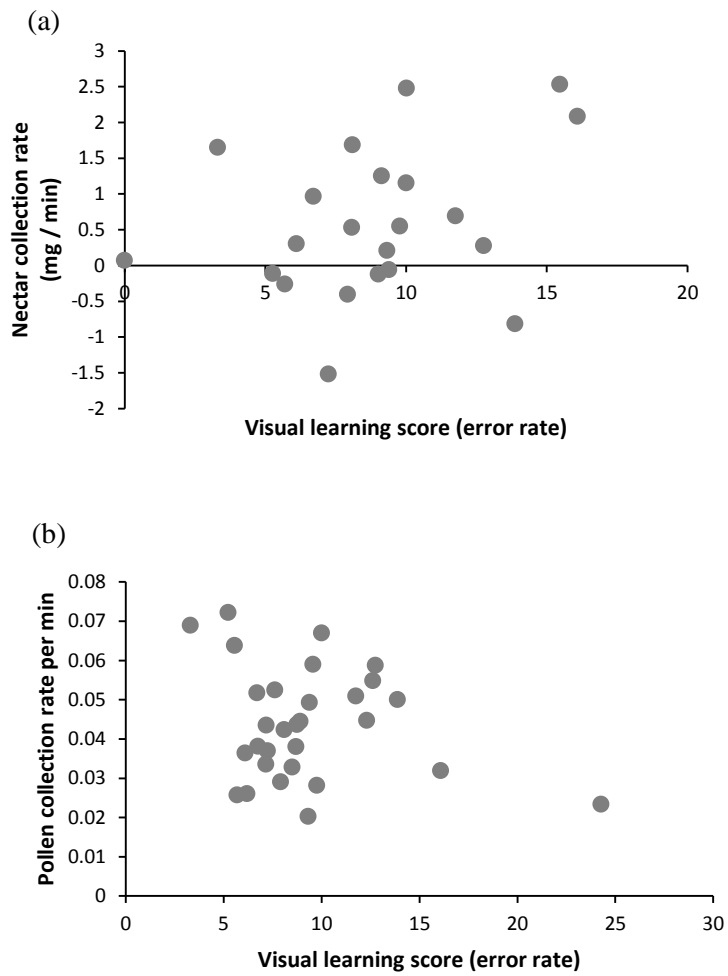


Figure 4.5. Data shown are (a) nectar collection rates of the bees that performed at least 3 nectar foraging bouts and their associated visual learning ability ($n = 22$). (b) Pollen collection rates of bees for which I observed at least 3 pollen foraging bouts and their associated learning ability ($n = 30$). Lower learning scores indicate that the bee was a better learner (i.e. made fewer errors).

4.4.1.2 Foraging effort of visual learners

On average, the 49 foraging bees performed 103 bouts (range: 6–253) in total, over 8 days (range: 1–22), 12 bouts per day (range: 3–32) lasting 48 minutes (range: 21–106). The best model to predict the number of days foraged was the basic + learning model (estimate \pm SE = 0.041 ± 0.012), providing a significant improvement on the next best model (Δ AICc = 7.97, see Table 4.2). Poorer learners foraged for a greater number of days (Figure 4.5a). The best model to predict bout duration and average number of bouts completed per day was colony age (estimate \pm -0.520 \pm 0.085 and 0.062 \pm 0.019 respectively, Table 4.2, Figure 4.5b & c); therefore learning performance did not predict bout duration or average number of bouts.

Table 4.2 Candidate models to predict the number of days foraged, average bout duration and average bouts per day response variables for the bees tested in the visual paradigm (n = 49). The AICc value of each model and the Δ AICc from the best model are presented. The model with the lowest AIC value from the top four models (indicated with an asterisk) had learning ability added to it to assess whether this significantly decreased the AICc value. The best model (based on the lowest AICc value) is highlighted in bold.

Model name	Days foraged		Bout duration		Bouts per day	
	AICc	Δ AICc	AICc	Δ AICc	AICc	Δ AICc
Basic	279.64*	7.97	163.69	7.97	331.90	26.13
Colony age	281.59	9.92	155.72*	0.00	305.77*	0.00
Worker age	280.26	8.59	157.96	2.24	328.52	22.75
Worker weight	281.91	10.24	165.74	10.02	331.96	26.19
Best model* + learning	271.67	0.00	158.14	2.42	308.25	2.48

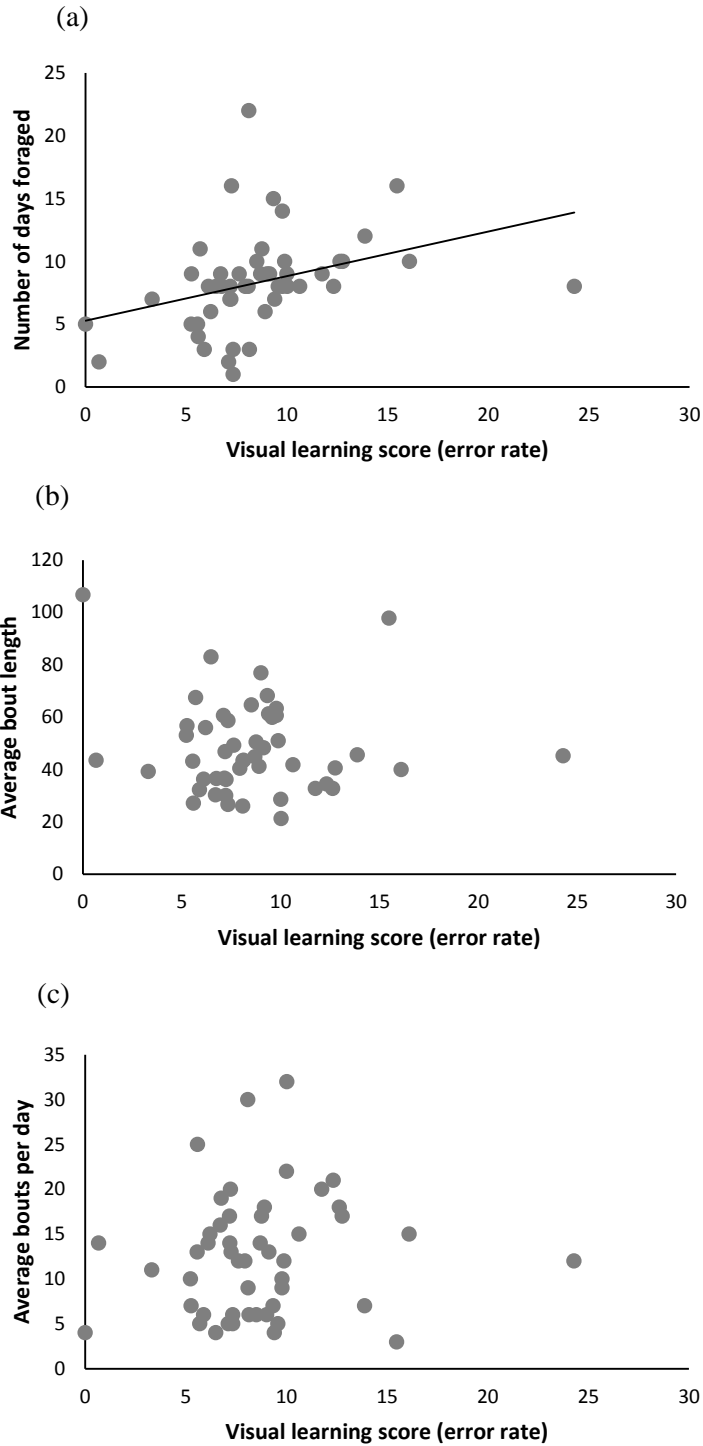


Figure 4.6 (a) Scatter plot of the significant positive relationship between visual learning ability and the number of days on which each bee foraged. Scatter plots showing no significant relationship between visual learning ability and either (b) average bout duration and (c) average number of bouts completed per day. Data presented are for all visually tested bees that foraged

once they were RFID tagged, with each dot representing a single bee ($n = 49$). Lower learning scores indicate that the bee was a better learner (i.e. made fewer errors).

4.4.2 Olfactory learning bees

A total of 80 bees from 5 colonies were tested in the olfactory task. I found no significant intercolony variation in olfactory learning (Kruskal–Wallis, $H_4 = 6.064$, $p = 0.194$, see appendix 4.3), and this was still the case when the bees that did not learn were excluded (Kruskal–Wallis, $H_4 = 3.418$, $p = 0.490$). All of these bees were RFID tagged and allowed to forage outside: 40 foraged, and the other 40 bees never returned to the colony once they left. On average, bees that never returned had a significantly lower learning score: i.e. they were better learners in the learning task (Mann Whitney U, $Z = 2.208$, $p = 0.027$, excluding non-learner bees, $n = 60$).

4.4.2.1 Foraging efficiency of olfactory learners

I observed at least one foraging bout from 33 of the 40 RFID tagged bees for which olfactory learning ability data were available (on average, 13% (range 4 – 40%) of the foraging bouts completed by each bee). As for the visual learning colonies, this was representative of the total number of bouts these individuals performed (Pearsons correlation, $t = 8.42$, $p < 0.001$). I found that learning performance neither predicted average nectar nor pollen collection rate (Table 4.3 and Figure 4.6).

Table 4.3 Candidate models to predict the nectar collection rate and pollen collection rate response variables for the bees tested in the olfactory paradigm that we observed more than 2 foraging bouts for including learning as a factor (n = 15 and 14 respectively). The AICc value of each model and the Δ AICc from the best model are presented. The model with the lowest AIC value from the top four models (indicated with an asterisk) had learning ability added to it to assess whether this significantly decreased the AICc value. The best model (based on the lowest AICc value) is highlighted in bold.

Model name	Nectar collection rate		Pollen collection rate	
	AICc	Δ AICc	AICc	Δ AICc
Basic	39.79*	0.00	49.59*	0.00
Colony age	43.25	3.46	53.38	3.79
Worker age	43.49	3.69	53.52	3.93
Worker weight	43.43	3.64	53.63	4.04
Experience	43.12	3.33	52.44	2.86
Best model* + learning	47.80	8.01	59.66	10.07

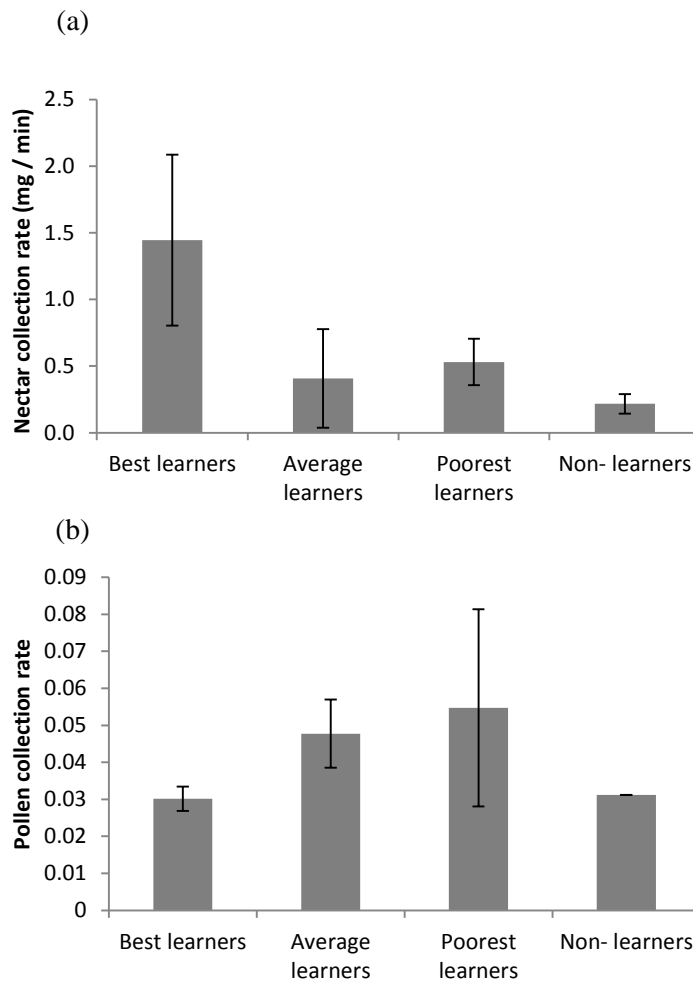


Figure 4.7. Data shown are mean \pm SE (a) nectar and (b) pollen collection rate of the bees that performed at least 3 nectar foraging bouts and their associated olfactory learning ability, in the 4 factor groups used in the model. Numbers in each group for the nectar and pollen collection rates respectively: best learners (n = 3 and 7), average learners (n = 4 and 4), poorest learners (n = 5 and 2), non – learners (n = 3 and 1).

4.4.2.2 Foraging activity

On average, the 40 foraging bees performed 69 bouts (range: 4–236) in total, over 6 days (range: 1–15), 10 bouts per day (range: 3–26) lasting 67 minutes (range: 29–184). The best model to predict the number of days foraged was colony age + learning ability, providing a significant improvement on the next best model (Δ AICc for colony age model = 3.31, see Table 4.4), therefore learning ability included as a factor significantly improved the prediction. Bees that did not learn the task foraged for significantly fewer days than the poorest learning bees, however there was no difference between the other groups (Figure 4.7a). I found that neither

bout duration, nor numbers of bouts completed per day, were best predicted by a model including learning performance (Table 4.4, Figure 4.7b and c).

Table 4.4 Candidate models to predict the number of days foraged, bout duration and bouts per day response variables for the bees tested in the odour paradigm using learning ability added as a factor (n = 39). The AICc value of each model and the Δ AICc from the best model are presented. The model with the lowest AIC value from the top four models (indicated with an asterisk) had learning ability added to it to assess whether this significantly decreased the AICc value. The best model (based on the lowest AICc value) is highlighted in bold.

Model name	Days foraged		Bout duration		Bouts per day	
	AICc	Δ AICc	AICc	Δ AICc	AICc	Δ AICc
Basic	224.41	4.93	167.42	9.37	257.46	30.34
Colony age	222.78*	3.31	158.05*	0.00	227.12*	0.00
Worker age	226.54	7.07	168.76	10.71	259.49	32.37
Worker weight	225.48	6.01	169.89	11.85	259.67	32.55
Best model* + learning	219.47	0.00	163.20	5.15	229.17	2.05

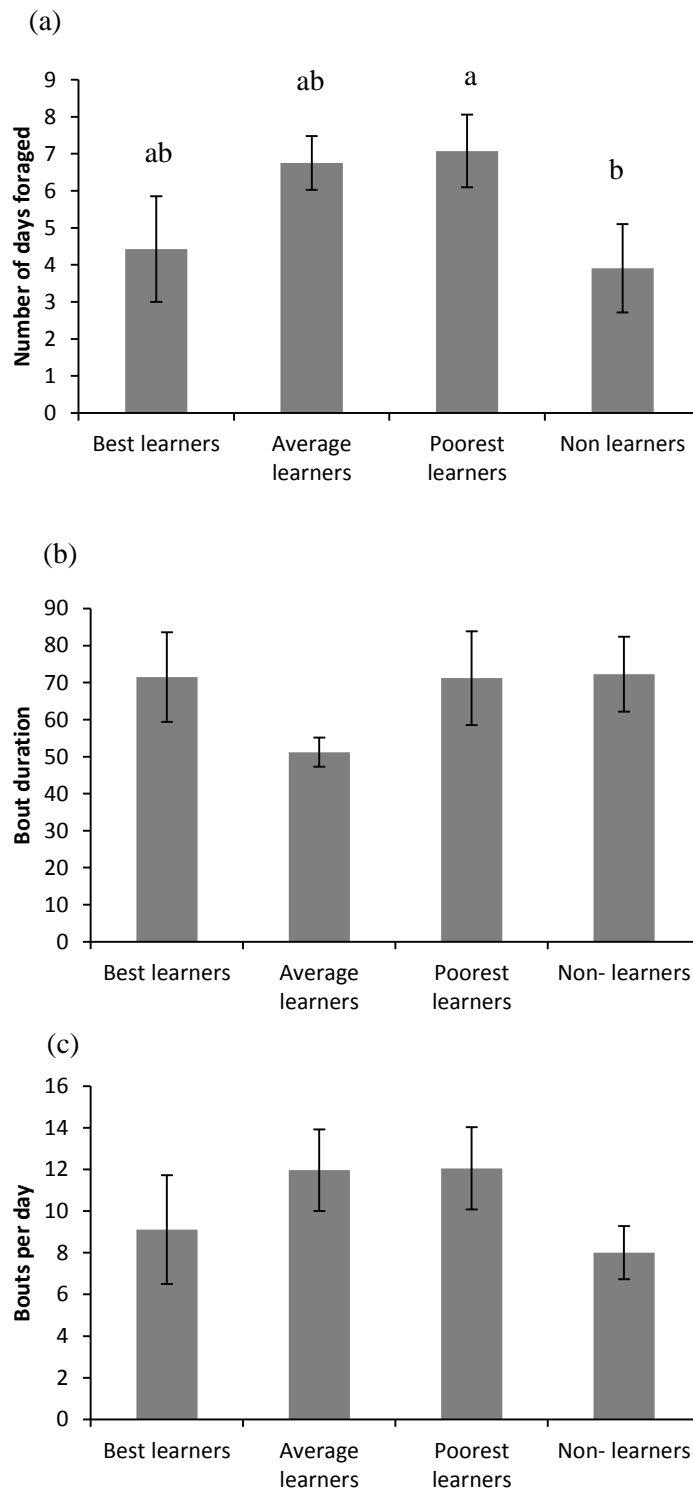


Figure 4.8. Mean (\pm SE) (a) number of days foraged, (b) bout duration and (c) number of bouts per day by the odour learning tested bees in the 4 groups used in the model using learning score as a factor. Significant differences ($p < 0.05$) are indicated by letters above columns (a). Best learners ($n = 7$), average learners ($n = 8$), poorest learners ($n = 13$), non-learners ($n = 11$).

4.5 Discussion

The work in this chapter is the first to investigate the relationship between individual bumblebees (*B. terrestris*) learning performance, in both a separate visual and olfactory task, with foraging efficiency and activity in a realistic field environment. I found that neither visual nor olfactory learning performance correlated with foraging efficiency. Visual learning performance did, however, predict foraging effort; bees that were poorer visual learners performed more foraging overall, by being active in this role for a greater number of days. The impact of variation in olfactory learning on foraging activity was less clear, as there appeared to be an underlying difference between learners and non-learners, with the non-learners foraging less actively. However, variation among those bees that learnt the relationship between odour and reward in the laboratory assay, suggests a similar pattern to that seen in the visual learning bees, whereby poorer learners performed a greater number of foraging bouts.

This work significantly adds to previous work that has addressed whether individual cognitive ability influences fitness related traits (Keagy, Savard & Borgia 2009; Cole *et al.* 2012). In this work I was able to control for previous experience of individuals, by testing the learning performance of naïve bumblebees in lab before they were allowed to forage outside. This is important as an individual's experience could affect how well they perform in a cognitive task, as there is potential for one individual to have some prior experience that helps them in the task that another individual does not have, therefore this is not testing cognitive ability across individuals fairly. Additionally, I used two cognitive tasks that are well-established at testing a specific area of cognition, whereas the use of problem solving ability to test cognition (Keagy, Savard & Borgia 2009; Cole *et al.* 2012) has received criticism of not testing a specific area (Rowe & Healy 2014). Finally, the result of finding a cost to enhanced learning ability challenges the view that learning will always be adaptive, and opens up questions to understand why this is not always the case.

One explanation for poorer visual learners performing more foraging could be that there is a trade-off between two traits: learning performance and longevity. The number of days bees in this experiment foraged for is a good proxy for longevity as 92% of bees foraged until their death; an increase in their foraging bout durations was also seen as they neared the end of their foraging careers, indicating that they were likely in physical decline. Better learners could be foraging for less time due to reduced longevity, a result consistent with work on *Drosophila* showing enhanced learning and memory being linked to decreased individual longevity (Mery & Kawecki 2004; Mery & Kawecki 2005). A more recent study has also shown an energetic

cost to enhanced learning in honeybees, with the bees giving more correct responses (better learners) having reduced survival following the learning task (Jaumann, Scudelari & Naug 2013). This suggests that individuals may have a limited energy /resource budget. The visually tested bees may either put this into enhancing their learning performance or extending their foraging career duration. Alternatively, the results could be linked to the environment in which the bees were foraging. Black capped chickadees born in harsh and changeable environments show enhanced cognitive performance (problem solving ability), compared to those born in less changeable / extreme environments (Roth, LaDage & Pravosudov 2010). This indicates that learning performance is more important in some environments compared to others, and that it can be selected for. The finding that better learning performance did not improve foraging effort or efficiency, could suggest that the environment in and around Royal Holloway did not favour better learners (perhaps as floral resources are plentiful), however under different environmental conditions this could change.

It has been shown that poor 'slow' learning bees, in the same visual learning task set up used in this study but with different colonies of bees, are more likely to sample different resources, than 'fast' learners that rarely sample other new flower types (Evans & Raine 2014). When observed in the lab, bees tested in the same visual task with poor learning ability have been shown to collect nectar at a higher rate per foraging bout (Burns & Dyer 2008; Evans & Raine 2014). However, I did not find poorer learning bees in this study collecting nectar or pollen at a higher rate. There could be a number of potential situations that explain this. Firstly, the two types of foraging strategy may have been equally effective in the test environment. Secondly, while all flowers in the lab experiments mentioned so far required the same flower handling skills, blooms in a natural environment are likely to vary, requiring bees to learn more than one set of flower handling skills. Pollen has been shown to take time for bees to learn to collect efficiently (Raine & Chittka 2007c). So although a poorer learning bee may be more likely to sample new flowers, it may take them longer to handle flowers, meaning that their efficiency may be comparable to a faster learning bee. Alternatively, as I only measured the quantity of the pollen and nectar collected (weight and size) there is another benefit that we did not measure, which is the quality of the pollen and nectar bees were collecting (i.e. protein content in the pollen and sugar concentration of the nectar). When quality of the pollen in the nest is higher more bees will go out and forage (Kitaoka & Nieh 2009) and individual bumblebees have been shown to discriminate pollen quality during collection, by associating the quality with a colour cue (Nicholls & de Ibarra 2014). Potentially the better learning bees could have been collecting higher quality resources, and this would be an interesting area to investigate further.

The fact that we have found that poorer visual learners do more foraging seems to contradict the result from Raine & Chittka (2008), who found that colonies containing better learning individuals in a colour learning task collected more nectar. Before conducting this experiment, I expected that on the individual level better visual learners would do more foraging. However, there are a few reasons why we have found what may seem to be a different result. The first could be that Raine & Chittka's result could have been confounded by a colony effect, for example that better learning colonies may have just been in better condition overall (e.g. parasite free, energy rich) and therefore had more resources to put into learning and foraging. Raine & Chittka (2008) also tested the learning ability of 15 bees from each colony and used this as a measure of learning ability for the colony, and the colonies were then allowed to forage outside where a different subset of workers were observed for all colonies. Therefore, they did not know the learning performance of the foragers that collected nectar in the field. Although it is likely that the better learning colonies would have included more 'better' learning bees in the rest of the colony that did not have their learning ability tested, this is not necessarily the case. Raine & Chittka (2008) also provisioned the colonies with *ad libitum* pollen with the intention that bees would collect only nectar, therefore this could have affected how they foraged compared to the bees in this chapter, where nearly 60% of the foraging bouts bees returned with pollen. Thirdly, and potentially most importantly, these experiments were performed in different locations, indicating that different learning abilities could perform better in different environments. Measuring the foraging ability of individuals in multiple environments would be an interesting area for further research, so we could quantify the characteristics of environments that favour different learning abilities.

The data from the olfactory tested bees are the first to test bees in this type of task, and then monitor their field foraging performance. These data are harder to interpret than the visual data due to a more restrictive sample size (number of bees for which foraging data are available), and there are potentially different processes underlying the bees that learn and do not learn, as I found non learners forage for less time. However, the overall outcome is essentially the same relationship as visually tested bees: foraging efficiency was not predicted by learning performance, but within bees that learn there was a trend for poorer learners to forage for longer. A lab study by Riveros & Gronenberg (2009) found that *Bombus occidentalis* bumblebees that had more foraging experience performed better in an olfactory PER learning task. They suggest this could indicate either that better learners do more foraging, and/or that learning ability may be improved by accumulating foraging experience. However, in this chapter I find the opposite effect when bees were allowed to forage in a natural environment. There is potential that the criteria were not stringent enough to ensure I tested only motivated foragers, and therefore perhaps some bees did not learn or forage as well

because they were not real foragers. However, the conclusions I can draw from the olfactory data are limited by the relatively small sample size I have for bees that foraged after being RFID tagged. Compared to the visually tested bees, I also found that on average olfactory tested bees performed fewer foraging bouts (mean number of bouts: 69 vs 103). This could be due to the different nature of the two learning tasks; the visual task gave individuals experience of foraging on artificial flowers and bees that were tested were definitely foragers. In contrast the olfactory task is potentially more stressful for the bees and the process may have therefore shortened their life.

Although finding only suggestive effects of variation in olfactory learning ability on foraging performance could indicate that olfactory learning is less important for foraging, I feel this is very unlikely. Flowering plants invest heavily in odour signals to attract pollinators that are frequently costly - if they were not useful they would not produce them. Bumblebees appear to learn better when there is both visual and olfactory cues present compared to either cue alone (Kulahci *et al.* 2008). The improvement in task performance is smaller when a visual cue is added to an olfactory cue, compared to adding scent to a visual cue. This indicates that olfactory cues are potentially more useful to foragers than visual cues, and could be detected from further away from their source. In addition, bees given multimodal cues (visual and scent) compared to visual alone, have been shown to be able to maintain accuracy by using the olfactory cues, when the visual cues became unreliable due to low light (Kaczorowski *et al.* 2012). Therefore, learning olfactory cues may be easier than learning visual cues in a natural environment, and the possession of some olfactory learning ability may be enough for bees to be able to use olfactory cues effectively.

Overall, the results in this chapter indicate that individual bumblebee visual and olfactory learning ability, as it was measured here, is not important in predicting foraging efficiency. Interestingly, poorer visual learners performed more foraging bouts – a result broadly supported by trends in the olfactory data also, challenging the view that learning will always be adaptive. Importantly, this work adds to and improves on the growing area of research on individual cognitive ability, and how this affects the fitness of the individual. Understanding the importance learning ability in different environments, and whether foraging quality may be predicted by learning performance, are interesting areas for future research.

Chapter 5

The impacts of an anthropogenic stressor on olfactory learning and memory

In this chapter I worked in collaboration with Dara Stanley (postdoctoral research assistant). We both contributed equally to the concept of the project both bringing different skill sets to the project, and due to the scale of the project this would have not been able to be done independently. We equally carried out experimental work and data analysis. I wrote this chapter independently which formed the basis of the submitted publication below, after comments and edits from Dara and my supervision team.

Accepted for publication on 14th October 2015 as: Stanley, D. A[†], Smith, K. E[†] & Raine, N. E. Bumblebee learning and memory is impaired by chronic exposure to a neonicotinoid pesticide, *Scientific Reports*, **5**, 16508 doi: 10.1038/srep16508. [†] Joint first authorship

5. The impacts of an anthropogenic stressor on olfactory learning and memory

5.1 Abstract

Pesticides are applied for crop protection, and bumblebees have the potential to be exposed to them while foraging for nectar and pollen on treated plants, in their natural environment. Although bees typically encounter these pesticides at sub-lethal levels, exposure can have impacts on factors such as reproduction or foraging behaviour with consequences for colony fitness. Bees face the challenge of navigating in complex environments and learning to manipulate many different flower types while foraging. Learning ability is essential in their survival and success as foragers. I assessed the impacts of two potential scenarios of exposure to the most widely applied neonicotinoid insecticide on oilseed rape crops in the UK, thiamethoxam, at field-realistic levels on bumblebee (*Bombus terrestris*) odour learning and memory using proboscis extension reflex (PER) conditioning. The first of these mimicked individual exposure during a foraging bout (acute exposure). The second mimicked colony-level exposure during the flowering period of a pesticide-treated crop (chronic exposure). Acute exposure had minimal effects on learning and memory, although bees exposed to field-realistic acute doses (10ppb thiamethoxam) showed fewer correct responses than controls, there was no difference in the proportion of individuals that could learn associate odour with reward. However, after field realistic chronic exposure bees learnt more slowly and their memory was impaired 3 hours after the learning task. This indicates that chronic exposure to pesticides has negative consequences on bumblebee learning and memory, which may have implications for behaviours such as foraging, navigation, brood care, and ultimately colony fitness.

5.2 Introduction

Bees are essential pollinators of many important agricultural crops and wild plants (Garratt *et al.* 2014), but declines in this group have been recorded worldwide (Biesmeijer *et al.* 2006; Potts *et al.* 2010). Bees can encounter a number of environmental stressors while foraging across the natural landscape, which are potential drivers of this decline. The ability to distinguish between the effects of these stressors on bees is difficult as they are likely to be interacting (Williams & Osborne 2009). The intensification of farming through increases in field sizes and hedge removal has decreased the complexity of habitat available for bees (Osborne *et al.* 2008). This has resulted in habitat loss for bumblebee nesting and foraging, which may mean that bees have to travel further when resources are scarce (Westphal, Steffan-

Dewenter & Tschardtke 2006). The species richness and diversity of bumblebees has been found to be lower in these intensively farmed areas (Williams 1988). The intensification of farming has also seen an increase in the use of pesticides applied to crops. Neonicotinoids are a major class of widely used pesticides, that act systemically when applied to the seeds of crops travelling through the plant tissues to target sucking pests (Elbert *et al.* 2008). Non-target organisms such as bees can be exposed to these pesticides in trace residues found in pollen and nectar, which can persist long after application (Rortais *et al.* 2005). Crops that have these neonicotinoids applied, like oilseed rape, flower for several weeks (Stanley & Stout 2014), and therefore individual bees may potentially be exposed to them for a substantial length of their foraging life. Although at these trace levels they should not be lethal to bees, there is growing evidence of sub-lethal effects. These effects range from impaired foraging ability (Gill, Ramos-Rodriguez & Raine 2012; Feltham, Park & Goulson 2014; Gill & Raine 2014), and decreased reproductive output (Whitehorn *et al.* 2012) to decreased navigation ability (Henry *et al.* 2012; Fischer *et al.* 2014).

Foraging bees in their natural environment have to use sophisticated behaviours to collect pollen and nectar from flowers, relying heavily on learning and memory. These include: navigating through a complex environment to find flower patches and returning to their nest site; learning which cues (such as colour, scent and texture) are reliable predictors of floral reward from a diverse array of flower species; and acquiring and fine-tuning the complex motor skills required to efficiently extract pollen and nectar from a variety of flower species (Raine *et al.* 2006a). Neonicotinoids act as agonists of insect nicotinic acetylcholine receptors (nAChRs) by binding to and activating these receptors (Nauen, Ebbinghaus-Kintscher & Schmuck 2001), affecting patterns of information transmission through the nervous system. It is therefore possible that an underlying cause for the sub-lethal behavioural effects reported to date, such as reduced pollen foraging efficiency, could be that the learning and memory abilities of workers have been impaired by neonicotinoid exposure.

Bumblebees are a key group of social bees that perform essential pollination services for a wide range of commercially important crops and wild plant species (Hayter & Cresswell 2006; Stanley, Gunning & Stout 2013). To date, studies investigating possible impacts of neonicotinoids on learning and memory have been performed exclusively on honeybees (Decourtye *et al.* 2004b; Williamson, Baker & Wright 2013; Williamson & Wright 2013). There are striking differences in biology between bumblebees and honeybees, which could mean that the sensitivity to pesticide exposure could be markedly different. In contrast to honeybee colonies, which are perennial, bumblebee colonies are much smaller and have an annual life cycle. While honeybee workers will become foragers for a relatively short period

at the end of their life (average 7 days (Dukas & Visscher 1994)), bumblebee workers may forage for their entire lifetime (2 - 3 weeks, (Brian 1952)). Furthermore, bumblebees seem less able than honeybees to metabolise the neonicotinoid imidacloprid (Cresswell *et al.* 2014), by clearing under 70% of assimilated imidacloprid each day, compared to honeybees, which were able to continuously metabolise. However, this study was not performed at field-realistic levels, and work on bumblebee metabolism capabilities is lacking as much of the work on toxicity to bees uses honeybees (Arena & Sgolastra 2014). Taken together, these factors could mean that individual bumblebee workers may be impacted differently, and are potentially at greater risk of pesticide exposure and associated sub-lethal effects than honeybees.

Most work on the sub-lethal effects of pesticides on bees has focussed upon the neonicotinoid imidacloprid. This was the first neonicotinoid to be used for pest control on agricultural crops. The work in this chapter instead focuses upon thiamethoxam, which has had growing use since 2005 in the UK, and is now the most widely used neonicotinoid seed dressing in the UK. In 2012, over 388 ha were treated with the pesticide (Garthwaite *et al.* 2012). There has now been a two year moratorium put in place for the use of three pesticides including imidacloprid and thiamethoxam in the EU, on crops attractive to bees. Therefore, during this time, it is important that research continues into their effects on bees, as their use will be reviewed in 2015. Although it is assumed that both thiamethoxam and imidacloprid have the same toxicity (Nauen *et al.* 2003), there is some evidence to suggest bumblebee microcolonies fed imidacloprid show reduced feeding and brood production at a lower concentration (1 and 2.5µg/kg) than thiamethoxam, where it took a higher concentration (39µg/kg) for these effects to be seen (Laycock *et al.* 2012; Laycock *et al.* 2014). This emphasises the need to study the effects of all neonicotinoid pesticides, as their impact may not always be comparable.

The aim of this chapter was to test whether acute and chronic exposure to the neonicotinoid pesticide thiamethoxam has an effect on the learning and memory of the bumblebee (*Bombus terrestris*). The levels of thiamethoxam we used were at a field-realistic level that are in the range of what bees would potentially encounter in the pollen and nectar of treated crops (Castle *et al.* 2005; Thompson *et al.* 2013). Learning performance and memory was tested using the olfactory proboscis extension reflex (PER) paradigm. In the first experiment, bees were fed a small volume of sugar water treated with pesticide, mimicking their consumption during a foraging bout visiting 10 – 12 seed treated oilseed rape flowers (acute exposure). In the second experiment, bees were fed sugar water treated with pesticide for 24 days, mimicking a situation in which a colony forages solely on a field of seed treated crop for its entire flowering period (chronic exposure), which is several weeks (Stanley & Stout 2014).

5.3 Methods

5.3.1 Pesticide exposure

Both experiments used the same concentrations of pesticide in sucrose solution. A stock solution of thiamethoxam (Sigma Aldrich, grade PESTANAL, analytical standard, brand: Fluka) was made by dissolving 10mg thiamethoxam in 100ml Acetone. Aliquots of this were added to 40% (v/v) sucrose to create solutions of the following concentration of pesticide: 250ppb (acute experiment only), 10ppb and 2.4 ppb thiamethoxam. The highest concentration (250ppb) was chosen as a positive control for the acute experiment, as at this high level (far above levels bees would be exposed to in the field), it would be expected to have an effect (approximately 42% of NOEL honeybee LD₅₀ European Food Safety Authority (2012)). The latter two solutions were chosen to be within field relevant ranges; the lower concentration (2.4ppb) was based on measurements of thiamethoxam found in nectar pots of *B. terrestris* colonies foraging on a field made of oilseed rape in the UK (Thompson *et al.* 2013), and the upper (10ppb) is at the top end the range in plant tissues regarded to be sufficient for control of pests, and therefore likely to be found in pollen and nectar (Castle *et al.* 2005; Godfray *et al.* 2014). A control solution was also made by repeating the process above, but using an aliquot of 10ppb acetone only. Fresh solutions were made up weekly.

5.3.2 Experiment 1: Acute exposure

Six bumblebee (*Bombus terrestris audax*) colonies were obtained, each containing a queen and a mean (\pm SE) of 66 (\pm 2.4) workers on arrival, from Biobest (Westerlo, Belgium) in March 2014. Each colony was transferred to a bipartite wooden nest-box (with a transparent Perspex lid) and connected to a flight arena (60 x 100 x 35 cm) by a transparent Perspex tube. Pollen (Frozen, honeybee collected - Koppert Biological systems: Weert, The Netherlands) was provided directly into the nest-box every 2 days, and 40% untreated sucrose solution (v/v) was provided *ad libitum* from a gravity feeder in the flight arena. After a week in which the colonies became acquainted with the feeder and flight arena, the experiment began.

The experiment lasted for four weeks, with the same procedures repeated weekly. Each week, the six colonies were randomly assigned to two groups to be tested in the learning task, on separate consecutive days. The day before each group was scheduled to have their learning ability tested, the colonies were blocked from entering the flight arena, and all bees were returned to the nest box, to increase motivation for sucrose foraging. After approximately two hours, bees were again allowed access to the flight arena; bees that landed and started to feed

from the gravity feeder, were captured using forceps, and harnessed as described in section 2.3.2. Ten bees were taken from each of the three colonies being tested the next day (on 5 occasions for there were not 10 bees seen on the feeder in this time and as many as possible were taken). Bees were harnessed in the early afternoon. Two hours later the bees were fed with 40% sucrose solution. Following feeding, the bees were placed in a horizontal position secured by plasticine, with the head over a piece of plastic-covered cardboard (Figure 5.1). Bees were left in the dark until the following morning.

The next morning, bees were fed a 10 μ l droplet of sucrose solution, containing either 250ppb, 10ppb or 2.4ppb thiamethoxam or the control solution. The 10 μ l drop mimics the volume of nectar found in approximately 10 – 12 oilseed rape flowers (unpublished data DS). This was fed to the bee by touching the antennae with 40% sucrose (untreated), and if the bee responded with a proboscis extension, the 10 μ l droplet of the solution for that bee was placed on the covered cardboard, so the bee could drink it. This allowed cross contamination between bees to be avoided. Bees were assigned to treatment groups randomly within each colony. Once the bee had drunk the droplet it was placed in an upright position, and learning performance was tested an hour after being fed. Only bees that consumed the full droplet were allowed to progress to the PER testing. Olfactory learning ability was tested using the same methods as described in section 3.3.2. Individual memory was assessed by giving each individual one presentation of the odour 3 hours after PER testing had finished, and their response noted. Following the experiment, the size of all the bees was measured using the widest part of the bee's thorax as the measurement point.



Figure 5.1 Photograph of harnessed bees positioned after feeding. Bee harnesses are held in place with a piece of plasticine and their head set above a piece of plastic covered cardboard used for easy disposal the next day.

5.3.3 Experiment 2: Chronic exposure

Twenty-one bumblebee (*Bombus terrestris audax*) colonies were obtained from Biobest (Westerlo, Belgium) in April 2014, each containing a queen and approximately 70 workers. Colonies were weighed on arrival to estimate size, and assigned sequentially to each of the three treatment groups (10 ppb Thiamethoxam, 2.4ppb Thiamethoxam and control), based on decreasing weight. Three colonies (one of each treatment) were assigned to treatment daily, until after 7 days, all colonies were receiving treated sucrose. This regime was chosen to allow for sequential daily PER testing later. Colonies were fed their treatment sucrose solution in a bird feeder inserted at the base of the colony box, every 2-3 days initially, and then every 1-2 days, when the colonies had grown significantly. Untreated pollen was fed to colonies every 2-3 days through the top of the box (colony feeding done by DS). All colonies had additionally been used for another experiment, where some of the bees from the colony had foraged on apple trees for a maximum of 1 hour (performed by DS).

After colonies had been exposed to treatments for an average of 24 days (range 22-26), they were tested using a PER conditioning protocol. To collect foragers the colony boxes were placed in a flight arena, and entrances opened. Colonies previously had experience of leaving the boxes to feed from a feeder in the flight arena, and therefore we assumed that bees leaving the colony were likely to be foragers. Six workers that had exited each colony were caught and harnessed as described in section 2.3.2. Two hours after harnessing bees were fed 40% sucrose, and then left in a dark room overnight. The following morning, the bee's responsiveness was tested, by touching their antenna with a droplet of 50% sucrose solution. Those bees responding with a proboscis extension, were fed a small droplet of this untreated solution, and testing began 15 minutes later. Five colonies were sampled on each day, and testing continued for 4 days, until 20 of the 21 colonies had been tested (one colony in the 2.4 ppb treatment produced large numbers of males earlier in the colony cycle than others, and so was excluded from testing). Following testing, bee size was measured as above.

5.3.4 Analysis

All analysis was performed in R version 3.1.0 (R Core Team 2014). A number of variables to describe learning performance were extracted from the olfactory PER results from both experiments including: 1) whether bees learnt the association between odour and reward (or not) over any of the 15 presentations, giving a binary response (0, 1); 2) total number of correct responses (proboscis extensions in anticipation of reward); 3) trial number when the first correct response occurred. Differences among treatment groups were tested for each of these

response variables using generalized linear mixed models (GLMM's), (glmer function in lme4 package (Bates *et al.* 2014)), assuming binomial distribution for Binary and Poisson for count variables. Treatment and size of the bee were included as predictor variables. In addition, colony membership (both acute and chronic experiments) and week of testing (acute experiment only) were included as random effects. I also tested for differences in worker body size among treatment groups, using a linear mixed effects model with colony as a random factor in the nlme package (Pinheiro *et al.* 2014). All models were tested for their fit by assessing normal Q-Q plots and residual vs fitted plots. To compare treatment groups to each other I used pairwise post hoc comparisons, to perform multiple comparisons, using the glht function from the multcomp package (Hothorn, Bretz & Westfall 2008). To assess impacts of treatment on memory after 3 hours, I compared correct responses on the 15th trial with those 3 hours later, by calculating the proportion of correct responses from each colony and then compared between colonies for each treatment group using a pairwise Wilcoxon test. Trial number of the first response and total number of learnt responses were analysed for all bees, and then reanalysed including only those bees that learnt (showed at least one learnt response).

5.4 Results

5.4.1 Experiment 1: Acute exposure

The olfactory learning ability of 171 individual bees from 6 colonies was tested. Bees that did not respond to more than 5 odour presentations with a proboscis extension when their antenna were touched with sucrose solution, were classed as unresponsive and were excluded from further analysis ($n = 29$). I found no difference in the size of bees selected for different treatment groups (Linear mixed effects model, $F_3 = 0.342$, $p = 0.795$). Additionally, I found that there were not a significantly different number of unresponsive bees between our treatment groups when each is compared to the control (Appendix 5.1).

Of the 142 responsive bees I found that more bees learnt the task in the control group, compared to the 250ppb treatment group and 10ppb treatment group (Figure 5.2A), but this was not significant for the latter (Table 5.1, $p = 0.002$ and $p = 0.164$ respectively). There was no difference between the control and 2.4ppb group, however using post hoc tests I found a significant difference between the 250ppb and 2.4ppb treatment groups (Tukey, Z value = 3.169, $p = 0.008$). Additionally, I found a significant effect of size with larger bees being better learners (Table 5.1, $p = 0.005$). When I used the total number of correct responses as the response variable, I found that the control group responded correctly significantly more times than the 250ppb and 10ppb treatment groups (Table 5.1, $p < 0.0001$ and $p = 0.004$ respectively). But, I found no difference between control and 2.4ppb (Figure 5.2B). I found additional significant differences in post hoc tests between 2.4ppb and 250ppb (Tukey, Z value = 5.694, $p < 0.0001$) and 10ppb (Tukey, Z value = 3.479, $p = 0.003$).

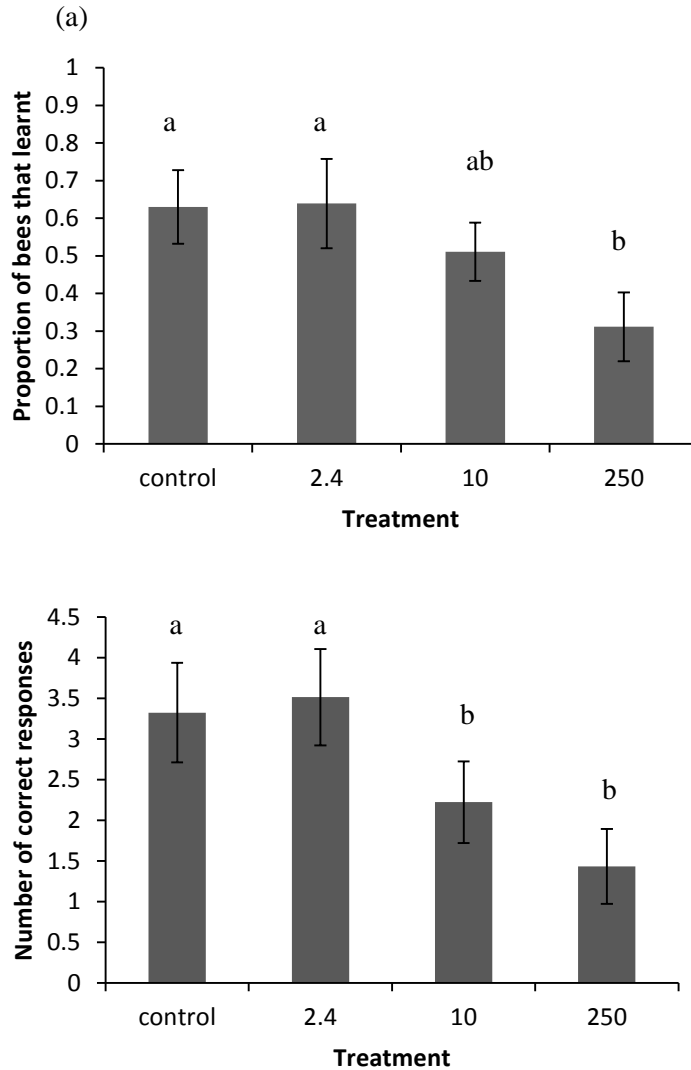


Figure 5.2 The mean proportion of bees in each acute treatment group that learnt (i.e. responded correctly to at least one odour presentation). (b) The mean number of correct responses bees gave in each treatment group. Data presented is mean \pm SE. Number of bees in each treatment: Control, n = 34; 2.4ppb, n = 35; 10ppb, n = 36; 250ppb, n = 37. Letters indicate significantly different pairwise comparisons from post-hoc tests ($P < 0.05$).

Finally, I compared the learning ability of the bees that responded correctly at least once during the 15 conditioning trials (n = 78). I found that learning ability was not affected by treatment (Figure 5.3a). The control group did not respond correctly more times than any of the other treatment groups (Table 5.2a), or learn the task quicker (Table 5.2b). The size of bees also no longer had an effect on learning ability (Table 5.2, Z value = 1.489, p = 0.136). In addition, the memory of the odour association 3 hours later was not significantly impacted compared to the responses on the 15th trial of the learning task for any treatment group (Related

samples Wilcoxon signed ranked test: 2.4ppb $p = 0.715$; 10ppb $p = 0.180$; 250ppb $p = 0.655$; control $p = 0.317$, Figure 5.3b).

Table 5.1 Generalized linear model for (a) the binary learnt response variable and (b) the number of correct responses variable. Parameter estimates are calculated with reference to the control group. Colony was included as a random effect ($n = 142$). Significant values are highlighted in bold.

	<i>Fixed effects</i>	<i>Parameter estimate</i>	<i>SE</i>	<i>Z value</i>	<i>P value</i>
<i>(a) Learnt response</i>	Intercept (Control)	-8.533	3.340	-2.555	0.011
	Treatment (250ppb)	-1.627	0.534	-3.047	0.002
	Treatment (10ppb)	-0.715	0.513	-1.393	0.164
	Treatment (2.4ppb)	0.070	0.531	0.131	0.895
	Bee size	1.860	0.669	2.782	0.005
<i>(b) Number of correct responses</i>	Intercept (Control)	-2.433	0.994	-2.447	0.014
	Treatment (250ppb)	-0.864	0.168	-5.157	<0.001
	Treatment (10ppb)	-0.426	0.146	-2.915	0.004
	Treatment (2.4ppb)	0.073	0.130	0.561	0.575
	Bee size	0.675	0.193	3.500	<0.001

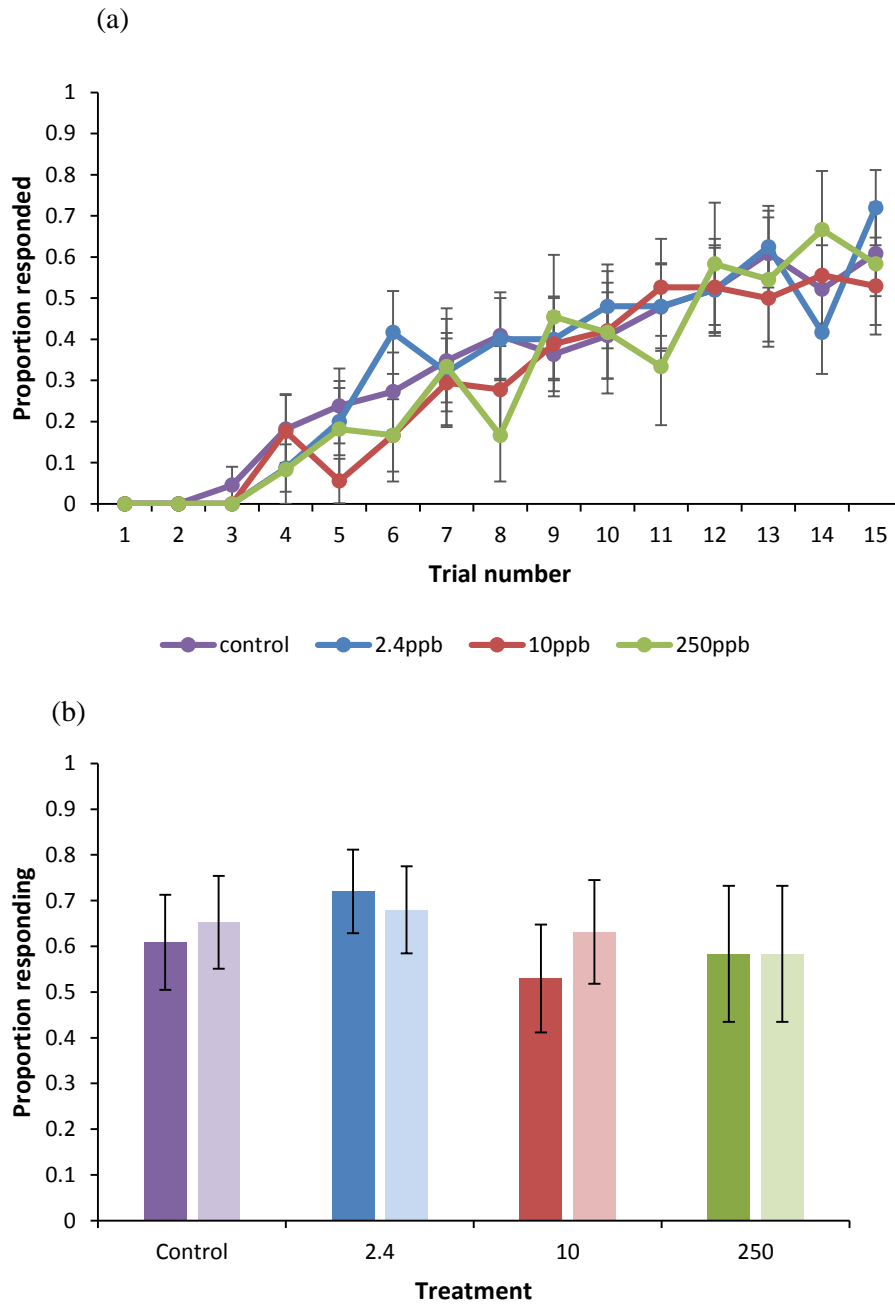


Figure 5.3 (a) Acquisition curves showing the proportion of acutely exposed bees responding correctly with a proboscis extension to the conditioned odour across the 15 conditioning trials. (b) Memory test data: Proportion of bees that responded correctly to the conditioned odour on trial 15 (dark bars) and 3 hours after the learning task (light bars). Only bees that responded correctly at least once were included: control, $n = 23$; 2.4ppb, $n = 25$; 10ppb, $n = 19$; 250ppb, $n = 12$ (both graphs show mean \pm SE).

Table 5.2 Generalized linear models for (a) number of correct responses variable (b) trial number of first correct response. Parameter estimates are calculated with reference to the control group. Colony was included as a random effect (n = 78).

	<i>Fixed effects</i>	<i>Parameter estimate</i>	<i>SE</i>	<i>Z value</i>	<i>P value</i>
<i>(a) Number of correct responses</i>	Intercept (Control)	0.079	0.954	0.082	0.934
	Treatment (250ppb)	-0.129	0.172	-0.750	0.453
	Treatment (10ppb)	-0.177	0.147	-1.205	0.228
	Treatment (2.4ppb)	0.041	0.132	0.309	0.758
	Bee size	0.286	0.192	1.489	0.136
<i>(b) Trial number of first correct response</i>	Intercept (Control)	1.75853	0.65128	2.700	0.00693
	Treatment (250ppb)	0.04956	0.12344	0.402	0.68808
	Treatment (10ppb)	-0.06935	0.11023	-0.611	0.54120
	Treatment (2.4ppb)	-0.03113	0.10431	-0.298	0.76534
	Bee size	0.06933	0.12846	0.540	0.58937

5.4.2 Experiment 2: Chronic exposure

I tested the olfactory learning ability of 100 bees from 20 colonies (5 bees tested from each colony), of which five bees were removed from the analysis as unresponsive. Again, there was no significant difference in size of bees between the three treatment groups (Linear mixed effects model, $F_3 = 2.83$, $p = 0.087$), however there was a trend for the 10ppb treated bees to be smaller. I found a trend for a decreasing proportion of bees learning the task with increasing thiamethoxam concentration, however this was not significant (Table 5.3a and Figure 5.4a). I additionally found that bees gave more correct responses in the control group compared to the 2.4ppb and 10ppb, however this was not quite significant (Table 5.3b and Figure 5.4b). I found a significant effect of size, with larger bees giving more correct responses (Table 5.3b, $p = 0.008$).

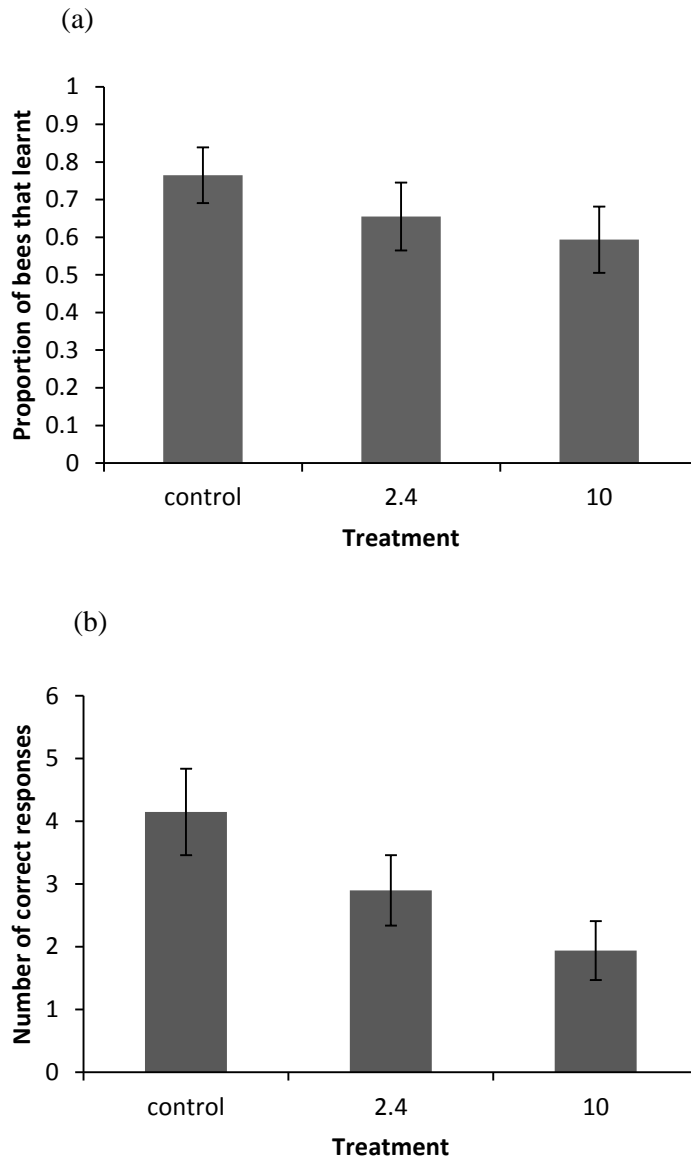


Figure 5.4 (a) The mean proportion of chronically exposed bees in each treatment group that responded correctly to at least one odour presentation (i.e. showed learning). (b) The mean number of correct responses bees gave in each treatment group. Data presented is mean \pm SE. Number of bees in each treatment: Control, n = 34; 2.4ppb, n = 29; 10ppb, n = 32.

Table 5.3 Generalized linear models for the (a) learnt response variable and (b) the number of correct responses variable for the chronically exposed bees. Parameter estimates are calculated with reference to the control treatment. Colony was included as a random effect (n = 95). Significant values are highlighted in bold.

	<i>Fixed effects</i>	<i>Parameter estimate</i>	<i>SE</i>	<i>Z value</i>	<i>P value</i>
<i>(a) Learnt response</i>	Intercept (Control)	-4.239	4.549	-0.932	0.351
	Treatment (10ppb)	-0.693	0.551	-1.258	0.209
	Treatment (2.4ppb)	-0.592	0.570	-1.039	0.299
	Bee size	1.083	0.910	1.190	0.234
<i>(b) Number of correct responses</i>	Intercept	-2.038	1.261	-1.616	0.106
	Treatment (10ppb)	-0.743	0.386	-1.924	0.054
	Treatment (2.4ppb)	-0.359	0.388	-0.923	0.356
	Bee size	0.642	0.243	2.638	0.008

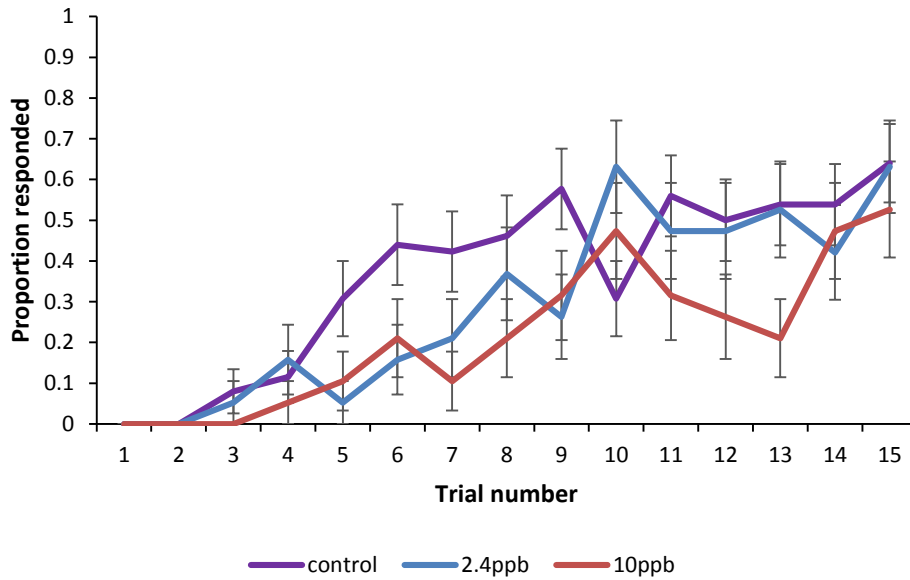
Table 5.4 Generalized linear models for the (a) total number of correct responses variable, (b) trial number of first correct response variable for the chronically exposed bees. Parameter estimates are calculated with reference to the control treatment. Colony was included as a random effect (n = 64). Significant p value are highlighted in bold.

	<i>Fixed effects</i>	<i>Parameter estimate</i>	<i>SE</i>	<i>Z value</i>	<i>P value</i>
<i>(a) Number of correct responses</i>	Intercept (Control)	-0.632	1.241	-0.509	0.611
	Treatment (10ppb)	-0.425	0.251	-1.692	0.091
	Treatment (2.4ppb)	-0.189	0.241	-0.787	0.432
	Bee size	0.431	0.242	1.784	0.074
<i>(b) Trial number of first correct response</i>	Intercept (Control)	1.728	0.837	2.064	0.039
	Treatment (10ppb)	0.333	0.111	2.990	0.003
	Treatment (2.4ppb)	0.238	0.108	2.205	0.027
	Bee size	0.039	0.164	0.235	0.814

Finally, I compared the learning ability of bees that showed at least one correct response in each treatment group; the total number of correct responses was not significantly different between the treatment groups (Table 5.4a). However, I found that control bees learnt significantly faster than bees from both the 2.4ppb and 10ppb treatment groups (i.e. on average, the first response for control bees happened earlier in the experiment than for pesticide treated bees) ($p = 0.027$ and $p = 0.003$ respectively, Table 5.4b and Figure 5.5a). Bee size no longer had a significant effect on learning ability (Table 5.4). By the end of the 15 trials, the learning performance between groups was comparable (Figure 5.5a). The 3-hour period between the end of conditioning and the memory test had no significant impact on the proportion of control bees showing learnt responses (Related samples Wilcoxon signed ranked test, $p = 0.317$; Fig 5.5b). However, the proportion of bees exposed to 2.4ppb pesticide that showed learnt responses was significantly lower after 3 hours in comparison to the end of the trial period (Related samples Wilcoxon signed ranked test, $p = 0.027$, Figure 5.5b), showing an impact of pesticide on memory. The proportion of bees remembering after 3 hours in the 10ppb was lower in comparison to the end of the trial period, but this difference was not quite

significant at the 5% level (Related samples Wilcoxon signed ranked test, $p = 0.066$, Figure 5.5b).

(a)



(b)

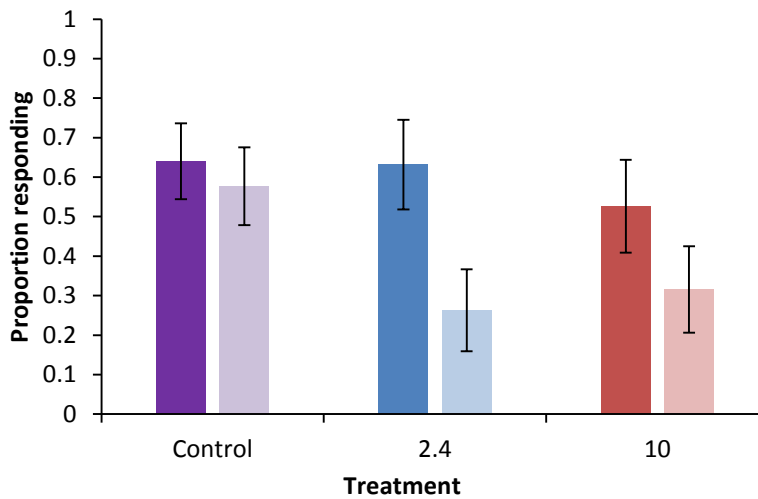


Figure 5.5 (a) Acquisition curves showing the proportion of chronically exposed bees responding correctly with a proboscis extension to the conditioned odour across the 15 conditioning trials. (b) Memory test data: Proportion of bees that responded correctly to the conditioned odour on trial 15 (dark bars) and 3 hours after the learning task (light bars). Only bees that responded correctly at least once were included: control, $n = 26$; 2.4ppb, $n = 19$; 10ppb, $n = 19$ (graphs show mean \pm SE).

5.5 Discussion

In this chapter, I investigated the effects of field-realistic exposure to the neonicotinoid pesticide thiamethoxam, on learning and memory of individual bumblebees (*B. terrestris*). Foraging bees have the potential to be exposed to pesticides applied to crops in different intensities. In my experiments I mimicked two levels of exposure, acute (a bee visiting multiple flowers of a systemically treated crop in one foraging bout) and chronic (a colony foraging on a systemically treated crop for 3 weeks). I found a minimal effect of the acute exposure on bumblebee learning and memory at field-realistic concentrations, however when given at a higher dose (250ppb – positive control) this significantly reduced the number of bees that could learn the task. In contrast, the bees that were chronically exposed were slower to learn the task and showed memory impairments after 3 hours, and there was a trend for fewer bees to be able to learn the task with increasing thiamethoxam concentration.

Reduced speeds of learning an association as a result of chronic neonicotinoid exposure could have significant knock-on effects for colony development in the field, as intercolony variation in visual learning speed has been shown to correlate with foraging performance in the field (Raine & Chittka 2008). Although I did not show the same in chapter 4 on an individual level in a different environment, however bees that did not learn during the olfactory task performed the least foraging. As such, colonies containing impaired learners could be more constrained in terms of the floral resources they can collect and invest into colony growth and reproduction. Impaired learning may be a consequence of the metabolism of pesticides taking up valuable resources, which may have alternatively been invested in learning. Honeybees have been suggested to show a similar trade off in learning ability, when bees were nutritionally stressed (Jaumann, Scudelari & Naug 2013).

Bees typically forage in an environment containing dozens of flower species that differ in colour, scent, morphology and also the quantity and quality of rewards they provide. Foraging bees need to learn to exploit the most rewarding floral sources, which will change over time. Hence, if a bee takes longer to learn floral cues as predictors of reward, they may miss out on more profitable flowers. The additional finding that a bee's 3 hour memory is significantly impaired following pesticide exposure, means that bees potentially have to spend additional time re-learning how to handle morphological complex flowers and/or the location of rewards. Although bees ended up with the same level of learning in the chronic experiment at the end of the conditioning period, the differences in rates of learning took place over a 3-hour test period. In chapter 4, I found that bumblebee foraging bouts lasted on average 48 – 67 minutes, so therefore pesticide impacts have the ability to impact multiple foraging bouts. Previous

work using the neonicotinoid imidacloprid, shows that exposed bees foraged less efficiently for pollen (Gill, Ramos-Rodriguez & Raine 2012; Feltham, Park & Goulson 2014). The work in this chapter suggests that these earlier findings could be related to impairment of their ability to learn and/or remember salient cue-reward associations. This is likely to be particularly true for pollen foraging, a more complex task than nectar collection, taking up to 3 times as many visits to learn to collect efficiently (Raine & Chittka 2007c). This gives us an example of how slower learning could negatively affect the dynamics and efficiency of bee foraging.

Work in this chapter, provides the first evidence that field-relevant neonicotinoid exposure impacts on learning abilities in bumblebees, complementing the growing body of evidence on the sub-lethal effects of pesticides on learning in honeybees (Decourtye *et al.* 2004b; Williamson, Baker & Wright 2013; Williamson & Wright 2013). Although some of these studies were not within the field-realistic exposure range. However, because honeybees and bumblebees vary appreciably in aspects of their physiology, ecology, life history and ability to metabolise pesticides (Cresswell *et al.* 2014), this could also mean they are differentially affected by similar pesticide exposure. While honeybees communicate information about profitable reward sites through the waggle dance, bumblebees primarily have to learn for themselves which flowers are rewarding. Although bumblebees can learn about the odour of rewarding flower species (Dornhaus & Chittka 1999; Dornhaus & Chittka 2001) from nest mates, they are unable to communicate about specific locations, meaning foragers need to explore and locate these rewarding flower patches themselves. In addition to the higher cognitive demands placed on bumblebee foragers, their physiology appears to mean they are less well equipped than honeybees to detoxify neonicotinoids following exposure (Cresswell *et al.* 2014). Honeybee physiology allows workers to continuously metabolise imidacloprid so it is not found in body residues. However, bumblebees have been shown to metabolise 70% of their daily intake (Cresswell *et al.* 2014), and therefore we might expect that bumblebees are more susceptible.

Overall, acute exposure to thiamethoxam showed no significant effects on learning and memory at field-realistic concentrations (up to 10ppb), although there was evidence of a trend for fewer bees being able to learn the task in the 10ppb group, and a significant trend for the 250ppb treatment, compared to control bees. While acute exposure of 250ppb is likely to be far in excess of that which might occur in the field, it is interesting that treated individuals able to learn the task show no apparent deficit in either learning or memory. There is evidence that honeybees can constantly metabolise (can break down as fast as its ingested) imidacloprid they ingest when they are fed at a level of 98 μ g kg (Cresswell *et al.* 2014), although bumblebees did not at this level. Bees in this chapter were only fed a 10 μ l droplet of 250ppb,

so this could indicate that bees that learnt the task had managed to deal with the effects of the pesticide, before or during the task. Although at a more field realistic dose (10ppb) I did not find significantly fewer bees learning the task, there is a trend for this to be lower than the control, and I also found that the number of learnt responses was lower. Bumblebee colonies are much smaller than honeybee colonies and therefore more vulnerable to pesticide exposure (Thompson 2001), even a small proportion of bees being unable to learn could affect the colony's ability to cope with changing conditions and ability to adapt. One reason for some of the data trends being non-significant, could be greater variability in learning ability among bumblebees, compared to honeybees. Most honeybees will learn to associate an odour with reward after only one or two conditioning trials (Williamson, Baker & Wright 2013), whereas bumblebees typically need more trials (Riveros & Gronenberg 2009). In this chapter, I found some bees learning as early as the 3rd trial, but others did not learn until the 15th trial, which is similar to chapters 2, 3 and 4. Additionally, having bees restrained between the pesticide exposure and the learning task (1 hour acute, 20 hours chronic), may have potentially impacted upon metabolism; in the field they would have been able to fly, and this may have further aided in the metabolism of thiamethoxam. Therefore, potentially in natural conditions the effects we see may be decreased further. Alternatively, there is potential for bees to consume a much higher volume of the acute dose during a foraging bout, or they could continue to forage before they are able to metabolise the pesticide, therefore the effects on learning and memory could be magnified

In this chapter, I have shown that field-realistic concentrations of thiamethoxam have minimal effects on bumblebee learning and memory when acutely exposed, but when exposed chronically for 3 weeks, bumblebees were slower to learn and their 3 h memory was impaired. This adds to the body of evidence showing sub-lethal effects of neonicotinoids on bumblebees, this being the first to show learning and memory deficits in bumblebees after chronic exposure. Deficits in learning and/or memory following chronic exposure has implications for many tasks bumblebees must carry out to successfully reproduce (including foraging, navigation, brood care), and could be a sub-lethal impact of pesticides not recorded in other studies. Key differences between results obtained from different bee taxa, indicate that results from honeybees cannot simply be extrapolated to bumblebees or solitary bees.

Chapter 6

General discussion

6. General discussion

6.1 Summary of chapters

It has been suggested that having a range of foraging strategies within a colony (e.g. fast and inaccurate vs slow and accurate), can reduce the variation in reward collection across different situations, compared to a colony with a single foraging strategy (Burns & Dyer 2008). In chapter 2, I found that there was neither a trade-off, nor a correlation, in learning performance between two sensory modalities (visual and olfactory). This may suggest that having a mix of learning abilities in different modalities, across individuals, may be beneficial for the colony to cope with changeable conditions. Variation in individual learning phenotypes (across sensory modalities) could allow a colony to exploit environments more effectively, where the flower species in bloom is not fixed. There is potential for the sensory modality that is the most reliable predictor of reward, to be different for different flower species, so a mix of learning phenotypes (specialising in different sensory modalities) within a colony, could reduce variation in foraging performance over time.

I followed on from this in chapter 3 by investigating a potential cause of this variation in learning ability. In honeybees, there is a large number of studies suggesting that there is a link between foraging specialisation and learning ability, pollen foragers learn better in olfactory and tactile tasks (both faster acquisition rates and higher final level of task performance) than nectar foragers (Scheiner, Erber & Page 1999; Scheiner, Page & Erber 2001a; Scheiner, Page & Erber 2001b). Learning performance is further correlated with sucrose responsiveness. I found that in two colonies, bees that foraged for pollen (pollen only and flexible foragers) were better at the olfactory learning task than the nectar foragers; however sucrose responsiveness and learning were obviously not linked. In chapter 3, I confirmed that pollen foraging is a skill that requires learning for performance to improve (Raine & Chittka 2007b), which could be a reason for enhanced learning performance by bees that select to forage for pollen. Among those individual bees that collected pollen, olfactory learning ability did not appear to be driving their pollen collection rate, therefore another learning process may have been important, such as motor learning. My results from chapter 2 indicate that learning in different sensory modalities is not correlated, suggesting that this may not also be the case for olfactory and motor learning (or indeed other learning processes) that may be driving pollen collection ability.

When foraging in the field, neither pollen nor nectar collection rates were linked to either the visual or olfactory learning ability of individual bees (chapter 4), providing further support

that these learning modalities may not be particularly important in learning efficient resource collection (chapter 3). This is not in agreement with a previous colony level experiment, suggesting a positive correlation between visual learning and nectar collection rate (Raine & Chittka 2008). However, as discussed in section 4.5 there is potential for the correlation in Raine & Chittka's (2008) study to be driven by a third colony-level factor (e.g. parasite infection). In addition, the two experiments (chapter 4 and Raine & Chittka 2008) were performed in different environments, which could have affected the use, and relative importance, of visual and olfactory learning ability to bees in these given environments. Whilst it may be assumed that enhanced learning will lead to better foraging, poorer learners have actually been shown to perform better than good learners in certain situations. The errors poorer learners make when foraging can allow them to discover new, more profitable, flowers more quickly and increase their nectar collection rate (Evans & Raine 2014). Therefore, differences in learning ability could be linked to bees using different foraging strategies, and in the environment used in chapter 4, bees of different learning abilities were able to perform comparably. This interesting future area of research is discussed in more detail in section 6.3.

In chapter 4, when I investigated whether learning was linked to foraging effort, I found that visual learning ability was predicting bees foraging activity. Poorer visual learners foraged for a greater number of days, and thus performed more foraging bouts overall. The results from the bees that learnt during the olfactory task (responded correctly at least once), were suggestive of the same pattern. As learning ability did not predict resource collection rate, this suggests that these poorer learning bees were contributing more work to the colony, and bringing back more food. This is consistent with the view that, enhanced learning ability in this situation was costly for individuals, and supports results from previous laboratory studies of fruit flies and honeybees, that have also shown learning ability to have an operating cost (Mery & Kawecki 2004; Jaumann, Scudelari & Naug 2013). These results suggest that having variation in learning ability among individuals within the colony will be beneficial, to give them the best chance to adapt to different environmental situations. These findings challenge the results of previous studies, that have suggested a positive correlation between cognitive ability and fitness related traits (Raine & Chittka 2008; Keagy, Savard & Borgia 2009; Cole *et al.* 2012). However, I have been able to address some of the issues that potentially affect these earlier studies by using both well-established tasks, that are accepted as testing specific cognitive abilities, and controlling for the individuals' previous experience, expanding our knowledge considerably in this exciting research area.

When foraging in their natural environment, bees have the potential to be exposed to pesticides while foraging on treated crops or flowers surrounding these crops. In chapter 5, I showed

olfactory learning ability is impaired at field realistic exposure levels of the neonicotinoid pesticide, thiamethoxam. Such a reduction in learning performance, following environmentally relevant pesticide exposure, could be a mechanism explaining reported behavioural effects, such as decreased foraging efficiency (Gill, Ramos-Rodriguez & Raine 2012; Feltham, Park & Goulson 2014; Gill & Raine 2014). My work is the first to assess the impacts of pesticides on learning and memory in bumblebees. These results show similar trends to the majority of honeybee learning studies (Decourtye *et al.* 2004a; Decourtye *et al.* 2004b; Williamson & Wright 2013), however bumblebees are less able to metabolise pesticides (Cresswell *et al.* 2014), therefore we cannot assume the effects on these two bee taxa will be the same, and more work is needed in this area. The use of field-realistic concentrations highlights pesticide exposure to bumblebee colonies foraging in the field is of real concern. Although acute exposure had minimal effects on learning ability, chronic exposure caused bees to learn slower and their 3 hour memory was impaired compared to controls. Comparing the variation in olfactory learning performance in chapter 4 and 5 (Appendix 6.1a & b), it can be seen that chapter 5 control exposed bees had a similar level of variation to those in chapter 4, but the pesticide exposed bees variation was considerably reduced. Throughout this thesis (chapters 2-4) I have found considerable variation in learning ability (both visual and olfactory) within colonies, suggesting that it is beneficial for the colony to have this inter-individual variation. If pesticide exposure decreases this variation it could be one driver behind the negative impacts pesticide exposure has been shown to cause.

6.2 The use of the proboscis extension reflex

Previous work using the proboscis extension reflex (PER) paradigm with bumblebees has highlighted that this taxon is less amenable to the technique than honeybees and that learning rates were lower (Laloi *et al.* 1999; Laloi & Pham-Delegue 2004; Riveros & Gronenberg 2009; Toda, Song & Nieh 2009). However, there also appeared to be differences among bumblebee species, with *B. terrestris* (Laloi *et al.* 1999; Laloi & Pham-Delegue 2004) performing poorer than *B. occidentalis* (Riveros & Gronenberg 2009). My work in this thesis, along with a recent study (Sommerlandt, Rossler & Spaethe 2014), has highlighted that PER is a viable technique for testing olfactory learning performance in bumblebees (*Bombus terrestris*), and that the learning rates achieved with this improved technique, are more comparable to other bumblebee species than previous reported. However, in this thesis I have found that there is considerable variation in learning ability both among individuals (Figure 2.5 & Appendix 6.1a) and colonies (Figure 2.4 & Appendix 4.3). This is an important factor that needs to be taken into account when designing experiments using this technique.

I have used the proboscis extension reflex conditioning paradigm for use in all of my research chapters, which shows the adaptability of this technique in testing different hypotheses. I chose to use absolute conditioning for the final three research chapters, as I found during pilot work that learning rates were comparable to the differential conditioning paradigm I used in chapter 2. Additionally, using absolute conditioning has the benefit of being able to test more bees at one time, which was useful for these three chapters. I found that a higher proportion of bees did learn after 15 trials of differential conditioning (chapter 2, 0.70 ± 0.05 , $n = 94$ bees, Figure 2.4), compared to absolute conditioning (chapter 4, 0.60 ± 0.06 , $n = 80$ bees, Appendix 4.3). However, there was one particularly good learning colony in chapter 2, in which 91% of the bees learnt by the end of the learning task and the other three colonies were between 58-66%. Absolute and differential conditioning results have been found to be comparable in another recent study (Sommerlandt, Rossler & Spaethe 2014), although these authors did not take colony membership into account. Therefore, my work suggests that future studies may benefit from using differential conditioning, but solid results can also be achieved using absolute conditioning. In chapter 5, I also tested the 3 hour memory retention of bees after completing the associative olfactory PER task, which shows another use of this technique, as memory can easily be tested alongside learning. Unless the bees were going to be fed again, memory testing is unlikely to be possible much past 3 hours, as honeybees start to die when harnessed for longer than this period (Jaumann, Scudelari & Naug 2013).

From my experience, I would still say that bumblebees can be less amenable to the PER conditioning technique, but there are a number of factors that are important in improving this. Clearly the harnessing technique is very important; the yoke method that I adapted from Riveros & Gronenberg's (2009) work transformed how the bees behaved in the harnesses, to a much calmer state, compared in constant wriggling and buzzing in honeybee-style harnessing. Whereas, with honeybees not as much care needs to be taken with the harnessing and bees tend to perform well however they are harnessed. Increasing the sucrose concentration bees are given during the PER task, compared to what they are fed in the colony, helps to increase motivation for the task. Motivation is also increased by giving the bees a small droplet of sucrose solution 15 minutes prior to when the PER task begins. Bumblebees show considerable size variation (unlike honeybees), and I have found that during pilot experiments that small bees do not perform as well during the PER: they are less responsive and motivated to take part in the task. Smaller bees have been observed to perform in nest tasks more often (Brian 1952; Free 1955; Goulson *et al.* 2002), and in the experiments presented in this thesis I chose to test only foragers. Size did not come out as a factor affecting learning performance in my analyses (with the exception of some models in chapter 5, where the pesticide exposure may have caused this effect), probably as I did not test the smallest bees

in the colony. I highlight this as potential body size impacts on learning and memory performance should be taken into account in future experiments.

A potential weakness of using PER conditioning with bumblebees (and potentially bees in general) is that it may shorten their lifespan. I found in chapter 4, that bees that had their olfactory learning tested foraged less, compared to bees that had their visual learning tested (average number of bouts 69 vs 103 bouts). This may indicate that the paradigm is stressful for bees. However, as different colonies were used for different modalities, it is therefore impossible to disentangle whether this is a colony effect, rather than an effect of the paradigm used to test learning. In chapter 2, I did not find the PER paradigm having a negative effect on the bees, as bees readily began foraging once they were returned to the colony following testing. This view is also supported by the finding that, there was no difference in visual learning performance of bees that were tested in the olfactory task or visual task first. In chapter 3, I tested the sucrose responsiveness of bees prior to them being tested in the olfactory PER task. I found in this experiment that learning performance was lower: by the end of the 15 trials 46% of bees had learnt the task, a level considerably lower than other chapters. This could be due to these two colonies being particularly poor olfactory learning colonies. Alternatively, it could be due to the sucrose responsiveness test being performed prior to the olfactory task, that could have potentially desensitised bees, as their antenna were touched multiple times without reward. My work in chapter 3 is the first time sucrose responsiveness and olfactory learning have been tested together in bumblebees. I based my protocol on previous work using honeybees in which the learning task started after the sucrose responsiveness test (Scheiner, Erber & Page 1999). I was still able to find differences between nectar and pollen foraging bees. However, I would recommend that if future studies test sucrose responsiveness and olfactory learning using PER, that they are either tested with more time in between or bees are tested using only one or the other protocol (i.e. sucrose responsiveness or odour learning).

Overall the work presented in this thesis show excellent promise of a range of future avenues for using proboscis extension reflex conditioning in learning and memory experiments using bumblebees (*B. terrestris*).

6.3 Future research areas

The research presented in my thesis opens up many questions for future research, and I will discuss some of the ideas that could lead on from each chapter.

Following on from the work in chapter 2 it would be useful to understand how, and indeed if, a mix of learning abilities across the two sensory modalities (visual and odour learning) is beneficial. Is this variation helping colonies adapt to the changing importance of cues in different modalities at different time periods? For example, individual bumblebees may pay more attention to visual cues over olfactory cues if they are a better visual learner. Alternatively, one cue may be favoured over the other for the majority of bees, which could potentially indicate innate preferences by bees to use particular cues irrespective of their individual learning ability. Additionally, it would be interesting to understand whether learning in other sensory modalities (e.g. tactile) is also uncorrelated with visual or odour learning performance, and what drives the allocation of resources into learning and memory in individual bees.

In chapter 3, I found that bees that foraged for pollen were better olfactory learners than nectar foragers, which may indicate that foragers are selected based on their learning ability. Being the first study to investigate the link between learning and foraging preference in bumblebees, I decided to test the foraging preferences in a simplistic set up in the lab. It would be interesting and relevant to follow up on this study, to see if this is also true in a field setting with more complex flowers. Additionally, I only used two colonies in this experiment, and although I found the same pattern for both colonies the more colonies tested the better understanding we will have about whether this is likely to be representative across all colonies, and the reasons behind such patterns.

Chapter 4 highlights that environment is potentially a very important factor in determining the use of learning ability by an animal. Theory predicts that learning will be more important in unpredictable and changeable environments (Stephens 1991), provided this level is not too high. It has been shown that animals living in different environments have different learning abilities; black capped chickadees in harsher environments are better problem solvers (Roth, LaDage & Pravosudov 2010); three spined sticklebacks from rivers and ponds used different cues to solve a spatial task (Odling-Smee & Braithwaite 2003). However, what we do not know is whether an individual's learning ability allows them to perform equally well across situations, or whether there are particular environment types that favour different learning phenotypes. This would involve both having detailed information on individual's learning performance but also on multiple test environments. Bumblebees would be a good model to test this as they can be placed in any environment, and as long as there is food they will forage and return to the colony – therefore foraging performance can be measured as it was in chapter 4. Colonies could also be moved between environments. As visual and olfactory learning do not appear to be correlated among individuals, then being able to test bees in both modalities

before testing foraging performance would be valuable. This would be challenging to achieve, although the split colony box set up I used in chapter 4 allowed me to be able to continue testing new individual's learning ability, once the colony was attached to the test environment. However, if all individual's learning ability needed to be tested before they were moved to the test environment this would be difficult. Collecting information on the flowers in bloom and the complexity of the environments would also take substantial work, potentially a number of years prior to the experiment being completed. This would be a large undertaking, with many issues that would need resolving, but an interesting area for future research.

My work in chapter 5 is the first in investigate how learning and memory in bumblebees is affected by pesticide exposure. Therefore, it is not known whether the same effects would be seen across different learning tasks such as visual or spatial. Additionally, throughout this thesis I have suggested that a colony having variation in individual learning performance is beneficial. It would be interesting to test whether the lower variation in the pesticide exposed colonies does have negative consequences for the colony. To test whether variation is important, selection experiments could be used once individual learning ability is known, to create experimentally manipulated colonies with greater and/or lower levels of variation in learning ability among individuals. These colonies could then have their foraging performance compared in the same, and/or a range of different, habitat types along a gradient of floral marketplace complexity.

6.4 Final conclusions

There has been a recent explosion of interest in individual differences in cognitive ability, alongside the growth in examples of animal showing personalities in the last decade. Social insects have been at the forefront of our understanding, due to the observed individual differences both within and between colonies. Bumblebees are a model study organism for increasing our understanding of this variation as they can be observed in the lab and field. In this thesis I developed a robust protocol using a modified proboscis extension reflex paradigm to test olfactory learning, and used this to explore some of the causes and consequences of variation in learning performance. My results highlight the complexity of individual variation in learning performance across tasks; suggesting that this variation may actually be beneficial for the colony to adapt to changeable environments and allocate foraging effort for pollen and nectar. The results also indicate that poorer learning bees may be more valuable to the colony under some environmental conditions, and that better learning bees may be too costly to the

colony. This challenges the widely held, and intuitively appealing, view that enhanced learning ability will always be adaptive in a wider context.

Acknowledgements

I would like to thank my supervisors, Nigel Raine and Elli Leadbeater, for their advice, feedback and support throughout my PhD. I would also like to thank my advisor, Mark Brown, for his help and advice. I have met some wonderful people while working at Royal Holloway, and been lucky to have a great lab group throughout, thanks to Rich Gill, Dara Stanley, Gemma Baron and Lisa Evans, for useful discussions, support and friendship. Also, everyone else who has worked in the 'Bees and Trees' office in the last three years, and made this a really enjoyable experience: Simon Morath, Eva Muiruri, Harriet Milligan, Matthias Furst and Catherine Jones. Finally, thanks to Sean O'Riordan for his love, patience and encouragement.

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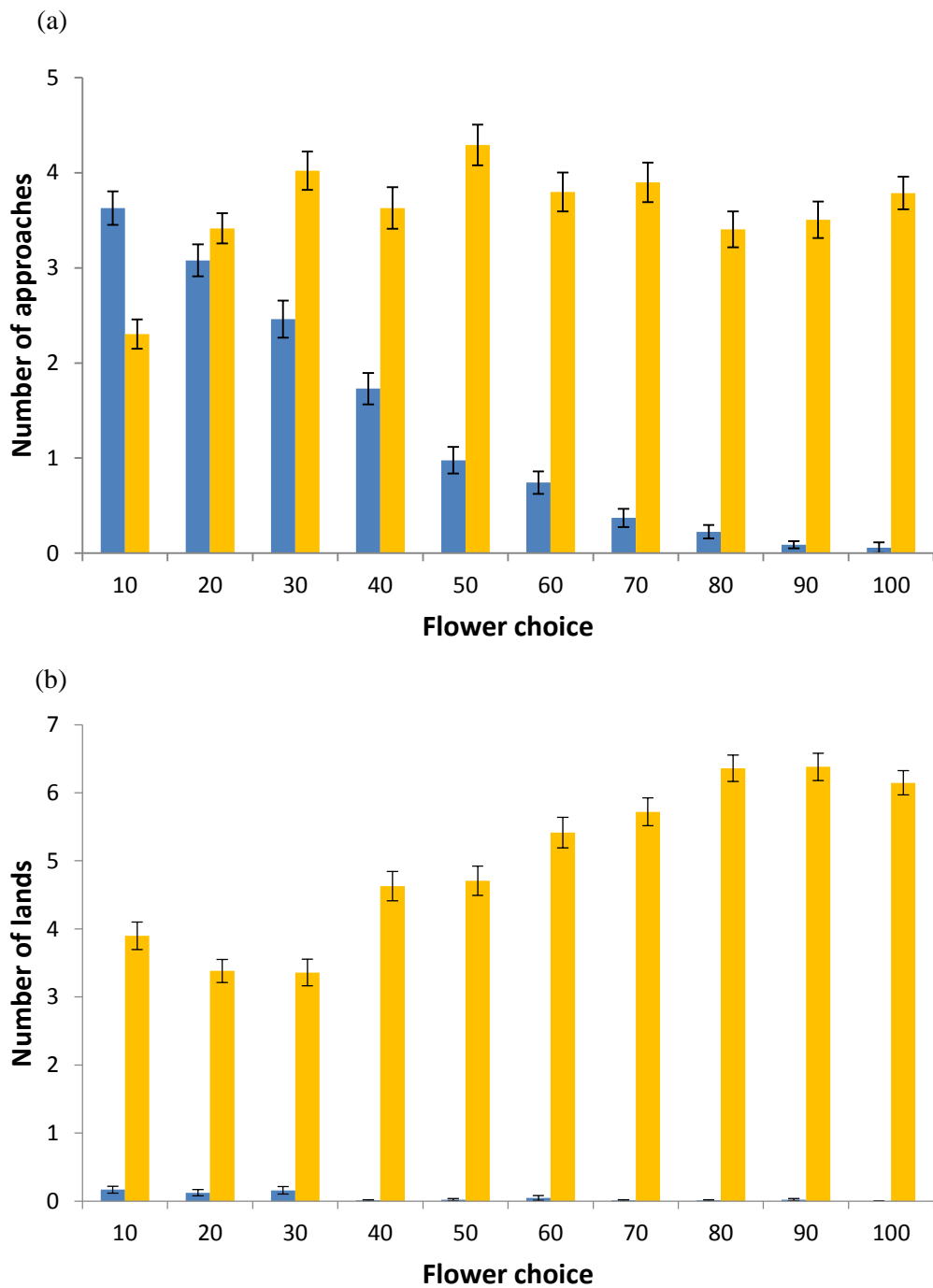
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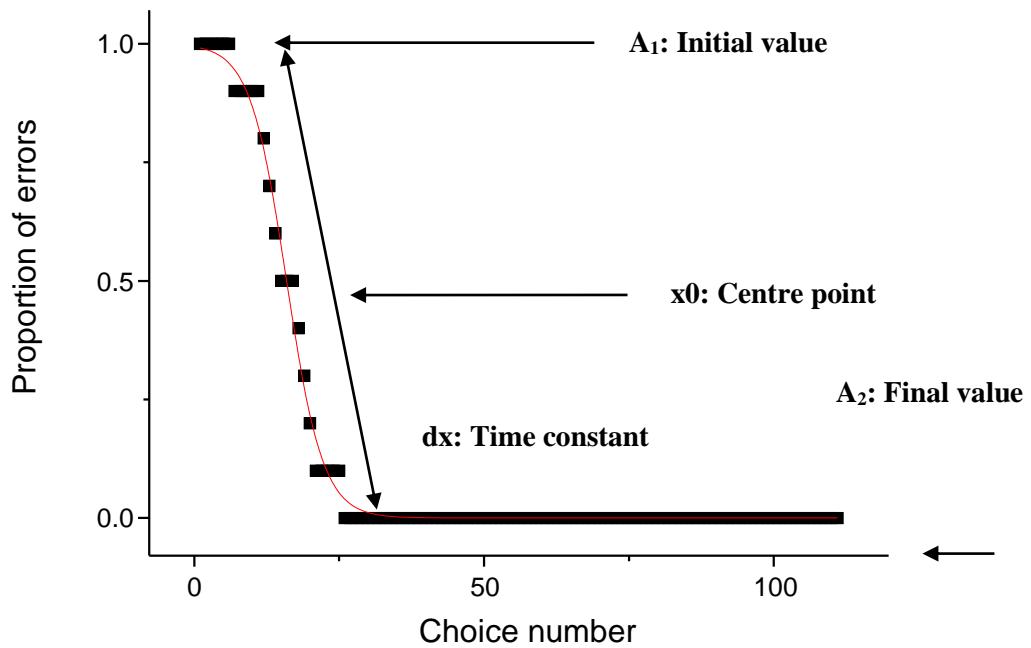
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Appendices



Appendix 2.1 Flower choices made by bees in the colour learning paradigm in Chapter

2. Choices are broken down into mean (\pm SE) numbers of blue and yellow approaches (panel a) and lands (panel b) made during consecutive bins of 10 flower choices. The flower choices begin from when each bee probed a yellow flower for the first time. Data presented are pooled across the four colonies, and include all 89 bees that completed the colour learning task.



Appendix 2.2 Boltzmann curve fit indicating the four parameters used in chapter 2: A_1 is the proportion of errors the bee makes at the start of the curve, and A_2 is the proportion of errors the bee makes when it reaches saturation learning performance. Bees that learnt the task well have an A_2 value ≈ 0 . x_0 is the centre point of the curve between the two asymptote values (A_1 and A_2), and is also the point at which this curve has the steepest slope. As such, lower values of x_0 indicate bees that improve their task performance more rapidly once the learning process begins. The parameter dx indicates the relative rate the curve transitions from one asymptote to the other: the lower the value, the more rapid the transition and therefore the quicker the bee learnt the task.

Appendix 3.1 Results from a pilot experiment for Chapter 3

In a pilot experiment I weighed the pollen loads of bees returning to the colony from a petri dish of pollen in the flight arena. Returning bees were caught in a marking cage and one of their pollen loads removed. I then classified the load size as small, medium or large by observation and then weighed the pollen (Table 1). I used the means, maximum and minimum values from this pilot experiment to give the approximate mass for each size class of pollen load. I decided to include more categories as there was appreciable variation in pollen load mass within just the three basic categories (Table 2).

Table 1 Mean, standard error, minimum and maximum mass for each of the three observed pollen load size categories (large, medium and small).

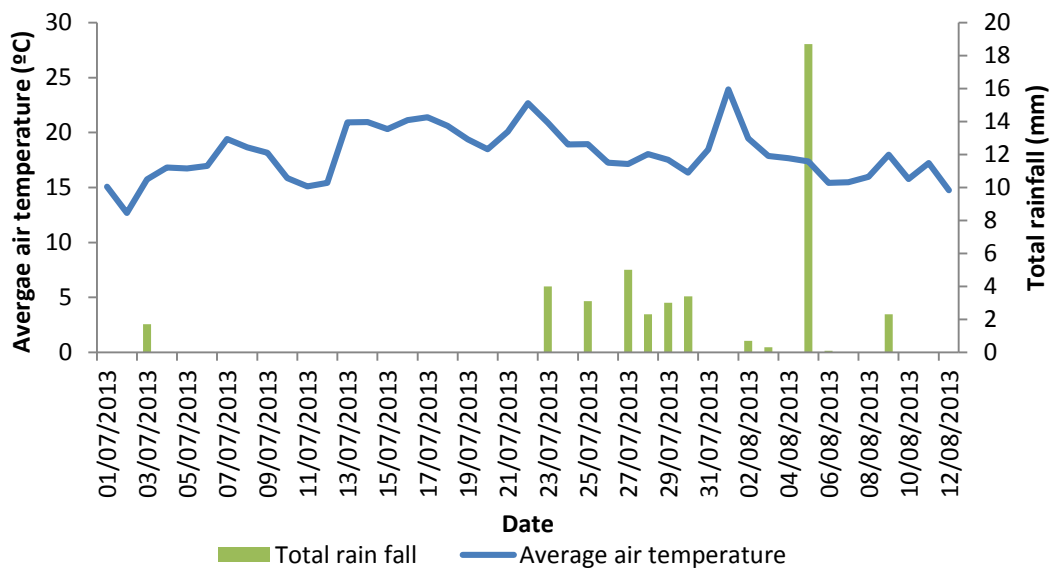
	Mass (mg)				n
	Mean	SE	Min	Max	
Large	14.2	0.5	11.6	18.3	16
Medium	8.5	0.3	6.5	11.6	28
Small	4.2	0.3	2.2	5.9	13

Table 2. Categories used for analysis of pollen collection with the total load (i.e. 2 symmetrical pollen loads) in mg which was assumed for each of these based on the pilot experiment.

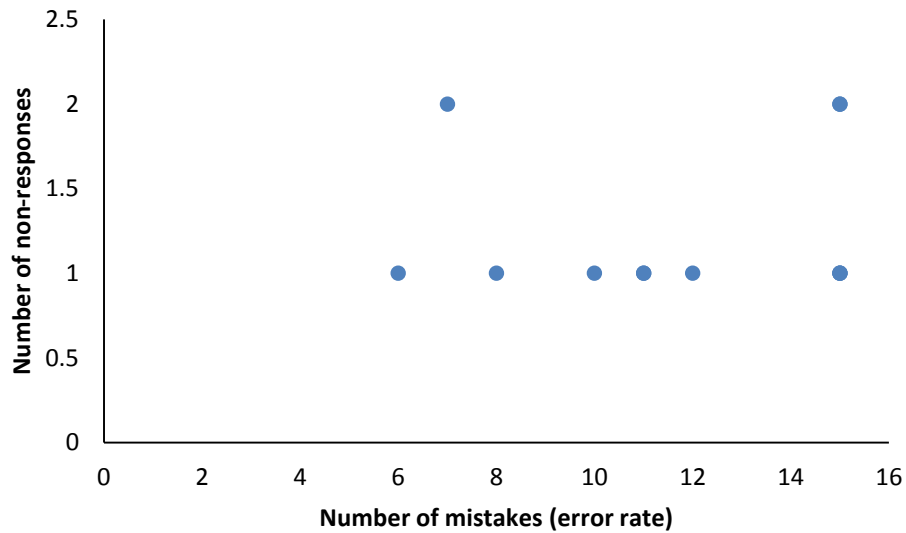
	Very small	Small	Small/med	Medium	Med/large	Large	Very large
Total load (mg)	4	8	12	17	23	28	40

Appendix 4.1 Weather conditions during Chapter 4 experiment

The conditions during the experiment were slightly above the long term average (1981 – present) for the time of year (July: 1.9°C above and August: 0.7 °C above) and we experienced a heat wave from the 3rd to 22nd July (Metoffice 2013). During the experiment weather measurements were recorded 6km away at Silwood Park meteorological station (data provided by Jim Culverhouse). The mean maximum air temperature ranged from 19.6 °C to 32.3 °C (mean = 24.9 °C), mean minimum air temperature ranged from 6.1 °C to 16 °C (mean = 11.4 °C). There was a total of 44.6mm of rain ranging from 0 to 18.7mm a day. The average air temperature and total rainfall each day are shown in the graph below.

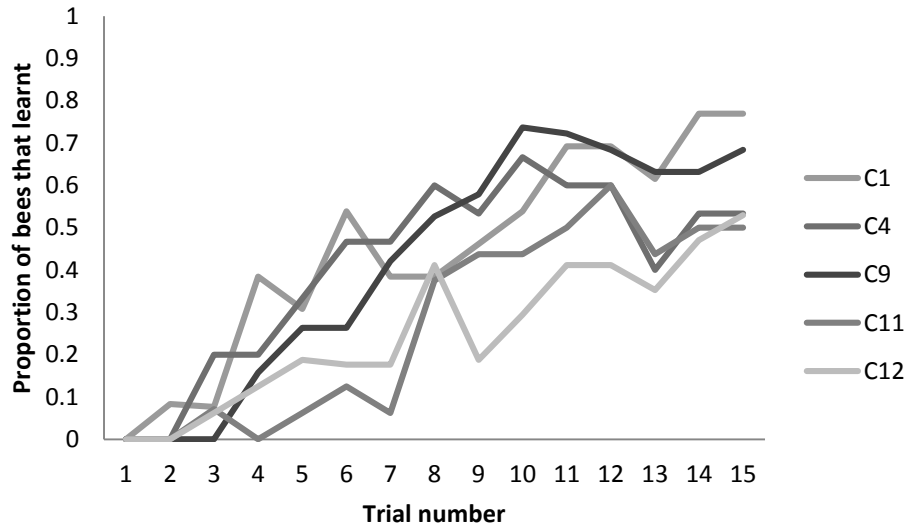


Appendix 4.2. Learning rate and non-response rate. Graph shows the learning rate of the 12 bees which were unresponsive on 1 or 2 of the learning trials (i.e. did not respond with a proboscis extension when their antenna were touched with sucrose). The higher the number of mistakes the poorer learner the bee was. The graph shows that the bees that were unresponsive on 1 or 2 trials had a variety of learning rates, and there is not a link with unresponsiveness and learning rate.



Appendix 4.3. Learning curves across the 15 trials during the olfactory task in chapter

4. Graph shows the proportion of bees in each colony learning to associate the conditioned odour with reward (determined by a proboscis extension to the rewarding odour prior to reward presentation). Data shown are for all bees tested in this paradigm: C1: n = 13, C4: n = 15, C9: n = 19, C11: n = 16, C12: n = 17.

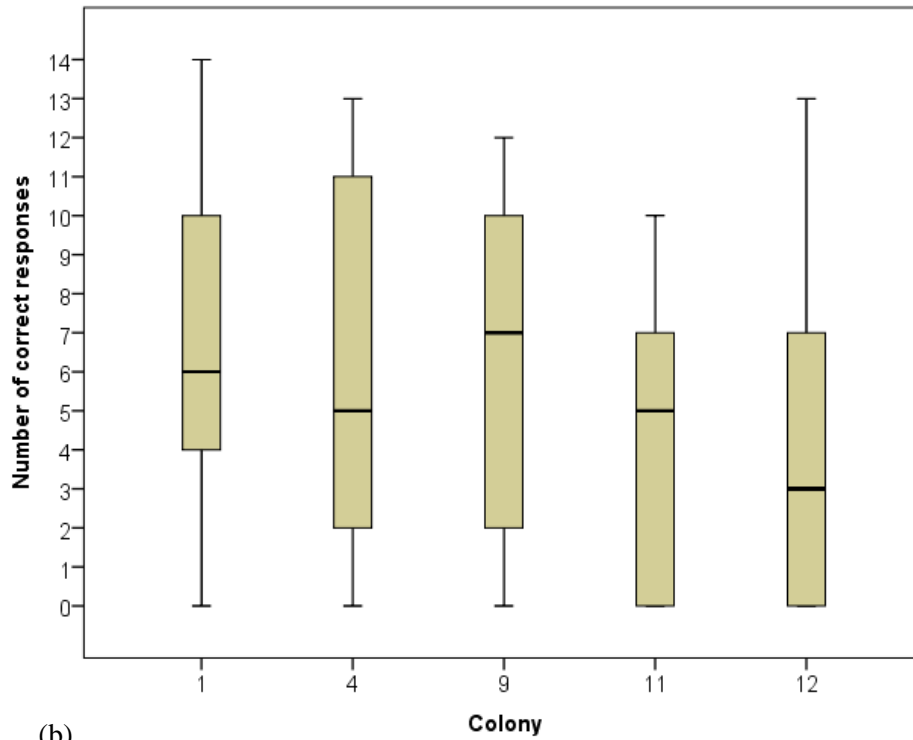


Appendix 5.1 Generalized linear model for number of unresponsive bees in the acute experiment in chapter 5. Parameter estimates are calculated with reference to the control group. Colony was included as a random effect (n = 171).

Fixed effects	Parameter estimate	SE	Z value	P value
Intercept (Control)	1.792	3.276	0.547	0.584
Treatment (250ppb)	0.495	0.580	0.855	0.393
Treatment (10ppb)	0.645	0.607	0.059	0.953
Treatment (2.4ppb)	0.031	0.530	1.063	0.288
Bee size	-0.092	0.650	-0.142	0.887

Appendix 6.1 Boxplots of the correct number of responses in olfactory learning task in
(a) the 5 olfactory colonies in chapter 4 and (b) the three treatment groups in chapter 5.

(a)



(b)

