The role of pollen as a reward for learning in bees

Submitted by **Elizabeth Nicholls** to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Psychology

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

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Abstract

In contrast to the wealth of knowledge concerning sucrose-rewarded learning mechanisms, the question of what bees learn when they collect pollen from flowers has been little addressed. Pollen-rewarded learning is of interest not only in furthering our understanding of associative conditioning pathways in the insect brain, it may also shed light on the role that cognitive processes may have played in shaping the early evolutionary relationship between plants and their pollinators, given that pollen is thought to have been the ancestral reward for flower visitors. Thus the central aim of this thesis was to demonstrate the conditions under which pollen may reinforce learning of floral features in two model species, the honeybee (*Apis mellifera*) and bumblebee (*Bombus terrestris*). Having developed a number of paradigms for the study of pollen-rewarded learning, here I ask what bees might learn during pollen collection, both in terms of the sensory characteristics of pollen itself and additional cues paired with this reward. Freely flying bees were shown to be sensitive to differences in the type of pollen offered for collection and were able to associate the presence of a coloured stimulus with both the availability and quality of the pollen reward. The sensory pathways involved in the evaluation of pollen were also investigated. When bees were restrained, in order to more tightly control exposure to the reward, pollen was not found to support learning in an olfactory conditioning task. Furthermore, when delivered in solution with sucrose, pollen was found to inhibit learning relative to bees rewarded with sucrose alone. It seems that pollen contains compounds which are perceived as distasteful by bees and that through the contamination of nectar, pollen may influence bees foraging decisions via differential learning and recognition of floral cues.

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Author's Declaration

Unless otherwise stated, the author was responsible for all data collection and analysis. Natalie Hempel de Ibarra, as the supervisor, provided advice in experimental design, analysis and interpretation of the data.

The use of the first person plural (i.e. 'we' as opposed to 'I') in Chapter 3 reflects a collaboration with Doreen Drenske, a former Msc student at the Freie Universität Berlin. Doreen collected the data with honeybees, under the supervision of Natalie Hempel. The author collected data with bumblebees and analysed the findings of both experiments.

Under the author's supervision, Jacob Dyer collected the antennal sensitivity data (Chapters 2, 6) and ran the extended conditioning experiment (Chapter 5) as part of his Msc dissertation. Adrienne Richter-Krefft, a research intern, collected some of the data in Chapter 6. All data was analysed by the author prior to inclusion in the thesis.

Chapter 1: Introduction

'That these [bees] and other insects, while pursuing their food in the flowers, at the same time fertilize them without intending and knowing it and thereby lay the foundation for their own and their offspring's future preservation, appears to me to be one of the most admirable arrangements of nature.' Christian Konrad Sprengel, 1793

Since Sprengel's characterisation during the eighteenth century of the mutually beneficial relationship between plants and insects, the mechanisms maintaining such associations, and the selective pressure each exerts over the other, have fascinated scientists. However, within such affiliations lies a conflict of interest, and indeed it might be better to adopt Westerkamp's (1996) portrayal of the relationship between plants and their pollinators as a 'balanced mutual exploitation'. Whilst plants wish to minimise the costs incurred by the provision of attractive rewards to insect visitors, it is the desire of pollinators to maximise their foraging efficiency through rewards gained. In short, plants do not give up their rewards easily, and as noted by Sprengel, insects do not actively pollinate flowers. In fact in the case of flowers which offer only pollen as a reward, plants and insects act as competitors for the same resource (Westerkamp, 1997). As a result, plants use multiple strategies to manipulate the behaviour of pollinators in order to maximise the transfer of pollen between flowers, with such tactics heavily dependent on the sensory systems and cognitive capabilities of their pollen vectors. In this thesis I have used various methods to investigate plant-pollinator relationships from the perspective of the insect with the aim to further the understanding of mechanisms underlying learning and memory formation in insects and how their cognitive processing might shape the interactions between plants and pollinators. Employing both honeybees and bumblebees as species which pollinate a wide variety of plant species and serve as well established models for the study of insect learning, I have attempted to elucidate the mechanism by which bees might assess pollen rewards and their capacity for learning about differences in flower quality.

1.1 Evolution of angiospermy, floral rewards and insect pollination

Angiosperms first appear in the fossil record early in the Cretaceous period, between 130 and 140 million years ago (mya) (Brenner, 1963; Crane, Friis, & Pedersen, 1995; Dilcher, 1979; Hickey & Doyle, 1977), although more recent molecular evidence suggests they may have had an earlier origin, with estimates ranging from the mid-Jurassic to as early as the Triassic (Bell, Soltis, & Soltis, 2005, 2010; Sanderson, Thorne, Wikström, & Bremer, 2004). The relationship between vascular plants and insects is thought to have evolved earlier, with fossil evidence dating back to the Palaeozoic era (see Labandeira, 1998 for review). Indeed there is considerable support for the notion that insect pollination, widely considered the basal mode of pollination in flowering plants (Endress, 1990; Faegri & Van der Pijl, 1971; Thien, Azuma, & Kawano, 2000) pre-dates angiospermy, with fossil evidence of pollen transfer by insect visitors to early gymnosperms first appearing around the late Jurassic period (Crepet, 1974; Klavins, Kellogg, Krings, Taylor, & Taylor, 2005; Labandeira, Kvacek, & Mostovski, 2007). Medullosan seed ferns, for example, possessed extremely large spores, thought to have been too heavy to be wind transported (Dilcher, 1979; Taylor, 1978; Taylor & Millay, 1979) and more recent evidence has been uncovered in the form of amber preserved thrips, covered in cycad pollen grains and possessing specialised hair-like structures postulated to function as an adaptation for pollen collection (Peñalver et al., 2012).

Herbivory of tissues probably represents the earliest relationship between living plants and insects, dating back as far as the mid-Devonian (Labandeira, 1998), with the consumption of spores having been traced to the early stages of the Permian period (Edwards, Selden, Richardson, & Axe, 1995; Krassilov & Rasnitsyn, 1996; Rasnitsyn & Krassilov, 1996a, 1996b). The shift from an antagonistic relationship to one of mutual benefits has several suggested origins, and given that insect pollination is thought to have evolved multiple times, different selective pressures may have been involved in each case (Pellmyr, 2002). Pellmyr (1992) suggests that as well as arising as a by-product of tissue feeding and seed parasitism, pollination may also have resulted from insects using plants as hosts for larval development. Any one of these interactions could have resulted in spore or pollen transport between individuals, and however minimal this may initially have been, in instances where the benefits from out-crossing

outweighed the costs incurred by the loss of gametes, selection for attraction rather than repellence of insects would be predicted to occur (Pellmyr, 2002). Current estimations suggest that plants have been providing nutritional rewards to lure pollinators from the mid-Mesozoic onwards (Labandeira, 1998, 2000).

Whilst some have suggested that sugary stigmatic exudates, akin to the pollination drops of gymnosperms, may represent the earliest reward for pollinating insects (Endress, 1994; Pacini & Nicolson, 2007), generally pollen is considered the ancestral reward in angiosperms (Crepet, 1979; Pettitt & Beck, 1967). Evidence from insect mouthpart morphology suggests that adaptations for spore or pollen eating first appeared late in the Pennsylvanian period (Labandeira, 1997, 2002; Rasnitsyn, 1977), with excess pollen being provided as a reward from the late Palaeozoic onwards (Labandeira, 1998). Reconstruction of the ancestral angiosperm flower has proven difficult due to gaps in the fossil record and discrepancies between interpretations of morphological and molecular data (for most recent reviews see Doyle, 2012; Endress, 2011; Friis, Pedersen, & Crane, 2010). Nevertheless it is widely agreed that the earliest flowers were simple, relatively small and that the perianth, if present at all, was highly reduced compared to modern flowers (Crepet, Friis, Nixon, Lack, & Jarzembowski, 1991; Endress, 2001; Friis, Crane, & Pedersen, 1986; Friis, et al., 2010). Since pollen is often both conspicuously coloured and fragrant, prior to the appearance of a well developed perianth it seems likely that in addition to providing a food reward, the androecium itself most likely served as the original advertisement for attracting pollinating insects (Crepet, et al., 1991; Faegri & Van der Pijl, 1971).

The dual function of pollen as an agent for gamete transmission and a food reward for pollinators does not mean that its production is any less costly to flowers. On the contrary, consumption or active collection of pollen by visiting insects results in a direct loss of reproductive potential for the plant. Several studies have shown that whilst pollinating insects are capable of removing nearly all of the pollen provided by an individual flower, only a miniscule fraction of that removed actually contributes to fertilisation (Harder & Thomson, 1989; A. Müller, 1996a; A. Müller et al., 2006; Westerkamp & Claßen-Bockhoff, 2007; Westrich, 1989). For example, Schlindwein et al. (2005) observed that solitary bees removed up to 95.5% of pollen produced by *Campanula rapunculus* flowers and yet only 3.7% of that was involved in pollination.

Many modern pollen-rewarding flowers have evolved packaging and dispensing mechanisms which serve to limit pollen consumption during each pollinator visit (Buchmann, 1983; Castellanos, Wilson, Keller, Wolfe, & Thomson, 2006; Harder & Thomson, 1989; Harder & Wilson, 1994). In contrast, flowers which place pollen in a prominent position, clearly on display to visiting insects, are likely to incur a high cost of pollen wastage (Lunau, 2006; Vogel, 1978). This may partially explain why, during the mid to late Cretaceous, the perianth, and corolla in particular, became far more conspicuous, superseding the androecium as the main attractant for pollinators (Crepet, 2008; Crepet, et al., 1991; Dilcher, 2000; Soltis et al., 2009; Specht & Bartlett, 2009). Stamens, in contrast, became reduced in number and size (Friis, et al., 2010).

During the late Cretaceous and early Tertiary period, *ca.* 65-70 mya, angiosperms experienced a major radiation, resulting in an increase in the complexity of flowers and an explosion in the breadth of floral forms. This sudden increase in diversity was famously described by Charles Darwin as an 'abominable mystery' since it contrasted with his theory of evolution based on gradual shifts in form and function (F. Darwin & Seward, 1903). Given the concurrent radiation in pollinating insects, particularly those of the order Hymenoptera (Grimaldi, 1999), some have suggested that selection by insect visitors may be responsible for the rapid diversification in floral form (Burger, 1981; de Saporta & Marion, 1881; Raven, 1977; Regal, 1977). However more recent molecular evidence would seem to caution against accepting a single explanation for the increase in dominance of the angiosperms during this period, invoking instead a complex interaction between multiple biological traits and environmental factors (Crepet & Niklas, 2009; Davies et al., 2004).

Following such diversification, many modern flowers have evolved morphological adaptations to limit pollen removal by insects (A. Müller, 1995; Westerkamp, 1997; Westerkamp & Claßen-Bockhoff, 2007). For example some flowers, such as those of the *Fabaceae* family, have their reproductive parts hidden within a keel which bees must manipulate with their legs in order to gain access to food rewards (Westerkamp, 1997). In other flowers, anthers are enclosed in narrow corolla tubes which require morphological adaptations of the mouthparts and proboscis for efficient pollen harvesting (A. Müller, 1995; Parker & Tepedino, 1982). Heteranthy is floral trait also known to have puzzled Darwin (1877). Two or more types of stamens are exhibited, some dedicated to producing fertile pollen and others producing pollen which serves as

a food reward to bees (F. Müller, 1883; H. Müller, 1881). Often this 'food pollen' is sterile, meaning it is less costly for the plant to produce (and less nutritious for the pollinator) (Vogel, 1978). Whilst anthers providing 'food pollen' are typically brightly coloured, 'fertilising anthers' are often cryptic and similar in colour to the petals of the flower (Vallejo-Marín, Da Silva, Sargent, & Barrett, 2010). In addition, fertilising anthers frequently dispense grains in positions on the insect body which are inaccessible and thus harder for the pollinator to groom and repackage grains for transport back to the hive, which may improve the likelihood of pollen reaching the stigma (Vallejo-Marín, Manson, Thomson, & Barrett, 2009). Though not a common phenomenon, heteranthy has evolved independently in several plant families and is considered to be an adaptation to minimise the necessity for excess pollen production in those flowers providing only pollen as a reward (Vallejo-Marín, et al., 2010; Vallejo-Marín, et al., 2009; Vogel, 1978).

The increase in conspicuousness of the corolla during the late Cretaceous was accompanied by the appearance of well-developed nectar producing organs (Crepet, et al., 1991; Friis, Pedersen, & Crane, 2006). Indeed, one of the main hypotheses regarding the origin of nectar is that it may have arisen as a by-product of plants' need to excrete the excessive levels of solutes which accumulate in flowers as a result of high rates of water consumption by floral organs (De la Barrera & Nobel, 2004). This evolutionary innovation has been extremely successful, given that today nectar represents the most common floral reward (Simpson & Neff, 1983). Several hypotheses have been proposed to explain the widespread switch from rewarding with pollen to the provision of nectar for insect visitors. The main argument stems from the fact that pollen is costly to produce, since it must contain a certain quantity of protein necessary for pollen tube growth and successful fertilisation (Colin & Jones, 1980; Roulston, Cane, & Buchmann, 2000; Simpson & Neff, 1983). In contrast, plants can be rather more flexible in terms of the energy they allocate to nectar production, and unlike pollen, nectar can be reabsorbed and reallocated to other parts of the plant depending on need and flower visitation rates (Nepi, Pacini, & Willemse, 1996; Nepi & Stpiczyńska, 2007; Pacini & Nepi, 2007). From the perspective of the pollinator, harvesting nectar requires fewer morphological and behavioural adaptations than pollen collection (Thorp, 1979, 2000) and given that most insects possess the capacity to metabolize sugars, nectar is also easier to digest (Huber & Mathison, 1976; Roulston & Cane, 2000). Additionally those insects which originally fed on honeydew secreted by aphids

may have been predisposed to search for sweet substances, as would those which foraged on the sugary exudates produced by gymnosperms (Simpson & Neff, 1983). In addition to sugars, nectar often contains additional solutes such as amino acids, meaning pollinators are able to meet a range of nutritional demands with this reward. However these explanations do not appear sufficient to account for the manner in which nectar so promptly out-competed pollen as reward, leading to the fast radiation of both plants and pollinating insects (Grimaldi, 1999).

One idea that has received little attention in this discussion is that nectar, through its immediate consumption by pollinators, may be better at rewarding learning of floral features such as petal colour or odour in foraging insects. In a similar vein, it may also be easier for insects to assess differences in the quality of reward provided by flowers when foraging for nectar rather than pollen. In both cases, from the perspective of the plant, nectar would not only be less costly to produce, it would also better promote constancy in the flowers visited by insects if foragers are better able to recognise highly rewarding plants of the same species, thus enhancing out-crossing potential. However, when entertaining this theory it is necessary to distinguish between pollinators that rely on nectar exclusively as a food source, such as butterflies which do not need pollen at all and holometabolous insects such as bees which collect pollen for brood, from those insects which consume pollen, such as flies and beetles. It could well be that the emergence of nectar-producing organs was driven by the successful recruitment of novel pollinator clades which possessed a predisposition not only to consume sucrose, but also to learn effectively when rewarded with sucrose about locations and specific plant cues, whilst at the same time being less dependent or specialised on pollen consumption. Competition between pollen-consuming and nectar-consuming insects for floral resources could have been a major factor in the success of nectar as a reward trait promoting more successful pollination through flexible spatial mobility and more effective learning of floral locations and cues. One way to explore this notion further, is to ask how pollinating insects perceive pollen rewards and if these reinforce learning, as well as comparing learning, foraging and flower constancy between pollen-collecting, pollen-feeding and nectar-feeding pollinators.

1.2 Assessment of reward quality

Based on the fact that beetles pollinate many extant species of basal angiosperm, Coleoptera have long been proposed as the earliest insect pollinators (H. G. Baker & Hurd Jr, 1968; Diels, 1916; Takhtajan, 1991). However, more recent evidence suggests that a varied assemblage of insect groups may have been involved in pollen transfer, notably those of the order Diptera (flies) and Thysanoptera (thrips) (Bernhardt, 2000; Grimaldi, 1999; Labandeira, 2005; Peñalver, et al., 2012; Thien, 1980; Thien et al., 2009). Bees, often considered the most important group of insect pollinators (Proctor, Yeo, & Lack, 1996), are thought to have arisen around the same time as angiosperms (Danforth, Sipes, Fang, & Brady, 2006; Engel, 2004; Grimaldi, 1999) though fossilised bees are extremely rare (R. A. Baker & Chmielewski, 2003). Derived from a carnivorous predator, bees switched to meeting their protein demands, and those of their larvae, with pollen instead of insect prey. As reviewed by Michez et al. (2011) the ancestors of bees, so called 'proto-bees', possessed a number of morphological adaptations which may have facilitated the shift from predation to pollination. Their mouthparts, for example, once designed for chewing and dissecting prey would have been well adapted to chewing pollen instead (Crepet, 1979; Simpson & Neff, 1983) and it is assumed that by switching from searching for cryptic prey, to the conspicuous pollen displays of early angiosperms, foraging efficiency would have been improved (Engel, 2004). Body hair, involved principally in thermoregulation (Heinrich, 1979), may have aided pollen collection (Simpson & Neff, 1983) prior to the evolution of more complex morphological and behavioural adaptations (Buchmann, 1983; Michener, 1999; A. Müller, 1995; Thorp, 1979, 2000).

Current evidence suggests that ancestral bees were oligolectic (Danforth, et al., 2006; Michez, Patiny, Rasmont, Timmermann, & Vereecken, 2008), specialising on a few, often closely-related pollen types. Over time, increases in the breadth of pollen diets appear to have been more common than restrictions (Danforth, Conway, & Ji, 2003; Michez, et al., 2008; A. Müller, 1996b) and a number of pollen generalist species are known to have evolved from oligolectic ancestors (A. Müller, 1996b). Pollen collection by modern bees is considered to be a behaviourally complex (Thorp, 1979, 2000) and cognitively demanding task (Raine & Chittka, 2007b) and so some have proposed that foraging efficiency would have been improved by sticking to one pollen host once the handling skills necessary for collecting this reward had been mastered (Michez, et al.,

2011). However, given that the earliest flowers, with which proto-bees are thought to have evolved, had a relatively simple structure compared to many modern flowers, pollen collection from flowers may not have always required the complex handling skills demonstrated by Apidae bees today (though see Proença, 1992). More likely the complexity of pollen as a food source meant that early bees were limited to those pollen species that their larvae were pre-adapted to digest. Similarly the presence or absence of key nutrients, essential for growth, is also likely to have been important in determining those pollen species suitable for rearing larvae. Sterols, for example, have been hypothesised to be particularly limiting of pollen choice, since bees cannot synthesise these compounds, and they are essential for the production of hormones, such as those which control moulting (Dötterl & Vereecken, 2010). As well as difficulties arising from digestion, many pollen species also contain secondary compounds, some of which are toxic to insects, which could further limit those pollen species suitable for consumption by larvae (Praz, Müller, & Dorn, 2008; Roulston & Cane, 2000; Sedivy, Müller, & Dorn, 2011). Pollen specialisation in ancestral bees, regardless of the underlying mechanism, provides some of the earliest evidence of the ability to distinguish between flowers on the basis of the reward they provide.

Given that considerable variation in the nutritional value of pollen exists amongst plant species (Roulston & Cane, 2000; Stanley & Linskens, 1974), within populations (Loper & Cohen, 1987; A. W. Robertson, Mountjoy, Faulkner, Roberts, & Macnair, 1999) and even between anthers on the same flower in those species displaying heteranthy (Vogel, 1978). As a result, we might expect bees to have some form of mechanism for preferentially selecting those pollen species most suited to rearing brood. Indeed, different pollen species have been shown to affect both the development and survival of young bees and larvae (Génissel, Aupinel, Bressac, Tasei, & Chevrier, 2002; Levin & Haydak, 1956; J. O. Schmidt, Thoenes, & Levin, 1987; Sedivy, et al., 2011; Tasei & Aupinel, 2008) as well as influencing the eventual body size of adult honeybees (Regali & Rasmont, 1995). In contrast to their oligolectic ancestors, today many social species such as honeybees are polylectic, meeting colony demands for protein with pollen from a variety of plant species. Despite this, individual bees have been shown to concentrate their foraging effort on a few select plant species rather than exploiting all available pollen sources, with more controlled choice tests also revealing preferences for certain pollen species over others (Boch, 1982; Boelter & Wilson, 1984; Cook, Awmack, Murray, & Williams, 2003; Doull, 1966; Free, 1993; Levin & Bohart, 1955; A. Müller,

1995; J. O. Schmidt, 1982; Wahl, 1966). Taken together, such evidence suggests bees have the ability to discriminate between plant species on the basis of the pollen reward provided, but the question of whether they base such preferences on nutritional differences between pollen species remains unanswered. Nectar rewards are known to be assessed on the basis of flow rate and sugar content (Núñez, 1966, 1970, 1982; Seeley, 1986; Waddington, 2001). However, with the exception of some members of the Colletid family, bees do not ingest pollen whilst foraging, but rather store grains externally, often in specialised leg structures, in order to carry pollen back to the colony (Michener, Winston, & Jander, 1978; Thorp, 1979, 2000). As a result, the mechanism of pollen assessment remains, as yet, unclear.

Since pollen is collected by bees primarily as a source of protein, nitrogen content has typically been used as the measure of nutritional quality (Pernal & Currie, 2000 and references therein). Attempts have been made to relate foraging preferences to the nitrogen content of pollen collected by bees (Levin & Bohart, 1955). An investigation of 23 British plant species, spanning nine families, revealed that those species whose pollen contained the highest concentration of protein received the greatest number of pollinator visits (Hanley, Franco, Pichon, Darvill, & Goulson, 2008). Roberston et al. (1999) offered bumblebees a choice between morphs of the monkeyflower, *Mimulus guttatus*, a species polymorphic in terms of the quality of pollen it produces. Some morphs produce copious amounts of sterile pollen, which is lacking in cytoplasm and therefore of little nutritional value to bees. A logistic regression revealed viability of pollen grains to be the single most important predictor of foraging preference, independent of the number of grains produced, which was actually found to negatively correlate with foraging preference. A number of studies have shown that the presence of certain essential amino acids (as defined by De Groot, 1953) has a greater implication on colony survival than total protein intake (Loper & Cohen, 1987; McCaughey, Gilliam, & Standifer, 1980), leading Cook et al. (2003) to hypothesise that the specific amino acids present in pollens may serve as a more accurate measure of nutritional quality. In contrast, Pernal and Currie (2000), observed that supplementing pollen with additional amino acids had little effect on hypopharyngeal gland development in worker bees, which serves as a measure of protein assimilation and the ability to produce food for brood. This led them to conclude that essential amino acids are present at sufficient levels in most pollen species, and that total protein content is more critical for gland development. Cook et al. (2003) gave honeybees a choice between two crop species,

oilseed rape and field bean, and found that bees preferred the pollen species which contained the highest concentration of the most essential amino acids, but only when they had previous experience of foraging on this pollen type. This was interpreted by Cook et al. (2003) as evidence of experience-based assessment of nutritional quality by bees. However given that pollen preference was defined by whichever species an individual landed on first, it is unclear whether bees actually spent a disproportionate amount of time collecting the more nutritious pollen. As conceded by the authors, in this and other studies discussed thus far, differences between pollen species in terms of other nutritional properties such as lipid content, or the presence and/or absence of essential vitamins and minerals were not accounted for.

Following the manipulation of colony-level pollen stores, Fewell and Winston (1992) observed that honeybees in pollen-supplemented colonies returned to the hive with pollen loads containing a higher percentage of nitrogen, relative to those from pollen-depleted colonies. Bees from pollen-depleted colonies responded via an increase in foraging rate and load size, so that overall there was no difference in the total nitrogen content of loads returned to each colony type. Over the course of the observation period, the volume of pollen stored in supplemented and depleted colonies converged, suggesting that honeybees maintain pollen stores around a homeostatic set point. Fewell and Winston (1992) interpreted this finding as evidence of individual foragers ability to respond flexibly to changes in pollen stores, preferentially seeking out and collecting only the best quality pollen when stored levels are high. In a similar experiment, Pernal and Currie (2001) compared the effect of manipulating both the quantity of pollen stored in colonies, and the quality, exchanging existing stores for combs containing either nitrogen-rich or nitrogen-poor pollen. They observed that bees responded in a similar manner to both kinds of manipulation, increasing the proportion of foragers allocated to pollen collection when supplies were depleted or the quality of stored pollen was poor. In contrast to the findings of Fewell and Winston (1992) there was no difference in the quality or breadth of species collected under either manipulation, leading Pernal and Currie (2001) to conclude that individual honeybee foragers lack the ability to assess the protein content of pollen whilst collecting, proposing instead that they may receive feedback about colony demand from the nurse bees which unload their pollen sacs. Interestingly, a difference in the protein content of loads collected by experienced and inexperienced foragers was observed, with inexperienced foragers returning with more nitrogen rich pollen. This was hypothesised to be the result of

inexperienced bees most likely being younger and less energy constrained and therefore better able to sample available resources. Pernal and Currie (2001) suggest that recruiting more inexperienced foragers under times of need, may provide a mechanism by which honeybee colonies might selectively increase the quality of pollen returned to the hive. However, this does not explain the discrepancy with Fewell and Winston's (1992) finding, given that in this study, bees in pollen-supplemented hives were observed to be more selective than those from pollen-depleted hives where additional, inexperienced pollen foragers were more likely to be recruited.

Waddington et al. (1998) directly manipulated the quality of pollen available for collection and observed that honeybees were less likely to perform a dance to alert their hive-mates to the location of pollen sources that had been diluted with inert, indigestible alpha-cellulose compared to sources of undiluted pollen, indicating that they perceived the diluted pollen to be an inferior food source. Using a similar method, Kitoaka and Nieh (2009) found that the more alpha cellulose present in the pollen sample provided for collection, the less likely bumblebee foragers were to return to the hive with pollen. When foraging on nectar, bumblebees have been shown to increase the temperature of their flight muscles with increasing sugar concentration, thus enabling them to forage more efficiently on high quality resources (Nieh, León, Cameron, & Vandame, 2006). Mapalad et al. (2008) observed a similar effect in pollen foraging bumblebees, with thoracic temperatures observed to correlate positively with the ratio of pollen to alpha cellulose. Such findings suggest that, in contrast to previous anecdotal reports of bees collecting non-food substances such as dried paint or fungal spores (Shaw, 1990), bees may at least have the ability to distinguish between pollen and other powdered substances offering little nutritional value. However, when considering foraging effort at the colony level, it should be noted that a variety of external factors, such as weather conditions or the season, can also have an impact on foraging motivation (Bergman, Molau, & Holmgren, 1996; Brian, 1952; Cartar, 1992; Free, 1955; Young & Owen, 1989). Thus studies of individual foraging preferences provide a more valuable measure of the capacity to discriminate between pure and nutritionally-poor pollen sources.

In all studies discussed thus far, it has not been possible to determine the exact component(s) of the pollen reward guiding foraging choices. In experiments where pollen has been diluted with alpha cellulose, Pernal and Currie (2002) suggest that collection preferences may simply have been guided by differences in the olfactory

intensity between pollen samples. In an experiment in which they used artificial pollen surrogates to separate the effects of odour, particle size, protein content and handling time, ensuring a single cue was varied at a time, Pernal and Currie (2002) found the presence of pollen odour to have the most influence over the probability of bees landing and collecting pollen. Olfactory cues are certainly highly salient attractants for foraging insects, and bees have been shown to be capable of distinguishing pollen odours from that of the whole flower itself (H. E. M. Dobson, 1987). However, when bees were given the highly unnatural choice between pollen samples producing an odour versus those lacking in fragrance, it is unsurprising that the fragrant samples were chosen more often by bees. When offered a source of protein, in the form of de-fatted soybean flour, diluted to varying degrees with alpha cellulose, Pernal and Currie (2002) observed no difference in the weight of loads collected by bees foraging on different samples, suggesting that honeybees did not discriminate between pollen samples on the basis of protein content alone. Both grain size and handling time were found to have an influence on behaviour. Since grain size may influence the manner in which grains pack in to the corbiculae (Vaissière & Vinson, 1994) bees may select pollen species in order to maximise foraging and packing efficiency. Interestingly, grain size has actually been shown to correlate with protein content for a number of species and so physical cues may provide bees with a reliable indicator of pollen quality (H. G. Baker & Baker, 1979; Batra, 1993; Roulston, et al., 2000; Simpson & Neff, 1983)

In most pollen species, the majority of nitrogen is found within the cytoplasm, concealed within the intine of the grain (Stanley & Linskens, 1974). Therefore a large proportion of the nitrogen contained within pollen grains may be inaccessible and thus undetectable without consumption and digestion of the cell wall. This may explain why honeybees in Pernal and Currie's (2002) study failed to discriminate between pollen analogues on the basis of protein content alone. Interestingly, pollen feeding experiments also found little evidence of bees compensating for a diet poor in protein by increasing pollen consumption, though it was observed that young honeybees avoided the extremely nutrient poor pollen of jack pine (*Pinus banksiana*), a wind-pollinated species (Pernal & Currie, 2000). Given that pollen is such a complex food source, consisting of vitamins, minerals (Herbert, 1992) and lipids (Solberg & Remedios, 1980), many of which are also essential for growth and development of larvae and young bees, it may be that individuals base their pollen preferences on other nutritional cues detectable at the surface of the grain. For example, the addition of lipids

or fatty acids to granular substances have been shown to have a phago-stimulatory effect and influence the decision to engage in pollen foraging (J. O. Schmidt & Hanna, 2006; Singh, Saini, & Jain, 1999). Others have suggested that deterrents, in the form of toxic secondary compounds, may also be influential in guiding pollen preferences (Sedivy, et al., 2011).

1.3 Putative sensory mechanisms for pollen assessment

Though bees are not thought to ingest pollen during collection, they have ample opportunity to sample grains with the main gustatory organs; the mouthparts and antennae. Bees are known to probe flowers with the antennae prior to landing (Lunau, 1992, 2006; Lunau, Unseld, & Wolter, 2009; Pohl, Watolla, & Lunau, 2008) and often grasp and scrape pollen from the anthers with their mandibles, collecting the dislodged grains with their proboscis (Casteel, 1912; Thorp, 1979; Thorp & Estes, 1975). Some species even have specialised hairs on the mouthparts (A. Müller, 1995; Parker & Tepedino, 1982; Shinn, 1967; Thorp, 1979), designed for collecting pollen from flowers with inaccessible anthers. To facilitate adherence of the pollen grains to each other and the corbiculae, bees add regurgitated fluids to the grains (Casteel, 1912) thus providing further opportunities for gustatory sampling.

Compared to what is known about both vision and olfaction in this species, the gustatory system of honeybees is poorly understood (see de Brito Sanchez, 2011 for a recent review). That honeybees possess so few gustatory receptor genes (10 genes, H. M. Robertson & Wanner, 2006) compared to other insects (60 genes- fruit flies, Dunipace, Meister, McNealy, & Amrein, 2001; 52 genes- mosquitoes, Hill et al., 2002), has been taken as evidence of their limited ability to detect gustatory compounds in their environment. However it is possible that as a result of splicing, each gene codes for multiple receptors (de Brito Sanchez, 2011). Additionally, receptor neurons may be widely tuned to a range of tastants and unique activation/inhibition patterns could produce a neural 'fingerprint' for each compound in the central nervous system (across-fibre pattern theory) (de Brito Sanchez & Giurfa, 2011; R. P. Erickson, 1968, 2008). Indeed Wright et al.'s (2010) investigation of conditioned taste aversions in bees found that the bitter compound quinine elicited a 'deterrent cell' pattern of responding in a number of types of receptor neuron.

Gustatory receptors sensitive to sugar are found on the antennae, mouthparts and the distal segment (tarsi) of the forelegs (Whitehead & Larsen, 1976) in honeybees. These sensory organs are also sensitive to salts (NaCl and KCl) though only the tarsi seem to possess a water-sensitive cell (Lorenzo, 2009). The mouthparts possess an additional receptor type, the sensitivity of which is unknown. Some have postulated that this may respond to either protein (Dethier, 1961) or amino acids (Goldrich, 1973; Shiraishi & Kuwabara, 1970), though this is yet to be tested at the physiological level in bees. Interestingly, when offered the choice between sucrose solutions, bees have been shown to preferentially imbibe those containing amino acids over those without, suggesting they are able to differentiate between the two rewards (Alm, Ohmeiss, Lanza, & Vriesenga, 1990; Bertazzini, Medrzycki, Bortolotti, Maistrello, & Forlani, 2010; Carter, Shafir, Yehonatan, Palmer, & Thornburg, 2006; Kim & Smith, 2000; Petanidou, Van Laere, N Ellis, & Smets, 2006).

An electrophysiological study of the gustatory receptors of flies led Shiraishi and Kuwabara (1970) to classify amino acids according to the responses generated by the different chemosensory cells of the labellar in fleshflies and blowflies. Four classes were identified, those which resulted in no response (Class 1), those which caused a general inhibition of both salt and sugar receptor cells (Class 2), those which stimulated just the salt receptors (Class 3) and those which stimulated the sugar receptors (Class 4). A subsequent behavioural study revealed that like bees, flesh flies also prefer to feed on solutions containing amino acids, though they show an avoidance of solutions containing histidine and lysine (Potter & Bertin, 1988). The ecology of these flies differs considerably from that of bees, and their gustatory repertoire is thought to be much more diverse. Nevertheless is interesting to note that amino acids, common to the nectar of almost all plant species (H. G. Baker & Baker, 1977) may have an influence on its perception or 'taste' for visiting pollinators. Whilst some amino acids may enhance the perceived sweetness of nectar (Gardener & Gillman, 2002) others could lead to the solution being perceived as salty or bitter, and potentially serve to deter to unwanted flower visitors.

When the antennae or tarsi of bees are touched with sucrose solution, an extension of the proboscis is observed, a behaviour which has been characterised as an unconditioned, appetitive response to stimulation with a food reward (Bitterman, Menzel, Fietz, & Schafer, 1983). A similar effect was observed in honeybees stimulated

at the antennae with hand-collected almond pollen (Scheiner, Page, & Erber, 2004). Very few individuals responded with proboscis extension to inert alpha cellulose, suggesting that bees are able to detect phago-stimulatory compounds in pollen at the level of the antennae. An electrophysiological study of the labellar chemoreceptors of hoverflies (*Eristalis tenax*), a species whose ecology resembles that of bees, found that when diluted in water, extracts of pollen stimulate the salt receptor cell (Wacht, Lunau, & Hansen, 2000). Unlike KCl or NaCl, which inhibit the water cell and are thus rejected at high concentrations (300mM KCl), pollen produced the same response in the water cell as that for pure water, meaning that flies were more likely to imbibe the aqueous pollen extract. Interestingly, though pollen is known to contain sugars (Stanley & Linskens, 1974), no stimulation of the sugar receptor was observed.

1.4 Learning in bees

Given that both the quality and availability of food reward offered to pollinators differs between plant species and can fluctuate over several time scales, depending for example on the season or nectar re-fill rates, flowers are considered to represent a highly variable food source (Heinrich, 1979). Thus the well-studied ability of bees to learn the location of profitable flower patches and the features, such as pollen odour and petal colour, associated with highly rewarding flowers is considered to be an adaptation to efficiently exploiting such an ever-changing resource.

Sir John Lubbock (1882) first demonstrated the ability of honeybees to associate a colour cue with a food reward, with Karl von Frisch (1914) subsequently confirming that this ability involved colour vision. In his classic experiments, von Frisch trained individual bees to fly to a feeding dish containing sugar water, located some distance from the hive. Once bees had learnt the feeding place he placed the dish on top of a square of blue cardboard. To determine whether bees were able to perceive this colour he subsequently added identical squares of cardboard to the feeding place in various shades of grey, white and black, to build up a multiple-choice scenario for the forager. The position of the various squares was randomized between trials and the reward removed during testing. Bees searched preferentially for food in the blue square,

demonstrating that they did indeed use colour vision rather than relying on brightness cues to identify the rewarding square.

Menzel expanded on von Frisch's original methods to produce individual learning curves for a range of spectral wavelengths (Menzel, Erber, & Masuhr, 1974). A free-flying paradigm was also used but cardboard squares were replaced with a table illuminated from below using monochromatic colours. Menzel et al. (1974) found that in a dual choice experiment, acquisition was independent of the alternative colour but that certain wavelengths were learnt more quickly. Giurfa et al. (1995) using colour-naïve individuals, showed that bees have in built colour preferences, but that these are easily overridden by training on an alternative colour. This suggests that there is no link between learning rate and the perception of different wavelengths, but that variation in acquisition of different colours can be explained as 'preparedness to learn'. Similar experiments with bumblebees have demonstrated the generality of sucrose-rewarded colour learning in free-flying bees (Gumbert, 2000; Heinrich, 1976, 1979; Heinrich, Mudge, & Deringis, 1977; Lunau, Wacht, & Chittka, 1996).

Both the order in which the stimulus and the reward are experienced and the overlap between presentations has been found to have an effect on acquisition (Menzel, et al., 1974). As first observed by Opfinger (1931) bees must see the stimulus on their approach to the table (at least 2 seconds before landing and for 0.5 seconds during feeding) for learning to take place. The subsequent development of the restrained paradigm, which permits full control over stimulus and reward delivery, allowed for a more in-depth investigation of the conditions required for learning.

When the antennae of a honeybee is touched with a sufficiently concentrated sucrose solution, the proboscis of the bee extends, a phenomenon known as the proboscis extension response (PER). By pairing this unconditioned response with a neutral odour (conditioned stimulus, CS), following a number of training trials, presentation of the previously neutral odour alone is sufficient to elicit the PER (Bitterman, et al., 1983; Takeda, 1961). Though it was already known that the PER could be conditioned (Kuwabara, 1957), Bitterman et al. (1983) were the first to use appropriate controls and analyse individual rates of learning. As with colour learning in free-flying bees, acquisition was found to be rapid, occurring within three trials (Menzel, et al., 1974). Again, timing was found to be important, as was the order of presentation. An inter-stimulus interval of 1-3 seconds was found to be optimal (Menzel & Bitterman, 1983)

and the conditioned response is only acquired if the odour precedes sucrose presentation. This paradigm has subsequently been adapted for the study of conditioning in bumblebees (Laloi & Pham-Delegue, 2004; Laloi et al., 1999; Riveros & Gronenberg, 2009).

Though the restrained paradigm may be argued to have reduced ecological validity in comparison to the free flying paradigm, it has the advantage of permitting a tighter control over stimulus presentation and inter-trial interval. In addition, *in vivo* recordings of nerve cells can be performed whilst bees are engaged in learning. However, attempts at using this paradigm to investigate conditioning in the visual modality in honeybees have been less successful. Kuwabara (1957) found that bees could only be trained to associate colours using PER if the antennae were cut, and still learning took between six and 38 trials. Kuwabara did not include controls such as unpaired presentations but Hori et al. (2006) replicated these experiments and found that bees had to be trained over two days, and that at least five trials were required to reach acquisition. A more recent study by Niggebrügge et al. (2009) found that by placing a bee in the centre of a cylindrical paper arena and presenting a projected light spot to a single eye it is possible to condition bees within a couple of trials to at least five different coloured stimuli. Discrimination varied according to the degree of chromatic similarity, with those colours that are more closely related being discriminated more poorly. Such a finding contrasts with results from the free-flying paradigm, where individuals were capable of making fine-grained discriminations between colours (Backhaus, Menzel, & Kreissel, 1987; Daumer, 1956; Kühn, 1924; von Helversen, 1972). This suggests that behavioural context may have an influence on perceptual processing, a fact which should be borne in mind when generalising results collected under one paradigm to another.

In the multiple investigations of appetitive learning in bees conducted to date, sugar water has typically been used as the rewarding stimulus. The question of whether pollen can reinforce learning has been little addressed. This is of interest when considering the fact that pollen is likely to have been the ancestral reward for pollinating insects. Indeed not all modern flowers provide a nectar reward and some species have secondarily reverted to rewarding with pollen alone. Might the earliest flower visitors have been capable of learning the features of flowers providing their preferred pollen reward? Could pollen promote flower constancy in the same way that has been demonstrated for nectar-rewarded bees?

1.5 Foraging specialisation and learning

Bees have the opportunity to experience different pollen odours when interacting with dancers in the hive (von Frisch, 1923) and von Aufsess (1960) demonstrated that bees can learn to use pollen odour as a cue to the location of a sucrose reward. More recently Cook et al. (2005) have shown that it is possible to condition bees to pollen odour using the PER paradigm. Bumblebees are known to use pollen odours to distinguish between pollen rewarding and non-rewarding flowers (H. E.M. Dobson, Danielson, & van Wesep, 1999) therefore it seems reasonable to suggest that bees might also learn something about the features of such flowers whilst foraging.

Following an experiment in which free-flying bees were trained to visit a pollen feeder, Arenas and Farina (2012) suggest that individual bees were able to learn odour cues associated with the pollen reward. Pollen feeders were scented with filter paper soaked in either linalool (LIN) or phenylacetaldehyde (PHE). Following training, when given a choice between the conditioned odour (either PHE or LIN) and the alternative, more bees were observed to visit the feeder paired with the trained odour. Olfactory preferences were tested either in the presence of pollen, as in training or provided with dishes containing chalk, which effectively served as an unrewarded test since bees failed to collect this resource. Whilst under both testing conditions bees did prefer to visit the conditioned feeder, the fact that experiments were conducted with experienced bees, under natural conditions means that it is not possible to rule out whether those marked individuals that returned to the feeder during testing may have learnt the conditioned odour whilst foraging on flowers in the surrounding area. Training naive bees, and testing learning under more controlled conditions is necessary to truly demonstrate that bees can learn when pollen alone serves as the reinforcer.

Scheiner et al. (2004) already demonstrated that honeybee foragers extend their proboscis when the antennae are touched with pollen, meaning it was relatively easy for Grüter et al. (2008) to adapt the PER paradigm in order to test whether pollen might substitute for sucrose as the unconditioned stimulus (US) in an olfactory conditioning experiment. Responsiveness to both pollen and sucrose was tested in the same individual with an inter-trial interval of 15 minutes. As in Scheiner et al.'s (2004) study, a high proportion of bees were found to extend the proboscis in response to pollen applied directly to the antennae, whereas no bees exhibited PER in response to the

pollen odour alone (immediately prior to physical application). 70% of pollen foragers and 48% of non-pollen foragers learnt to associate pollen with the olfactory CS after three trials, leading Grüter et al. to conclude that bees readily learn to respond to an odour where pollen acts as the US. However, this studied lacked an appropriate control group, in which the presentation of the CS and US was unpaired. As a result, it is not possible to conclude whether the observed increase in response to the CS was the result of bees learning the predictive relationship between CS and US delivery (associative learning) or rather due to the multiple stimulations of the antennae with pollen leading to a non-associative increase in sensitivity to the CS.

The question of whether pollen can reinforce learning is also of interest when one considers that within honeybee colonies, individual foragers have been shown to have a tendency to collect one type of reward exclusively over their lifetime (Seeley, 1995). As a result, even when visiting flowers which produce both pollen and nectar, some individuals may receive only pollen as a reward. The exact mechanisms underlying such foraging task specialisation are still to be determined, although foraging role has been shown to correlate with sensitivity to external stimuli. Page, Erber and Fondrk (1998) adapted the paradigm for conditioning the PER, to develop a method for measuring response thresholds (RT) to sucrose. The antennae are touched with an increasing series of sucrose concentrations and the RT is defined as the lowest sucrose concentration eliciting PER. By testing the PER-RT of bees returning to the hive, Page et al. (1998) demonstrated that pollen foragers have a lower RT to sucrose compared with nectar foragers. Pankiw and Page (2000) subsequently confirmed the link between sucrose responsiveness and foraging role by demonstrating that an individual's future foraging behaviour could be reliably predicted by their response threshold during behavioural development within the hive.

It may at first appear paradoxical that bees which forage for pollen are more sensitive to sucrose than those which collect nectars, but Page et al. (2006) argue that such specialisation could be adaptive for the colony, since nectar foragers would only collect from flowers producing highly concentrated nectar, thus returning to the hive the best quality resource currently available. Scheiner et al. (2004) also suggest that sucrose responsiveness is unlikely to be directly responsible for the differences in pollen and nectar forager behaviour, rather that variation in sucrose RT's may represent general differences in sensory processing. This view is supported by the fact that sucrose

sensitivity is also known to correlate with sensitivity to other modalities such as pollen (Scheiner, et al., 2004) and light (Erber, Hoormann, & Scheiner, 2006; Tsuruda & Page, 2009).

Differences in sensitivity to external stimuli have been demonstrated to have an impact on differences in learning between forager types. Scheiner et al. (1999) found that pollen foragers acquired a tactile conditioning task more rapidly, reached a higher asymptote and greater resistance to extinction than nectar foragers. An analogous result was found for olfactory conditioning (Scheiner, Barnert, & Erber, 2003), though it should be noted that more recent findings suggest that behaviours observed under restrained conditions may not accurately reflect the behaviour of free flying bees (Mujagic & Erber, 2009), yet again suggesting that behavioural context may have an impact on sensory perception and learning. Differences in the learning performance of foragers reinforced with their respective rewards, has yet to be tested, and it may be that variation in reward perception by foragers could lead to the differential reinforcement of behaviours and thus contribute to the maintenance of foraging task specialisation in honeybee colonies.

1.6 Thesis preview

It has long been acknowledged that pollen represents the original floral reward for the ancestors of many of today's insect pollinators, though the question of whether this food resource can reinforce the learning of floral features has been little addressed. Thus the central aim of my thesis is to determine whether pollen can reward learning in two well-studied species of generalist pollinator, *Apis mellifera* and *Bombus terrestris*. After describing the development of a method for studying learning in pollen-collecting bees (Chapter 2), I first compare and contrast pollen-rewarded learning with the well established features of sucrose-rewarded learning, using colour-naive free-flying bees housed under controlled conditions (Chapter 3). Studies of bees foraging on flowers in their natural setting have led to the suggestion that individuals may have the ability to discriminate between plant species and varieties on the basis of the nutritional quality of pollen they provide. In Chapter 4 I investigate whether individual bumblebees display preferences for pollen samples of differing nutritional value and test whether differences

in pollen quality might promote floral constancy in bees, as has been shown for nectar. In order to explore the nature of the pollen reward in more detail, in Chapters 5 and 6 I examine learning in restrained honeybees in an olfactory conditioning paradigm, substituting the traditional sucrose reward with pollen. This method permits the dissociation of the different components of the pollen reward, and so I attempt to determine both the sensory organs and chemical cues which might be involved in the pollen-reward pathway.

Chapter 2: Method development

Sucrose-rewarded learning mechanisms have been widely studied in honeybees (e.g. Lubbock, 1882; Menzel, 1967, 1968, 1969; von Frisch, 1914, 1967) using a variety of experimental paradigms. In contrast, very few attempts have been made to study learning where pollen serves as the reward. The development and refinement of experimental methods to study pollen-rewarded learning has formed an important part of the research for this thesis. In this chapter I will describe in detail and critically discuss how the methods used in subsequent chapters were derived.

2.1 Conditioning free-flying bees with pollen

Except where stated, all experiments with free-flying bees were conducted inside the laboratory, which permitted tight control over the sensory experience of bees. The pollen used in all experiments was mixed-species honeybee-collected pollen (Werner Seip, Germany), freshly ground to a fine powder prior to each experiment. Small colonies of honeybees or commercial bumblebee colonies (Koppert Biological Systems, Suffolk, UK) were kept inside flight cages (1 m^3) with bees were permitted to fly freely and collect pollen and sucrose solution *ad libitum*. The living cage was connected via a Perspex corridor to an experimental flight cage of the same size. Flight cages previously used to study colour learning in nectar-rewarded bees have in many cases been more limited in height than those used in the current experiments, and often in the case of bumblebees the experimental arena has been connected directly to the nest box (e.g. Ings, Raine, & Chittka, 2009; Lotto & Chittka, 2005 ; Raine & Chittka, 2005 all used experimental arenas measuring 120 x 100 x 35 cm). The low height in particular makes such cages unsuitable where pollen collection is involved. Upon accumulating pollen on the legs and body, bees are typically observed to leave and hover above the flower, grooming and repackaging pollen into the corbiculae whilst in flight (Michener, et al., 1978). The same behaviour is also observed when bees collect pollen from petri dishes in the current studies. Thus an important advantage of using a larger flight cage for studying pollen-rewarded learning is that bees have sufficient space to fly/hover above

the stimuli whilst manipulating pollen into their corbiculae for transport back to the hive.

2.1.1 Vertical stimulus presentation, hidden pollen

In the learning experiments presented in Chapter 3, a comparison was made between nectar and pollen foraging honeybees in terms of the rate of learning with their respective rewards. It was necessary to keep the visual characteristics of the pollen reward hidden, since it was hypothesised that such cues might interfere with learning of the coloured stimuli in the discrimination task. Four coloured stimuli (8 cm diameter) were presented vertically on the outer surface of a grey plastic box (20 x 10 x 15 cm). In order to obtain a pollen reward bees were required to crawl through small tubes positioned in the centre of the coloured stimuli. Pollen was presented on the floor of the training box in a large petri dish.

For nectar foragers, a drop of sucrose solution was presented inside the tubes. Netting across the end of the tube prevented access to the pollen inside the box, but still permitted the odour to diffuse out, thus standardising the olfactory environment during testing of each forager type. Tubes were cleaned and replaced after each training trial in order to remove any olfactory cues left by previously tested individuals. Bees were trained and tested in a differential conditioning task, in which one of the two presented colours (either blue or yellow) was rewarded. For the non-rewarded colour, tubes either had netting to prevent access to the pollen, or in the case of sucrose-rewarded bees, they contained a droplet of water near the entrance instead of sucrose solution.

This method was designed to compare bees rewarded with pollen and nectar under the same experimental conditions. After training to two colours alternately over twenty trials, a difference in the colour preference of the two forager types was observed when tested following a one hour delay. This finding suggests that the underlying processes of colour learning and memory recall may differ according to the type of reinforcement received. An alternative explanation however could be that pollen foragers, which have to enter the grey test box, experience a longer delay between viewing the coloured stimulus and experiencing the reward than sucrose-rewarded bees. The timing of

stimulus presentation and reward is known to be important in the development of learnt associations in honeybees (Opfinger, 1931, Menzel et al. 1974).

Given the relatively complex experimental nature of the learning task in which pollen was hidden, and that the processes of encoding and retrieval involved in the formation of memories associated with pollen are currently unknown, it was necessary to adopt a more simplified method when attempting to determine the duration of learnt associations between visual stimuli and pollen.

2.1.2 Horizontal presentation, exposed pollen

Coloured stimuli (15 cm diameter) were presented horizontally and small petri dishes (5.5 cm diameter) of pollen were fixed in the centre of each. Bees were thus exposed to the visual properties of the pollen reward. This is akin to the original style of stimulus presentation used by von Frisch (1914, 1967) when training sucrose-rewarded honeybees, and subsequently adopted by others such as in the early tests of colour learning by Menzel et al. (1967, 1968, 1969). The method was successfully piloted outdoors with free flying honeybees trained to collect pollen early in the flowering season (February 2010) when naturally occurring pollen was sparse. All subsequent experiments using this method were conducted in the laboratory with bumblebees.

I first tested whether bumblebees could learn to associate coloured stimuli with the presence of pollen (Chapter 3). Two colours were presented, but only one was rewarded. For the unrewarded colour the pollen dish was present but had a clear covering to prevent access to pollen. The covering had small openings which permitted the diffusion of pollen odour, meaning that the visual and olfactory cues were constant between rewarded and unrewarded stimuli. After testing for colour preferences immediately following training, I re-tested bees following delays of one and twenty four hours to examine consolidation of pollen-rewarded associations to the mid and long-term memory respectively. The horizontal presentation method was also used to test whether bees can learn to associate differences in pollen quality with a coloured cue (Chapter 4).

To determine which sensory organs make direct contact with pollen grains during collection and therefore may be involved in sensing and assessing pollen, I closely observed the collecting behaviour of honeybees and bumblebees. The most recent review of pollen collection in bees was published 12 years ago by Thorp (2000), with much of this data based on real-time observations of bees at flowers. Several historical accounts of pollen collecting behaviour suggest that contact between the proboscis and pollen is common in honeybees (Casteel, 1912 and references therein) and so I attempted to verify such claims, by filming bees at high speed (250 frames/ second) to determine whether any fine scale or rapid behaviours may have been overlooked in previous accounts of pollen collection by bees.

As well as filming bees collecting pollen from flowers (*Brassica napus* and *Potentilla fruticosa)* I also made video recordings of individuals engaged in pollen collection from petri dishes, as they were required to do in many of the free-flying experiments presented in this thesis (Chapters 3 and 4). This allowed me to study the exact behaviours involved in pollen collection in the current experimental set up. Both honeybees and bumblebees were observed to probe pollen with their antennae as they alighted on flowers or moved around the pollen dish and often used the proboscis and mandibles to collect and manipulate grains. Flying away from the dish and engaging in bouts of grooming were common during the collection process, and the tarsi were frequently used to clean grains from both the antennae and proboscis.

2.1.3 Discussion

Vertical presentation of stimuli has been favoured in visual discrimination experiments of recent decades since it permits the experimenter to control the angle of approach (Wehner, 1981; Wehner & Lindauer, 1966) and in the case of the Y-maze (Srinivasan & Lehrer, 1988), the distance at which bees view the stimuli. The vertical presentation method (Chapter 3) did however require extensive training, since bees had to learn first how to enter (and leave) the pollen box. The low light levels and restricted size of the boxes used in this presentation method made foraging quite difficult for bees, reducing their ability to fly up and groom pollen into the corbiculae. Given that the priority of this thesis was to identify learning mechanisms rather than investigate visual perception,

the method was simplified. Stimuli were presented horizontally and the pollen reward was visible. Because pollen was on display, this method permitted close observation of the behaviour of bees engaged in pollen collection and led to some interesting observations regarding the extent to which bees pay attention to the visual characteristics of the pollen they collect. In addition, horizontal presentation more closely resembles the natural pollen presentation mode of certain flowers, particularly those considered to have appeared early in the evolutionary history of angiosperms (e.g. *Magnolids, Asteraceae*) (Crepet, et al., 1991).

The comparison of two closely related, but ecologically and behaviourally quite distinct species, engaged in comparable learning tasks, under similarly controlled conditions offers the valuable opportunity to make some tentative generalisations about pollen-rewarded learning in Apidae bees. Bumblebees are commercially reared and much easier to keep in flight cages than honeybees. In general they experience a lower mortality and it is easier to manipulate a colony's foraging motivation. However, they are less well studied than honeybees and given that they do not show such rigid division of labour, there are some limitations to the questions one can ask. Nevertheless, bumblebees are suitable candidates for testing whether individuals are able to assess pollen quality whilst foraging (Chapter 4), given existing evidence to suggest that colony-level foraging effort fluctuates according to changes in the quality of pollen available for collection (Kitaoka & Nieh, 2009). Bumblebee colonies are considerably smaller, have fewer workers and less space to store pollen than honeybees, and so pressure to maximise the quality of pollen brought back to the hive is presumably higher. Indeed when Leonhardt and Blüthgen (2011) compared the pollen collected by honeybee and bumblebee colonies located in the same habitat, they observed that the pollen stored in bumblebee colonies was twice as rich in protein. Bumblebees do not use precise information to recruit fellow foragers to food patches and foragers sample colony stores individually whilst unloading their pollen sacs. As a result, bumblebee foragers receive less feedback from in-hive bees about colony pollen needs than honeybees and so their foragers may be more likely to possess the ability to individually assess differences in pollen quality.

2.2 Restrained Methods

Pollen is a complex stimulus comprising of visual, tactile and multiple chemical components. As a result, the exact mechanism by which pollen may reinforce learning is unclear. Foraging bees do not collect pollen for their own nutritional needs and therefore pollen cannot be classed as an appetitive reward in the traditional sense. Pollen grains are occasionally found in the guts of adult bees (Crailsheim et al., 1992), but these are likely to have been ingested by chance, either whilst grooming pollen from the body or through imbibing pollen-contaminated nectar. As a result it seems unlikely that post-ingestive processes are involved in learning during pollen collection.

Bees have ample opportunities to sample pollen during collection, via gustatory receptors on the antennae, mouthparts and tarsae. Free-flying experiments closely resemble the natural foraging situation, however pollen collecting behaviour is complex. Though observing bees engaged in pollen collection permits identification of those organs which may potentially be involved in pollen-rewarded learning, given the multiple sites of pollen contact during such behaviour, it is not possible to determine what represents the exact reinforcing component(s) of the reward. Consequently I used the proboscis extension response (PER) paradigm to give more precise control over pollen contact with sensory organs, and to examine the relative importance of antennal and mouthpart stimulation. All PER experiments were conducted with honeybees. The conditioned stimulus (CS) consisted of pure hexanol (a compound commonly present in floral odours), rather than coloured stimuli as used in free-flying experiments, since olfactory stimuli are more commonly used in the PER paradigm and are learned more quickly than visual stimuli in this experimental set up (Hori, et al., 2006; Kuwabara, 1957; Niggebrügge, Leboulle, Menzel, Komischke, & de Ibarra, 2009).

In the PER paradigm, bees are restrained in metal harnesses which permit free movement of the head, antennae and mouthparts. Following delivery of the olfactory CS the antennae are stimulated with sucrose solution (unconditioned stimulus) which leads to the reflexive extension of the proboscis. Bees are then permitted to feed on the sucrose solution for a few seconds, though the feeding component is not necessary for learning to take place (G. A. Wright, Mustard, Kottcamp, & Smith, 2007). Following multiple pairings of the CS and US presentation of the CS alone becomes sufficient to elicit PER. Here, I substituted the sucrose US for pollen, since antennal stimulation with

pollen grains also leads to proboscis extension (Scheiner, et al., 2004). Pollen was either delivered as is, or mixed with water to produce a solution. The relative merits of each delivery method will be discussed below.

Prior to conditioning, the motivation of individuals to respond to sucrose solution is usually tested and only those which respond with proboscis extension are included in subsequent experiments. Similarly, I pre-tested bees to ensure they were motivated to respond to pollen and only attempted to condition those individuals that responded with proboscis extension to antennal stimulation with both pollen and sucrose. Bees were first permitted to drink water until satiated so that when pollen was delivered in solution I could rule out the influence of thirst on responding to the pollen-water mixture. Although bees may collect water under natural conditions, water on its own seems not to reinforce learning in the PER paradigm (Ayestaran, Giurfa, & de Brito Sanchez, 2010). Therefore any evidence of learning in bees rewarded with pollen in solution can reasonably be attributed to the pollen component of the reward.

2.2.1 PER conditioning, dry pollen

The conditioning protocol followed the standard forward-paired method elucidated by Bitterman et al. (1983). The CS preceded US delivery and there was an overlap in stimulus delivery of one second, since this has been shown to facilitate learning. The CS for all experiments was 1-hexanol (98% purity) diluted in mineral oil to 2.5 molar (M). 20 ml of the odour solution was placed in a 60 ml glass bottle which was connected to an air pump with silicone tubing. The air stream was gated by a PLC-controlled valve to deliver uniform odour puffs (see also Smith, 1998). The odour stream was directed frontally at the head of the bee, and removed via a constant air stream, emitted from an extractor system located behind the animal.

Where pollen served as the US in its natural state it was delivered to the antennae via a small oval sponge (1 cm length) attached to a plastic stick. The commercially available sponges had a surface texture capable of holding fine powders, and were replaced after each experiment. Pollen could not be delivered to the proboscis, because grains had a tendency to stick to the mouthparts which subsequently impacted on the motivation of bees to exhibit the proboscis extension response. Antennal stimulation has been shown

to be sufficient to reinforce learning in conditioning experiments with sucrose reward, therefore the US was delivered to the antennae only.

Typically, in PER conditioning bees are rewarded with a toothpick dipped in sucrose solution. Stimulating bees with a dry toothpick alone does not reinforce learning (Ayestaran, et al., 2010), hence mechanical stimulation can be ruled out as a potential component of rewards delivered in this manner. Stimulation with softer substances, such as sponge, have yet to be tested and so it was necessary to include a control group in which bees were stimulated with the sponge alone. To rule out the possibility that bees were responding with proboscis extension to the odour of the pollen stimulus rather than antennal stimulation, an attempt was made to standardise the olfactory environment by placing a dish of pollen (*ca.* 3 g) between the subject and the site of CS delivery. In addition, the US delivery apparatus consisted of two sponges taped together in a cross formation, one coated in pollen and one clean. Pollen-rewarded bees received stimulation with the pollen bud whereas control bees were stimulated with the clean bud but experienced the same olfactory cues as pollen rewarded bees.

An interesting result was observed in the olfactory conditioning experiment, in that bees receiving stimulation with a clean sponge (control group) demonstrated a higher than expected response to the CS (Chapter 5, Fig. 5.1). It was hypothesised that this relatively high level of responding might be due to olfactory cues emanating from the pollen dish arousing bees and leading to a greater probability of proboscis extension. In order to test this I repeated the previous experiment but exposed only half of the bees to the pollen dish during conditioning (control groups). I also tested the effect of pollen exposure on bees rewarded with sucrose solution (30% sucrose weight/weight (w/w), sucrose groups), meaning that four groups were conditioned in total. In the sucrose-dish and control-dish groups, a 3 g dish of pollen was placed between the bee and the odour delivery site as in the previous experiment. In the sucrose and control groups, the dish was present but covered with a lid to prevent the diffusion of olfactory cues.

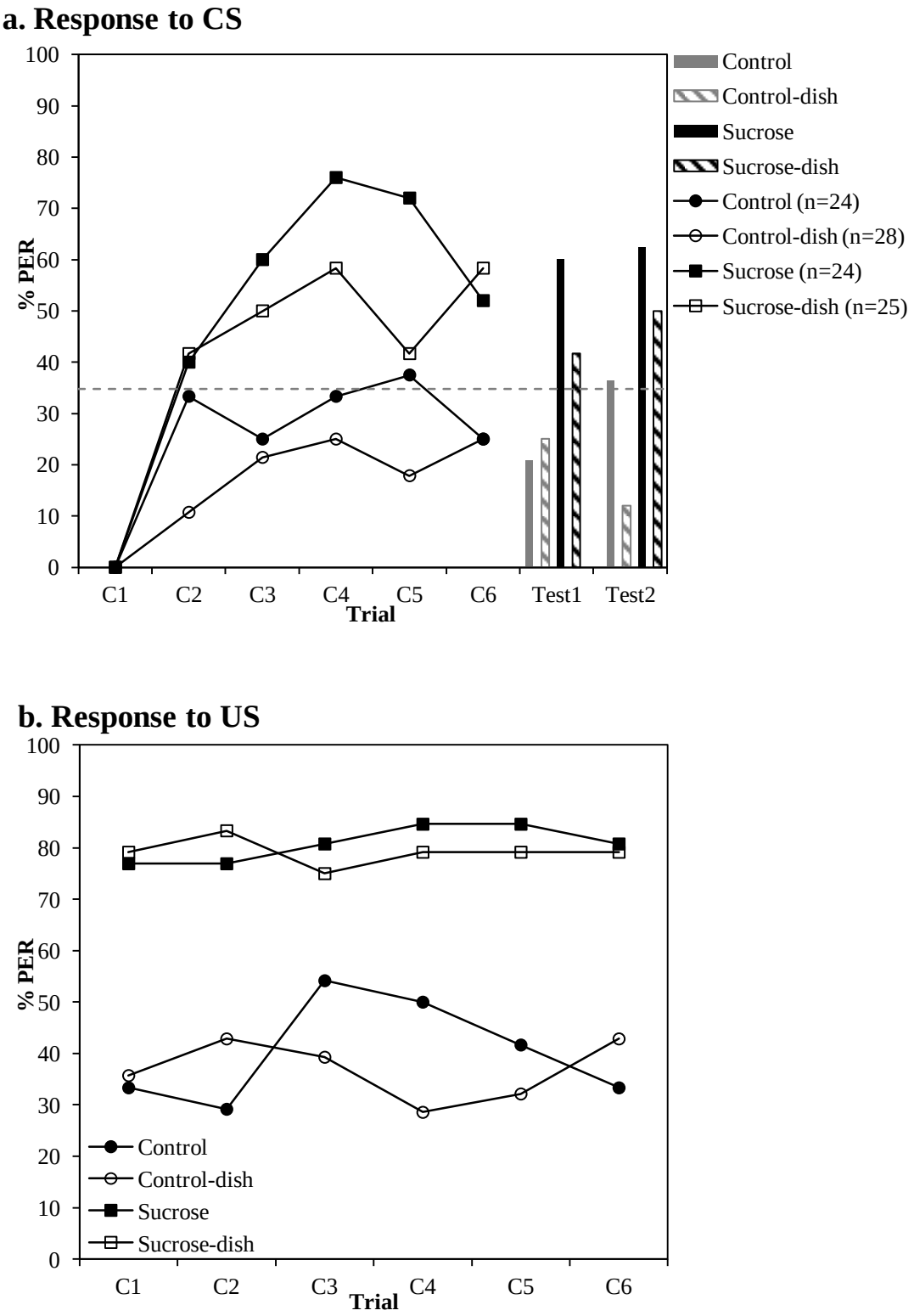


Figure 2.1 Proportion of bees responding to the CS (a) or US (b) on each trial. The US was either 30% sucrose solution (Squares) or a clean sponge (Circles). Bees were either exposed to an open dish of pollen during conditioning (Open shapes) or a closed dish (Black shapes). Bars represent the number of bees responding to the CS in the unrewarded tests. (Black bars=Sucrose, Grey bars=Control, Striped=Exposed to pollen; Striped= Non-exposed) In Test 1 olfactory conditions were matched to those experienced during training and in Test 2 the pollen dish was covered for all bees. The dashed line represents the overall spontaneous response to the CS on the first trial for all bees tested.

Bees received six conditioning trials and two unrewarded test trials. In Test 1 bees experienced the same conditions as in training (i.e. dish was uncovered for 'dish' groups and covered for remaining groups). In Test 2 the pollen dish was covered for all treatment groups, to test whether pollen odour might act as a contextual cue for the recall of the CS–US association. If so, I expected to see decline in response between test one and two for bees pre-exposed to pollen (sucrose-dish and control-dish groups)

Overall, sucrose-rewarded bees outperformed control bees in terms of overall acquisition (Fig. 2.1a GEE, Treatment X^2_1=17.659, p<0.001). The presence of the pollen dish had no impact on the overall level of acquisition (GEE, Condition X^2_1=2.680, p=0.102) and there was no interaction between the type of reward received and whether or not bees were exposed to pollen during training (GEE Treatment x Condition X^2_1=0.074, p=0.785). Given that there was no difference in responding between pollen-exposed and non-exposed control bees, (LSD contrast, Control-dish *vs.* Control p=0.149) it seems that pollen odour was not responsible for the higher than expected response to the CS and US seen in the initial conditioning experiment. Interestingly, acquisition of bees exposed to the pollen dish actually seems to be slower than that of non-exposed bees, with the interaction between trial number and training condition approaching significance (GEE, Trial x Condition X^2_4=8.440, p=0.077). Possibly the presence of the pollen odour interfered with detection of the olfactory CS, thus leading to a lower level of responding.

Analysis of the unrewarded test reveals a significant effect of the type of antennal stimulation received during training (GEE, Treatment X^2_1=17.861, p<0.001). Bees that were rewarded with sucrose were more likely to respond to the CS than those stimulated with the dry sponge (Fig. 2.1a). The overall difference in responding to the CS in bees that experienced the pollen odour versus those that didn't is nearing significance (GEE, Condition X^2_1=3.459, p=0.063). Similar to the slightly slower rate acquisition observed during training, pollen exposed bees showed a lower level of response to the CS relative to unexposed bees, with the exception of the pollen-exposed control group (control-dish) in the first test. Overall there is no significant difference in responding between the first test, where bees experience the same olfactory environment as they did during training, and the second test where the pollen dish is absent for all bees (GEE, Test, X^2_1=0.060, p=0.806). Likewise, no significant interaction between olfactory conditions experienced during training and responding in each test were observed (GEE, Test x

Condition, X^2_1=1.193, p=0.275), suggesting that the absence of this cue does not affect recall in those bees trained in the presence of pollen odour.

As expected, bees stimulated at the antennae with sucrose show a higher level of responding than those stimulated with a dry sponge (Fig. 2.1b GEE Treatment X^2_1=28.55, p<0.001). The presence of the pollen dish had no significant effect on responding to the US (GEE, Condition X^2_1=0.075, p=0.784). In fact, control bees that were exposed to the pollen dish showed a slightly lower level of response to the US compared to bees that weren't exposed. Overall responding to antennal stimulation remained constant throughout the course of the experiment (Trial X^2_5=2.583, p=0.764; Treatment x Trial X^2_5=3.795, p=0.579). In the case of control bees, this finding is somewhat surprising, given that one might expect to see habituation to repeated antennal stimulation in an absence of an effective reward.

In summary it appears that the higher than expected responding to stimulation with the dry sponge was not the result of arousal induced by the pollen odour. Rather it seems that simply stimulating the antennal mechanoreceptors of hungry bees may be sufficient to lead to proboscis extension in some cases.

2.2.2 Antennal sensitivity to pollen in solution

In a previous study of pollen-rewarded learning in which the PER paradigm was used (Grüter, et al., 2008) pollen was mixed with water and delivered to both the antennae and proboscis. Adding water to pollen places grains under osmostic shock, causing them to burst and release the cytoplasm, which is presumed to be where most of the nutritional content of pollen grains lies. Grüter et al. (2008) state that they used a dilution of between 50-70% pollen and water (w/w) however when trying to replicate such a dilution I found the solution to be extremely viscous. Applying the solution to the antennae or proboscis resulted in the cocktail stick becoming completely coated in the mixture, and led to the formation of clumps which adhered to the antennae. The strongest solution I was able to produce which didn't have this effect was a mixture of 30% pollen and water (w/w). The problem of pollen grains sticking to the antennae and proboscis persisted however, so filter paper was employed to remove the larger grains from solution.

Although bees may not be able to detect the presence of pollen in weaker solutions, the strongest concentration may not necessarily be that which is most preferred by bees. It was therefore useful to determine what constitutes an appropriate dilution of pollen and water to use in conditioning experiments. Using Page et al.'s (1998) method, for assessing the gustatory responsiveness of honeybees to sucrose I tested antennal sensitivity to solutions containing increasing concentrations of pollen. Samples ranged from 0.1% pollen (w/w) to 30% pollen (w/w) and were all passed through filter paper in order to remove the largest grains.

Bees returning to the hive were collected, with pollen and non-pollen foragers indentified by the presence/absence of corbiculae loads. Thus it was possible to examine whether differences exist between the two forager types in terms of antennal sensitivity to pollen, as has previously been suggested by Scheiner (2004) for bees stimulated with dry pollen. As in PER conditioning experiments, bees were restrained in metal harnesses and a small piece of tape was added to the base of tubes containing pollen foragers. This was hidden from the view of the experimenter, to avoid any potential bias in the coding of behaviour. 15 bees of each forager type were tested in parallel.

Bees were tested with ascending pollen concentrations from pure water to 30% pollen, and proboscis extension to antennal stimulation was recorded on each trial. Solutions were delivered via a toothpick and water via a syringe. On the first trial, in which bees were stimulated with water alone, individuals were permitted to drink until satiated, in an attempt to minimise the influence of thirst on the response to the pollen-water solutions. Between each pollen stimulation the antennae were stimulated with water to avoid cross-contamination. The ITI was ten minutes. Finally the antennae were stimulated with 30% sucrose, to demonstrate that bees were still motivated to feed by the end of the experiment and that any decline in response to the pollen-water solution was not the result of a general lack of motivation or fatigue.

Although the response to antennal stimulation with pollen in water was consistently greater than the response to water alone (Fig. 2.2), a concurrent decline in response to stimulation with pure water and pollen-water mixtures ranging from 0.1% to 3% pollen (w/w) was observed. When stimulated with 10% and 30% pollen respectively, the level of responding was observed to increase, and was found to be significantly different from the response to water alone (GEE, 10% Pollen, Treatment X^2_1=38.460, p<0.001; 30% Pollen, Treatment X^2_1=28.30, p<0.001). Thus 30% pollen was selected as the

concentration to be used in subsequent conditioning experiments. Pollen and non-pollen foragers responded in a similar manner to antennal stimulation with the various solutions over the course of the experiment, suggesting there is little difference between forager types in terms of sensitivity to pollen in solution.

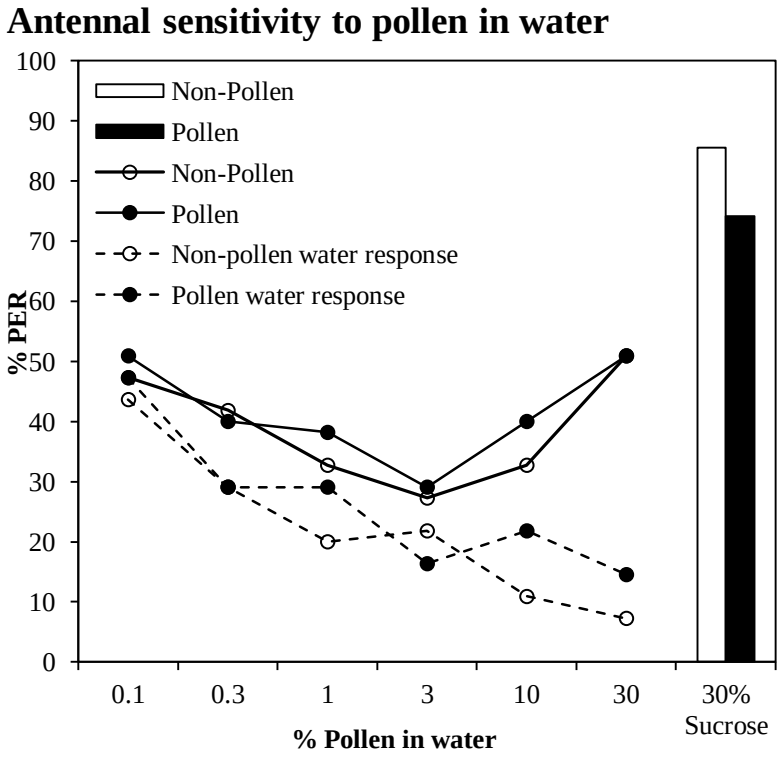


Figure 2.2 Proportion of bees extending the proboscis to antennal stimulation with a series of pollen-water mixtures, tested in order of ascending pollen concentration. Bees were separated according to forager type (Black circles=Pollen foragers, *n*=53 Open circles=Non-pollen foragers, *n*=55). Bars represent the response to a final stimulation with 30% sucrose (White=pollen foragers, Black=non-pollen foragers). The dashed lines represent bees responses to water stimulation prior to the stimulation with each pollen concentration (Black triangles=pollen foragers, Open triangles=non-pollen foragers).

2.2.3 PER conditioning, pollen in solution

As in previous conditioning experiments, a standard forward-paired conditioning protocol was used where pollen was presented in solution (either water or 15% sucrose). Pollen solutions were delivered via a toothpick. Since it is known that bees cannot learn an association where the US precedes the CS (Bitterman, et al., 1983), a control group

was trained in which bees received a reverse pairing of CS and US, delivered ten seconds apart, ensuring there was no overlap between stimuli as in the forward-paired condition. The control group served to demonstrate that any observed increase in responding to the CS in bees rewarded with pollen in solution resulted from learning of the predictive relationship between the CS and US and not simply as a result of arousal stemming from repeated stimulation of the antennae.

Initially bees were stimulated first at the antennae and then at the proboscis and permitted to feed for up to two seconds, as is typical in PER conditioning experiments. Interestingly, in bees rewarded with a mixture of pollen and 15% sucrose solution, I observed a decline in response to the CS and to a lesser extent to the US itself over the course of training. This led me to speculate as to whether the pollen-sucrose mixture was perceived as being distasteful to bees. Since the effect seemed to build over time, it was hypothesised that such a pattern of response may result from some form of post-ingestional malaise as observed in bees exposed to toxic substances such as amygdalin (G. A. Wright, et al., 2010). To test for this, the experiment was repeated with the feeding component omitted and bees stimulated at the antennae alone. If the same pattern of responding was observed, then this would suggest that bees are able to detect distasteful compounds present in pollen pre-ingestively.

2.3.4 Discussion

Whilst the proboscis extension response paradigm might be argued to have less ecological validity than the free-flying method when exploring the mechanisms underlying pollen-rewarded learning, it does permit closer control over the delivery of the pollen reward. Therefore this method is better suited to determining the sensory organs and exact components of the pollen reward responsible for reinforcing behaviour in pollen-collecting bees. Previous studies (Arenas & Farina, 2012; Grüter, et al., 2008) claim to have demonstrated that pollen is a suitable unconditioned stimulus for the reinforcement of learning in an olfactory conditioning task. However these studies lack any suitable control groups, such as bees receiving unpaired, or randomised presentations of the CS and pollen US. The authors' conclusion, that an increase in response to the CS following multiple pairings with pollen results from an associative

learning process, is consequently lacking in supporting evidence. It is therefore crucial to rule out alternative explanations such as potential non-associative effects of repeated antennal stimulation on responsiveness to the CS.

Based on my observations of honeybees and bumblebees engaged in pollen collection, the antennae and proboscis were targeted as the sensory organs most likely to be involved in sensing pollen whilst collecting. Applying pollen grains directly to the proboscis over several trials proved difficult, given the tendency of grains to adhere to the surface of the mouthparts and inhibit proboscis extension, the very measure of learning used in the olfactory conditioning paradigm. Arenas and Farina (2012) apparently applied hand-collected pollen to the proboscis only, but give little detail as to how grains were applied. Applying pollen in solution is one way to resolve the issue of grains sticking to either the antennae or proboscis, though clearly this does not resemble the conditions experienced during natural collection. Grüter et al. (2008) report using mixtures containing up to 70% pollen (w/w), presenting the US to both the antennae and proboscis. I found mixtures containing such high concentrations of pollen to be extremely viscous and quite difficult to apply to the sensory organs. I consequently tested bees sensitivity to antennal stimulation with a range of pollen concentrations from 0.1% to 30% pollen, which was found to be the most concentrated mixture that could still applied without clogging the antennae and mouthparts. 30% pollen elicited a response significantly different to that produced by water alone, suggesting that the pollen component was detected by bees, and was therefore selected for use as the US in subsequent conditioning experiments.

The proboscis and antennae may well be responsible for sampling pollen during collection, but the legs are principally involved in gathering pollen from the anthers of flowers, grooming excess grains from the head and body and packaging grains into the corbiculae. The tarsi also possess gustatory receptors, but the role they might play in the reinforcement of pollen-rewarded behaviour was not investigated here. A method in which tarsal stimulation with sucrose solution is substituted for antennal stimulation in the PER paradigm has already been described (de Brito Sanchez et al., 2008), and so it would be relatively easy in future studies to adapt this method to test whether stimulation of the tarsi with pollen might also release proboscis extension and serve to reinforce learning in an olfactory conditioning paradigm.

Chapter 3: Can pollen serve as a reward for visual learning in bees?

Abstract

Pollen is the ancestral floral reward and yet the role it plays in shaping the learnt behaviour of pollinating insects is little understood. Colour-naive honeybees and bumblebees were trained to discriminate between coloured stimuli associated with the presence or absence of a pollen reward. The visual characteristics of pollen were either hidden or exposed. In the former condition, the performance of pollen-rewarded honeybees was compared with that of sucrose-rewarded individuals trained in a reversal learning task. The reward assignment was switched between two colours in four successive training bouts. One hour after training, pollen-rewarded bees chose colours randomly indicating that they were able to recall the memory for both conditioned colours. Sucrose-rewarded bees showed a preference for one colour only, despite having learnt both during training. This indicates that colour learning and memory recall may differ depending on the type of reinforcement received, which may affect the decision-making of individual foragers and potentially influence the regulation of task partitioning in social bees. When the visual characteristics of pollen were exposed, bumblebees learnt to associate this reward with the presence of a coloured stimulus, and retained such memories for at least 24 hours. Our findings support the idea that, prior to the emergence of nectar rewards, pollen-reinforced behaviours may have played a significant role in insect-plant relationships, both prior to and throughout early angiosperm evolution.

3. 1 Introduction

Many plants rely on pollen-eating and pollen-collecting insects for their reproduction. Some even secure their services by offering pollen abundantly, though nectar is the energetically cheaper resource to produce (Colin & Jones, 1980; Simpson & Neff, 1983). Considered to have been the prevailing reward for ancestral plant-visiting animals, pollen pre-dates both the production of nectar and the evolution of

angiospermy (Crepet, 1979; Pettitt & Beck, 1967). Pollination mutualisms involving diverse insect groups are thought to have first evolved in association with gymnosperms, with mouthpart adaptations for pollen and nectar feeding present in the fossil record from the late Pennsylvanian and late Jurassic respectively (Labandeira, 1997). However rapid diversification in the pollinating insect groups is not observed until the appearance of the angiosperms in the early Cretaceous (Pellmyr, 2002). Ancestral flowers were small, and the perianth, if present at all, was highly reduced in comparison to modern flowers, meaning that the conspicuously coloured androecium most likely provided distinctive signals used to attract insect pollinators (Crepet, et al., 1991; Faegri & Van der Pijl, 1971). During the late Cretaceous, form and complexity of flowers diversified enormously over a short period of time, and was accompanied by the appearance of well-developed nectar producing organs (Crepet, et al., 1991). The shift from rewarding with pollen to nectar undoubtedly had a pronounced impact on plant-pollinator relationships, but could insect pollinators have exerted selective pressures on plants which facilitated this switch from pollen to nectar as the predominant reward trait in modern angiosperms?

From an insect's perspective, nectar contains sufficient nutrients to sustain the metabolism of the adult form and requires fewer adaptations for digestion than pollen (Roulston & Cane, 2000). Whilst this might partially explain the proliferation of nectar as a floral reward, ease of digestion probably does not account solely for the success of this innovation in flowering plants. The emerging separation between pollen-feeding and pollen-provisioning insects may have been mediated by a differential ability to handle more complex demands on spatial orientation in order to navigate between breeding and feeding locations, as well as variations in the capacity to make choices between a diverse selection of rewarding flowers. Modern pollen-provisioning insects are typically flower-constant foragers who are able to the learn the features of floral displays and the handling skills necessary for complex flower morphologies. Aside from the advantage to pollinators in terms of improved foraging efficiency, flower-constancy benefits plants since it better promotes the transfer of pollen between individuals of the same species (Chittka, Thomson, & Waser, 1999; Waser, 1986), as well as reducing the likelihood of plants receiving heterospecific pollen which may block receptive sites on the stigma (Waser & Price, 1983). Flower-constant foraging strategies most likely emerged in conjunction with fast, robust learning mechanisms.

The question then arises as to what extent sucrose-sensitive learning mechanisms in the brains of insect pollinators were implicated in the acceleration of the success of the nectar reward and the appearance of more diverse floral forms. Were pollen-feeding insects less effective pollinators than nectar-rewarded pollinator and did sugary nectar represent a more easily processed reward variable, better suited to the support of sophisticated neural mechanisms?

Currently little is known about what insects learn during pollen collection, but such information is crucial in determining whether the learning of floral features in pollen-rewarding plants might have been less robust than in those providing nectar, and therefore less likely to promote pollinator behaviour beneficial to plants. Here we attempt to further understanding of the evolution of nectar production, focusing on bees as a group of insect pollinators known to be remarkably good at learning when rewarded with nectar-like sucrose solutions.

Previous studies have focussed on bee preferences for different pollen types (Boelter & Wilson, 1984; Levin & Bohart, 1955; Pernal & Currie, 2002; J. O. Schmidt, 1982), how they learn to manipulate pollen-rewarding flowers (Raine & Chittka, 2007b) or learn pollen odours (Cook, et al., 2005; Von Aufsess, 1960). In the latter experiments bees were rewarded with sucrose. That bees develop preferences for particular species during pollen collection, suggests they are able to perceive differences between pollen types. Since pollen is not ingested by foragers, but carried externally on the body back to the hive or nest, the mechanism of discrimination between different pollen types remains open to debate (Cook, et al., 2003; Fewell & Winston, 1992; Pernal & Currie, 2001, 2002; Waddington, et al., 1998), though it seems plausible that learning might be involved, with pollen itself acting as the reinforcer.

Here we tested whether bees can learn to associate a coloured stimulus with a pollen reward. Since olfactory cues are known to influence the development of pollen preferences (Arenas & Farina, 2012), we controlled for the sensory experience of individuals by rearing and testing bees in the laboratory. To determine whether pollen is an effective reinforcer of learning, we compared the performance of pollen-rewarded bees with that of bees rewarded with sucrose, using identical visual stimuli in the same spatial, visual and olfactory context. Pollen is a reward that is not ingested and therefore may lead to a slower acquisition of behavioural responses, as evinced for olfactory

conditioning where bees are prevented from imbibing the sucrose reward (Bitterman, et al., 1983; Sandoz, Hammer, & Menzel, 2002; G. A. Wright, et al., 2007). We therefore expected to find pronounced differences between sucrose and pollen-rewarded bees, both in their ability to learn the task and their memory for colours.

3.2 Methods

3.2.1 Vertical Presentation

Small colonies of honeybees were housed in a flight net (1 m^3) within a greenhouse at the Institute of Neurobiology of the Free University of Berlin. Sucrose solution was offered via a transparent feeder with a grey base. The pollen reward consisted of commercially available honeybee-collected pollen pellets (Werner Seip, Ebersgöns, Germany), ground to a fine powder and supplied inside a dark box, to limit any learning of the visual cues associated with each reward. Individually-marked bees were allowed to fly into a test cage and forage in a grey, pollen-containing training box (20 x 10 x 15 cm) for five trials (pre-training). Bees could enter and leave the training box via two tubes (diameter 1.5 cm, 2.5 cm deep), which were replaced after each trial. Pollen-rewarded bees collected pollen from petri dishes positioned on the floor of the box. Sucrose reward was administered at the dark end of the tubes, where wide-meshed netting prevented sucrose-rewarded bees from contacting the pollen, but allowed odour to diffuse out as in the pollen-rewarded condition. To avoid any potential guidance as a result of water vapour in sucrose-rewarded bees, a water drop was placed at the end of the unrewarded tube. Following pre-training, spontaneous colour preferences were tested individually. A solid grey panel, featuring a yellow and blue disc (8 cm diameter), was mounted to the front of the training box. Approaches (within 2 cm of stimuli) and contacts to each disc were counted over a period of two minutes.

During training, sucrose and pollen feeders were removed from the flight net. Bees were presented with two grey pollen-containing training boxes, stacked vertically. The four entrance tubes were surrounded by blue and yellow circular collars (8 cm diameter), two of each colour. The configuration of the coloured stimuli was changed after each trial. Given that bees learn colours very quickly (Menzel, 1967) two colour-reversal tasks

were used to detect potential differences in performance between pollen and sucrose rewarded bees. Experiments consisted of either a ten or twenty-trial training session. Following the pre-test, bees in the first experiment were trained individually to find rewards at the blue stimulus, which was presented simultaneously with the non-rewarding yellow stimulus. For each trial we recorded search time and choices. After five training trials colour preferences were re-assessed in an unrewarded test. This was followed by a second, five-trial training bout in which yellow was the rewarded colour, and a final unrewarded colour preference test.

In a second experiment, the reward was swapped between the colours after every five-trial training bout, with blue the first rewarded colour. Colour preferences were tested immediately after the fourth bout (in which yellow was rewarded) and bees were released back into the flight cage without reward. No food sources were available in the flight cage. After a delay of one hour, colour preferences were re-tested. To check for potential switches in motivation following the delay, we trained bees in another five-trial bout in which yellow was rewarded, and gave bees a final unrewarded test of colour preference.

3.2.2 Horizontal Presentation

Bumblebee colonies were obtained from Koppert Biological Systems (Suffolk, UK) and housed inside a flight net (L x W x H: 80 x 80 x 100 cm) within a laboratory at the University of Exeter. The flight net was connected to a test cage via a Perspex corridor. When not engaged in experiments, bees were provided with sucrose solution and pollen *ad libitum*. Bees observed collecting pollen were marked with individual coloured number plates (Opalithplättchen, E.H. Thorne Limited, UK).

In order to train bees to visit the test cage, a large petri dish of pollen (9 cm diameter) was placed inside the flight cage, near the entrance to the corridor. Once marked bees started collecting pollen, the dish was gradually moved through the open corridor and into the experimental arena. A large disc of grey paper (75 cm diameter) inserted between two identically sized Perspex discs horizontally level with the corridor, served as the background surface on which pollen was presented during training. Once bees had learnt to visit the grey disc, the single large petri dish was replaced by a number of

smaller (5.5 cm diameter) petri dishes. Once an individual had been observed to visit several different petri dishes, on two separate foraging trips, colour training was commenced.

First the colour preference of each bee was tested (pre-test). Upon entering the test cage, bees were presented with four discs (15 cm diameter) of laminated coloured paper (two of each colour, pairings were as follows; Blue/Green, White/Orange, or Blue/Yellow). A petri dish (9 cm diameter) containing *ca.* 3 g of pollen was placed in the centre of each coloured disc. Transparent covers prevented access during this unrewarded test but pollen was still visible and a number of small holes in the surface of the lids ensured that the pollen odour diffused out. Test trials lasted between two and five minutes and the number of approaches, contacts and landings made to each coloured disc was recorded. An approach was classified as when a bee's whole body crossed from the grey background to the coloured stimulus. A 'contact' was classed as any physical connection between the bee's body and the petri dish or coloured stimulus. A 'landing' was differentiated from a 'contact' by the splaying of the bee's legs.

During training the sucrose and pollen feeders were removed from the living cage. One colour of each pair tested was rewarding and provided petri dishes filled with pollen. The unrewarding colour had petri dishes with transparent covers, as in the pre-test. Bees were permitted to forage *ad libitum* and the trial ended once a bee returned to the hive and unloaded their corbiculae. Between training trials the coloured stimuli were cleaned with ethanol to remove any odour cues, as were the lids of petri dishes following unrewarded tests. Bees received five training trials in total and the inter-trial interval (ITI) was between ten and fifteen minutes.

To determine whether an individual had learnt the rewarded colour, following the fifth training trial, their colour preference was re-assessed in an unrewarded test. An individual was then tested once more following a delay of 24 hours in order to check for consolidation to the long-term memory. Between the immediate learning test and the 24 hour test, pollen and sucrose were placed back inside the flight net, meaning bees were exposed to pollen during this period, but in the absence of any additional colour cues. Bees were given a single reminder trial prior to the 24 hour test. Four small petri dishes were presented in the absence of the coloured discs. This was to motivate bees to make approaches and contacts in the final memory test.

Videos of test trials were coded blind with respect to the rewarded colour during training. Observers noted the number of approaches, contacts and landings made to each of the coloured discs during the first minute of testing.

3.2.3 Statistical Analysis

Two-tailed paired t-tests were used to detect colour preferences prior to training. To test whether training to one colour led to a preference for that colour during the unrewarded test, we used one-tailed paired t-tests to compare the number of approaches, contacts and landings to each colour. As an additional measure of preference, we also analysed the number and direction of transitions between stimuli. A t-test was used to compare the number of transitions made towards the rewarded colour versus those towards the unrewarded colour during each test. In cases where data were not normally distributed (Shapiro & Wilk, 1965) Wilcoxon's signed-rank test was used. To rule out the potential influence of positional biases on sample choice, the number of approaches to each dish location was compared using repeated measures ANOVA. In all cases there was no significant difference between locations.

In experiments where stimuli were presented vertically, we tested whether bees adjusted their search behaviour towards the rewarded colour within each 5-trial bout and between bouts using GEE (Generalized Estimating Equation) modelling. The GEE approach is an extension of the Generalized Linear Model (GZLM) which permits a non-normal distribution of the dependent variable and accounts for repeated measurements of the same individual (Hardin & Hilbe, 2002). Search time was coded as the response variable with forager type and either training trial or bout number included as factors. Significance tests were based on Wald approximations of the likelihood ratio test. Post-hoc least significant difference contrasts (LSD) were used to compare treatment groups.

3.3. Results

3.3.1 Vertical Presentation

Overall, both nectar and pollen foragers initially showed a strong preference for the Blue stimulus (Fig. 3.3, Pre-test, Pollen-reward n=15 t_{14}=3.262 p=0.006; Sucrose-

reward n=12, t_{11}=5.836 $p<0.001$), and contact ratios closely resembled that of approaches. Whilst in the ten trial experiment, both nectar and pollen foragers initially had a blue preference, only five out of seven pollen foragers engaged in the pre-test. The choices of nectar-rewarded bees, but not pollen foragerss were significantly different from random in the pre-test (Fig. 3.1a, b, Pre-test, Pollen-reward, Approaches t_4=1.336, p=0.253, Contacts p=1.000; Sucrose-reward, Approaches, t_5=3.284, p=0.017, Contacts, t_5=2.697, p=0.043). In the unrewarded tests conducted after each bout of five trials, pollen-rewarded bees (n=7) selected the rewarded colour (Fig. 1a, Trained to Blue Approaches t_6=5.753, p=0.001, Contacts z=-2.232, n=7, p=0.013 Trained to Yellow: Approaches t_6=2.652, p=0.038, Contacts z=-1.609, n=7 p=0.054) and sucrose-rewarded bees (n=6) performed similarly well (Fig. 3.1b Trained to Blue: Approaches t_5=6.369, p=0.001, Contacts z=-2.201, n=6, p=0.014; Trained to Yellow: Approaches t_5=8.436, $p\leq0.001$, Contacts t_5=3.836, p=0.012).

Overall, pollen foragers had significantly longer search times than nectar foragers (Fig. 3.2, GEE, Forager type, X^2_1=16.176, p<0.001), though for both forager types, latency to finding the correct rewarding stimulus was observed to decrease significantly within the first training bout to Blue (LSD Contrast, T_1 *vs.* T_5, Pollen foragers, p=0.028; Sucrose foragers, p=0.009), and during re-training to Yellow (LSD Contrast, T_6 *vs.* T_{10}, Pollen foragers, p=0.001; Sucrose, p=0.020). By the end of each bout, both forager types exhibited similar search times (LSD Contrast, Pollen *vs.* Nectar, T_5 p=0.095; T_{10} p=0.051)

In the twenty-trial experiment bees showed similar reduction in search time during training bouts (Fig. 3.4) and preferred the last rewarded Yellow stimulus in the unrewarded test conducted immediately after training (Fig. 3.3 Immediate test, Pollen reward: n=6, t_5=3.376, p=0.020 Sucrose reward: n=6, t_5=4.417, p=0.010). After a one hour delay, pollen-rewarded bees chose both colours (t_5=1.452, p=0.210) whereas sucrose-rewarded bees preferred blue (t_5=5.531, p=0.032). Following a final bout of training, in which yellow was rewarded, both groups of bees preferred yellow (Pollen-reward, t_5=4.596, p=0.030; Sucrose-reward t_5=4.992, p=0.035).

The overall search times of each forager type were not significantly different in the twenty-trial test, (GEE, Forager type, X^2_1=0.808, p=0.369), and as before, by the fifth trial of each training bout there were no differences in the latency to reaching the correct stimulus between bees rewarded with pollen or sucrose (Fig. 3.4, LSD Contrast Pollen

vs. Sucrose, Trials $_{5, 10, 15, 20}$ and $_{25}$, p=n.s.). Sucrose-rewarded bees exhibited little change in search times between the first trial of a new colour training bout, and the last trial of the previous bout (LSD Contrast, T_5 *vs.* $T_{6,}$ p=0.776; T_{10} *vs.* $T_{11,}$ p=0.337, T_{15} *vs.* $T_{16,}$ p=0.053), whereas following all but the final reversal in training colour, pollen foragers showed a significant increase in their latency to finding the rewarded colour following a switch (LSD Contrast, T_5 *vs.* T6, p=0.009; T_{10} *vs.* T_{11}, p=0.031 T_{15} *vs.* $T_{16,}$ p=0.493). The fact that pollen foragers search times did not increase following the third colour reversal, suggests that these bees may have become adapted to dealing with switches in the rewarded colour over time. Despite the differences in memory recall in the one hour test, on the first trial of the final bout, both groups of forager were equally fast in finding the last trained yellow stimulus (LSD Contrast, T_{21}, Pollen *vs.* Sucrose p=0.424).

3.3.2 Horizontal Presentation

It was evident from the previous experiments that honeybees learnt which colour permitted access to pollen, however it remained unclear whether they could learn this task when immediately trained against their initial colour preference. We conducted a second set of experiments with bumblebees, which are easier to keep and test within indoor flight nets. In contrast to the previous set of experiments, stimuli were presented horizontally and pollen was visible to bees.

In the unrewarded pre-test, bumblebees showed a preference for Green (*vs.* Blue), Orange (*vs.* White) and Yellow (*vs.* Blue) respectively (Fig. 3.5 Pre-test). Bees choices were significantly different from random (Approaches, Blue/Green z=-2.207, n=6, p=0.027; White/Orange t_5=2.587, p=0.049; Blue/Yellow z=2.366, n=5, p=0.043), with individuals displaying a preference for Green, Orange and Yellow respectively. Bees had a tendency to make more touches and landings on the colour that they preferentially approached, though this distribution was not statistically significant (Fig. 3.5b) (Contacts: Blue/Green z=-0.368, n=6, p=0.713; White/Orange z=-4.22, n=6, p=0.673; Blue/Yellow t_4=2.366, p=0.077).

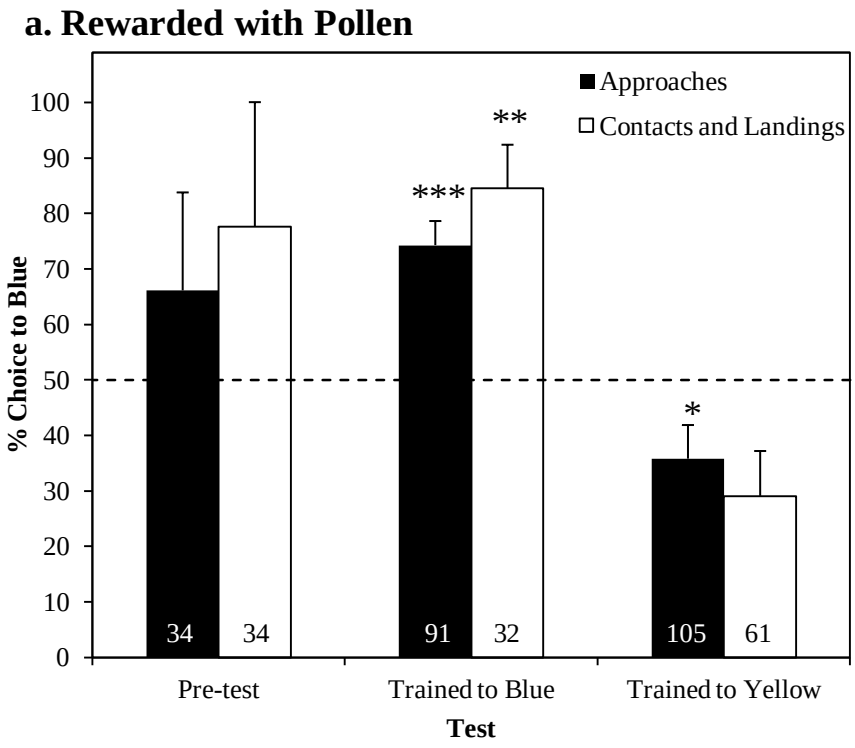


Figure 3.1 Ten-trial colour learning experiment. Proportion of approaches (Black bars, mean ±SE) or contacts and landings (White bars) to blue, made by (a) pollen (n=7) and (b) sucrose (n=6) rewarded honeybees. Unrewarded tests were conducted prior to training (pre-test) and following training to blue and then yellow. The total number of approaches or contacts and landings are included on each bar. Asterisks denote a significant deviation from random choice (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

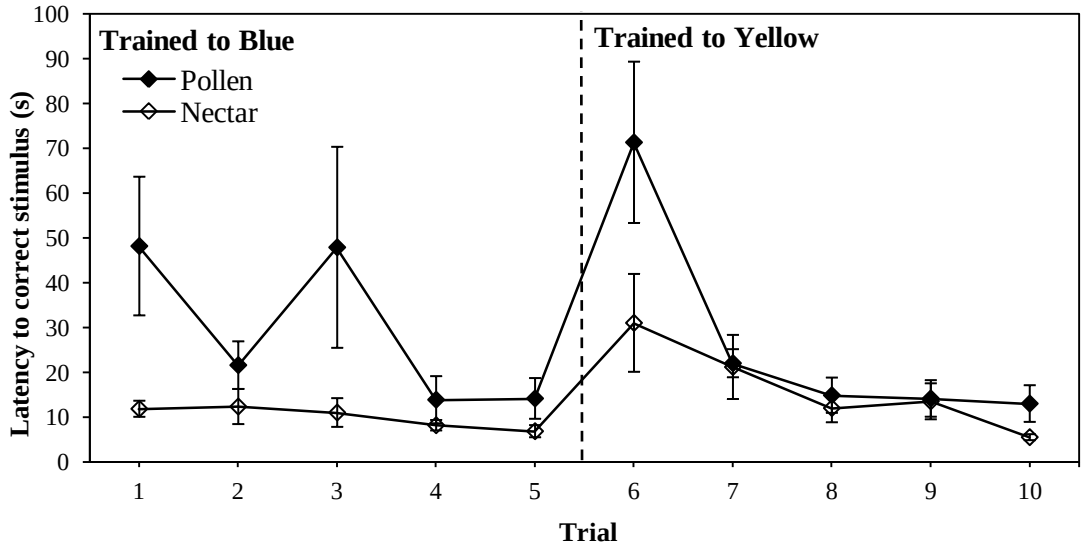


Figure 3.2 Latency to correct stimulus choice in colour learning experiment. Both pollen (Black diamonds, Mean ±SE, n=7) and nectar-rewarded bees (White diamonds, n=6) reduced their search time during each five trial bout. The dotted line denotes the unrewarded test conducting following training to blue. Another unrewarded test was conducted following the tenth trial.

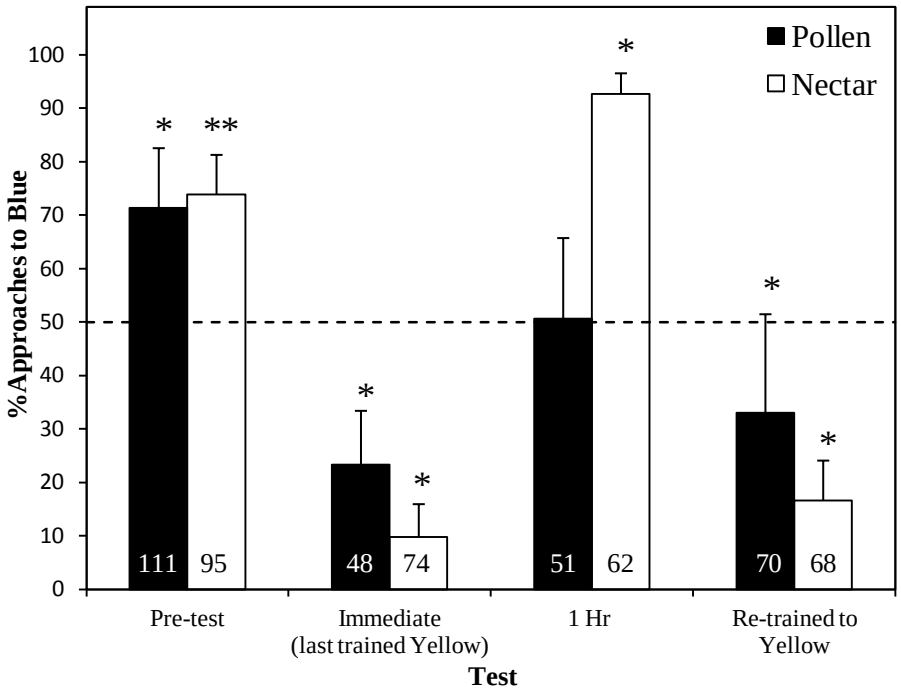


Figure 3.3 Twenty-trial colour reversal experiment. Proportion of approaches made to the blue stimulus by pollen (Black bars, mean ±SE, *n*=6) and sucrose-rewarded bees (White bars, *n*=6). Contact ratios resembled the ratio of approaches. Starting with Blue as the rewarded colour, after each five-trial training bout, the reward was swapped between colours. Colour preferences were re-tested immediately following the fourth bout (Yellow rewarded) and after a delay of one hour. Bees were trained for a further five trials, rewarding on yellow, and colour preferences re-tested a final time. The total number of approaches or contacts and landings are included on each bar. Asterisks denote a significant deviation from random choice (*$p \leq 0.05$, **$p \leq 0.01$).

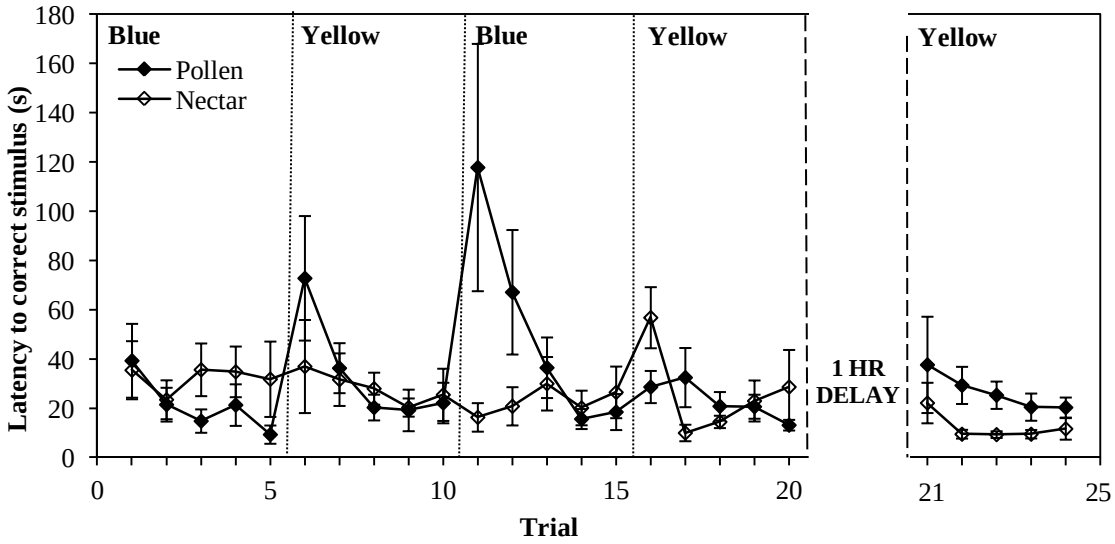


Figure 3.4 Latency to correct stimulus choice in colour reversal experiment. Both pollen (Black diamonds, Mean ±SE, *n*=6) and nectar-rewarded bees (White diamonds, *n*=6) reduced their search time during each five-trial bout, though pollen-rewarded bees appear to exhibit longer search times following each switch. Bees colour preferences were tested immediately following Trial 20, following a delay of one hour and again after Trial 25.

During training only one coloured stimulus (Blue or White) provided access to pollen. Following the fifth training trial, colour preferences were re-tested and it was evident that bees had shifted their preference towards the rewarded colour (Fig. 3.5, Immediate test). For each colour combination except Blue^{+}/Yellow^{0}, the number of approaches made to the rewarded stimulus was significantly different from those made to the unrewarded colour (Blue^{+}/Green^{0} t_5=3.931, p=0.011; Orange^{+}/White^{0} z=-1.802, n=5, p=0.036; Blue^{+}/Yellow^{0} z=-1.414, n=4, p=0.079). Bees also made significantly more touches and landings on the rewarded colour (Blue^{+}/Green^{0} z=-1.826, n=6, p=0.034; Orange^{+}/White^{0} z=-2.207, n=5, p=0.014). Bees trained to Blue^{+}/Yellow^{0} did not show a significant preference for the rewarded colour, although the proportion of approaches to Blue increased considerably from 19% to 54% suggesting that they had learnt the task.

After 24 hours colour preferences were re-tested (Fig. 3.5). The pattern of responding was similar to that displayed in the immediate test for bees trained to Blue^{+}/Green^{0} and Orange^{+}/White^{0}. The number of contacts and landings made to the rewarded colour remained significantly different from 50% (Blue^{+}/Green^{0} z=-2.023, n=6, p=0.022; Orange^{+}/White^{0} z=-1.753, n=6, p=0.04). A preference was not observed in terms of approaches to the rewarded colour (Blue^{+}/Green^{0} t_5=2.238, p=0.27; Orange^{+}/White^{0} t_5=1.508, p=0.192), but bees did not revert to their original preference for the unrewarded colour either. These results suggest that bees recalled the rewarding colour following a 24 hour delay. Only bees trained to Blue^{+}/Yellow^{0} reverted to their original preference for the unrewarded colour, making significantly more approaches to the Yellow stimuli than expected by chance (t_4=2.993, p=0.04).

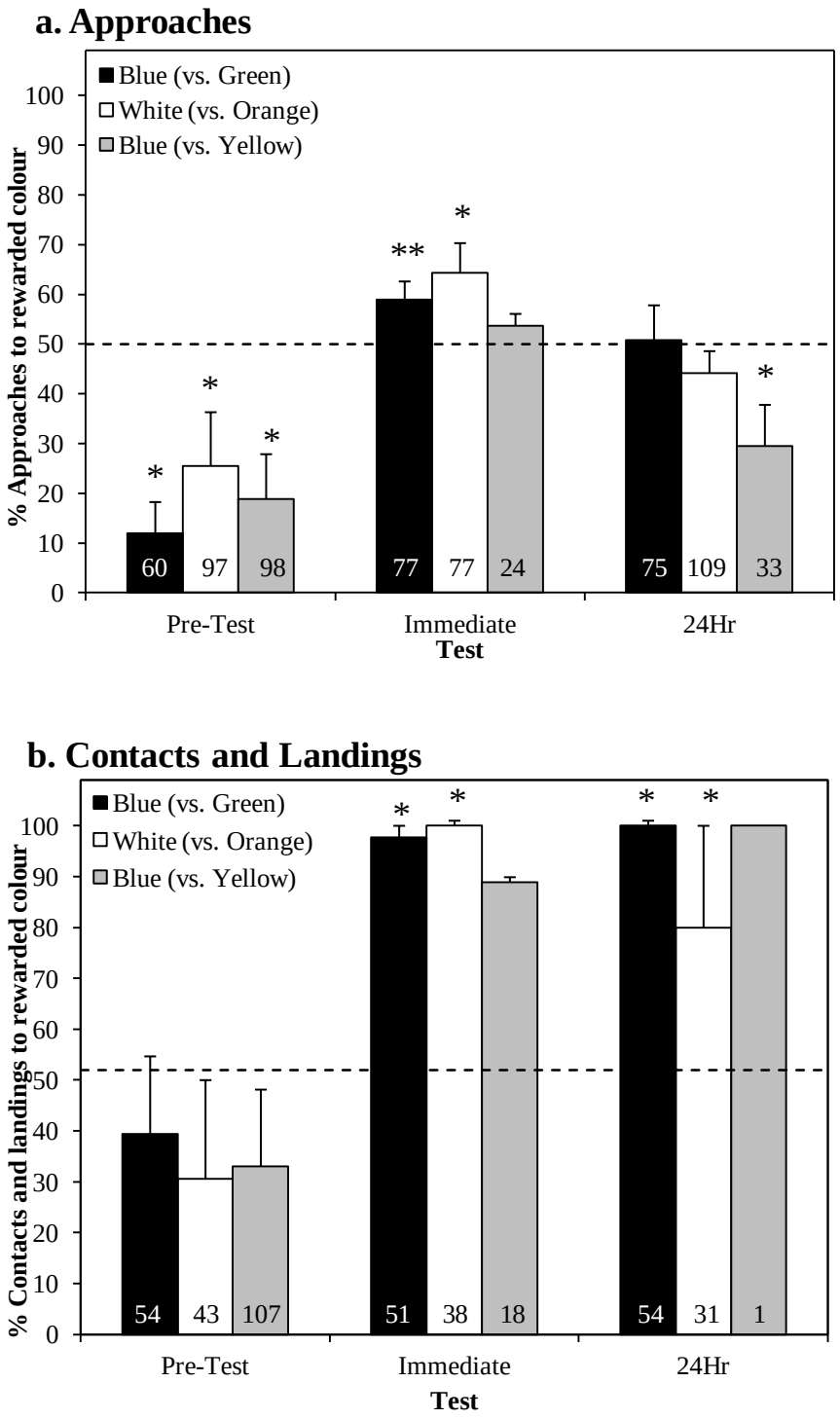


Figure 3.5 Discrimination of horizontally presented colours by bumblebees. The proportion of (a) approaches or (b) contacts and landings made to the rewarded colour prior to training (pre-test), immediately following training, and following a delay of 24 hours. Bars represent different colour combinations of Blue^{+} *vs.* Green^{0} (Black bars, Mean ±SE, n=6,), White^{+} *vs.* Orange^{0} (White bars, n=6) and Blue^{+} *vs.* Yellow^{0} (Grey bars, n=5, Immediate test n=4). The total number of approaches or contacts and landings are included on each bar. Asterisks denote a significant deviation from random choice (*$p \leq 0.05$, **$p \leq 0.01$).

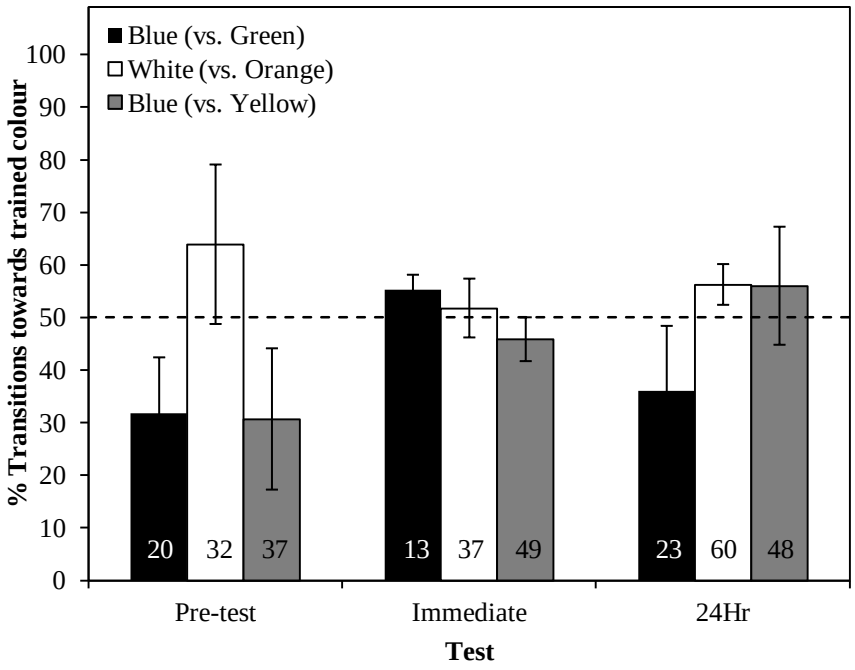


Figure 3.6 Transitions towards the rewarded colour by bumblebees. The proportion of transitions made to the rewarded colour (e.g. Blue$^+$ to Blue$^+$ and Green^0 to Blue$^+$ transitions) prior to training (pre-test), immediately following training, and following a delay of 24 hours. Bars represent different colour combinations of Blue$^+$ *vs.* Green^0 (Black bars, Mean ±SE, n=6,), White$^+$ *vs.* Orange^0 (White bars, n=6) and Blue$^+$ *vs.* Yellow^0 (Grey bars, n=5, Immediate test n=4). The total number of transitions are included on each bar.

On average bees made 6.38 (±0.60) transitions between stimuli during the unrewarded tests. There were no significant differences in the number of transitions towards the rewarded versus the unrewarded colour in the pre-test (Fig. 3.6, Transitions pre-test, Blue/Green t_5=-1.472, p=0.201, White/Orange t_5=1.936, p=0.111, Blue/Yellow t_4=-1.0, p=0.374), however for bees offered the choice of Blue *vs.* Green or Blue *vs.* Yellow, a larger proportion of transitions (*ca.* 70%) were made towards the unrewarded colour (Green or Yellow). Following training, there was an increase in the tendency of bees in these two groups to make transitions towards the rewarded colour, meaning that bees made an almost equal number of transitions in either direction during the immediate test (Immediate test Blue$^+$/Green^0 t_5=-1.746, p=0.141, Blue$^+$/Yellow^0 z=-1.0, n=4 p=0.317). Bees offered the choice of Orange vs. White actually made a greater proportion of transitions towards the rewarded colour in the pre-test, however they also made an equal number of transitions in both directions during the immediate test (White$^+$/Orange^0 z=1.511, n=6 p=0.131). Following a delay of 24 hours, the pattern of responding remained largely unchanged from that observed in the immediate test, with the exception of bees trained to discriminate Green from Blue, which reverted to making

slightly more transitions (*ca.* 65%) towards the unrewarded colour, as in the pre-test. The difference in the number of transitions towards the rewarded versus unrewarded colour was not significant however (24 Hr test, $Blue^{+}/Green^{0}$ t_5=0.725, p=0.501, $Blue^{+}/Yellow^{0}$ z=-0.577, n=5 p=0.564, $White^{+}/Orange^{0}$ t_5=1.581, p=0.175).

Analysis of bee's approaches towards and transitions between stimuli yield very similar results, with bees showing an increase in their preference for the rewarded colour between the pre-test and immediate test conducted post-training. The 'transition' measure confirms that bees do indeed sample both stimulus-types during training, and that re-landings on the same stimulus are unlikely to have biased the 'approach-based' measure of preference. Nevertheless, the shift in colour preference is greater when the 'approach' measure is used, suggesting that the decision to re-approach the same stimulus may be of importance. Since choices were limited to one of four locations, it is unsurprising that bees make re-approaches (5.8 ±0.82 on average/test), and indeed a high number of consecutive re-approaches may indicate that an individual has a strong motivation towards a particular colour.

3.4 Discussion

In order to secure repeated visits from pollinators, most flowering plants provide a food resource as an incentive reward. Whilst nectar is the most common reward trait in modern angiosperms, flower visitors also collect pollen from many species. A minority of plants provide only pollen as a reward for pollen-feeding and pollen-collecting insects. The dual function of pollen as an agent for gamete transmission and reward for pollinators does not mean that its production is any less costly to flowers. On the contrary, consumption or active collection of pollen by visiting insects results in a direct loss of reproductive potential for the plant, meaning that in nectar-less species flowers and insects essentially act as competitors for the same resource (Westerkamp, 1997). Accordingly plants use various strategies to manipulate the behaviour of pollinators in an attempt to maximise the transfer of pollen between flowers, and such tactics are heavily dependent on the sensory systems and cognitive capabilities of their pollen vectors.

Reinforcement with pollen is a potential mechanism by which insects might learn the floral characteristics of pollen-rewarding plants, benefiting the pollinator by enabling the recognition of palatable pollen types and the plant by increasing the likelihood of pollen transfer between flowers of the same species. Here we show that bees are able to learn both cues provided by pollen itself, and those of a coloured stimulus paired with the pollen reward. Honeybees and bumblebees were observed to change their preference towards the rewarded colour after training, indicating that pollen can serve to reinforce learning in a visual conditioning paradigm.

Pollen is the ancestral floral reward (Crepet, et al., 1991; Pettitt & Beck, 1967), with mouthpart adaptations for spore or pollen eating first appearing in the fossil record late in the Pennsylvanian period (Labandeira, 1997, 2002; Rasnitsyn, 1977). When the evolutionary history of plant-pollinator relationships are considered in line with the findings presented here, one might hypothesise that even the earliest insect pollinators learnt the location and visual features of flowers, with pollen serving as the reinforcing stimulus. Though it remains to be tested, pollen-rewarded learning may well represent a widespread trait amongst the orders of pollinating insects.

The earliest flowers are thought to have been simple in structure, with a reduced perianth, and pollen itself most likely providing the visual and olfactory cues which guided pollinators to flowers (Crepet, et al., 1991; Faegri & Van der Pijl, 1971). In contrast with the blue preference typically reported for bees housed and tested under similar conditions to those used here, bumblebees in our experiments displayed a preference for yellow over blue, (Chittka, Ings, & Raine, 2004; M. Giurfa, et al., 1995; Gumbert, 2000; Ings, et al., 2009). We suggest that pre-exposure of bumblebees to the visual characteristics of pollen in the horizontal condition may have led to an association between the yellow colour of pollen and its reward value during pre-training. Pollen-collecting honeybees tested in the vertical condition, where pollen was concealed from view, displayed the typical blue preference in the pre-test, indicating that spontaneous colour preferences are independent of the motivation to collect a particular food resource. Interestingly, the responses of bumblebees trained to discriminate Blue stimuli from Yellow in the horizontal presentation paradigm were also inconsistent with the findings for other colour pairings. Visual memories formed via reinforcement with pollen were found to persist for at least 24 hours when bees were trained to discriminate Orange from White and Blue from Green. However, bees trained

to discriminate Blue from Yellow reverted to their original preference for yellow in the memory test. Again, given that the pollen reward was visible in this paradigm, one explanation for this discrepancy is that the yellow colour of pollen was reinforced both prior to and during the experiment, and since bees were permitted to collect pollen between the immediate and 24 hour test, this may interfered with recall of the rewarding blue stimulus following a delay of 24 hours.

During the mid-Cretaceous, the perianth and corolla in particular became far more conspicuous in flowers, eventually superseding the androecium as the main attractant for pollinators (Crepet, et al., 1991; Endress, 2011; Friis, et al., 2010). The fact that bees pay attention to the visual properties of pollen could partially explain the evolutionary trend away from the prominent pollen displays of early angiosperms towards concealed rewards in pollen-only flowers (Vogel, 1978). Whilst complex floral morphologies and specialised pollen dispensing mechanisms, such as poricidal anthers, may help to limit pollen removal and wastage during insect visits (Buchmann, Jones, & Colin, 1977; Castellanos, et al., 2006; Harder & Thomson, 1989), perhaps such morphological adaptations also serve to reduce pre-alighting assessment of pollen type or abundance by pollinators (Harder, 1990).

An increase in the diversity of floral form and complexity during the late Cretaceous radiation was accompanied by the appearance of well-developed nectar producing organs (Crepet, et al., 1991; Friis, et al., 2006). Given the primary role of pollen as an agent for the transfer of genetic material, to permit pollen-tube growth and successful fertilisation, individual grains must contain a certain concentration of nitrogen-based compounds (Roulston, et al., 2000). In contrast, rewarding pollinators with nectar permits flowers to be more flexible in terms of the energetic resources they allocate to attracting insects, and unlike pollen, nectar can be reabsorbed and reallocated to other parts of the plant depending on the rate of flower visitation (Nepi, et al., 1996; Nepi & Stpiczyńska, 2007). From the perspective of the pollinator, harvesting nectar requires fewer morphological and behavioural adaptations than pollen collection (Thorp, 1979, 2000) and is also easier to digest (Roulston & Cane, 2000). When considering the proliferation of nectar as the primary floral reward, one idea that has received little attention is the notion that nectar-rewarded learning might better promote floral constancy and spatial learning than when pollen serves as the reinforcer. To test this theory we compared learning and memory recall between pollen and sucrose-rewarded

honeybees. Given that individuals do not consume the pollen reward during training, we expected to find pronounced differences in acquisition between groups. However, in general, pollen-rewarded visual learning was observed to be fast and memories were seen to persist over time.

Within each five-trial bout, a decrease in search times was observed for bees collecting both types of reward, though pollen-rewarded bees tended to exhibit longer search times in the first few trials following a switch in rewarded colour. Differences in the memory recall of pollen and nectar-rewarded bees were also observed. After extended colour-reversal training over twenty trials, colour preferences were re-tested following a one hour delay. Sucrose-rewarded bees preferentially selected blue, despite the fact that yellow was the last trained colour. Pollen foragers, in contrast, selected both colours equally, indicating that they recalled both memories. It seems unlikely that sucrose-rewarded bees had forgotten the learnt association with the yellow stimulus and simply reactivated their spontaneous preference for blue. Their response in the final training session with yellow was reliable, indicating that they did not need to relearn the yellow colour. Colour learning in bees is fast: a coloured stimulus can be reinforced by sucrose after a single reward, and long-term memory is formed within three trials following reward exposures of more than five seconds in duration (Menzel, 1968). In addition Menzel (1967, 1969) showed that sucrose-rewarded bees can learn several colours in the same context, retaining such memories for the rest of their life. Therefore it seems most likely that sucrose-rewarded bees in our experiments formed associations of different strengths for the blue and yellow stimuli, thus affecting their choice ratios in the unrewarded memory test.

Both sucrose-rewarded honeybees and bumblebees (*Bombus bimaculartis*) have been observed to show little decay in their memory for rewarded colours when re-tested the following day (Dukas & Real, 1991; Menzel, 1968). In contrast, we observed that pollen-rewarded bumblebees show a weaker response to the rewarded colour following a delay of 24 hours, compared to when tested immediately following training. Nevertheless, bees did not revert to their original colour preference indicating that they still recalled the trained colour, suggesting that pollen-rewarded learning is robust over time.

Scheiner et al. (2003) observed consistent differences in the performance of pollen and nectar foragers in an olfactory conditioning task. Whereas in the current experiments

sucrose-rewarded bees exhibited reduced search times relative to those rewarded with pollen, Scheiner et al. found that when both forager types were rewarded with sucrose, pollen foragers showed the more rapid acquisition. Though we are unable to rule out the possibility that those bees seeking pollen differed inherently from sucrose foragers in terms of their learning ability, we consider this to be an unlikely explanation for the observed differences in memory recall, especially given more recent evidence from Mujagic and Erber (2009) that results obtained using PER conditioning do not necessarily predict the choices of free-flying bees.

The variation in memory recall between pollen and sucrose-rewarded bees hints at a difference in the time course and/or strength of memory consolidation for these two reinforcers. Further studies exploring the dynamics of pollen-reinforced learning may eventually lead to the characterisation of the reward pathways involved in the bee brain. In social bees, where individuals may switch between a variety of food and colony-related behavioural tasks, differential learning processes mediated by the two reward types could potentially influence the decision making and foraging dynamics of individual foragers, and thus may also contribute to the regulation of task partitioning (see also Scheiner, et al., 2004).

Whilst both species of bee tested here are used widely in studies of insect learning and may therefore be considered well-studied and equivalent models, they show some clear differences in their behaviour and life-history traits. Bumblebee colonies are much smaller than those of honeybees, and foragers use less precise information to recruit their nest-mates to food sources (Goulson, 2010; Heinrich, 1979). Given that bumblebees store smaller quantities of pollen, we might expect individuals to be more selective in their foraging decisions (Leonhardt & Blüthgen, 2011), which may also result in differences in the sensory evaluation of pollen in the two species. We therefore deem it worthwhile to consider both species in future investigations of the reinforcing function of pollen.

Chapter 4: Assessment of differences in pollen quality by a generalist pollinator, *Bombus terrestris*

Abstract

The fact that bees exhibit preferences for the pollen of certain plant species, provides preliminary evidence that they are capable of discriminating between pollen species. Bees are able to associate the availability of pollen with the presence of a visual signal, and so the question arises as to whether variations in pollen quality could also support learning of coloured cues. Here I investigated if individual bees can sense the difference between pure pollen and that diluted with an inert, indigestible powder, alpha cellulose, and whether they could learn to distinguish between visual stimuli that have been paired with pollen samples diluted to differing degrees. Whilst it was predicted that bees would have a preference for samples containing a higher concentration of pollen and thus the more nutritionally valuable resource, in fact bees varied in their preference and appeared to be influenced by both the degree of similarity between samples and the type of pollen experienced previously. Pollen preferences were observed to change over time, with bees more readily collecting samples containing weaker concentrations of pollen following repeated exposure. Bees became more constant to one pollen type in the presence of an additional visual cue, providing that samples differed sufficiently in terms of pollen concentration. The findings presented here thus suggest that bees may be able to identify flowers providing their preferred pollen type on the basis of floral cues such as petal colour, and I discuss the potential implications for both the evolution of plant-pollinator relationships and the provisioning of rewards by flowers.

4.1 Introduction

A substantial amount of the pollen produced by flowers is lost during visitation by pollinating insects, which both passively and actively remove pollen grains. Typically pollen which is actively removed fails to contribute to fertilisation thus constituting a considerable reproductive cost for plants (Harder & Thomson, 1989; A. Müller, 1996a;

A. Müller, et al., 2006; Westerkamp & Claßen-Bockhoff, 2007; Westrich, 1989). Schlindwein et al. (2005), for example, found that bees remove 95.5% of the pollen produced by the flowers of *Campanula rapunculus*, and yet just 3.7% of this contributes to pollination. As a result, plants face pressure to avoid wastage and excessive consumption or collection by visiting insects. Nectar-less plants in particular, have evolved various strategies to control pollen removal and maximise the degree of pollen exchange between individuals (see Harder & Thomson, 1989 for review). Some species (e.g. *Solanum*) have gone so far as to produce two or more kinds of stamen (heteranthy) which specialize in the production of different types of pollen; that which serves as a reward for pollinators (food pollen) and that intended for out-crossing fertilisation (fertilisation pollen). Often feeding pollen is sterile and in many cases contains little or no cytoplasm and so is not particularly nutritious for insects (Simpson & Neff, 1983; Vogel, 1978). Plant species also differ with regards to the nutritional value of pollen they provide (Roulston & Cane, 2000) and in some cases variation exists between morphs of the same species (A. W. Robertson, et al., 1999). This raises the question as to whether such variations in nutritional quality might affect the foraging decisions of insects which collect pollen from a variety of plant species.

Bumblebees are a well studied group of generalist pollinator and, alongside honeybees, have been shown to have preferences for different pollen types, both under natural foraging conditions and in more controlled choice tests (Boch, 1982; Boelter & Wilson, 1984; Cook, et al., 2003; Doull, 1966; Hanley, et al., 2008; Levin & Bohart, 1955; A. W. Robertson, et al., 1999; J. O. Schmidt, 1982; Wahl, 1966; Wolfe & Barrett, 1987). Whilst in both groups, pollen species can differentially influence the development and survival of brood and young bees, thus impacting on overall colony success (Génissel, et al., 2002; Levin & Haydak, 1956; J. O. Schmidt, et al., 1987; L. S. Schmidt, Schmidt, Rao, Wang, & Xu, 1995; Tasei & Aupinel, 2008), the simple fact that bees display preferences does not imply that individual foragers have the capacity to determine the suitability of a particular pollen type as a food source and that they use this information to guide their foraging decisions. Bees do not ingest pollen rewards whilst collecting and as yet the underlying sensory mechanisms guiding pollen choices remains to be determined, as does the degree to which learning is implicated in the development of such preferences. Indeed the extent to which individual foragers might be able to assess differences in pollen quality, both prior to and during collection, is little understood.

Pollen is a complex food source for pollinators. Whilst it contains a variety of nutrients, few are not easily accessed or digested (for review see Roulston & Cane, 2000). Many factors may determine the quality of pollen for a pollinator, but typically variation in nitrogen content has been considered the best equivalent measure, since pollen is usually the only source of protein for bees (De Groot, 1953; Roulston & Cane, 2000). In some instances foraging preferences have been shown to correlate with the protein or amino acid content of pollen (Cook, et al., 2003; Hanley, et al., 2008; A. W. Robertson, et al., 1999) though this is not always observed to be the case (Levin & Bohart, 1955; J. O. Schmidt, 1982; Wahl, 1966). The following examples serve to illustrate the considerable ambiguity which exists between the findings of studies designed to test whether bees have the ability to assess differences in pollen quality.

Field experiments with bumblebees have shown that individual foraging preferences correlate with both the availability, and in some cases, the protein and amino acid concentration of pollen provided by flowers (Armbruster & Herzig, 1984; Armbruster & Webster, 1982; Buchmann & Cane, 1989; Cresswell & Robertson, 1994; H. E.M. Dobson, et al., 1999; Eckhart, 1991; Galen & Plowright, 1985; Gori, 1989; Harder, 1990; Pellmyr, 1988; Wainwright, 1978; Zimmerman, 1982). Robertson et al. (1999) observed bumblebee visits to *Mimulus guttatus*, a species polymorphic with regards to the quality of pollen it provides, with some morphs producing large quantities of poor quality grains which are lacking in cytoplasm. In dual-choice field assays, the best predictor of foraging preference was found to be protein content, and bees were also more likely to forage, and foraged for longer, in patches of *Mimulus* plants that produced better quality pollen.

Experiments with honeybees have yielded contradictory results. Waddington et al. (1998) showed that foragers were less likely to dance, or danced less vigorously, for pollen that had been diluted with indigestible alpha cellulose, suggesting they deemed diluted pollen to be a poorer resource than pure pollen. Pernal and Currie (2001) however maintain that honeybee foragers lack the ability to individually assess pollen protein content whilst foraging and must rely on feedback from the nurse bees which unload their corbiculae. When the quality of pollen stored in honeybee hives was manipulated, they observed that honeybees responded by increasing foraging effort, rather than opting to collect pollen from plants with more protein rich pollen (Pernal & Currie, 2001).

In laboratory tests, bumblebees are less likely to forage for pollen which has been diluted with alpha cellulose (Kitaoka & Nieh, 2009), and individuals collecting pure pollen have been shown to have higher thoracic temperatures than those collecting diluted pollen (Mapalad, et al., 2008), similar to bees that forage on more highly concentrated sugar solutions (Nieh, et al., 2006). Though these results suggest that foragers can distinguish between pollen samples of differing quality, it must be noted that Kitoaka and Nieh (2009) measured fluctuations in the probability of pollen foraging at the colony level, rather than observing the choices of individual bees. Simply observing changes in the number of individuals foraging at a given time could prove to be misleading, since colony needs for resources, and the subsequent foraging motivation of individuals can fluctuate over short time periods (Bergman, et al., 1996; Brian, 1952; Cartar, 1992; Free, 1955; Shelly, Buchmann, Villalobos, & O'Rourke, 1991; Young & Owen, 1989). Thoracic temperature can differ between groups of foragers for a number of reasons (Heinrich, 1979) and so it is possible that social, environmental or motivational factors were the primary reasons underlying the fluctuating rates of foraging effort in these studies.

So far, no study has attempted to investigate, under controlled conditions, the ability of individual bees to distinguish between pollen of differing qualities. Therefore I conducted a set of experiments in which bees were pre-exposed to pollen diluted with alpha cellulose and then offered a choice between two pollen samples. First it was tested whether individuals could tell diluted and undiluted pollen apart, with the expectation that bees would display a preference for undiluted pollen, since this is of a higher quality in terms of nutritional value. I subsequently explored the choices of bees exposed to pollen diluted to varying degrees, and tested their ability to associate such differences in quality with coloured stimuli.

4.2 Methods

4.2.1 General methods

Bumblebee colonies were obtained from Koppert Biological Systems (Suffolk, UK) and housed inside a flight net (L x W x H: 80 x 80 x 100 cm) in a laboratory at the University of Exeter. The flight net was connected to a test cage of the same size via a

Perspex corridor. Bees inside the flight net were provided with sucrose solution *ad libitum.*

The 'quality' of honeybee-collected pollen was manipulated by diluting samples with inert, indigestible alpha cellulose. During pre-training, and when not engaged in experiments, bees were provided with 90% pollen (w/w).

In order to train individually-marked bees to visit the test cage, a large petri dish of 90% pollen (9 cm diameter) was placed inside the flight cage, near the entrance to the corridor. Once marked bees started collecting pollen, the dish was gradually moved through the open corridor and into the experimental arena. A large disc (75 cm diameter) of grey paper (HKS 92N, K+E Stuttgart, Stuttgart-Feuerbach, Germany) inserted between two identically sized Perspex discs was placed horizontally level with the corridor and served as the background surface for pollen presentation. Between trials the surface was wiped with ethanol to remove olfactory cues and any remaining pollen grains. Once bees had learnt to visit the single large petri dish, it was replaced by a number of smaller (5.5 cm diameter) petri dishes. When an individual had been observed to visit several different petri dishes, on two separate foraging trips, the ability to discriminate between pollen of differing qualities was tested.

4.2.2 Single-trial choice tests

Following pre-training, individual bees were given a rewarded test in which they were presented with four small petri dishes, two containing 3 g of undiluted (100%) pollen and two containing 3 g of 90% pollen. Bees were permitted to forage *ad libitum* and the trial ended once a bee returned to the hive and unloaded their pollen sacs. The number of landings and duration of time spent foraging in each dish was recorded. The number of re-landings on the same individual dish was also measured. I hypothesised that if bees are able to distinguish between pollen qualities, an individual would be more likely to re-land in the same dish when foraging on their preferred pollen sample, rather than switch and sample other dishes.

New groups of bees were presented with a choice of two diluted pollen samples. Bees were given a choice between 90% and 80%, 90% and 70% or 90% and 60% pollen. One

group of bees was also tested with undiluted and 80% pollen, neither of which were experienced during pre-training, to determine their preference between two novel samples.

4.2.3 Multiple-trial choice tests

As in the single-trial experiments, bees were pre-trained to collect 90% pollen and were then exposed to two pollen qualities over five trials. The location of the four petri dishes was varied between trials, since it is known that bees are strongly guided by positional cues. The number of landings and the total time spent foraging on each dish was compared between the first and last trial to see if overall preference changed over the course of several exposures to both types of pollen. Bees were tested with 90% *vs.* 70% and 90% *vs.* 60% pollen.

4.2.4 Matched-cue choice tests

Adding white, odourless alpha cellulose changes the appearance and olfactory intensity of pollen samples. Therefore I tested whether bees could distinguish between samples of differing quality (90% *vs.* 70%) prior to alighting, by relying on information regarding the odour and/or colour of the pollen. After being pre-trained to 90% pollen, bees were presented with four petri dishes, which were covered with transparent lids to prevent access to the pollen. Small holes permitted the odour of the pollen to diffuse through. By manipulating the mass of the different pollen samples it was possible to alter the visual or olfactory characteristics of the samples independently.

In the olfactory discrimination test, bees were exposed to dishes containing 90% pollen to keep the visual cues identical. To vary the olfactory cues, two of the dishes contained 3 g and two contained 2.33 g, matching the odour intensity of 3 g of 70% pollen. In the visual discrimination test, two dishes contained 3 g of 90% pollen and two dishes contained 3.86 g of 70% pollen, to match the odour intensities of the samples. Tests lasted until bees gave up their search and were limited to a maximum of five minutes. The number of approaches, contacts and landings made to each dish was recorded. An

approach was classified as when a bee's whole body crossed within 2 cm of the petri dish. A 'contact' was classed as any physical connection between the bee's body and the petri dish. A 'landing' was differentiated from a 'contact' by the splaying of the bee's legs. The order in which the two tests were presented was varied between individuals. Bees received a reminder trial between the two tests, during which they were permitted to collect 90% pollen, as in pre-training.

This experiment was subsequently repeated to determine whether bees could discriminate between 90% and 60% pollen on the basis of visual or olfactory cues alone. In the olfactory discrimination test, dishes contained either 3 g or 2 g of 90% pollen, the latter matching the odour intensity of 3 g of 60% pollen. In the visual discrimination test, dishes contained either 3 g of 90% pollen or 4.5 g of 60% pollen.

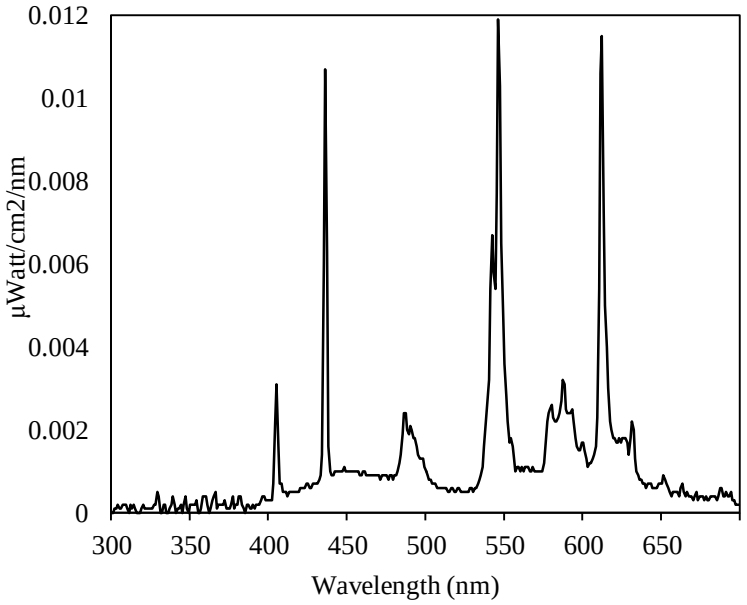
Spectra of the illuminating light and the pollen samples were measured with a calibrated photo-spectrometer (Avaspec2048, Avantes, USA) (Fig. 4.1 a, b). Using the receptor-noise limited model of bee colour vision (Vorobyev, Brandt, Peitsch, Laughlin, & Menzel, 2001), and spectral sensitivity data measured intra-cellularly in *Bombus terrestris* (Peitsch et al., 1992), the 90%, 80% and 70% were predicted to be similar in colour for bees. The distances in the perceptual colour space were between 0.1 and 1.5 times the standard deviation of receptor noise in bee photoreceptors, meaning that these colours would be difficult for bees to distinguish (Fig. 4.1c). The difference in chromatic contrast between the samples and the background was between 5.6 and 7.1, providing a cue that could potentially be used by bees for discrimination. The undiluted pollen (100%) and 60% pollen differed more strongly in both colour and chromatic contrast from the 90% and 80% samples (colour distances between 3.2 and 4.6). Undiluted and 90% pollen had equal brightness, but represented the dimmest samples for the bees compared to more diluted samples which were brighter (L-receptor contrasts to the grey background between 1.8 and 3).

4.2.5 Differential colour conditioning

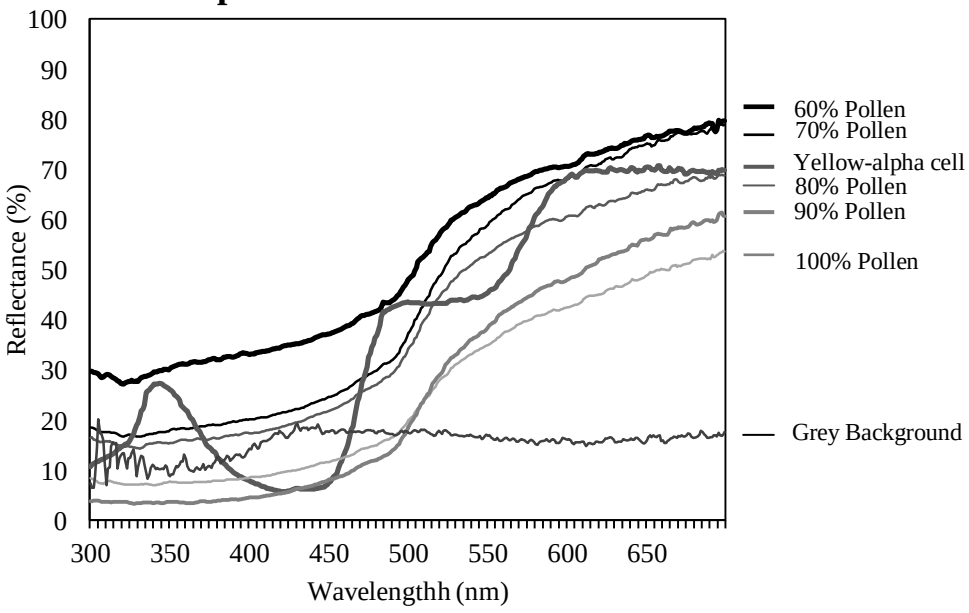
Prior to colour training, bees were pre-trained to petri dishes (5.5 cm in diameter) containing 90% pollen. During training dishes were placed on top of four coloured discs (15 cm in diameter, blue *vs.* green and orange *vs.* white). Both colours were rewarding,

but offering different dilutions of pollen: 90% *vs.* 70% or 90% *vs.* 60%. The colour preferred in the spontaneous test provided the weaker concentration of pollen during training (either Green or Orange). Bees were permitted to collect pollen *ad libitum* and a trial ended once a bee returned to the hive to unload their corbiculae. Between training trials the laminated coloured stimuli were wiped with ethanol. Bees received five training trials in total and the location of the stimuli was varied between trials.

a. Illuminating light



b. Pollen sample reflectance



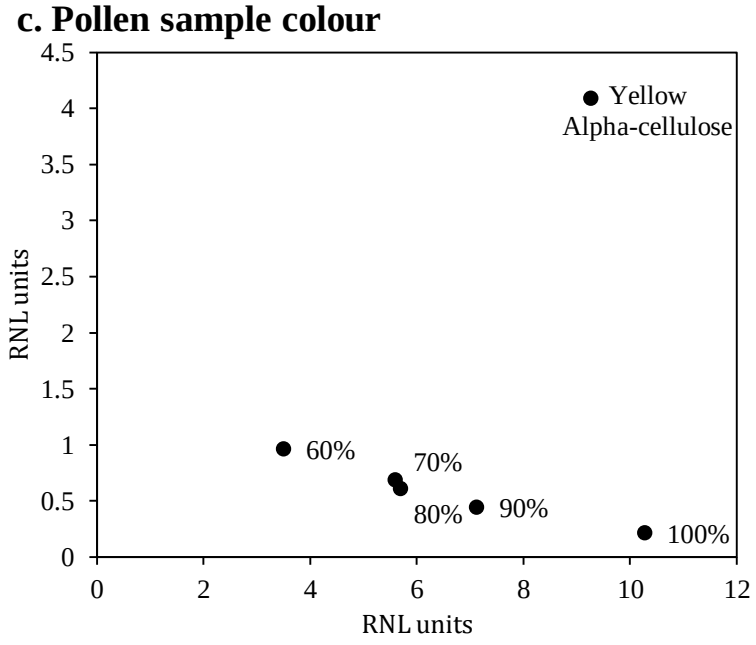


Figure 4.1 (a) Spectra of the illuminating light (b) and reflectance of the pollen samples. Yellow-dyed alpha-cellulose was used in the test of differential conditioning (see below section 4.2.5) and differed in colour from all pollen samples (colour distances between 4.0 and 6.6 Receptor-noise limited (RNL) units. 1 RNL unit=1 standard error of receptor noise) (c) (Receptor-noise limited model of bee colour vision Vorobyev, et al., 2001).

Colour preferences were tested prior to and following differential conditioning to determine whether training had any effect on preference. Both tests were unrewarded. Prior to training dishes contained 3 g of 90% pollen and were covered with transparent lids with small holes to permit odour diffusion, meaning that both visual and olfactory cues could be used by bees, but access to pollen was precluded. Test trials lasted until the bees gave up their search and were limited to a maximum of five minutes. The number of approaches, contacts and landings made to each coloured disc was recorded.

In the unrewarded test following training, all dishes contained alpha cellulose, which had been dyed yellow using food colouring, to standardise olfactory and visual cues. The colour of the yellow-dyed alpha-cellulose differed strongly from all pollen samples (Fig. 4.1c) and thus represented a novel pollen colour. Bees therefore had to make their choices on the basis of the colour of the discs as opposed to using the visual or olfactory characteristics of pollen.

After the alpha cellulose test, bees received a rewarded reminder trial and a second unrewarded test. To determine whether bees paid more attention to the coloured stimuli

or the visual and/or olfactory differences between the pollen samples themselves, the colour of the pollen sample was put in conflict with the colour of the disc by swapping the pollen samples between discs. For example, if 90% was paired with Blue during training, in this test it would be paired with Green.

4.2.2 Statistical Analysis

Differences in collecting behaviour were compared using a two-tailed paired t-test. In rewarded trials (single trial and multiple trial experiments) the duration of time spent foraging on each pollen type was compared. For unrewarded tests, the number of approaches and the number of contacts and landings made to each sample or coloured disc were compared. In cases where the data was not normally distributed (Shapiro & Wilk, 1965) Wilcoxon's matched-pairs test was used.

A preference index was calculated for each individual, the duration of time spent foraging on the more diluted pollen sample was subtracted from the time spent foraging on the less diluted sample and divided by the total amount of time spent foraging. This calculation was also repeated for the number of landings made to each sample. Scores below zero to -1 indicate an increasing preference for the more diluted pollen sample, whereas scores above zero to 1 represent an increasing preference for the less diluted sample. A score close to zero means that an individual foraged equally on both pollen types. The preference indices for duration of foraging and number of landings were found to be highly correlated in all cases (Spearman's correlation coefficient 100 *vs.* 90 r=0.860, p<0.001; 100 *vs.* 80 r=0.975, p<0.001; 90 *vs.* 70 r=0.879, p=0.001; 90 *vs.* 60 r=1.000, p<0.001), therefore duration of foraging was selected as the measure of preference.

To determine whether bees were able to discriminate between samples, a two-tailed paired samples t-test was performed to compare the time spent foraging on the preferred versus non-preferred samples. The preferred sample was defined as whichever a particular individual spent the longest time foraging on. To compare the strength of pollen preferences between experiments, the proportion of time spent foraging on the preferred sample was calculated for each individual. A one-way ANOVA was

performed on arc-sin transformed data to test whether the difference in pollen content between the samples had an effect on the overall strength of bees preferences.

Using a linear regression, I tested whether the first landing predicted the proportion of time a bee spent foraging on that particular sample, to determine whether the sample on which bees first alighted influenced pollen preferences.

The proportion of re-landings on the preferred and non-preferred sample was determined by dividing the number of repeated landings on the same individual dish by the total number of landings on the same sample (e.g. 90% pollen) and was compared using a paired samples t-test or Wilcoxon's signed-rank test. As an additional measure of discrimination, I also analysed the number and direction of transitions between pollen samples, and compared the number of transitions towards the preferred versus non-preferred sample.

To rule out the potential influence of positional biases on sample choice, the number of approaches to each dish location was compared using a repeated-measures ANOVA. For rewarded tests I compared the number of landings and the duration of time spent foraging at each location. In all cases there was no significant difference between locations.

4.3 Results

4.3.1 Can bees discriminate between undiluted and differentially diluted pollen samples?

After pre-training to diluted pollen (90%) bees were able to discriminate between this and undiluted pollen (Fig. 4.2), though individuals clearly differed in their preferences. A comparison of the duration of time spent foraging on 90% and 100% pollen yields a non-significant result (Duration, t_{11}=1.239, p=0.241) since there were both individuals that strongly preferred undiluted pollen and those that preferred diluted pollen, leading to a mean preference score close to zero (0.116 ±0.149 SE). However, individuals spent significantly more time collecting their preferred sample (t_{11}=6.180, p<0.001), and made a significantly greater proportion of re-landings to dishes containing the preferred

pollen type relative to those containing the sample that was less preferred (Fig. 4.3 z= -3.059, n=12, p=0.002).

When given the choice between samples containing 90% and 80% or 90% and 70% pollen (Fig. 4.2), the majority of bees preferred 90% pollen. However, in both cases the duration of time spent foraging on 90% pollen did not differ significantly from the time spent collecting the more diluted pollen samples (90% *vs.* 80% z=-1.580, n=10, p=0.114; 90% *vs.* 70% z=-1.682, n=10, p=0.093). One individual in particular had a strong preference for 70% pollen. When the preferred sample for each individual was compared against the less preferred, bees spent a significantly longer time collecting the preferred pollen type (90% *vs.* 80% z=-2.803, n=10, p=0.005; 90% *vs.* 70% z=-2.803, n=10, p=0.005), and made a greater proportion of re-landings to this sample (Fig. 4.3 90% *vs.* 80% t_8=2.714, p=0.026; 90% *vs.* 70% t_9=3.457, p=0.007), suggesting that individuals also discriminated between pollen samples that had been diluted with alpha cellulose to varying degrees. Eight out of ten bees made a first landing on 90% pollen when offered against 80%, whilst six out of ten chose the previously experienced pollen type (90%) over 70% pollen.

All bees showed a strong preference for 90% over 60% pollen, and three out of six bees tested made no landings on the weaker dilution at all (Fig. 4.2). This result suggests that bees are able to make foraging decisions prior to landing, and can assess differences between samples on the basis of visual and/or olfactory cues. The amount of time spent foraging on 90% pollen was significantly different from the time spent foraging on 60% (z=-2.201, n=6, p=0.028). No re-landings were made to dishes containing 60% pollen (Fig. 4.3)

When bees were offered two novel samples (100% and 80% pollen) the duration of time spent foraging on each was not significantly different (t_7=-1.107, p=0.330). Three out of the eight bees tested had a strong preference for the undiluted pollen, two bees strongly preferred 80% pollen and the remaining bees collected both types of pollen more or less equally (Fig. 4.2). As in previous tests, overall bees spent significantly longer foraging on their preferred sample as compared to the less preferred sample (z=-2.521, n=8, p=0.012). Whilst a greater proportion of re-landings were made to the preferred sample (Fig. 4.3 *ca.* 46% compared to 23% for less preferred), this difference was not significant (100 *vs.* 80 t_4=1.171, p=0.307).

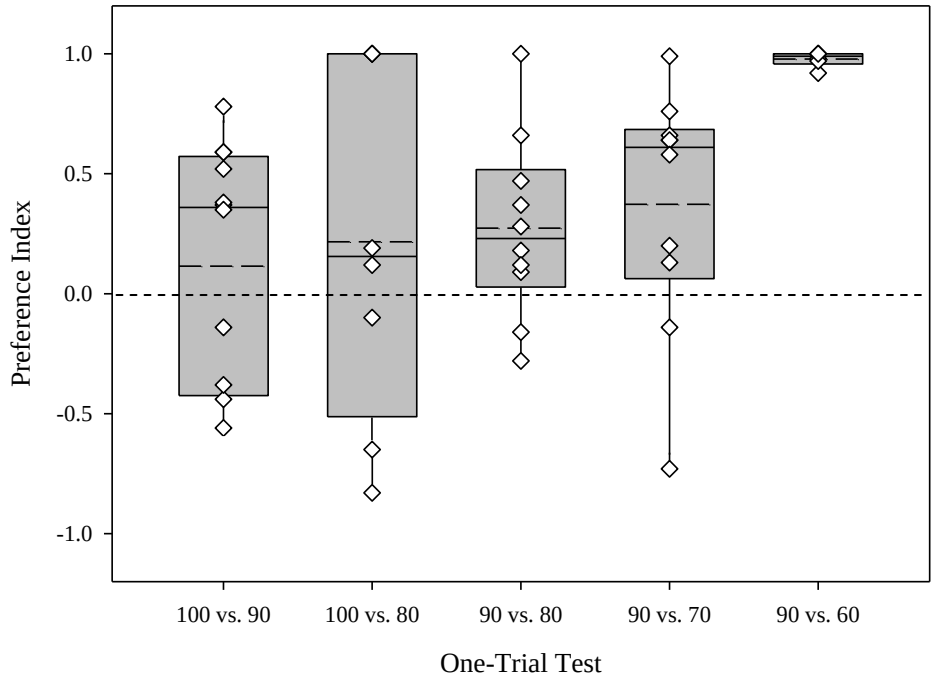


Figure 4.2 Preference index scores (open diamonds) based on the duration of time spent foraging on each pollen sample. Scores greater than zero indicate a preference for the sample with the higher ratio of pollen to alpha-cellulose. Quartiles are represented by box limits, solid lines represent the median preference score, dashed lines the mean. Whiskers depict the range. On a single trial, bees were either given a choice of pure pollen (100%) *vs.* 90% (n=12), pure pollen *vs.* 80% (n=8), 90% *vs.* 80% (n=10), 90% *vs.* 70% (n=10) or 90% *vs.* 60% pollen (n=6).

On average bees made 12.61 (±1.22) transitions between samples during the one-trial tests. Whilst for all pairs of samples tested bees made a greater proportion of transitions towards their preferred sample type (Fig. 4.4), there was no significant difference in the number of transitions made in each direction (100 *vs.* 90, z=-1.233, n=12, p=0.218, 100 *vs.* 80 t_9=-0.814, p=0.434, 90 *vs.* 80 t_7=1.823, p=0.111, 90 *vs.* 70 t_8=1.985, p=0.082, 90 *vs.* 60 z=-1.826 n=6, p=0.068). This most likely reflects the fact that once bees made the decision to switch locations they were unable to visually discriminate between samples and identify their preferred pollen type, except where differences between samples were large (90 vs. 70 and 90 vs. 60 are both nearing significance).

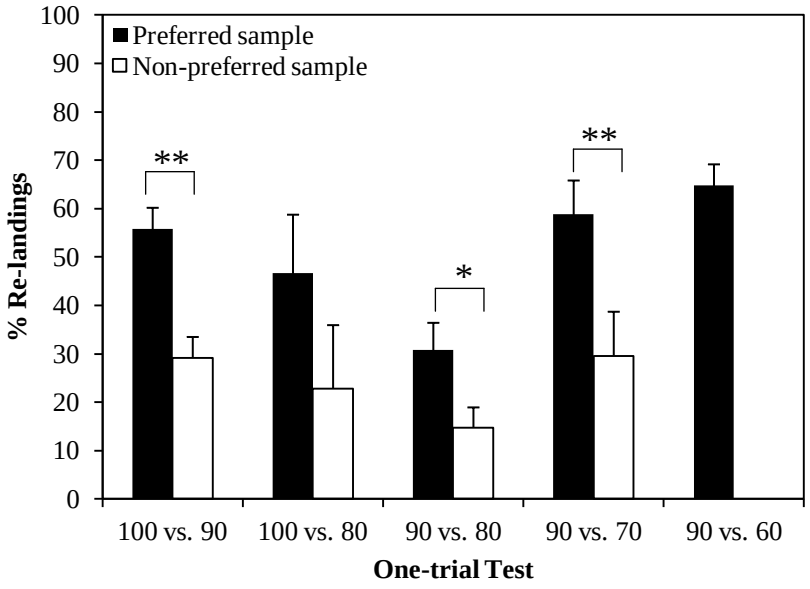


Figure 4.3 Proportion of landings on each sample (Black= preferred sample, Grey= non-preferred sample) that were re-landings to the same dish. Bees were either given a choice of pure pollen (100%) *vs.* 90% (*n*=12), pure pollen *vs.* 80% (*n*=8), 90% *vs.* 80% (*n*=10), 90% *vs.* 70% (*n*=10) or 90% *vs.* 60% pollen (*n*=6). Asterisks denote a significant difference in the proportion of re-landings made to each sample (*$p \leq 0.05$, **$p \leq 0.01$).

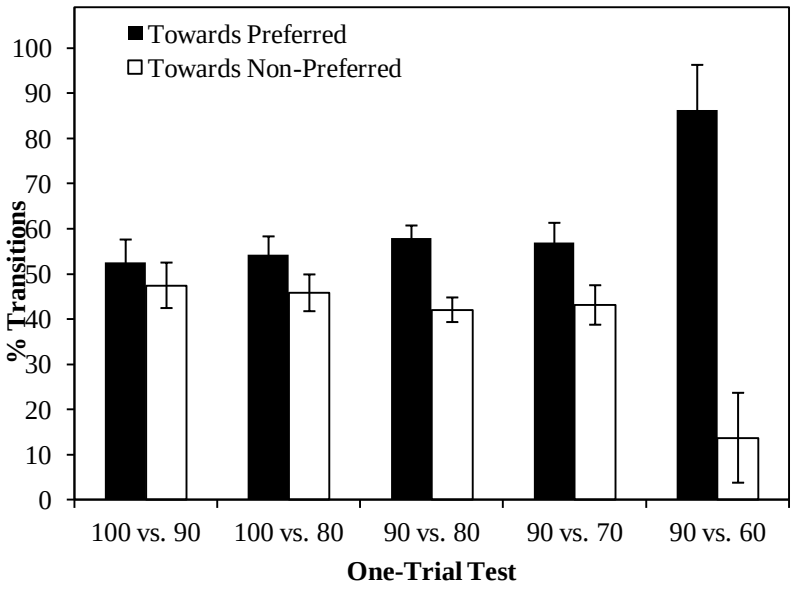


Figure 4.4 Proportion of transitions made to the preferred (Black bars) and non-preferred sample (White bars). Bees were either given a choice of pure pollen (100%) *vs.* 90% (*n*=12), pure pollen *vs.* 80% (*n*=8), 90% *vs.* 80% (*n*=10), 90% *vs.* 70% (*n*=10) or 90% *vs.* 60% pollen (*n*=6). Asterisks denote a significant difference in the proportion of transitions made towards each sample type (*$p \leq 0.05$, **$p \leq 0.01$).

The difference between samples, in terms of the degree of dilution with alpha cellulose, had a significant effect on the overall strength of preference for one sample over an another ($F_{4,45}$=5.046, p=0.002). Bees offered a choice between 90% and 60% pollen had significantly higher preference scores than bees offered the choice of 90% *vs.* 100%, 90% *vs.* 80% and 90 *vs.* 70% pollen (Dunnett's T3 test, all $p \leq 0.001$), but not the two novel samples (100% *vs.* 80%, p=0.168).

Whilst it seems that bees distinguish between pollen samples, they do not necessarily adjust their foraging behaviour to preferentially visit the sample containing a greater volume of pollen. Furthermore, there was no correlation between bees preference scores and the total amount of time they spent foraging, suggesting that individuals which specialised in collecting the more diluted pollen samples did not compensate for the lower concentration of pollen grains by collecting for longer.

For some groups, the chance of making the first landing on one dish over another was fairly equal, and did not predict the duration of time spent foraging on that sample (100 *vs.* 90%, 7/12 landed on 90%, t_{11}=0.512, p=0.620; 90% *vs.* 70%, 6/10 landed on 90% t_9=0.549, p=0.598). However, bees in one group (90% *vs.* 80%) preferred the sample type that they first encountered during the test (t_9=2.662, p=0.029), though the relatively small number of bees tested may mean that this result represents a statistical skew, especially considering that only two out of ten bees made their first landing on 80% pollen. It is unlikely that in the bias towards landing on 90% pollen in this group resulted from active selection of that particular pollen type prior to alighting. 80% and 70% pollen are observed to be quite similar in appearance (Fig. 4.1c), and yet no skew in choices was observed in the 90% *vs.* 70% group. When offered against 60% pollen, bees had a strong preference for the 90% sample, with five out of six bees making their first landing on this preferred pollen type and, unlike bees in other groups, individuals rarely landed on the alternative sample during testing. Interestingly, when bees were offered two novel samples (100% *vs.* 80%, n=8), first landings were equally distributed between both samples and predicted bees subsequent foraging preferences (t_7=3.859, p=0.008), suggesting that both current and prior experience may differentially influence collection decisions during a foraging bout

4.3.2 Did pollen preference strengthen with longer exposure to different samples?

New groups of bees were pre-trained to collect 90% pollen and exposed to either 90% *vs.* 70% or 90% *vs.* 60% pollen over five trials (Fig. 4.5). As before, the total amount of time spent foraging on each sample did not differ significantly between 90% and 70% pollen on the first trial (z=-1.572, n=6, p=0.116), although bees did have a significant preference for one pollen sample over the other, with the majority preferring the less diluted sample (Fig. 4.5 z=-2.201, n=6, p=0.028). Contrary to my expectations, preference for 90% pollen decreased following multiple exposures to the two samples, and some bees even switched from preferring 90% to preferentially collecting 70% pollen by the fifth trial. Although the total time spent foraging on each sample was the same (t_5=0.123, p=0.907), individual bees still preferred one sample type over the other (t_5=3.277, p=0.022).

When offered the choice between 90% and 60% pollen, all bees had a strong preference for 90% pollen on the first trial (Fig. 4.5 t_5=4.426, p=0.007). After five training trials, the preference for 90% had weakened considerably for one individual and two had switched to collect 60% pollen preferentially. For the remaining three individuals the preference for 90% pollen over 60% was maintained throughout the course of training. By the fifth trial the total amount of time spent collecting each sample was no longer significantly different (Duration z=-0.524, n=6, p=0.600), and individual preferences were much weaker than on the first trial (t_5=2.238, p=0.075).

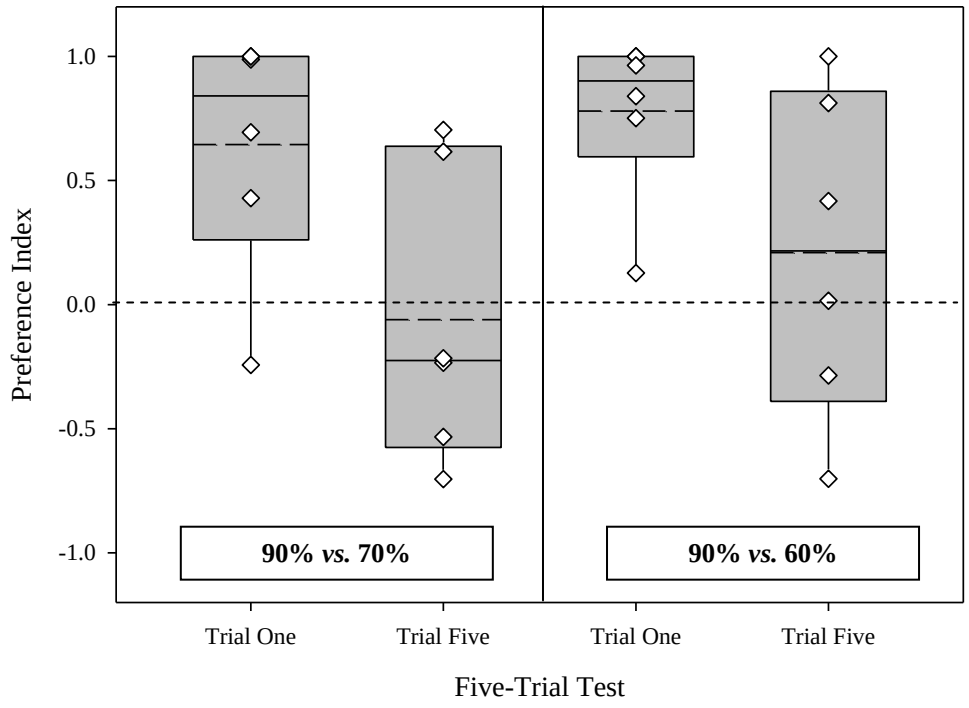


Figure 4.5 Preference index scores (Open diamonds) based on the duration of time spent foraging on each pollen sample, on the first and fifth trial of the multiple-trial experiment. Scores greater than zero indicate a preference for 90% pollen. Quartiles are represented by box limits, solid lines represent the median, dashed lines the mean preference score. Whiskers depict the range. Bees were either given a choice of 90% *vs.* 70% (*n*=6) or 90% *vs.* 60% pollen (*n*=6).

4.3.3 Did bees detect differences in colour and/or odour between samples?

The fact that bees avoid 60% pollen during their first exposure to this sample indicates that they assess pollen samples prior to landing and possibly learn their sensory characteristics. In a third set of experiments, I tested bee's preferences for pollen samples that were matched in either appearance or olfactory intensity.

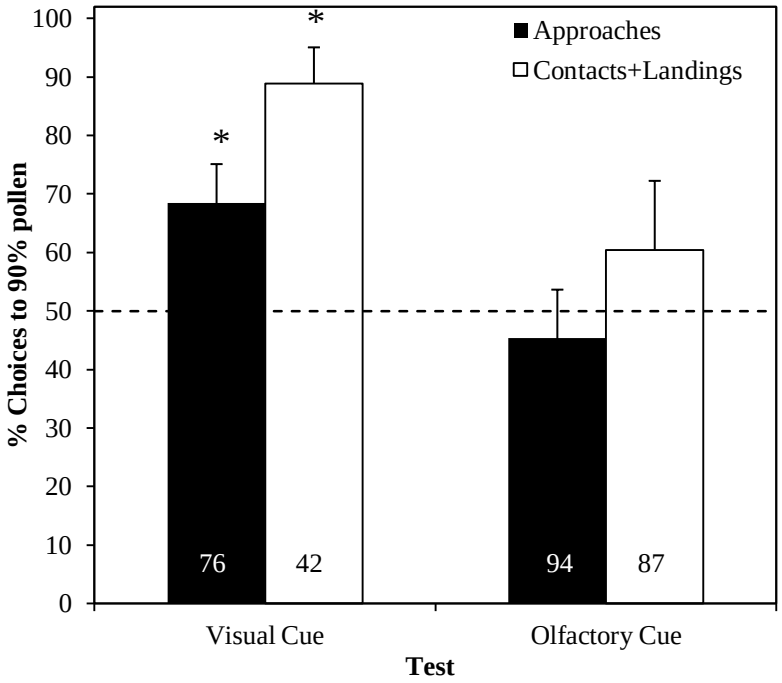
When the two pollen samples were visually different, bees demonstrated a clear preference for 90% pollen over 70%, both in terms of the number of approaches and the number of contacts and landings (Fig. 4.6a Approaches t_5=3.144, p=0.026; Contacts and Landings z=-2.023, n=6, p=0.043). The visual appearance of pollen samples thus provided sufficient sensory information on which to base their choice. When samples were matched in terms of visual appearance and only the intensity of the odour varied,

the same bees showed no preference for one pollen type over the other (Fig. 4.7 Approaches t_5=0.479, p=0.470; Contacts and Landings t_5=0.547, p=0.607) suggesting that individuals did not distinguish between samples on the basis of odour alone. Likewise, bees were able to discriminate between 90% and 60% pollen on the basis of visual differences between the samples, at least when the number of contacts and landings are considered (Fig. 4.6b Approaches t_6=1.543, p=0.174; Contacts and Landings t_6=2.594, p=0.041). Again, when samples differed in terms of olfactory intensity no preference between samples was observed (Approaches t_4=0.550, p=0.612, Contacts and Landings t_4=0.394, p=0.713).

4.3.4 Can bees associate coloured stimuli with differential pollen rewards?

When bees were offered a choice between different pollen samples over multiple trials, the general preference for the more concentrated sample (90%) was observed to weaken, despite the fact that bees are able to discriminate between samples visually (Exp 4.3.2, Fig. 4.5). In a subsequent experiment I tested whether the addition of an extra visual (coloured) cue to discriminate between samples might lead to bees foraging more constantly on 90% pollen. In an unrewarded colour preference test, approximately 70% of approaches were made to green discs, significantly more than to blue (Fig. 4.7 t_6=-2.595, p=0.041). Contacts and landings were distributed equally between green and blue (t_6=-2.201, p=0.712). Following five training trials with 90% pollen associated with the blue stimulus, bees continued to make significantly more approaches to the green discs (z=-2.201, n=7, p=0.028) meaning they did not shift their preference towards blue despite the fact that this stimulus had been paired with the sample containing the higher concentration of pollen. Approximately 70% of all contacts and landings were made to green, although presumably as a result of the relatively small number of contacts made by bees, the difference is not significant (z=-0.949, n=7, p=0.343).

a. 90% *vs.* 70% Matched cues test



b. 90% *vs.* 60% Matched cues test

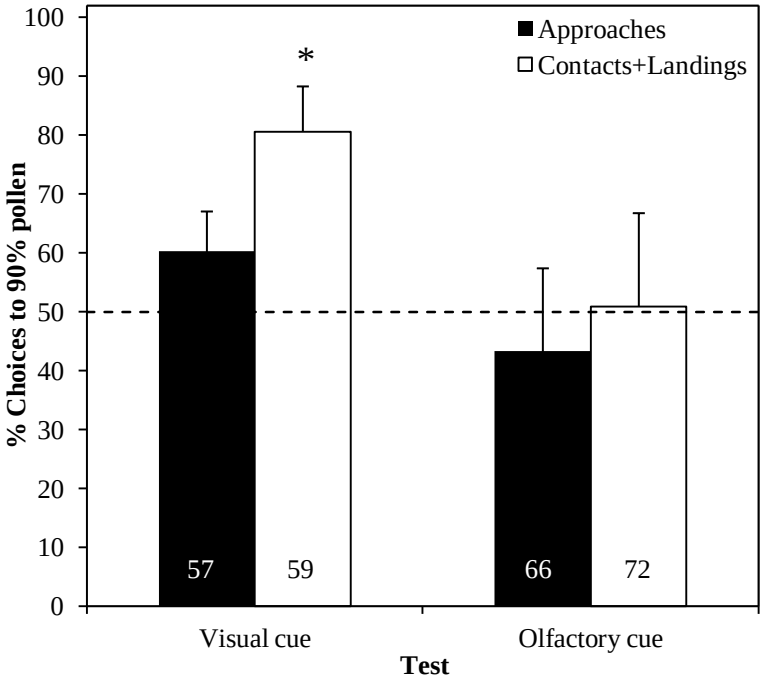


Figure 4.6 Discrimination performance of bees presented with either a visual or olfactory cue with which to differentiate between pollen samples. Bees were offered either (a) 90% *vs.* 70% pollen (Visual cue n=6, Olfactory cue n=6) or (b) 90% *vs.* 60% pollen (Visual cue n=7, Olfactory cue n=5) in an unrewarded test. Black bars represent the proportion of approaches to 90% pollen and white bars the proportion of contacts and landings. The total number of approaches or contacts and landings made by bees are included on each bar. Asterisks denote a significant deviation from random choice (*$p \leq 0.05$).

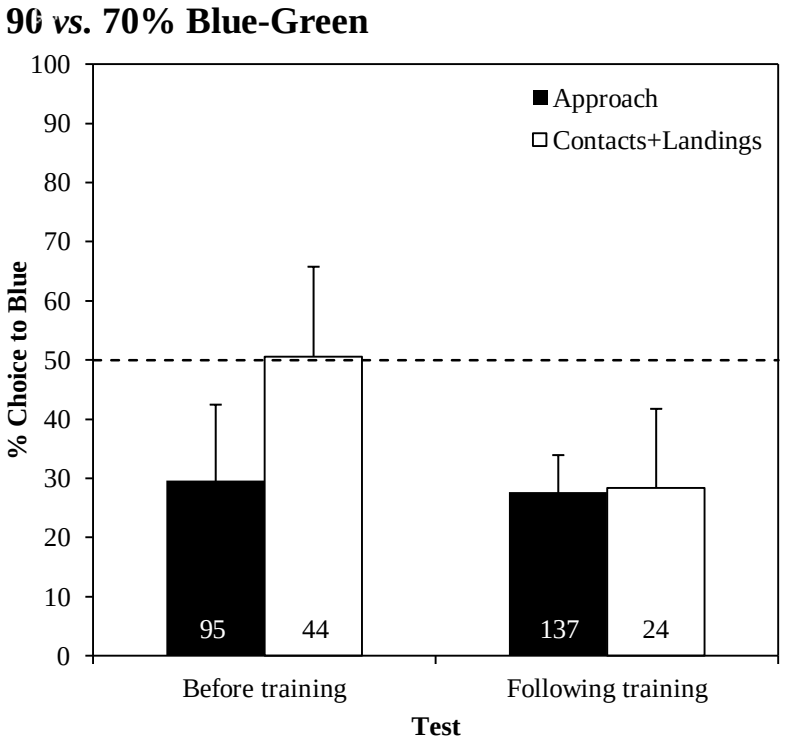


Figure 4.7 Proportion of approaches (Black bars) and contacts and landings (White bars) made to Blue in an unrewarded test, both prior to and following training (*n*=6). During training, blue was rewarded with 90% pollen. In the test before training, all dishes contained 90% pollen whereas in the test after training all dishes contained yellow, odourless alpha cellulose. The total number of approaches or contacts and landings made by bees are included on each bar. Asterisks denote a significant deviation from random choice (*$p \leq 0.05$).

Similarly, the next group of bees tested had a significant preference for green (*ca.* 70% of approaches) in the initial colour preference test, (Fig. 4.8a z=-2.207, n=6, p=0.027), though contacts and landings were randomly distributed between green and blue discs (z=0.106, n=6, p=0.916). Following five training trials, in which blue was rewarded with 90% and green with 60% pollen, the proportion of approaches to green discs clearly decreased, with an equal number of approaches made to both blue and green. This indicates that bees learnt to associate the blue stimulus with the less diluted pollen type (z=-1.219, n=6, p=0.223). An even greater proportion (*ca.* 85%) of contacts and landings were made to blue after training, though this difference is not significant (z=-1.633, n=6, p=0.102) and in fact only three bees out of six made any contacts with the stimuli, reflecting the generally observed trend that bees make fewer contacts with

the sample dishes when they contained alpha cellulose compared to the initial preference test where dishes contained pollen.

This experiment was repeated with another combination of colours, orange and white, to confirm that the shift in colour preference in bees rewarded with 90% *vs.* 60% pollen was not dependent on the particular set of coloured stimuli used in the previous experiment. Prior to training, bees displayed a significant preference for orange, with *ca.* 85% of approaches made to this stimulus (Fig. 4.8b z=-2.023, n=5, p=0.043). The difference in the number of contacts and landings made to orange versus white is approaching significance and as with approaches, a greater proportion of contacts were made to orange (z=1.841, n=5, p=0.066). During training, the white disc was rewarded with 90% pollen and orange with 60%. As with blue and green, bees made a similar number of approaches to both orange and white following training (t_4=0.623, p=0.567). Whilst the proportion of contacts and landings made to white increased considerably, from less than 10% before training to *ca.* 90% afterwards, the difference was not significant (z=-1.604, n=5, p=0.109). Again only three out of the five bees tested made any contact with the stimuli.

When collection preferences were compared between the first and fifth training trial, bees collected only 90% pollen and made no landings on the 60% sample on either trial. This contrasts with the findings of the previous experiment (section 4.3.2). When bees collected pollen in the absence of any additional colour cues, individuals were observed to collect both 60% and 90% by the fifth trial, with some individuals even switching to preferentially collect 60% pollen (Fig. 4.5). Bees offered the choice between 90% and 70% pollen sampled both pollen types consistently throughout colour training.

In the reversal test, pollen samples (90% vs. 70% or 90% vs. 60%) were switched between stimuli in order to put the colour of the less-diluted pollen sample and the surrounding colour in conflict with the trained association. Given that during training, bees offered the choice between 90% and 70% pollen failed to shift their colour preference towards blue, I predicted that in the reversal test bees would demonstrate a strong preference for 90% pollen, since it was now offered in association with the preferred colour, green. This was indeed the case (Fig. 4.9), although for only one of the three bees tested was the preference for 90% pollen significant (One-tailed binomial test, Approaches p=0.001; Contacts and Landings p=0.031).

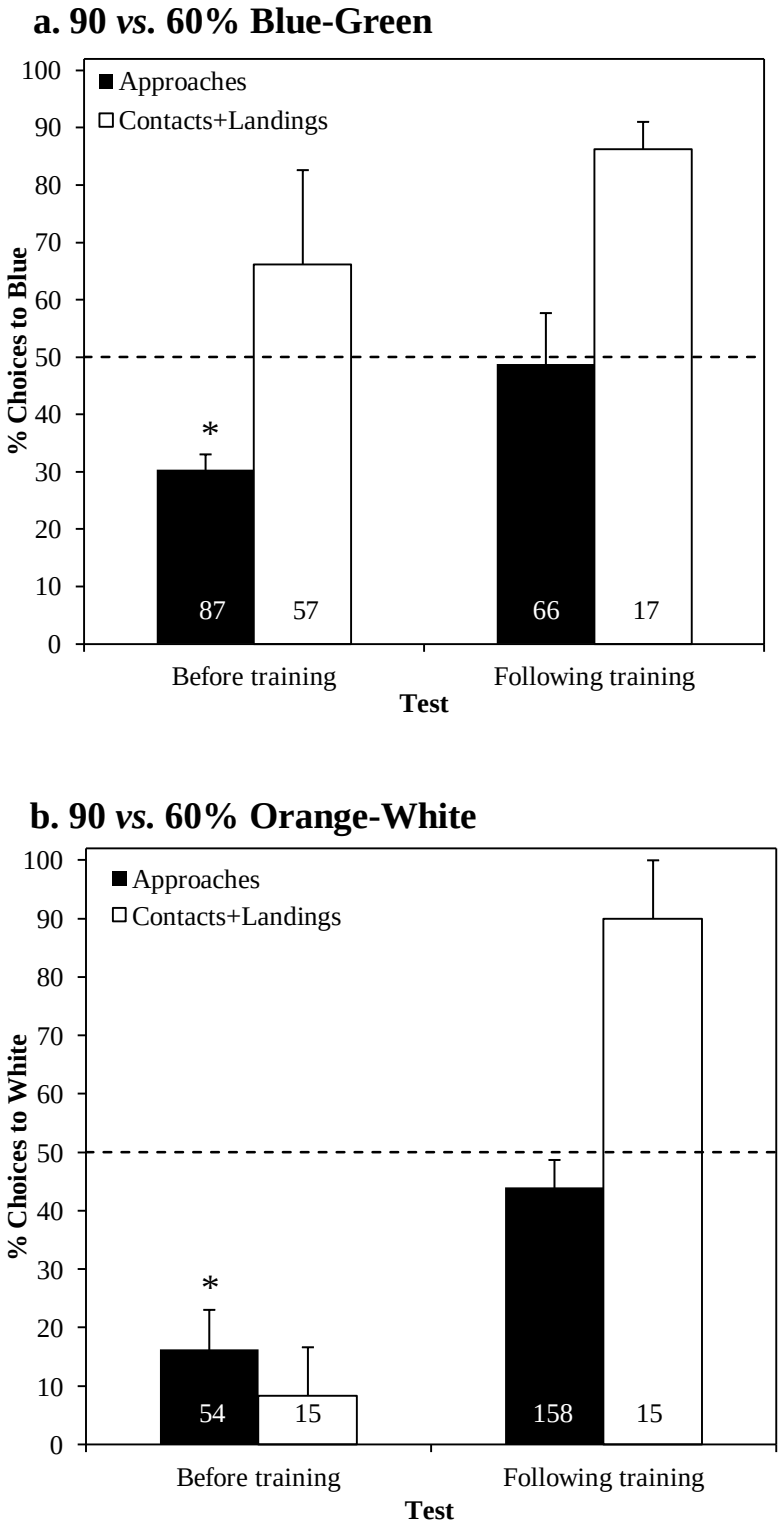


Figure 4.8 Proportion of approaches (Black bars) and contacts and landings (White bars) made to Blue (a) or White (b) in tests conducted prior to and following training. Tests were unrewarded. During training, either blue (n=6) or white (n=5) were rewarded with 90% pollen. In the test prior to training, all dishes contained 90% pollen, whereas following training dishes contained yellow, odourless alpha cellulose. The total number of approaches or contacts and landings made by bees are included on each bar. Asterisks denote a significant deviation from random choice (*$p \leq 0.05$).

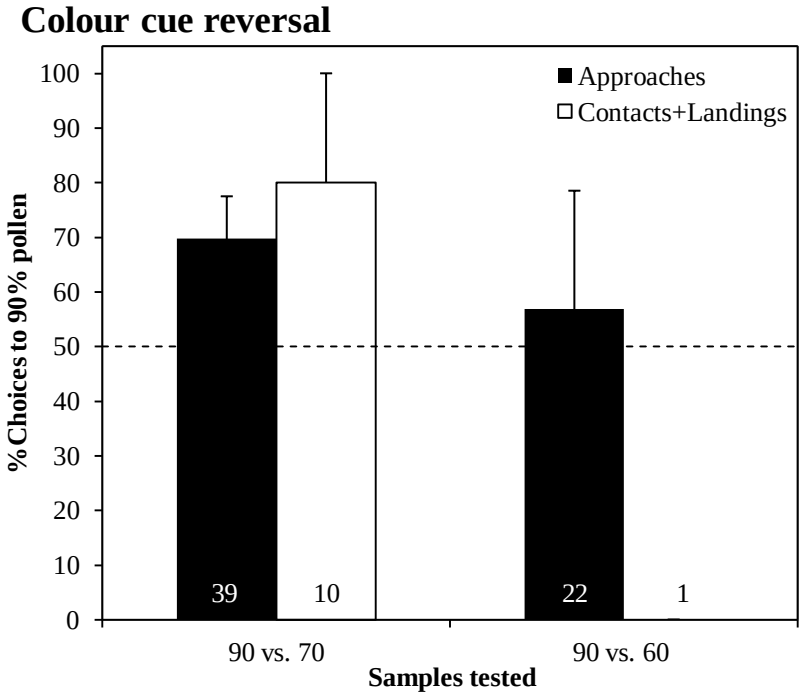


Figure 4.9 Proportion of approaches (black bars) and contacts and landings (white bars) to the 90% pollen sample when pollen samples were reversed between stimuli, so that the colour previously paired with the less diluted sample (trained colour) was paired with the more diluted sample and *vice versa*. Tests were unrewarded. Bees were either offered the choice of 90% *vs.* 70% pollen (Green *vs.* Blue n=3) or 90% *vs.* 60% pollen (Orange *vs.* White n=2; Green *vs.* Blue n=1). The total number of approaches or contacts and landings made by bees are included on each bar. Only one contact, a landing on 60% pollen, was made throughout the whole experiment (bar not shown).

Bees offered the choice between 90% and 60% pollen visited both samples almost equally in the reversal test (Fig. 4.9) providing further evidence that they had learnt the colour of the discs during training. For each of the three bees tested, there was no significant difference in both the number of approaches and the number of contacts and landings made to the 90% and 60% pollen samples (Two-tailed binomial test). Two out of the three bees tested made between 62-66% of their approaches to the white stimulus, even though it was now paired with 60% pollen, which had been completely avoided by bees during training. The third individual made only two approaches in total, and both of these were to green, the colour paired with 70% in training and with 90% in the reversal test, suggesting that this individual may have been guided more by the visual characteristics of the pollen samples.

Only a small number of bees were available for the reversal test and therefore I am cautious in placing too much emphasis on the response patterns observed. Nevertheless, the results indicate that it would worthwhile to explore this question further.

4.4 Discussion

The pollen produced by different plant species varies widely in terms of its suitability as a source of protein for developing brood (Roulston, et al., 2000), therefore one might question as to whether it would be adaptive for bees to have some form of mechanism for assessing pollen quality, which might in turn maximise foraging efficiency in terms of the total amount of protein returned to the colony or nest. The majority of studies designed to test for such an ability have either monitored the pollen collection preferences of naturally foraging bees, or observed fluctuations in colony foraging effort when different pollen types are made available for collection. Such studies have thus far yielded contradictory findings, which can be resolved by assessing the sensory and learning capabilities of individual bees in terms of their evaluation and discrimination between different pollen species. Here I show that individual bees distinguish between different pollen samples and can associate differences between samples with both coloured stimuli and the visual characteristics of pollen itself.

The present experiments show that whilst bees are able to distinguish between pure and diluted pollen, preferences for one over the other are not uniform. Whilst one would predict that bees should have a preference for pure pollen, given that this is of higher quality in terms of nutritional content, nearly half of the bees tested had a preference for the diluted pollen which they had experienced during pre-training. Thus under the conditions tested here, it seems that pre-exposure to pollen of a certain quality can substantially influence individual pollen preferences.

Foraging decisions in experiments in which bees were given the choice between diluted and undiluted pollen may have simply been based on the presence or absence of alpha cellulose, thus making discrimination relatively easy. However bees also showed clear preferences between pollen samples diluted to varying degrees, typically with the predicted preference for the less diluted sample. Interestingly, not all bees preferred the less diluted, more familiar pollen type and patterns of preference were seen to change

with repeated exposures to different pollen samples. After departing from a particular sample dish, bees were more likely to re-land on the same dish and continue collecting pollen when they had been foraging on their preferred sample type, whereas upon departing from the less preferred pollen type, individuals were more likely to switch and sample pollen from another dish. This finding further reinforces the notion of individual bees being capable of discriminating between samples and making active choices regarding which type of pollen to collect.

The strength of preference for one pollen type over another was dependent on the difference in pollen content between the samples offered. Bees offered a choice between 90% and 60% pollen showed stronger preferences than those offered samples with a smaller difference in pollen content. Where the difference in pollen content was large, individuals were reluctant to make landings on the weaker sample, suggesting that bees are able to assess pollen cues prior to alighting. Adding alpha cellulose to pollen leads to a change in both the appearance and the odour emitted by the samples. Under the current experimental set up, bees seemed to rely mainly on visual cues to discriminate between samples, suggesting that visual differences provided a more salient cue than differences in olfactory intensity.

This is in line with the findings of Dobson et al. (1999) who observed that in experiments in which the anthers of *Rosaceae* flowers were removed, bees assessing pollen availability were more reluctant to land on these manipulated flowers relative to intact controls, Whilst supplementation of antherless flowers with pollen odours improved landings relative to manipulated flowers lacking in pollen odour, the number of landings never reached that of intact flowers unless the visual component of the pollen display was also restored, as evidenced through comparison with the number of visits made by pollinators to odour supplemented flowers, in which the anthers had been emptied of pollen. Similarly, when artificial pollen odours were added to 2-day old flowers, thus producing a mixed-age androecium, bees appeared to be confused and spent longer hovering above the flower prior to alighting, again suggesting that visual signals from the androecia may be important in guiding pollen foraging behaviour.

In ancestral angiosperms, pollen served as one of the primary attractants to insects, with flowers typically possessing a showy androecium, with pollen clearly on display to flower visitors (Crepet, et al., 1991). Today, in contrast, many modern flowers have

pollen which is hidden from the view of the pollinator. Concealment of pollen, such as in the poricidal anthers of buzz-pollinated species, undoubtedly serves to limit pollen removal by individual insects and minimise wastage (Buchmann, 1983; Buchmann, et al., 1977; Castellanos, et al., 2006; Harder & Barclay, 1994; Harder & Thomson, 1989; Vogel, 1978). The current findings, coupled with suggestions from the previous chapter that bees may be conditioned to the visual characteristics of pollen itself, suggest that pressure to curtail pre-alighting assessment of pollen availability or perhaps even quality, may have also contributed to the trend towards pollen concealment in the evolution of floral morphology (Harder, 1990).

When pollen samples in the matched-cues test differed only in the intensity of odour emitted, there was no evidence of a preference for one sample over another, suggesting that bees did not distinguish between samples on the basis of olfactory cues alone. This may be because the relatively large volumes of pollen on offer led to a saturation of the olfactory environment, though studies with moths have shown that insects have the capacity to locate an olfactory target in a saturated environment (Balkenius & Dacke, 2010). Under natural conditions, it seems quite likely that the odour of pollen would provide bees with an additional cue to both the quantity, and possibly the specific nature of pollen offered by different flowers (H. E.M. Dobson, et al., 1999; H.E.M. Dobson, Groth, & Bergstrom, 1996). Dobson (1988) has shown that pollen species posses a unique signature scent, which originates from the neutral lipids which make up the outer layer of the grains. However, whilst several studies have shown that bees can discriminate between pollen species on the basis of olfactory cues (H. E. M. Dobson, 1987; Von Aufsess, 1960; von Frisch, 1923), this does not necessarily mean that bees either can or indeed do use such cues to inform foraging decisions prior to landing. Conclusive evidence still needs to be provided that the signature scent of a particular pollen type provides an additional cue which bees learn to associate with the collection of pollen. The present study indicates that in the presence of salient visual differences between pollen samples, bees choices were unaffected by variations in odour intensity.

There was no correlation between individual preference scores and the total duration of time spent foraging, suggesting that those bees which displayed a preference for the weaker pollen type did not collect larger pollen loads to counteract the higher concentration of nutritionally poor alpha cellulose grains. However, caution should be taken in assuming that foraging time equates to the amount of pollen collected, since a

high degree of variability in the collection times of individual bees was observed across all experiments. von Frisch (1942) also noted of bees collecting pollen from dishes in his experiments that 'whereas one accumulates huge masses of pollen in a short time, another needs five times as long for the same amount, and a third shows a very poor achievement after having worked as long as the other two together'. Thus a more accurate, if laborious measure, would be to remove the pollen loads of foragers and compare the ratio of pollen to alpha cellulose grains to determine whether the ratio is consistent across bees preferentially collecting different sample types.

The generally observed preference for 90% pollen over weaker dilutions may not result from selection of the most concentrated sample, but rather individuals may have chosen to collect 90% pollen because they had more experience of collecting this sample during pre-training. When offered the choice between two novel pollen samples, preferences were more variable between individuals. Whilst some bees collected only pure pollen, the same number of individuals collected equally from both samples. Some bees preferred 80% pollen, possibly because they had generalised from the diluted pollen experienced during pre-training. Pollen collection is a complicated behaviour which takes time to learn (Raine & Chittka, 2007b), and therefore bees may prefer to collect pollen with which they have had previous experience of packing. Switching between flower species has been shown to incur a cost for nectar foragers (Heinrich, 1979; Laverty, 1980), and the same is likely to be true for pollen foragers and thus could explain why some individuals in the current experiment remained constant to diluted pollen even when offered pure pollen as an alternative.

Following multiple exposures to diluted pollen samples, the generally observed preference for 90% pollen was seen to decline, with the majority of bees beginning to collect more of the alternative pollen sample (either 70% or 60% pollen) over the course of the experiment, leading to a mean preference score close to zero by the fifth trial, for both groups tested. Even the weakest dilution available (60% pollen) contained a relatively large volume of pollen and therefore may have been deemed an acceptable reward by bees once they had experience of collecting it. However, the degree to which bees experimented with the alternative sample over the course of five trials depended somewhat on the level of dilution. For half of the bees tested, the preference for 90% over 60% pollen was still fairly high following multiple exposures, whereas when offered the choice of 90% *vs.*70% pollen, the majority of bees had a moderate to strong

preference for 70% pollen by the fifth trial. Rasheed and Harder (1997) have argued that bumblebees tend to maximise pollen collection efficiency, accounting for the associated temporal and metabolic costs as opposed to simply maximising the amount of pollen collected per flower. It may well be that the higher proportion of alpha cellulose in 70% pollen means it easier to pack into the corbiculae, making collection more efficient and thus driving the switch in preference following multiple exposures.

I also tested whether bees could learn to associate a coloured stimulus with the quality of the pollen reward provided. I expected that the presence of an additional visual cue to pollen identity would lead to bees foraging constantly on one sample, improving the recognition of the different pollen types, potentially through an increase in the complexity of the signal (Gegear, 2005; Leonard, Dornhaus, & Papaj, 2011). Bees offered the choice between 90% and 70% collected from both sample types throughout the course of training, and were not observed to shift their colour preference towards the colour paired with the more concentrated sample (90%, Blue). In contrast to the previous experiment, in which bees were observed to more readily collect 60% pollen following repeated exposure, in the presence of additional coloured cues bees became constant to 90% pollen over 60%, making few visits to the weaker pollen sample over the course of training. Furthermore, unlike bees offered the choice between 90% and 70% pollen, bees in the 90% vs. 60% group were observed to shift their colour preference towards that of the stimulus which was paired with 90% pollen. This result suggests that, dependent on the differences in the quality of the pollen rewards offered by flowers, the learnt relationship between visual floral cues and pollen quality might limit the exploration of novel food sources to a certain extent, thus promoting flower-constant behaviour.

Grüter et al. (2011) suggest that in order to observe pollen constancy in the laboratory, it is necessary to use realistic rewards, of a similar value to those offered naturally by flowers. Again the difference in foraging behaviour between bees offered the choice of 90% *vs.* 70% and 90% *vs.* 60% most likely stems from the fact that 70% pollen, as the weaker concentration, still contains a large volume of pollen. However, given that bees collected from both sample types over the course of training, the question remains as to why bees didn't learn both coloured cues and respond to them equally in the unrewarded test. Dual-reward experiments with nectar-foraging bees indicate that, despite their excellent colour learning abilities (Menzel, 1967, 1968), under certain

conditions, initial colour preferences are not easily overridden. Heinrich (1977), for example, found that whilst bumblebees could easily switch from visiting white flowers to blue when the reward offered by white flowers was diminished, in the reciprocal experiment bees found it extremely difficult to curb their visits to blue flowers. Likewise, Banschbach (1994) observed that the ability of honeybees to learn about artificial flowers offering different rewards is dependent on the difference in sugar concentration between flowers. Bees showed no shift in colour preference away from blue towards yellow when flowers contained 10% and 20% sucrose respectively, but when flowers contained 10% and 30% sucrose, preference for yellow increased. This is analogous to the current finding that when two types of pollen reward were available, a shift away from the initial colour preference is apparent only when the pollen content was sufficiently different between samples.

When cues from the coloured surround and pollen itself were put in conflict, by switching pollen samples between the coloured stimuli with which they were associated during training, it seemed that bees may have been guided more by the coloured surround than the visual appearance of pollen itself, though these observations need to be confirmed by testing more individuals. In summary, these results suggest that additional floral features, such as petal colour, might aid bees in indentifying sources of their preferred pollen type and could potentially influence foraging choices, as has been shown for nectar foraging bees.

The question as to exactly how individual bees discriminated between samples remains open. Previous studies have found that the protein and amino acid content of pollen correlates with the collection preferences of bees (Cook, et al., 2003; Hanley, et al., 2008) and it has been suggested that foragers can selectively increase visits to flowers providing pollen with the highest protein concentration in response to colony demand (Fewell & Winston, 1992). However, other tests of preference have failed to corroborate these findings (Levin & Bohart, 1955; Pernal & Currie, 2001, 2002; J. O. Schmidt, 1982; Wahl, 1966). Kitaoka & Nieh (2009) observed that a bumblebee colony is more likely to hoard pollen when that available for collection contains a higher ratio of pollen to alpha-cellulose. The present results confirm that individual bees are indeed sensitive to differences in the ratio of pollen to alpha cellulose, although with increasing experience, bees are more likely to collect samples containing weaker concentrations of pollen. As a result, it seems unlikely that bees in this study based their preferences on

the availability of protein. It is possible that alpha cellulose has specific characteristics, such as mechanical properties which affect the way in which diluted pollen packs into the corbiculae. Bees are known to be sensitive to various physical properties of pollen, such as grain size, moisture content and the electrostatic charges of the grain surface (Chaloner, 1986; E. H. Erickson & Buchmann, 1983; Stanley & Linskens, 1974; Vaissière & Vinson, 1994; Vaknin, Gan-Mor, Bechar, Ronen, & Eisikowitch, 2000) and so perhaps differences in physical attributes such as these were used to distinguish between samples. Batra (1993) observed that bumblebees would avoid foraging on potato cultivars providing shrivelled, non-viable pollen grains, and therefore even if bees are unable to determine the protein content of pollen samples directly, sensitivity to physical properties could still aid bees in the selection of higher quality pollen, assuming that such properties serve as reliable signals of nutritional value, as has been shown for grain size (Roulston, et al., 2000).

The results presented in the previous chapter, coupled with the findings of several field experiments, indicate that bees have the capacity to discriminate between flowers on the basis of pollen availability (Armbruster & Herzig, 1984; Armbruster & Webster, 1982; Buchmann & Cane, 1989; Cresswell & Robertson, 1994; H. E.M. Dobson, et al., 1999; Eckhart, 1991; Galen & Plowright, 1985; Gori, 1989; Harder, 1990; Pellmyr, 1988; Wainwright, 1978; Zimmerman, 1982). The present experiments show that bumblebees may also be able to associate differences in pollen quality with salient floral features such petal colour. This would permit bees to identify those plants providing their preferred pollen type over a longer range than that permitted by the olfactory or visual cues emitted by pollen itself. Field evidence in support of this comes from Robertson et al.'s (1999) observation that bees foraging on *Mimulus guttatus*, a species polymorphic in the quality of pollen it produces, were able to discriminate between patches of flowers producing high and low quality pollen. Flowers producing pollen of intermediate quality located in patches of low quality flowers were visited preferentially by bees, but intermediate morphs dispersed in a high quality patch received far more visits than would be predicted by chance, suggesting that differences in pollen quality must be sufficiently great in order for bees to discriminate between morphs, a result mirrored in the colour learning experiments presented here.

The ability to discriminate between flowering plants on the basis of perceived pollen quality could enable pollen foraging bees to select and efficiently exploit highly

rewarding flowers. Such behaviour could potentially lead to selection on flowering plants in terms of the quality of pollen they provide for visiting insects (Hanley, et al., 2008) In general, plant species which are obligately pollinated by insects do possess pollen that is richer in protein than those that rely on wind pollination, though the primary role of pollen as an agent for gamete transmission most likely imposes constraints on the degree of selection by pollinators (Roulston, et al., 2000; Simpson & Neff, 1983). Hanley et al. (2008) found that across a number of habitats, plants which provide more protein in their pollen receive more visits from bees, regardless of pollen abundance. Robertson et al. (1999) observed that protein content, but not abundance or grain size, most strongly predicted the foraging preferences of bumblebeess. Flower constancy for pollen collection seems to involve decision-making processes that are mediated by a variety of sensory mechanisms, potentially including mechano-sensory feedback during pollen packing in the corbiculae and the ability to associate floral features with different types of pollen during collection. Further studies are needed to reveal more about perceptual mechanisms underlying the evaluation of pollen and the sensory organs involved in pollen-rewarded learning in bees.

Chapter 5: Does pollen reinforce learning in a classical conditioning paradigm?

Abstract

The proboscis extension response (PER) paradigm was adapted in an attempt to isolate the sensory organs involved in pollen-reinforced learning. Though bees were observed to extend the proboscis when the antennae were touched with pollen, I found no evidence that they learnt to associate this reward with the delivery of a neutral odour, either when pollen was applied in its natural state or when mixed with water. Responsiveness to antennal stimulation with pollen was observed to decline slightly over the course of the experiment, even when bees were prevented from consuming the reward, suggesting that honeybees may use a pre-ingestive mechanism for detecting pollen compounds. Previous studies have found that supplementation of the traditional sucrose reward with certain amino acids leads to an improvement in olfactory learning, thus I asked whether adding pollen, an important source of nitrogen-based compounds for bees, to sucrose might also improve acquisition in an olfactory conditioning task. The addition of pollen actually resulted in an inhibition of responding to the conditioned stimulus, relative to bees rewarded with pure sucrose, leading to the suggestion that distasteful compounds present in pollen may actually devalue the sucrose reward.

5.1 Introduction

Bees have been shown capable of learning visual cues associated with a pollen reward (Chapters 3 and 4) but the sensory and learning mechanisms involved in this reinforcement pathway remain as yet unknown. Since bees collect pollen primarily as a source of protein for developing brood, it has been suggested that nitrogen-based compounds might represent a core reinforcing component of the pollen reward. However, as discussed in the previous chapter, considerable debate exists as to whether bees are even able to detect the presence of protein or amino acids in pollen during

collection (Fewell & Winston, 1992; Kitaoka & Nieh, 2009; Mapalad, et al., 2008; Pernal & Currie, 2001, 2002; A. W. Robertson, et al., 1999). Such compounds may not necessarily be the only compounds present in pollen that bees are able to sense. Pollen also contains lipids, fatty acids and various vitamins and minerals (Day, Beyer, Mercer, & Ogden, 1990; McLellan, 1977; Solberg & Remedios, 1980; Somerville & Nicol, 2002; Stanley & Linskens, 1974; Todd & Bretherick, 1942), many of which are essential for growth and development (Brodschneider & Crailsheim, 2010; Manning, 2001). Whilst some evidence suggests that bees prefer lipid-rich pollens (Singh, et al., 1999), the ability to detect such substances remains largely untested (though see Pernal & Currie, 2002). Many pollen species also contain small quantities of sugar (Stanley & Linskens, 1974), and the bee-collected pollen used in the current experiments may contain substantially more, given that bees often add nectar to pollen in order to ensure that grains adhere to each other and the corbiculae (Casteel, 1912; Thorp, 1979). The only study to date in which differences in the sugar content of hand-collected and bee-collected pollen have been estimated systematically found that the amount of sugar added by bees is highly variable (Roulston, et al., 2000). Should bees possess the capacity to detect sugars that are not dissolved in water, then it is possible that pollen may activate the same reward pathway as nectar.

Bees have gustatory receptors located on the antennae, mouthparts and tarsi (see de Brito Sanchez & Giurfa, 2011 for review) and so are likely to have ample opportunity to sample pollen during the process of collection. Foragers appear to probe the anthers of flowers with their antennae and have been observed to remove dislodged pollen with the proboscis (Casteel, 1912). Thorp (1979) notes that biting behaviour is not uncommon during attempts to manipulate anthers, and describes a particular behaviour termed ‘milking’ where bees grasp the base of the anthers with their mandibles and scrape upwards, thus removing pollen grains from the surface (Thorp & Estes, 1975). Direct contact between the proboscis and pollen grains thus seems to occur frequently during pollen collection (Casteel, 1912 and references therein)

Scheiner et al. (2004) have shown that stimulation of the antennae with hand-collected pollen grains leads to an extension of the proboscis, an appetitive reaction termed the proboscis extension response (PER). The PER can be also elicited when the antennae are touched with a sufficiently concentrated sucrose solution and this has frequently been used as the unconditioned response in a learning paradigm in which honeybees are

restrained to restrict movement. The unconditioned stimulus (US) is paired with a neutral odour (conditioned stimulus) and following several training trials, presentation of the previously neutral odour alone becomes sufficient to elicit the PER (Bitterman, et al., 1983; Takeda, 1961). Substituting pollen for the typical sucrose reward, it is possible to test whether stimulation at the antennae or proboscis reinforces learning in this well established olfactory conditioning paradigm.

When visiting flowers pollinators frequently dislodge pollen grains from the anthers, and groom pollen from their bodies, some of which subsequently ends up in the nectar of those flowers which provide both rewards. Thus bees may also encounter pollen as a contaminant of nectar. When pollen grains are mixed with sucrose solution they burst as a result of osmotic shock, rapidly releasing their nutritional contents (Buxbaum, 1927; Erhardt & Baker, 1990; Gottsberger, Schrauwen, & Linskens, 1984). Whilst nectar naturally contains traces of chemical compounds other than sugars (H. G. Baker & Baker, 1973, 1977), contamination with pollen often leads to a considerable change in chemical composition, most notably an increase in the concentration of amino acids present (H. G. Baker & Baker, 1986; Erhardt & Baker, 1990; Gottsberger, et al., 1984). Feeding preference tests have shown that nectars containing certain amino acids are more attractive to bees (Alm, et al., 1990; Carter, et al., 2006; Kim & Smith, 2000) and the supplementation of artificial sugar solutions with amino acids has been shown to improve learning. Kim and Smith (2000) for example, found that the addition of glycine, a common component of both nectar and pollen, to the sucrose US in an olfactory PER paradigm, led to an enhanced conditioned response and resistance to extinction relative to a group which received sucrose alone. Wright et al. (2009) also demonstrated, via differential reinforcement, that supplementing the sucrose US with proline leads to an improvement in learning. In the present experiments I compared the acquisition of pollen-rewarded bees to those rewarded with sucrose in an olfactory learning task.

5.2 Methods

5.2.1. Subjects

Departing honeybee foragers (*Apis mellifera)* were collected from colonies located at Washington Singer Laboratories, University of Exeter. Individual bees were captured in small glass vials and placed on ice until they stopped moving. Bees were then transferred and restrained in metal harnesses which permitted free movement of the antennae and proboscis. Each subject was fed 30% (w/w) sucrose solution until satiated and then left for approximately 20 hours prior to conditioning.

5.2.2 Stimuli

The conditioned stimulus for all experiments was 1-hexanol (98% purity) diluted in mineral oil to 2.5 M. 20 ml of the odour solution was placed in a 60 ml glass bottle which was connected to an air pump via silicone tubing. The air stream was gated by a PLC-controlled valve, in order to ensure delivery of uniform odour puffs (see also Smith, 1998). The odour stream was directed frontally at the head of the bee, and removed via a constant air stream, emitted from an extractor system located behind the animal. The pollen reward consisted of commercial honeybee collect pollen (Werner Seip, Germany), ground to a fine powder and either delivered as a dry powder, or mixed with water or sucrose solution (30% pollen w/w). Pollen solutions were passed through filter paper to remove the larger clumps which were found to stick to the antennae and interfere with the experiment. The sucrose reward was either a 30% or 15% (w/w) sucrose and water mixture which was also filtered.

5.2.3 Pre-training sensitivity test

Prior to conditioning, bees were tested for their motivational state and sensitivity to pollen. The antennae of each subject were touched first with water, with those

individuals displaying proboscis extension permitted to drink until satiated. Antennae were then touched with 30% pollen-water solution, followed by water, 15% sucrose, and finally 30% sucrose, each delivered at five minute intervals. For those bees conditioned with dry pollen, this was substituted for the 30% pollen-water solution. Dry pollen was delivered via a small sponge (1 cm length) which was replaced following each experimental block. Only bees that responded to both pollen and sucrose solutions were included in subsequent experiments. Conditioning began approximately twenty minutes after the sensitivity test to allow any potential effects of sensitization to 30% sucrose to subside (Menzel, Hammer, Braun, Mauelshagen, & Sugawa, 1991).

5.2.4 General conditioning protocol

An individual bee was placed in the experimental arena at a distance of 4.5 cm from the odour delivery tube and left to habituate for 15 seconds prior to the conditioning procedure. The odour (CS) was delivered to the antennae for three seconds prior to US delivery and overlapped with the US for a further second. US delivery then continued for a further two seconds. Depending on experimental design, the US was either presented first to the antennae and then the proboscis or to the antennae alone. Bees were left in the arena for a further 15 seconds before being removed. The inter-trial interval was ten minutes for all experiments.

5.2.5 Dry pollen conditioning

To determine whether antennal contact with pollen is sufficient to serve as a reward for learning, the CS (2.5 M hexanol) was paired with delivery of dry pollen at the antennae only (US). To control for the effects of pollen odour, one clean and one pollen-coated sponge were taped together in a cross formation. Bees received either stimulation with the pollen sponge (pollen group) or the clean sponge (control group). In addition, a small petri dish (5.5 cm diameter) containing *ca.* 3 g of pollen was also placed between the bee and the syringe delivering the CS to create a standardised olfactory environment for all groups. A third group of bees received 30% sucrose, which was presented via a

cocktail stick. Performance of this group provided a baseline against which to compare the performance of both control bees and those rewarded with pollen.

After six conditioning trials bees received an unrewarded test trial, in which only the CS was presented. Proboscis extensions were noted prior both the presentation of the CS alone and during US delivery.

5.2.6 Pollen in water

To determine whether pollen in solution can serve as a US, three groups of bees were tested. One group were exposed to a forward pairing of the olfactory CS and pollen US. This US consisted of 30% pollen in water (w/w), which was found to be the strongest concentration that can be applied without clogging the antennae (see Chapter 2 for more details). A control group, which received unpaired presentations of the CS and pollen US, was included to rule out the possibility that any observed increase in response to the CS was simply due to non-associative effects of repeated antennal stimulation. As a positive control, a third group received forward pairing of the same olfactory CS with 30% sucrose as the US. This group served as a baseline against which to compare learning performance.

In forward paired trials, the odour (CS) was delivered first followed by the US (30% pollen-water or 30% sucrose solution), with an overlap of one second. The US was first presented to the antennae to elicit the proboscis extension response (PER) and then to the proboscis for consumption. In control trials, the pollen-water US was delivered first for three seconds. Following a delay of ten seconds, the odour (CS) was presented for four seconds.

All bees received six conditioning trials. In the pollen-US group, bees initially received three forward paired trials as described above. In the control group, bees were exposed to three unpaired presentations of pollen. Both groups then experienced three further trials where 30% sucrose solution (US) was forward paired with the CS, to control for the possibility that any absence of learning observed when bees were rewarded with pollen was the result of reduced motivation or fatigue. The third group of bees were rewarded with 30% sucrose solution on all six trials.

5.2.7 Pollen in sucrose

The conditioning procedure was similar to that described previously, except that the US was consistent across all six trials. Bees received either 30% sucrose solution, 15% sucrose solution, 30% pollen and water, or a mixture of 15% sucrose and pollen (30% Pollen w/w). Bees rewarded with 15% sucrose were expected to show a slower rate of acquisition compared with those receiving 30% sucrose, since sucrose concentration is known to influence learning (Loo & Bitterman, 1992). Thus a comparison against this group would permit the detection of any improvement in learning in bees rewarded with a mixture of 15% sucrose and pollen. In the first experiment bees were rewarded first at the antennae and then the proboscis. In a second experiment bees were rewarded at the antennae only, to distinguish between pre and post-ingestive effects of the pollen US.

5.2.8 Statistical Analysis

Subject responses were scored as binary variables. To scrutinise the learning effects, bees that spontaneously responded to the odour on the first trial were excluded from the analysis where possible, i.e. in the dry pollen and pollen in sucrose experiments. GEE (Generalized Estimating Equation) modelling was used to compare the acquisition curves of bees trained with different unconditioned stimuli (G. A. Wright, et al., 2007). The GEE approach is an extension of the Generalized Linear Model (GZLM) which permits a non-normal distribution of the dependent variable (i.e. binary distribution) and accounts for repeated measurements of the same individual (Hardin & Hilbe, 2002). Response to the CS was coded as the response variable with treatment and conditioning trial included as factors. Significance tests were based on Wald approximations of the likelihood ratio test. Post-hoc least significant difference contrasts (LSD) were used to compare treatment groups. Bees performance in unrewarded tests were compared using Generalised Linear Models (GZLM).

5.3 Results

5.3.1 Does antennal stimulation with dry pollen reinforce learning?

In all treatment groups an increase in response to the CS over the course of training was observed (Fig. 5.1a), though there was a significant difference in the overall level of acquisition between treatment groups (GEE, Treatment X^2_2= 6.480, p=0.039). Post-hoc analysis revealed a significant difference between bees rewarded with dry pollen and those rewarded with 30% sucrose solution (LSD contrast p=0.009). Likewise, in the unrewarded test, there is a significant difference between treatment groups (GZLM, Treatment X^2_2=10.919, p=0.004). Of those bees conditioned with sucrose, significantly more responded with proboscis extension during the test than bees in the other two groups (LSD contrast, Sucrose *vs.* Pollen p<0.001, Sucrose *vs.* Control p=0.015). There was no significant difference in responding between control bees and those rewarded with pollen (LSD contrast, Control *vs.* Pollen p=0.311), suggesting that dry pollen does not reinforce learning in an olfactory conditioning paradigm.

To determine whether the coating of pollen had any effect on bees in this experiment, responses to antennal stimulation with the clean and pollen-coated sponge were compared (Fig. 5.1b). Whilst pollen initially elicited a higher proportion of proboscis extensions than the clean sponge (*ca.* 70% for pollen *vs.* 40% for clean sponge, GZLM, Trial 1, Treatment X^2_2=6.349, p=0.042, LSD contrast p=0.016), over the course of the first three exposures, responses converged to a level of between 60-70% (GZLM, Trial 6, Treatment X^2_2=6.349, p=0.042, LSD contrast p=0.20). The response of bees stimulated with 30% sucrose remained consistently high throughout the course of the experiment.

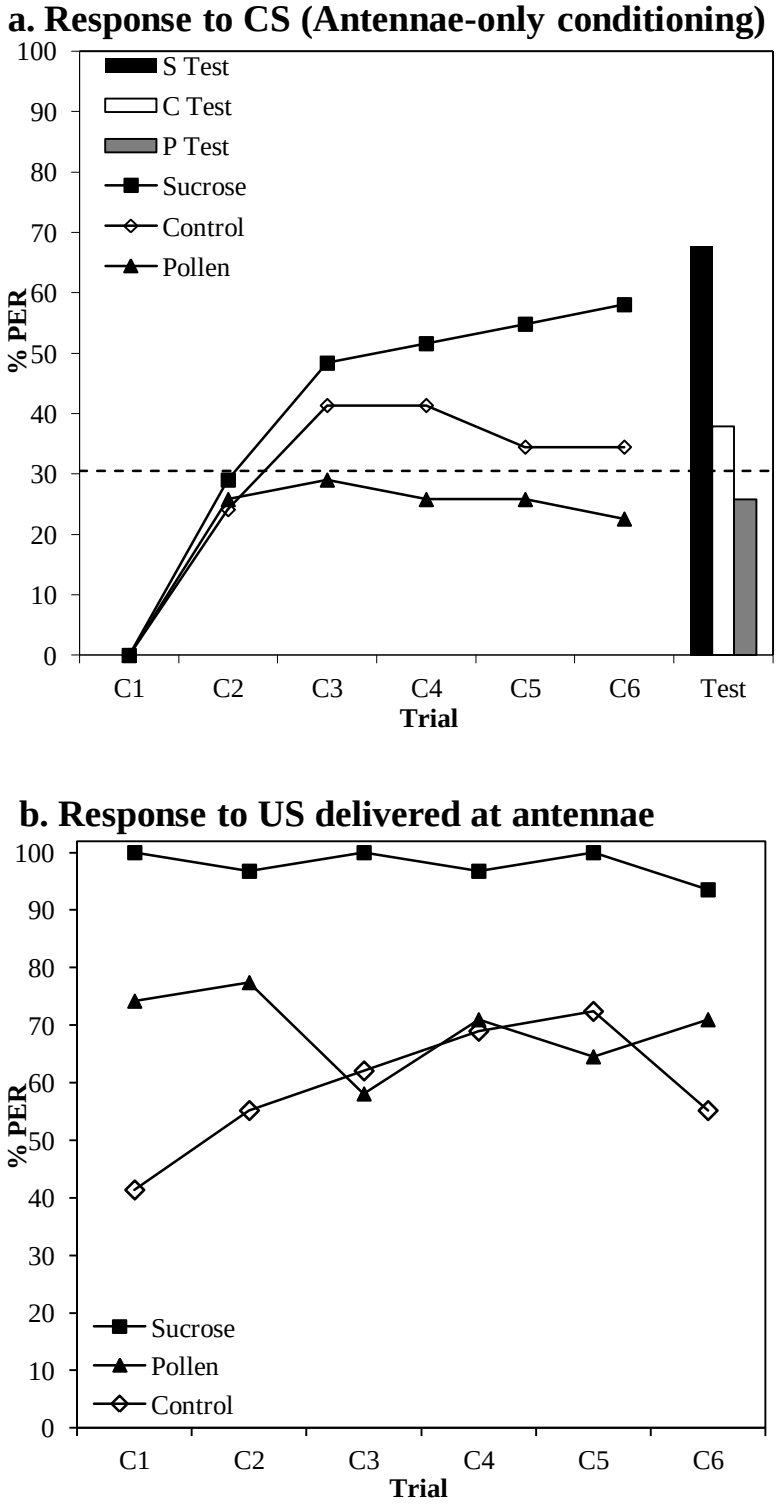


Figure 5.1 (a) Acquisition curves and (b) PER response to US stimulation at the antennae in bees rewarded at the antennae only with 30% sucrose solution (Black squares, *n*=31), dry pollen (Black triangles, *n*=31) or a clean sponge (Open diamonds, *n*=29). Bars represent the number of bees responding to the CS in the final, unrewarded test (Black=Sucrose, White=Control, Grey=Pollen). Dashed line represents the overall spontaneous response to the CS on the first trial for all bees tested.

The high level of response to both the CS and US in bees stimulated with the clean sponge was unexpected. One potential explanation is that this was driven by spontaneous responding to olfactory cues from the pollen dish placed in front of bees on the arena floor. The purpose of the dish was to standardise the olfactory environment between the three treatment groups, though the pollen odour may also have aroused bees, leading to a higher than expected response to the CS and US. However, in additional tests, no significant difference was observed between bees conditioned in the presence or absence of the pollen dish (see Fig. 2.1, Chapter 2), in terms of their response to either the CS or US. This was true for both sucrose-rewarded and control bees. Whilst sucrose-rewarded bees learnt the association, bees stimulated with a clean sponge showed little change in their response to the CS over time, yet displayed a relatively high level of US response as in the present experiment. Thus olfactory cues provided by the pollen dish can be ruled out as an explanation for the higher than expected CS and US response in control bees (see Chapter 2 for more details).

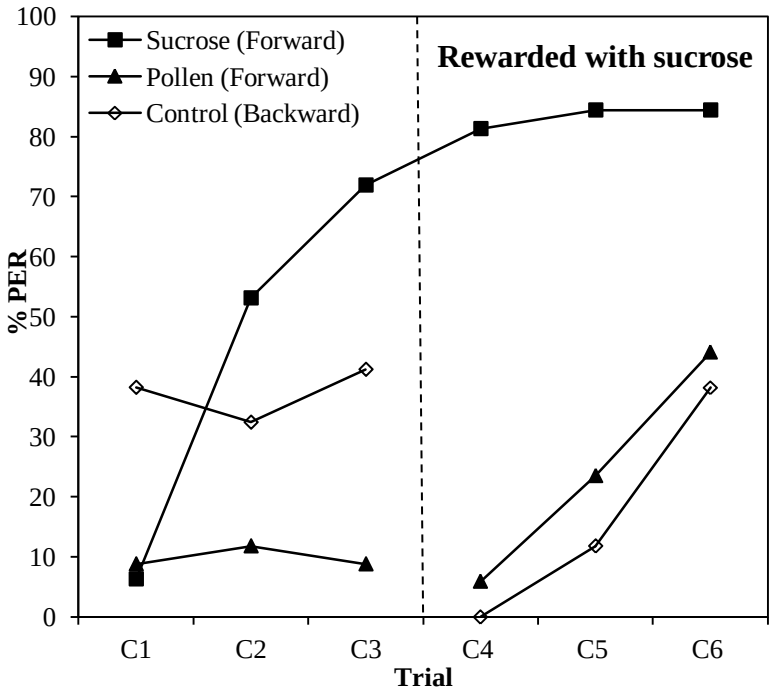


Figure 5.2 Proportion of bees extending the proboscis to a previously neutral odour following pairing with different unconditioned stimuli. For trials 1-3, the US was either 30% pollen (w/w) solution (Black triangles, forward paired, *n*=34, White diamonds, backward paired, *n*=34) or 30% sucrose (w/w) solution (Black squares, *n*=32). All bees received 30% sucrose on trials 4-6. The US was applied to both the antennae and proboscis.

5.3.2 Does pollen in solution reinforce learning?

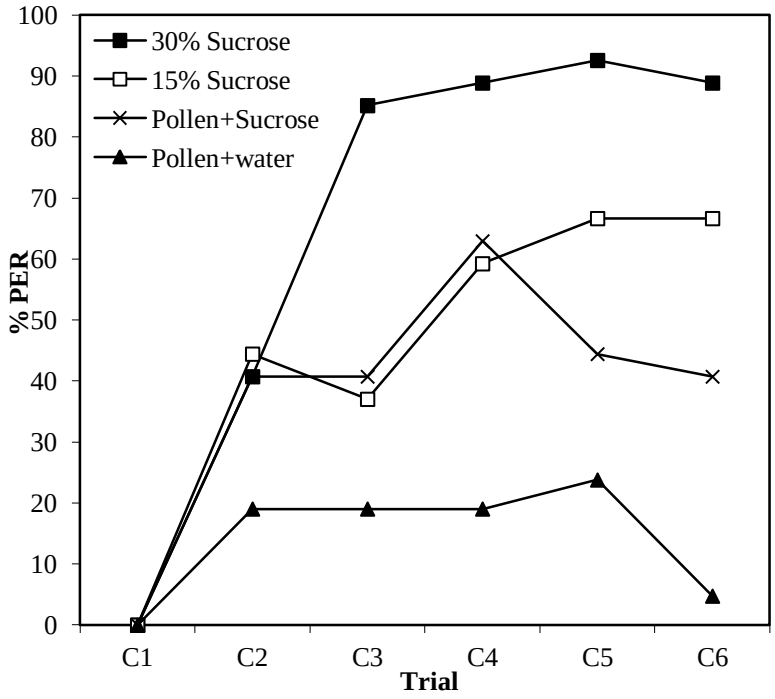
There was a significant effect of treatment on responding to the CS over the first three conditioning trials (Fig. 5.2, GEE, Treatment X^2_2=14.393, p=0.001; Treatment x Trial X^2_4=8.894, p=0.001). Whilst bees rewarded with sucrose showed a significant increase in response to the CS between trials one and three (Sucrose, C_1 *vs.* C_3, p<0.001), bees rewarded with pollen did not show any change in response over these three trials (Pollen (paired), C_1 *vs.* C_3, p=1.000; Control (unpaired), C_1 *vs.* C_3, p=0.479), suggesting pollen failed to support learning of the predictive relationship between CS and US delivery. The high level of spontaneous response to the CS displayed by control bees can be explained by the reversed presentation of stimuli. Since control bees were initially stimulated with the pollen US, often bees already had the proboscis extended when the CS was presented. However, on the first forward-paired trial with 30% sucrose (C_4) no response to the CS is observed, confirming that bees in this group had not acquired an association between the CS and pollen US.

In the last three training trials (4-6), bees in all groups were rewarded with sucrose and received forward pairings of the CS and US. Bees that were previously rewarded with pollen (control and pollen groups) showed a significant increase in responding to the CS over the course of the final trials (GEE, Trial X^2_1=19.384, p<0.001; Pollen p=0.001; Control p=0.005), thus demonstrating their capacity to learn the contingency between the CS and US. I observed a significant effect of treatment when comparing CS responding in pollen-exposed bees over trials 4-6, with the response of sucrose-rewarded bees over trials 1-3 (GEE, Treatment, X^2_2=10.953, p=0.004). Whilst there was no difference in response between pollen and control bees over the final trials (LSD contrast p=0.706), the overall level of acquisition in bees rewarded with sucrose over the first three trials was significantly greater than those bees previously exposed to pollen (LSD contrast, Sucrose *vs.* Control, p=0.001; Sucrose *vs.* Pollen, p=0.005). It seems that pre-exposure to pairings of the CS and pollen US may have retarded acquisition in bees in the pollen and control groups, potentially via a process known as latent inhibition, whereby repeated exposure to the CS, in the absence of a reward (or in this case, an effective US), impairs subsequent acquisition (Lubow & Moore, 1959)

5.3.3 Does supplementing sucrose solution with pollen lead to an improvement in acquisition?

When the performance of bees rewarded at the antennae and proboscis with either sucrose, pollen-water, or a mixture of pollen and sucrose were compared, the type of reward was found to have a significant influence on both the rate and overall level of acquisition (Fig. 5.3a GEE, Treatment X^2_3=27.503, p<0.001; Treatment x Trial X^2_{12}=34.6, p=0.001). Post-hoc analysis revealed no difference in acquisition between bees reinforced with 15% sucrose solution and those experiencing the pollen-sucrose solution (LSD contrast, 15% Sucrose *vs.* Pollen-sucrose solution p=0.453) suggesting that the addition of pollen does not lead to an improvement in learning. In fact, from the fifth trial onwards, bees reinforced with the pollen-sucrose solution show a decline in response to the CS, relative to those receiving the 15% sucrose alone. Analysis of the final training trial reveals that the response of bees rewarded with the pollen-15% sucrose mixture is significantly lower than that of bees rewarded with 15% sucrose alone (LSD contrast, p=0.048).

a. Response to CS (Antennae+proboscis conditioning)



b. Response to US delivered at antennae

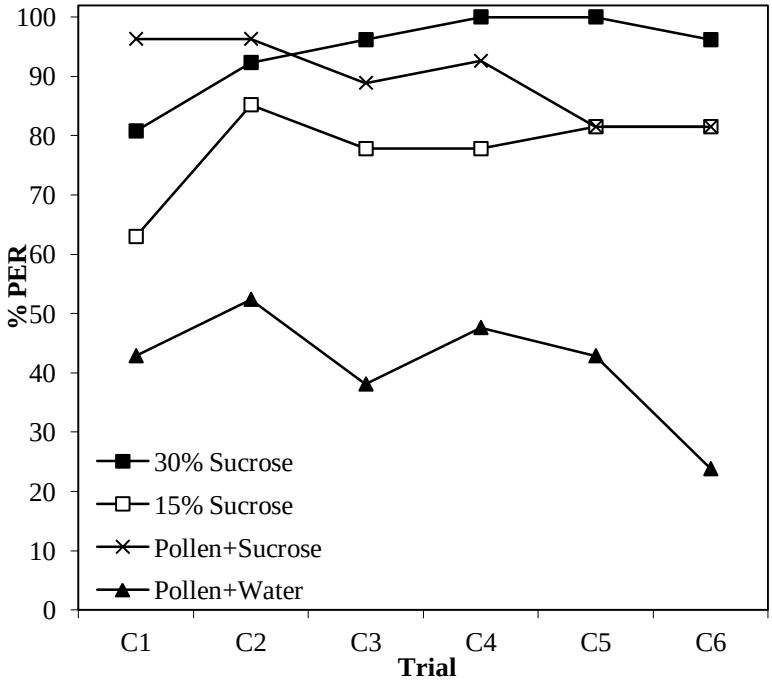
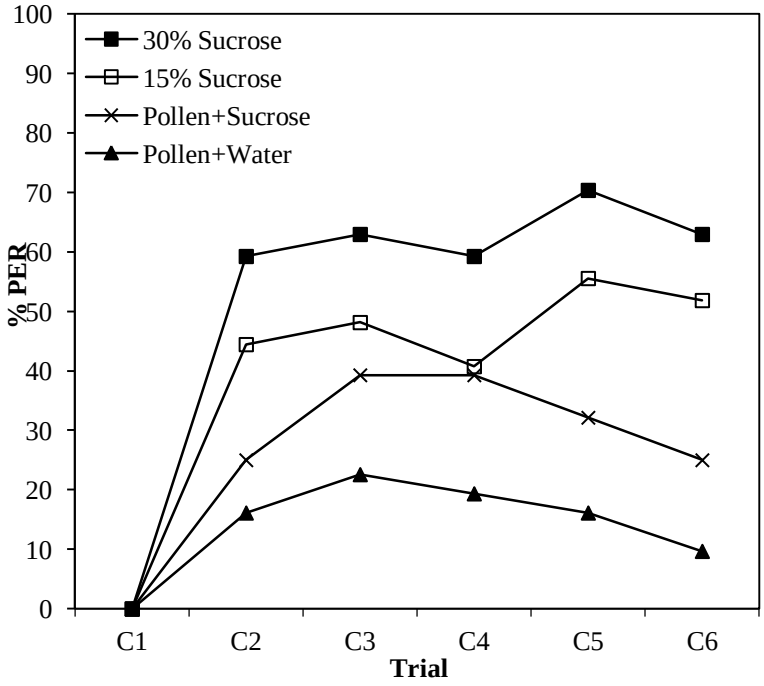


Figure 5.3 (a) Acquisition curves and (b) PER response to antennal stimulation in bees rewarded at the antennae and proboscis with 30% sucrose solution (Black squares, *n*=26), 15% sucrose solution (Open squares, *n*=27), a mixture of 15% sucrose solution and pollen (30% w/w) (Crosses, *n*=27) or 30% pollen in water (Black triangles, *n*=21)

a. Response to CS (Antennae-only conditioning)



b. Response to US delivered at antennae

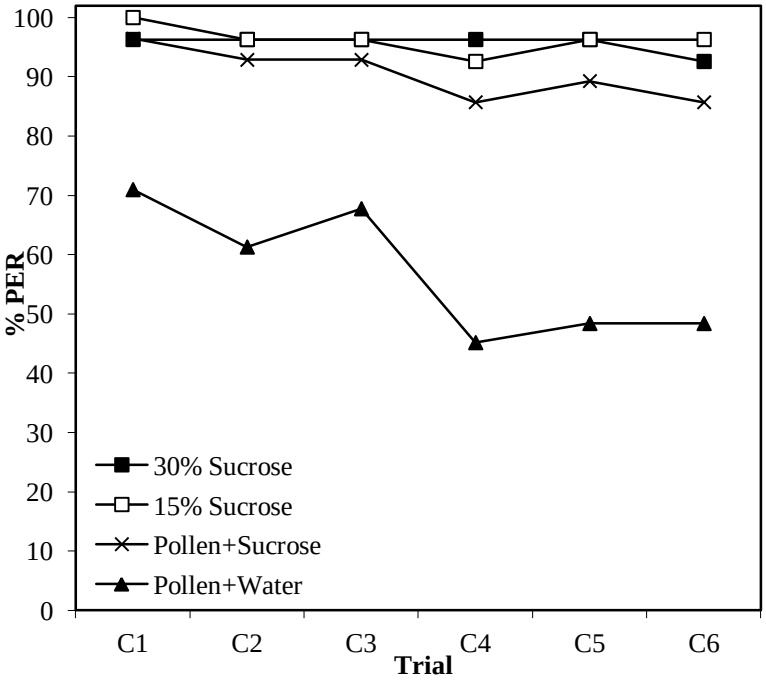


Figure 5.4 (a) Acquisition curves and (b) PER response to antennal stimulation in bees rewarded at the antennae only with either 30% sucrose solution (Black squares, *n*=27), 15% sucrose solution (Open squares, *n*=27), a mixture of 15% sucrose solution and pollen (30% w/w) (Crosses, *n*=28) or 30% pollen in water (Black triangles, *n*=31).

It may be that the apparent decline in response to the CS in bees rewarded with the pollen-sucrose mixture resulted from a lower motivation to extend the proboscis. Therefore I analysed differences between groups in terms of PER to US application at the antennae, prior to when bees were fed at the proboscis (Fig. 5.3b). On the first trial, a significant effect of treatment was observed (GZLM, Trial 1, Treatment, X^2_3=13.495, p=0.004), and interestingly the mixture of pollen and 15% sucrose actually elicited a higher level of responding than 15% sucrose alone (*ca.* 95% compared with 62%, LSD contrast p=0.002). By the sixth trial there was no difference in responding between bees in both groups (p=0.160), and those rewarded with the pollen and sucrose mixture were observed to show a slight decline in response to the US over the course of the experiment (from *ca.* 95% to 80%). On the first trial, *ca.* 40% of bees stimulated with the pollen-water US, responded with PER, significantly less than in all other groups with the exception of those rewarded with 15% sucrose (approaching significance) (GZLM, Treatment, Trial 6, X^2_3=25.210, p<0.001, LSD contrast, 30% Sucrose *vs.* Pollen-water p=0.004, Pollen-sucrose vs. Pollen-water, p<0.001; 15% Sucrose vs. Pollen-water p=0.091). By the final trial, the response of pollen-water bees declined to 25%, significantly lower than that of all other groups, where bees maintained a level of responding of between 80-95% (Trial 6, LSD contrast, all pairings p<0.001).

5.3.4 Are pre-ingestive mechanisms responsible for the inhibition of learning?

The previous experiment was repeated, stimulating bees at the antennae only, to determine whether pre- or post-ingestive effects were responsible for the differences in acquisition between groups. The overall pattern of responding was similar to that observed previously and the type of US received had a significant effect on acquisition (Fig. 5.4a GEE, Treatment X^2_3=25.065, p<0.001). Again there was no difference in terms of overall responding between bees receiving 15% sucrose and those stimulated with a mixture of pollen and sucrose (LSD contrast, p=0.113), though as before, by the final trial the response of sucrose-rewarded bees was significantly greater than that of bees receiving the pollen-sucrose mixture (LSD contrast, p=0.033).

As in the previous experiment, bees receiving sucrose alone maintained a high level of responding to the US over the course of training (Fig. 5.4b). On the final conditioning

trial, a significant difference in level of responding to the US was observed (GZLM, Trial 6, Treatment X^2_3=20.124, p<0.001), driven by the low level of responding in bees stimulated with 30% pollen in water (LSD contrast, 30% Pollen *vs.* 30% Sucrose p<0.001, 30% Pollen *vs.* 15% Sucrose p<0.001, 30% Pollen *vs.* Pollen-sucrose mix, p=0.001). No difference between bees stimulated with the pollen-sucrose mixture and those receiving sucrose alone was observed (LSD contrast, 15% Sucrose *vs.* Pollen-sucrose mix, p=0.161), though again a similar trend of a slight, but steady, decrease in responding to stimulation with pollen and sucrose was observed.

5.4 Discussion

Whilst pollen has been shown to reinforce visual learning in free flying bees, isolating the sensory and learning mechanisms involved in pollen-rewarded learning has proven more difficult. In the experiments presented here I took advantage of a paradigm in which bees are restrained, permitting tighter control over stimulus delivery, with the aim of identifying those sensory organs most likely to be involved in transmitting sensory information about the pollen reward, separate from other processes that may occur during pollen collection in free flying bees.

Antennal stimulation with dry pollen did not support learning of a contingent relationship between this US and an olfactory CS. Nor did bees learn to associate the CS with pollen when it was added to water and delivered in a soluble form. As reported by others (Grüter, et al., 2008; Scheiner, et al., 2004), the proboscis was observed to extend when the antennae were touched with pollen, however multiple pairings of this stimulation with a neutral odour did not, over time, lead to an increased response to the odour alone, regardless of whether or not the proboscis was also stimulated. Therefore I conclude that neither dry pollen, nor pollen in solution reinforces learning in the PER paradigm, and that the proboscis-releasing function of pollen appears to be dissociated from that of the reinforcing function (Menzel, Heyne, Kinzel, Gerber, & Fiala, 1999).

The current findings contrast with those of an early study in which pollen rewarded learning was also investigated using the PER paradigm. Grüter et al. (2008) claim that following three pairings of an olfactory CS and pollen US, bees would learn to respond with PER to the CS alone. Pollen was diluted with water to a concentration of up to

70% (w/w) and was delivered to both the antennae and proboscis. However, this study lacks the appropriate controls necessary to rule out the influence of non-associative effects resulting from repeated stimulation, which could may also lead to an increase in responsiveness to the CS. To fully demonstrate that the explicit pairing of the CS and pollen reward causes an increase in response to the olfactory CS as a result of a bees having learnt the predictive relationship between the two stimuli, it is crucial to include a control group where bees which receive random or unpaired presentations of the CS and pollen US and fail to show an increase in responsiveness to the CS over time. Furthermore I found that low dilutions (> 30% pollen w/w) of pollen in water result in a sticky mass that easily clogs the antennae. It is therefore unclear how the 70% pollen mixture was repeatedly delivered to the antennae and proboscis of bees in Grüter et al.'s study (2008).

Approximately 30% of all bees tested responded with proboscis extension to antennal stimulation with pollen in the first experiment. This is less than that observed by Scheiner et al. (2004) who found that *ca.* 45% of bees responded when stimulated with hand-collected almond pollen. This discrepancy might be explained by the fact that Scheiner et al. (2004) pre-selected bees which showed a high sensitivity to sucrose. Sucrose sensitivity is thought to correlate with foraging role, with those bees that are more responsive to sucrose being more likely forage for pollen (Page & Mitchell, 1998). It may be that in this pre-selected group, a higher proportion of bees were pollen foragers compared to those tested in the current study, where bees were collected upon departing the hive, and thus the foraging preferences of individuals were unknown. It is possible that pollen foragers are more sensitive to pollen (though this does not appear to be supported by preliminary studies of antennal sensitivity- see Chapter 2) thus explaining the higher likelihood of PER to antennal pollen stimulation. However, seasonal differences and genotypic variation in pollen hoarding behaviour between colonies could also account for such differences in responding.

Gustatory receptors on the proboscis, maxillae or mandibles could potentially be more important than those on the antennae for receiving reward input. Whilst there was little evidence for learning in bees stimulated at the proboscis with pollen in solution, clearly this stimulus does not replicate absolutely the natural situation for foraging bees. Under the current experimental set up it was not possible to deliver dry pollen grains to the proboscis, since they tended to adhere to it. When bees were restrained, they were

unable to clean the proboscis with the forelegs as they are observed to when foraging naturally (Casteel, 1912; Thorp, 1979). Future experiments might incorporate a re-design of the metal harnesses in order to permit free movement of the forelegs of bees. Given that the tarsi also possess gustatory receptors (de Brito Sanchez, et al., 2008; Marshall, 1935; Whitehead & Larsen, 1976), a control group would be required which receives tarsal stimulation alone. Since bees are known to use their forelegs to groom pollen from the body, there is actually the potential to receive input about the pollen reward through this pathway, and so it may be of interest to test whether tarsal stimulation elicits the PER, adopting the method previously developed by de Brito Sanchez et al. (2008) to condition the tarsi with sucrose.

One simple explanation for bees failure to exhibit learning in the restrained paradigm, is that the proboscis extension response is not an appropriate measure of the reinforcing properties of pollen. Unlike nectar, pollen is not thought to be ingested directly by foragers, thus bees may only learn to associate pollen with a CS when they are permitted to move freely and experience pollen under more natural conditions.

Adding pollen to sucrose did not lead to an improvement in acquisition as predicted. Previous studies have found that the addition of certain amino acids to nectar leads to an increase in feeding behaviour and an enhancement of learning (Kim & Smith, 2000; Wright et al. 2009). Release of the nitrogen-rich cytoplasm as a result of placing grains under osmotic shock was predicted to lead to an increase in reward value and a faster rate and overall level of acquisition. However quite the opposite was observed. By the end of training, bees reinforced with a mixture of pollen and sucrose showed a lower response to the CS than sucrose-rewarded bees. The same result was observed when bees were prevented from ingesting the reward, meaning that a pre-ingestive process, operating at the level of the antennae, must be responsible for the inhibition of olfactory learning.

Rather than improving the perceived quality of the US, the addition of pollen may have led to a decrease in the palatability of the sucrose solution. Whilst it is true that most pollen contains phago-stimulants in the form of lipids and fatty acids (J. O. Schmidt & Hanna, 2006) the pollen of many plant species also contains secondary metabolites such as phenolic compounds (Hagler & Buchmann, 1993; F. L. Liu, Zhang, Chai, & Yang, 2006) and pyrrolizidine alkaloids (Boppré, Steven, & Edgar, 2005; Detzel & Wink, 1993) thought to act as anti-herbivory defences and which may also be distasteful to

bees (Inouye & Waller, 1984; Reinhard et al., 2009). Almond pollen, for example, is known to contain high concentrations of amygdalin (London-Shafir, Shafir, & Eisikowitch, 2003) a substance shown to cause post-ingestional malaise in honeybees (G. A. Wright, et al., 2010). Even some amino acids, such as serine, have been shown to have a negative effect on feeding rate, particularly when present at high concentrations (Bertazzini, et al., 2010; Inouye & Waller, 1984). One factor that confounds this explanation is that the response to antennal stimulation (US response) remained rather high in pollen-sucrose rewarded bees and did not differ significantly from the response of bees rewarded with sucrose alone. If the pollen-sucrose mixture was distasteful, one would predict that individuals would begin to withhold proboscis extension following antennal stimulation (G. A. Wright, et al., 2010). However, perhaps because individuals were starved prior to conditioning, their motivation to respond to the sucrose component of the reward may have outweighed the negative influence of the distasteful pollen compounds. Previous studies have shown the degree of satiation to affect responsiveness to toxins in bees (Tan et al., 2007; G. A. Wright, et al., 2010).

An alternative explanation for the decline in CS response is that pollen grains present in the mixture resulted in physical damage to the antennae or led to clogging of the olfactory receptor pores, meaning that bees were less able to perceive the CS and therefore less likely to respond. Alternatively, compounds present in pollen might influence peripheral interactions between odorant molecules and the olfactory receptor cell, again affecting the ability to perceive the CS. The former explanation seems somewhat unlikely given that solutions were filtered and bees presumably experience much larger grains adhering to their antennae whilst collecting pollen naturally. However both explanations do predict a decrease in perception, and therefore response to the CS in the absence of a concurrent decline in responsiveness to the US.

The fact that pollen was observed to support colour learning in free flying bees, but not olfactory learning under restrained conditions suggests that the behavioural context and methods used to examine learning should be carefully considered in future explorations of pollen-rewarded learning. The question as to which factors and processes are necessary and sufficient to mediate pollen-rewarded learning in bees remains to be answered.

Chapter 6: Inhibitory effect of pollen compounds on olfactory learning in honeybees

Abstract

Floral nectar contains more than just sugars. Nitrogen-based compounds found frequently in this food reward, either as a result of direct secretion or due to contamination with pollen, have been shown to affect the behaviour of bees. In the previous chapter I hypothesised that nutrients released as a result of adding pollen to sucrose would also lead to an increase in the rate of acquisition in an olfactory learning task. However, in fact the addition of pollen was found to lead to an inhibition of learning relative to bees rewarded with sucrose alone. Here I investigated this inhibitory effect further. First I demonstrated that the inhibition of learning associated with the addition of pollen was persistent over an extended training period and that it was resistant to changes in the intensity of the conditioned stimulus, and the concentration of pollen added to sucrose. I ruled out potential mechanical effects, such as clogging of antennal sensilla pores, by adding an inert pollen substitute to the sucrose reward. In Chapter 5, a slight declining trend in terms of responding to antennal stimulation with the US was observed for bees stimulated with the pollen-sucrose mixture, suggesting that pollen compounds are perceived pre-ingestively as distasteful. When bees were rewarded with a mixture of sugar and salt (NaCl), a similar inhibitory effect on learning and a reduced US response was expected, but bees in both groups showed a similar level of performance. It was hypothesised that starved bees, used in all previous experiments, may have had a motivation to respond to the sucrose component of the reward that outweighed the aversive effect of distasteful pollen compounds and salt. In a final experiment I manipulated the hunger state of bees and found that when moderately satiated prior to the onset of training, bees displayed a much weaker response to the CS and a decline in responding to antennal stimulation with the pollen-sucrose reward over the course of the experiment. Such findings support the hypothesis that sucrose solutions containing pollen compounds are less attractive to bees than pure sucrose, leading to a lower strength of the association with the CS. Variations in the composition of floral nectars and pollen contamination may therefore modulate behavioural

responses to available flowers through differential recognition and learning of floral cues.

6.1 Introduction

During the process of pollen collection by flower visitors, contamination of nectar with pollen is common in some plant species, leading to a change in its chemical composition and more specifically a notable increase in the concentration of amino acids (Buxbaum, 1927; Erhardt & Baker, 1990; Gottsberger, et al., 1984). Amino acids also occur naturally in nectar, constituting the second most abundant compound aside from sugars (H. G. Baker & Baker, 1973, 1975, 1977). Within a certain range of concentrations, bees have been shown to prefer feeding on nectars or artificial sugar solutions containing amino acids over than those containing sugars alone (Alm, et al., 1990; Carter, et al., 2006; Kim & Smith, 2000; Petanidou, et al., 2006), leading to the suggestion that amino acids serve as an additional nutritional reward to visiting pollinators (H. G. Baker & Baker, 1973, 1975, 1986; Gottsberger, et al., 1984; Nicolson, 2007). In Chapter 5 I hypothesised that adding nitrogen-rich pollen to the typical sucrose reward would result in an improved rate of learning in an olfactory conditioning paradigm, as has been observed for the addition of glycine and proline (Kim & Smith, 2000; G. A. Wright, et al., 2009). Far from the predicted effect, I found that over the course of training, the presence of pollen appeared to inhibit responding to the conditioned stimulus (CS).

The positive influence of amino acids on honeybee feeding preferences is not universal. Inouye and Waller (1984) observed that feeding preferences were concentration dependent, and that for all amino acids tested, with the exception of phenylalanine, consumption was seen to decline with increasing concentration. Bertazzini et al. (2010) compared feeding responses to sucrose solutions containing 10 mM of proline, alanine or serine. Whilst solutions containing proline or alanine were preferred over a sugar control, the opposite effect was found for serine. A general negative effect on pollinator visits was also found for higher concentrations of serine and asparagnine in Mediterranean flower species (Petanidou, et al., 2006).

In addition to amino acids, pollen also contains secondary metabolites, including phenols, alkaloids (e.g. caffeine, nicotine) and glycosides (Boppré, et al., 2005; Detzel & Wink, 1993; London-Shafir, et al., 2003; Singaravelan, Nee'man, Inbar, & Izhaki, 2005) hypothesised to deter herbivory and nectar thievery (for review see Adler, 2000). Such compounds have also been postulated to aid in the maintenance of flower constancy (H. G. Baker & Baker, 1975; Masters, 1991; Rhoades & Bergdahl, 1981) since it is assumed that only certain pollinators will be able to tolerate their presence. When added to sucrose, some have been shown to deter feeding in bees, though only when present at concentrations exceeding the range typically observed in floral nectars (Detzel & Wink, 1993; Hagler & Buchmann, 1993; Inouye & Waller, 1984; Reinhard, et al., 2009; Singaravelan et al., 2006). Their effect appears to be dependent also on the availability of alternative nectar sources (Gegear, Manson, & Thomson, 2007) and the concentration of the sucrose solution in which they are presented (F. Liu et al., 2007). Given that sucrose solutions containing these types of compound can have a phago-inhibitory effect, perhaps the most plausible explanation for the inhibitory effect of pollen on olfactory learning is that bees experienced the mixture of pollen and sucrose as distasteful.

The gustatory receptors of honeybees are currently less well understood than those of other insects such as flies. An electrophysiological study of the labellar chemoreceptors of hoverflies (*Eristalis tenax*), whose ecology resembles that of bees, found that extracts of pollen in water stimulate the salt receptor cell (Wacht, et al., 2000). However unlike KCl or NaCl, which inhibit the water cell and are thus rejected at high concentrations (300mM KCl), the response of the water cell remained the same as that for pure water during pollen stimulation, meaning that flies were more likely to imbibe the aqueous pollen extract. Bees are capable of perceiving salt-water at the antennae (de Brito Sanchez, Giurfa, De Paula Mota, & Gauthier, 2005) and it has often been used as negative reinforcer in PER conditioning (Linander, Hempel de Ibarra, & Laska, 2012; G. A. Wright, et al., 2009). Nectars containing high levels of salts are less preferred by bees (Afik, Dag, Karem, & Shafir, 2006; von Frisch, 1942; Waller, 1972) For example, von Frisch (1942) found that supplementing 0.5 M sucrose solution (equivalent to 15% sucrose w/w) with 0.025 M NaCl led to a reduction in the likelihood of bees informing their nest-mates about this food source by around 30%, relative to the proportion of bees which danced for pure sucrose.

If the decline in response to the CS occurs as a result of the distasteful nature of pollen compounds leading to less effective reinforcement of the olfactory CS, then bees should withhold the proboscis extension when stimulated at the antennae with the US (G. A. Wright, et al., 2010). However, in those experiments presented in the previous chapter, responding to antennal stimulation with pollen and sucrose remained relatively high throughout the course of conditioning, showing only a slight decline (Figs. 5.3b, 5.4b, Chapter 5). Potentially these bees, which were starved prior to the onset of the experiment, were highly motivated to respond to any reward containing sucrose, and this may have outweighed the repellent effect of distasteful pollen compounds present in the mixture. Wright et al. (2010) found that the degree of satiation had an influence on the likelihood of bees imbibing aversive substances such as quinine. Furthermore, Tan et al. (2007) observed that caged honeybees would be willing to drink the toxic nectar of *Tripterygium hypoglaucum* if no other food was available. A similar effect has been shown for bumblebees offered nectars rich in secondary metabolites (Gegear, et al., 2007) and for lepidopteran species provided with a mixture of bitter alkaloids and sugars (Glendinning, Nelson, & Bernays, 2000; Shields & Mitchell, 1995). The presence of sucrose may be sufficient to override the negative influence of pollen compounds in hungry bees.

An alternative hypothesis is that pollen grains may have limited the detection of the CS odour, either via a physical impact on the antennae, through clogging of the olfactory sensilla pores for example, or as a result of chemical interactions between pollen compounds and odour-binding proteins and/or olfactory receptor molecules.

In the experiments presented here, I first tested whether the decline in response to the CS was transient or persistent by training bees over ten trials rather than six. I also determined whether inhibition of learning was consistent for lower CS and US strengths, reducing either the olfactory intensity of the CS or the concentration of pollen in sucrose. To establish whether the presence of a granular substance, in the absence of chemical cues, has an influence on responding to the CS, bees were trained with a mixture of sucrose and an inert powder, alpha cellulose. I also compared the learning performance of bees rewarded with a mixture of sucrose and salt (NaCl) a substance known to be distasteful to bees. Finally I tested whether satiated individuals would become less responsive to the pollen-sucrose mixture and thus show a reduced learning performance, relative to starved bees tested in previous experiments

6.2 Methods

6.2.1 Pollen in sucrose solution, extended conditioning

Bees were conditioned as before (Chapter 5, Exp. 5.2.4) for ten trials (instead of six). Bees were stimulated at the antennae only. Four groups were tested; bees rewarded with 30% sucrose (w/w), 15% sucrose (w/w), a mixture of 15% sucrose and pollen (30% w/w) and pollen in water (30% w/w).

The conditioned stimulus for all experiments was 1-hexanol (98% purity) diluted in mineral oil to 2.5 M. 20 ml of the odour solution was placed in a 60 ml glass bottle which was connected to an air pump with silicone tubing. The air stream was gated by a PLC-controlled valve to deliver uniform odour puffs (see also Smith, 1998). The odour stream was directed frontally at the head of the bee, and removed via a constant air stream, emitted from an extractor system located behind the animal. The pollen reward consisted of commercial pollen pellets (Werner Seip, Germany), ground to a fine powder and mixed with water or sucrose solution (30% pollen w/w). Pollen solutions were passed through filter paper to remove the larger grains. The sucrose reward was either a 30% or 15% (w/w) sucrose and water mixture which was also filtered.

Prior to conditioning, bees were tested for their motivational state and sensitivity to pollen. The antennae of each subject were touched first with water, and those bees which responded with proboscis extension were permitted to drink until satiated. Antennae were then touched with 30% pollen, with water a second time to prevent cross contamination, with 15% sucrose and finally with 30% sucrose solution. The interval between antennal touches was five minutes and only those bees responding to both pollen and sucrose solutions were included in conditioning experiments. Conditioning began approximately twenty minutes later to allow any potential effects of sensitization to 30% sucrose to subside.

An individual bee was placed in the experimental arena at a distance of 4.5 cm from the odour delivery tube and left to habituate for 15 seconds prior to the conditioning procedure. The odour (CS) was delivered to the antennae for three seconds prior to US delivery and overlapped with the US for a further second. US delivery (at the antennae only) then continued for a further two seconds. Bees were left in the arena for a further

15 seconds before being removed. The inter-trial interval was ten minutes for all experiments.

6.2.2 Effect of pollen concentration on inhibition of the conditioned response

In this experiment bees were trained for six trials only following the procedures described above. The amount of pollen in the sucrose solution was reduced from 30% to 10%. In a test of antennal sensitivity to pollen, a mixture of pollen water containing 10% pollen (w/w) was found to elicit a greater degree of proboscis extension than water alone, suggesting that bees are still able to detect the presence of pollen compounds at such a concentration (Fig. 2.2, Chapter 2).

6.2.3 Effect of CS strength on inhibition of the conditioned response

Following pre-testing for sensitivity to pollen and sucrose, bees were conditioned to either 0.025 M or 0.0025 M hexanol for six trials and given a final unrewarded test. These odour concentrations are weak but still above the detection threshold for hexanol (G. A. Wright & Smith, 2004). Using concentrations close to the detection threshold may reveal potential differences between pollen and sucrose-rewarded bees in terms of their perception of the olfactory CS. For each odour concentration, bees were either rewarded with a mixture of pollen and 15% sucrose (30% pollen w/w) or 15% sucrose alone. All groups were tested in parallel. Following training, bees response to the CS alone was tested

6.2.4 Alpha cellulose and NaCl mixed with sucrose reward

To separate the gustatory and mechano-sensory effects of the pollen grains, the inert, granular substance alpha cellulose (5% w/w) was added to 15% sucrose solution. The mixture was passed through filter paper as in previous experiments. A second group of bees were rewarded with a mixture of salt (NaCl) and sucrose. An equal weight of

sucrose and salt was mixed with water (30% w/w in total) and passed through filter paper (2.5M NaCl in 0.64 M Sucrose solution). A third group of bees were rewarded with 15% sucrose (w/w) alone and served as a baseline against which to compare performance of bees in other groups.

Following pre-testing for sensitivity to pollen and sucrose, bees were conditioned for six trials according to the protocol described above. The conditioned stimulus was 1-hexanol (98% purity) diluted in mineral oil to 2.5 M. Following training, bees response to the CS alone was tested

6.2.5 Effect of satiation on US response

Following pre-testing for sensitivity to pollen and sucrose, and one hour prior to the onset of training, individuals were fed 4 µl of 30% sucrose solution (w/w) using a 2 ml capacity Gilmont syringe for precise delivery. This volume is based on the observation by Friedrich et al. (2004) that feeding 15 µl of 1 M sucrose solution four hours prior to conditioning leads to moderate satiation in honeybees. Two groups were conditioned, bees rewarded either with 15% sucrose or a mixture of 15% sucrose and pollen (30% w/w). The conditioned stimulus was 1-hexanol (98% purity) diluted in mineral oil to 2.5 M. Following six training trials, bees response to the CS alone was tested. Finally bees in both groups were stimulated with 30% sucrose solution (w/w) in order to test their motivation to feed.

6.2.6 Antennal sensitivity to stimulation with increasing concentrations of pollen

Returning foragers were collected and indentified by the presence/absence of corbiculae loads. Thus it was possible to examine whether differences exist between the two forager types in terms of antennal sensitivity to pollen in sucrose. As in conditioning experiments, bees were restrained in metal harnesses and a small piece of tape was added to the base of tubes containing pollen foragers. This was hidden from the view of the experimenter, to avoid any potential bias in the coding of behaviour.

Bees were tested with ascending pollen concentrations from pure sucrose (15% w/w) to 30% pollen in sucrose, and proboscis extension to antennal stimulation was recorded on each trial. Solutions were delivered via a toothpick and water via a syringe. Prior to testing, bees were stimulated with water and allowed to drink, again to control for thirst. Between each pollen-sucrose stimulation the antennae were touched with water to avoid cross-contamination. The ITI was ten minutes. On a final trial the antennae were stimulated with 30% sucrose, in order to show that bees were still motivated to feed by the end of the experiment and that any decline in response to the pollen-sucrose solution was not the result of a general lack of feeding motivation or fatigue.

6.2.7 Statistical Analysis

Subject responses were scored as binary variables. To scrutinise the learning effects, bees that spontaneously responded to the odour on the first trial were excluded from the analysis where possible, i.e. in the dry pollen and pollen in sucrose experiments. GEE (Generalized Estimating Equation) modelling was used to compare the acquisition curves of bees trained with different unconditioned stimuli (G. A. Wright, et al., 2007). The GEE approach is an extension of the Generalized Linear Model (GZLM) which permits a non-normal distribution of the dependent variable and accounts for repeated measurements of the same individual (Hardin & Hilbe, 2002). Response to the CS was coded as the response variable with treatment and conditioning trial included as factors. Significance tests were based on Wald approximations of the likelihood ratio test. Post-hoc least significant difference contrasts (LSD) were used to compare treatment groups. Differences between groups in unrewarded tests were compared using Generalised Linear Model (GZLM). Performances in unrewarded tests were compared using Generalised Linear Models (GZLM).

6.3 Results

6.3.1 Does adding pollen to sucrose inhibit acquisition of the conditioned response?

The unconditioned stimulus had a significant effect on both the rate and overall level of acquisition (Fig. 6.1a, GEE, Treatment X^2_3=21.907, p=0.000; Treatment x Trial X^2_{24}=50.102, p=0.001*).* Post-hoc analysis reveals that over the course of ten trials, bees rewarded with 15% sucrose show significantly higher acquisition of the conditioned response compared with bees receiving a mixture of pollen and 15% sucrose (LSD contrast, 15% Sucrose *vs.* Pollen-Sucrose Mixture p=0.043). Over the first four conditioning trials, the rate of acquisition for bees receiving the pollen and sucrose mixture appears to have been much greater than for bees rewarded with pollen in water (Fig. 6.1a), with the difference in the overall level of response to the CS approaching significance (LSD contrast Pollen *vs.* Pollen-Sucrose Mixture p=0.054). Thus results obtained in previous experiments (Chapter 5) are persistent over a longer training duration.

Whilst there is no significant difference in responding to the various unconditioned stimuli on the first trial (Fig. 6.1b GZLM, Trial 1 X^2_3=5.039, p=0.169), by the tenth trial bees rewarded with pollen in water show a significantly lower response than bees in all other groups (GZLM, Trial 10 X^2_3=24.448; p<0.001; LSD contrast Pollen-water *vs.* all pairings p<0.001). Bees rewarded with a mixture of pollen and sucrose also show a declining trend in responding over the course of the experiment with just over 70% of bees exhibiting PER on the tenth trial, compared to 90% on the first trial (McNemar test X^2_1=3.125 p=0.070). The proportion of bees responding to pure sucrose remained consistently high (85-100%) over the course of training.

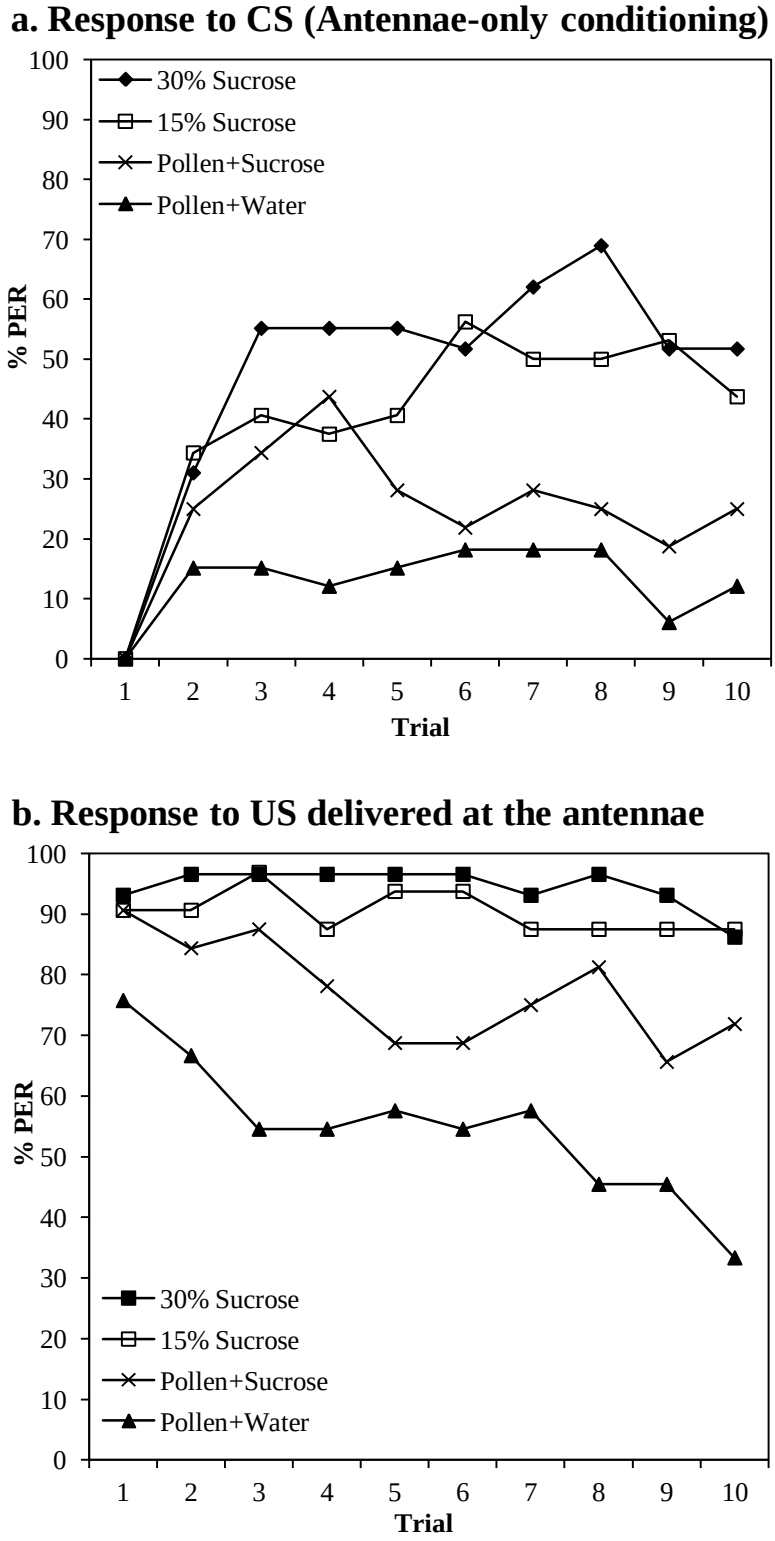


Figure 6.1 (a) Acquisition curves and (b) PER response to antennal stimulation in bees rewarded with either 30% sucrose solution (Black squares, *n*=29), 15% sucrose solution (Open squares, *n*=32), a mixture of 15% sucrose solution and pollen (30% w/w) (Crosses, *n*=32) or 30% pollen in water (Black triangles, *n*=33).

6.3.2 Is the inhibition of learning dependent on the concentration of pollen added to sucrose?

As before, the type of unconditioned stimulus significantly affected the overall level of acquisition (Fig. 6.2a, GEE, Treatment X^2_3=33.713, p<0.001), with bees rewarded with 15% sucrose outperforming all other groups, including those that were rewarded with a mixture of sucrose and pollen (10% w/w). Thus, even at weaker dilutions pollen inhibits olfactory learning in restrained honeybees (LSD contrast, Sucrose *vs*. Pollen-sucrose p=0.010; Sucrose *vs.* Pollen-water p<0.001; Sucrose *vs.* Water p<0.001).

Bees rewarded with a mixture of pollen and sucrose showed a higher level of acquisition than those rewarded with pollen and water (LSD contrast, Pollen-water *vs.* Pollen-sucrose p=0.033), whilst acquisition of bees rewarded with pollen-water did not differ from those rewarded with water alone (LSD contrast Pollen-water *vs.* Water p=0.782), supporting the conclusion from previous experiments (Chapter 5) that pollen does not support learning in an olfactory conditioning paradigm. Unexpectedly, there was no significant change in responding to the CS across successive trials, and there was no significant interaction between treatment group and trial (GEE, Trial X^2_4=5.761, p=0.218; Treatment x Trial X^2_{12}=12.030, p<0.443). This can mostly likely be explained by a plateau in the learning curve reached shortly after the second trial in the sucrose and pollen-sucrose groups, and the complete lack of learning in bees rewarded with either pollen-water or pure water.

Initially, all treatment groups displayed a response to antennal stimulation with their respective reward that was greater than that elicited by stimulation with pure water (Fig. 6.2b GZLM Trial 1 X^2_3=11.234, p=0.011, LSD contrast Water *vs.* all pairings p<0.05). Over the course of the experiment, bees rewarded pollen and water showed a considerable decline in response (*ca.* 40% difference between T1 to T6) and by the final trial, their response was no different to that of bees stimulated with water alone (GZLM Trial 6 X^2_3=26.251, p<0.001, LSD contrast Pollen-water *vs.* Water, p=0.209). Bees rewarded with pure sucrose or a mixture of pollen and sucrose showed a consistently high level of responding throughout the course of training (85-95%).

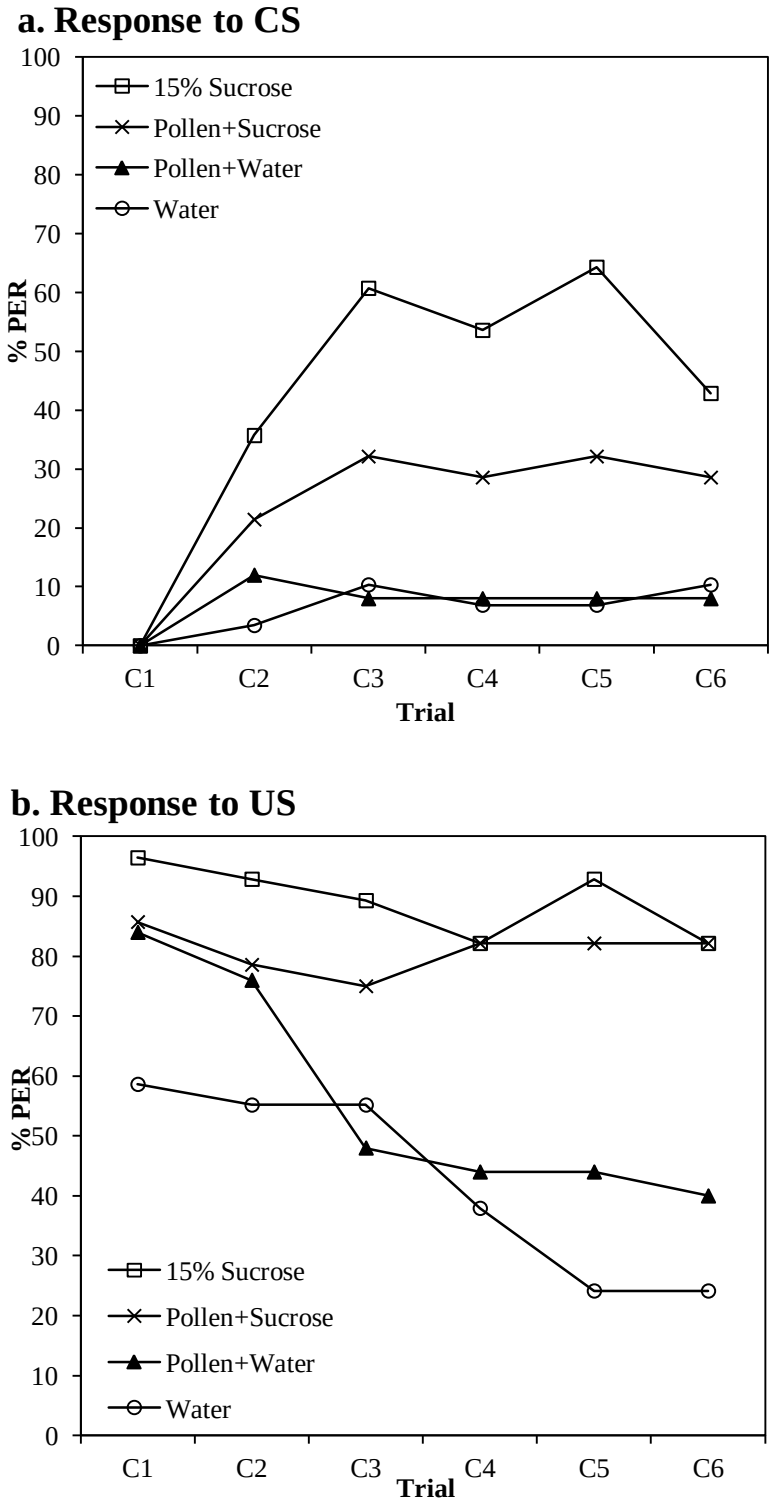


Figure 6.2 (a) Acquisition curves and (b) PER response to antennal stimulation of bees rewarded with either 15% sucrose solution (Open squares, *n*=28), a mixture of 15% sucrose solution and pollen (10% w/w) (Crosses, *n*=28), 30% pollen in water (Black triangles, *n*=25) or water alone (Open circles, *n*=29).

6.3.3 Is the inhibition of learning in bees rewarded with a mixture of pollen and sucrose dependent on the strength of the olfactory CS?

The strength of the olfactory CS had a significant effect on the overall level of performance during training and in the unrewarded test (GEE, CS Strength X^2_1=13.728, p=0.000; GZLM (Test), CS Strength X^2_1=14.066, p<0.001). As expected, bees conditioned to the more concentrated odour (0.25 M) showed a higher level of responding to the CS, relative to those conditioned with the odour closer to the olfactory detection threshold (0.0025 M, Fig. 6.3a). There was no interaction between the strength of the CS and the type of reward received (GEE CS Strength x Treatment X^2_1=0.890, p=0.346; GZLM (Test) CS Strength x Treatment X^2_1=0.011, p=0.916) indicating that bees rewarded with pure sucrose and pollen-sucrose had similar olfactory detection thresholds.

The overall effect of reward type is approaching significance (GEE; Treatment X^2_1=3.289, p=0.070; GLZM (Test) Treatment X^2_1=3.667, p=0.055, LSD contrast p=0.046), suggesting that there are consistent differences in the level of responding to the CS in bees rewarded with sucrose and those rewarded with a mixture of pollen and sucrose, independent of the strength of the CS. Response to antennal stimulation with the US remained high (*ca*. 80-90%) in all bees over the course of training (Fig. 6.3b GZLM, Trial 6, Treatment X^2_1=2.402, p=0.121).

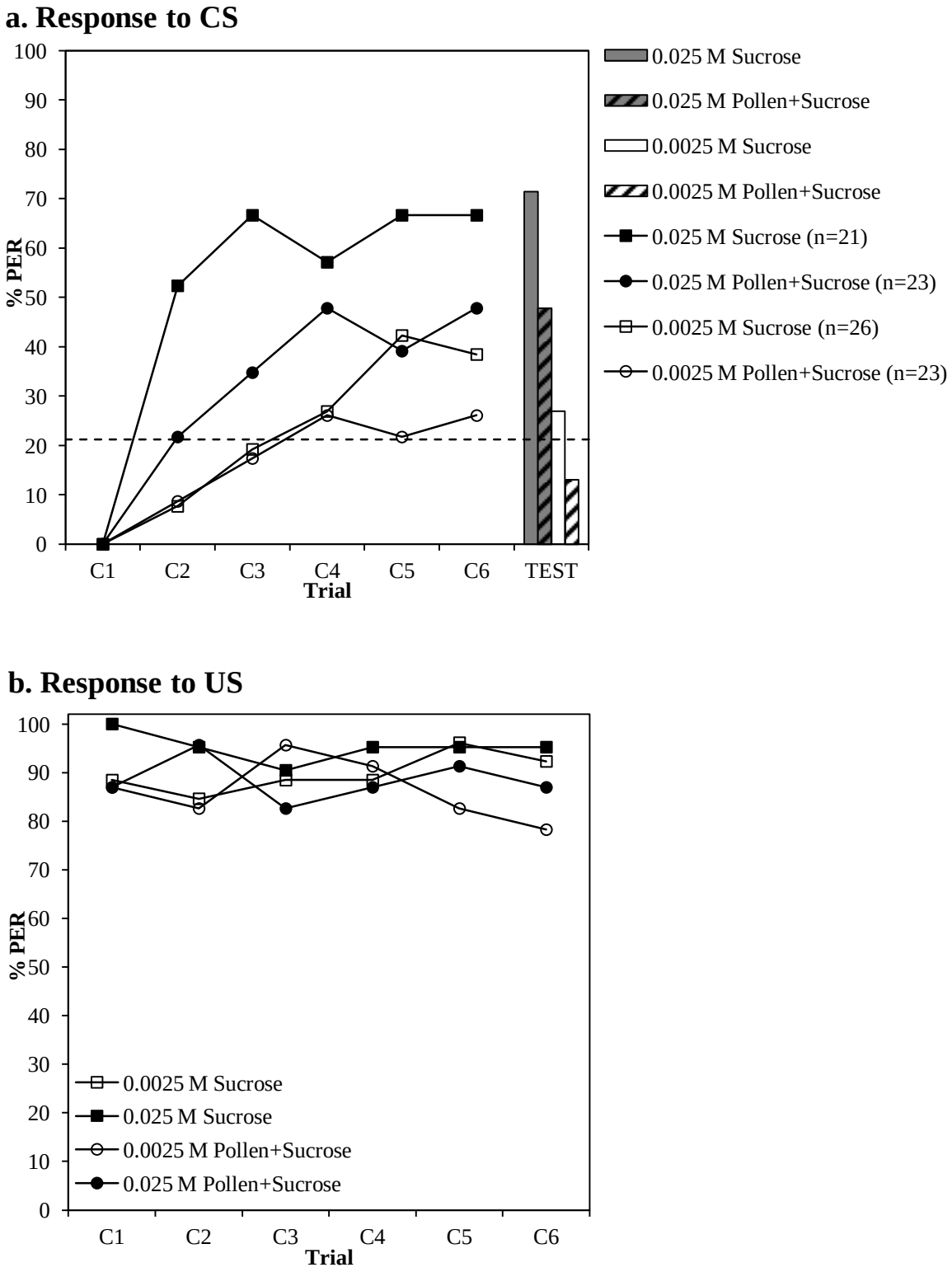
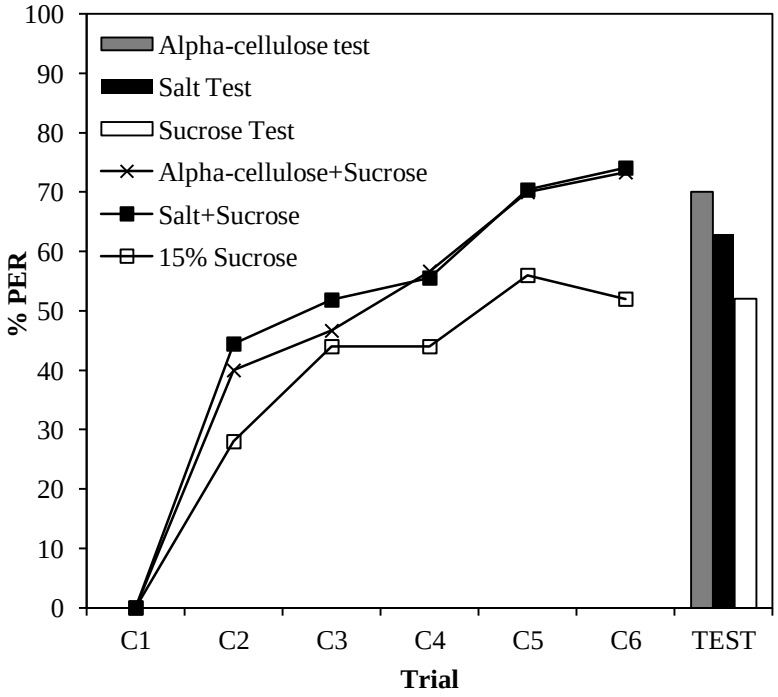


Figure 6.3 (a) Acquisition curves (b) and response to antennal stimulation of bees rewarded with 15% sucrose solution (Squares, *n*=47) or a mixture of 15% sucrose solution and pollen (30% w/w) (Circles, *n*=46). Bees were either conditioned to 0.025 M hexanol (Black symbols) or 0.0025 M hexanol (Open symbols). Dashed line represents the overall spontaneous response to the CS on the first trial for all bees tested. Bars represent the proportion of bees responding to the CS in the final, unrewarded test (Solid bars= 15% sucrose solution, Striped bars=15% sucrose solution and pollen. Grey= 0.025 M hexanol, White= 0.0025 M hexanol).

a. Response to CS



b. Response to US

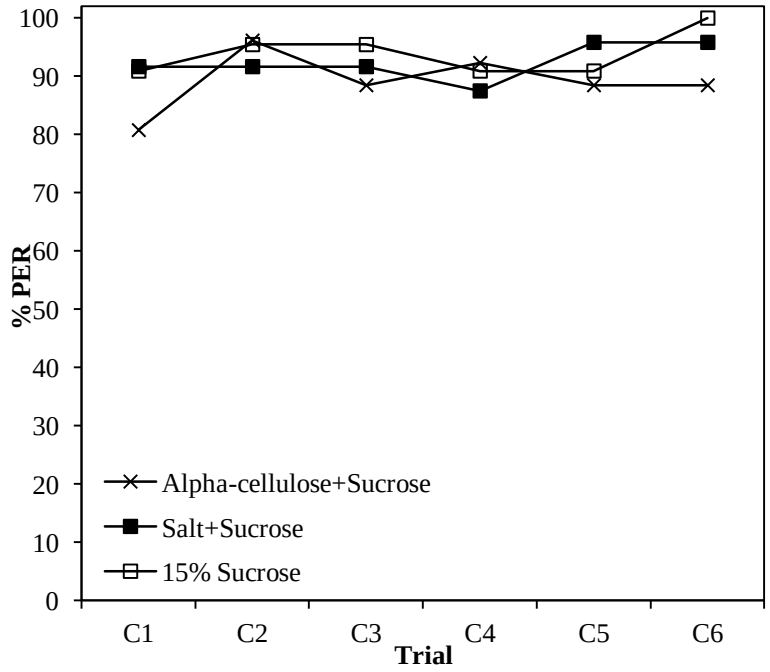


Figure 6.4 (a) Acquisition curves and (b) response to antennal stimulation in bees rewarded with either 15% sucrose solution (Open squares, *n*=25), salt and sucrose in water (30% w/w in total) (Black squares, *n*=24) or a mixture of alpha-cellulose in 15% sucrose solution (5% w/w) (Crosses, *n*=30). Bars represent the proportion of bees responding to the CS in the final, unrewarded test (Black=NaCl+Sucrose, White=15% Sucrose, Grey=Alpha cellulose+Sucrose).

6.3.4 Separating the chemical and mechano-sensory components of the pollen reward

Supplementing 15% sucrose solution with either salt (NaCl) or alpha-cellulose had no impact on the rate or overall level of acquisition (Fig. 6.4a). On the contrary, bees rewarded with these mixtures slightly outperformed those rewarded with sucrose alone, though no significant differences were found either during the course of training (GEE Treatment X^2_2=3.207, p=0.201; Treatment x Trial X^2_2=1.728, p=0.988) or in the unrewarded test (GZLM Treatment X^2_2=1.863, p=0.394). All bees showed a consistently high response to antennal stimulation with their respective rewards over the course of training (Fig. 6.4b, GZLM, Trial 6 X^2_2=0.850, p=0.654). The marginally better performance of bees receiving a mixture of salt and sucrose can most likely be explained by the higher molarity of sugar in this solution relative to pure sucrose (0.64 M compared to 0.5 M). Since alpha cellulose is not soluble, and all sugars should have been removed during the refining process, the elevated performance of bees in this group is more difficult to account for.

6.3.5 Does hunger state affect acquisition and the willingness to respond to antennal stimulation with pollen and sucrose US?

When bees were moderately satiated prior to the onset of conditioning, the overall level of acquisition was dependent on the reward received. Both over the course of training and in the unrewarded test, the level of acquisition of sucrose-rewarded bees was significantly higher than that of bees rewarded with pollen and sucrose (Fig. 6.5a GEE, Treatment X^2_1 =6.431, p=0.011; GZLM (Test), Treatment X^2_1=5.031, p=0.025). Both groups showed an identical response to the US on the first trial (*ca.* 80%, Fig. 6.4b) and though responding on the final trial was not significantly different between groups (X^2_1=2.237, p=0.135), bees stimulated with a mixture of pollen and sucrose showed a decline in proboscis extension to the US over the course of training, (from 80 to 62%), whereas bees rewarded with pure sucrose show the same level of responding on the first and last trial, again suggesting that pollen-rewarded bees found the mixture distasteful.

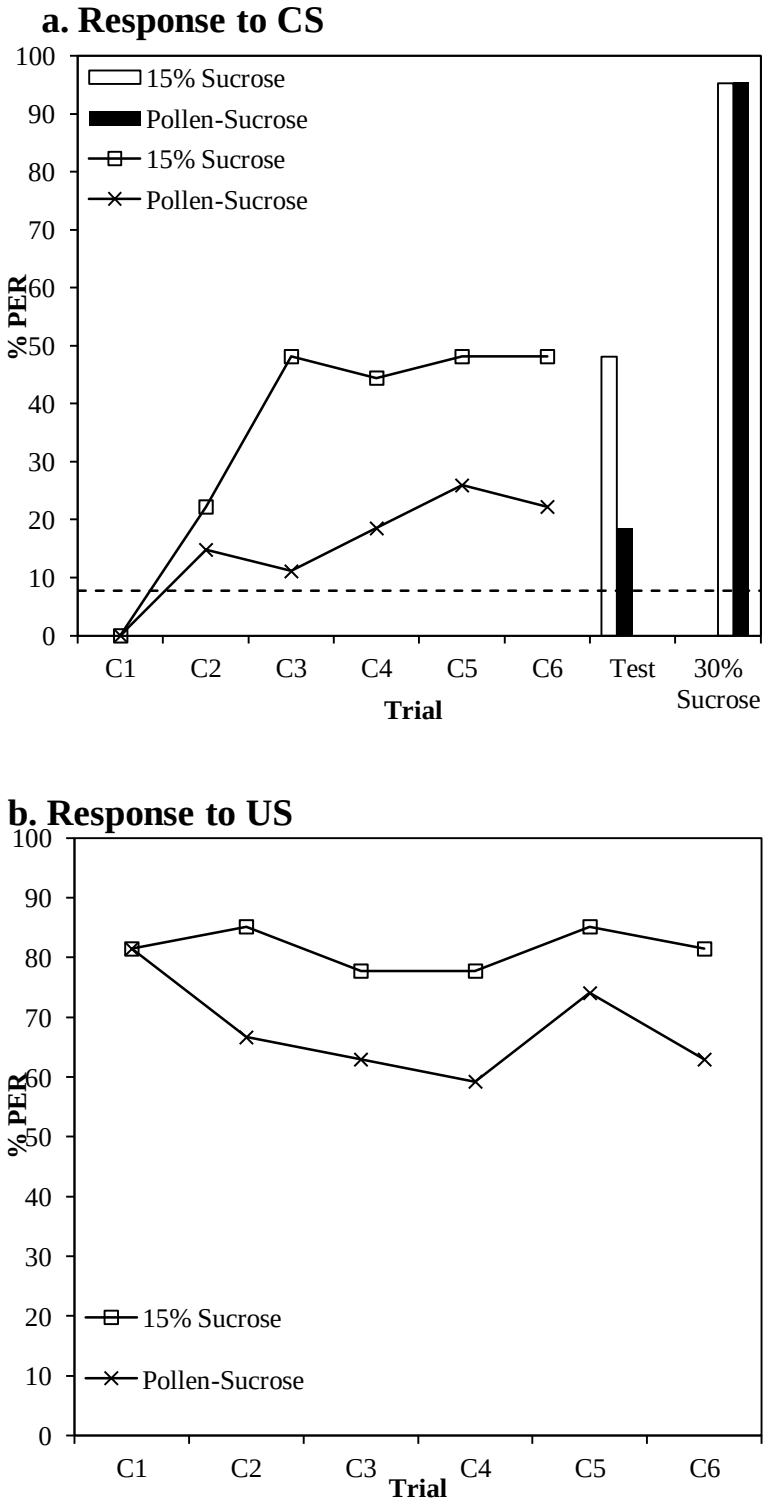


Figure 6.5 (a) Acquisition curves and (b) response to antennal stimulation in moderately satiated bees rewarded with either 15% sucrose solution (Open squares, *n*=27), a mixture of 15% sucrose solution and pollen (30% w/w) (Crosses, *n*=27). In order to moderately satiate bees, each animal was fed 4 µl of 30% sucrose solution, one hour prior to the onset of the experiment. Dashed line represents the overall spontaneous response to the CS on the first trial for all bees tested. Bars represent the proportion of bees responding to the CS in the final, unrewarded test, and when stimulated with 30% sucrose to test for feeding motivation (White=15% sucrose, Black= 15% sucrose and pollen).

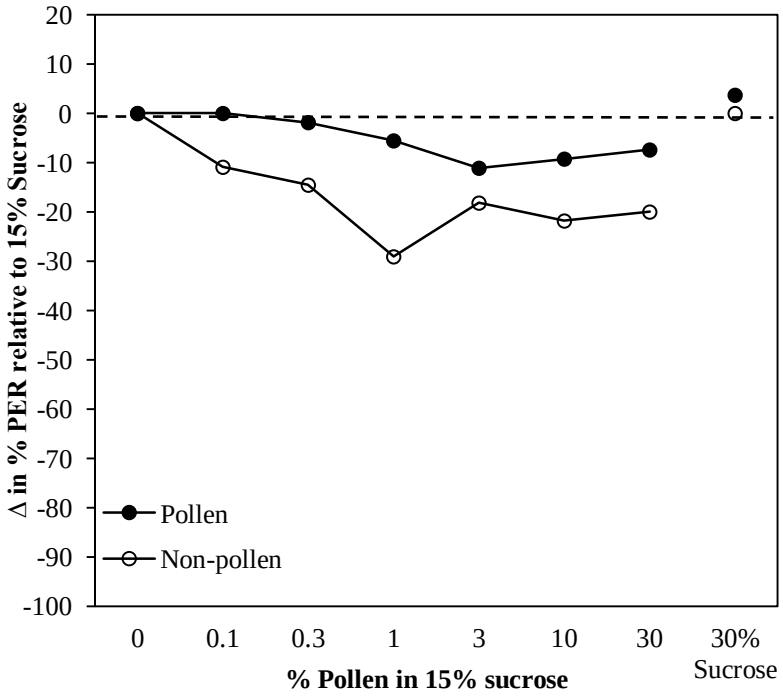


Figure 6.6 Relative change in the proportion of bees exhibiting PER to antennal stimulation with a series of pollen-sucrose mixtures, compared to their original response to pure sucrose (15% w/w). Solutions were tested in order of ascending pollen concentration. Bees were separated according to forager type (Pollen foragers=Black circles, *n*=54; Non-pollen foragers=Open circles, *n*=55) and no differences between foragers were observed in terms of responsiveness to pure sucrose on the first trial. On the final trial bees were stimulated with 30% sucrose solution (w/w).

6.3.6 Is the inhibitory effect of pollen persistent at weaker concentrations?

Pollen and nectar foragers displayed an identical level of responding to the initial stimulation with 15% sucrose (Fig. 6.6). Whilst bees in both groups were less likely to exhibit PER as the concentration of pollen in sucrose was increased (GEE, Group x Trial, X^2_1=10.081, p=0.121), the response of non-pollen foragers was consistently lower than that of pollen foragers, with the overall difference in responding approaching significance (GEE, Group X^2_1=3.372, p=0.066). Trial number had a significant effect on the level of response across all bees (GEE, Trial X^2_6=17.742, p=0.007), though whilst for all pollen concentrations non-pollen foragers showed a significant reduction in PER relative to their response to sucrose on the first trial (0.1-30% w/w), (LSD contrast 0% pollen vs. all other concentrations, $p<0.05$), in pollen foragers only the response to 3% pollen was significantly different from the initial response to 15% sucrose. The response

to the final stimulation with 30% sucrose (w/w) was high (*ca.* 80%) and there was no significant difference between forager groups (X^2_1=0.033, p=0.856), suggesting that the decline in responding over the course of the experiment was not simply due to fatigue.

6.4 Discussion

Whilst previous findings have suggested that supplementing nectar with amino acids such as proline can lead to an increase in the perceived value of such a reward (Kim & Smith, 2000; G. A. Wright, et al., 2009), I observed that adding pollen, a major source of nitrogen-based compounds for bees, to an artificial nectar source actually hinders learning. Here I have demonstrated that the previously observed impairment of CS responding in bees rewarded at the antennae with a mixture of pollen and sucrose is consistent when bees are trained over a greater number of trials, and that the inhibition of learning appears to be resistant to changes in the strength of the olfactory CS and the concentration of pollen present in the solution.

One hypothesis for the reduced level of responding to the CS over time, is that the presence of pollen somehow impairs bees ability to detect the olfactory stimulus. However, I found that that when tested with two weak CS concentrations, bees rewarded with either pure sucrose or a mixture of pollen and sucrose showed a similar relative decline in responding when tested with the weaker of the two concentrations. The acquisition curve of bees rewarded with pollen and sucrose rewarded bees in the stronger CS condition did not overlap with the learning curves of bees tested with the weaker CS, thus it seems that pollen compounds do not affect CS perception. Similarly, bees rewarded with a mixture of sucrose and the inert, granular substance, alpha cellulose did not show any impairment in learning relative to those rewarded with pure sucrose, indicating that the physical presence of grains in solution is unlikely to be responsible for the lower CS response over the course of the experiment.

The learning curves of bees rewarded with the pollen-sucrose mixture closely resemble the pattern of acquisition observed for bees stimulated at the proboscis with a mixture of quinine and sucrose (G. A. Wright, et al., 2010). Whilst responding to the CS was seen to increase slightly over the first few conditioning trials, following several exposures response declined and was consistently lower than that of bees rewarded with sucrose

alone. Wright et al. (2010) attributed the low level of responding to the pre-ingestive detection of bitter-tasting quinine during stimulation at the proboscis, given that bees quickly began to reject the reward. Subsequent intracellular recordings from proboscis sensilla revealed that quinine elicits a specific 'deterrent cell' pattern of responding. In previous experiments, where individuals were trained over six trials, bees appeared not to withhold their response to antennal stimulation, as predicted if the pollen-sucrose mixture is indeed perceived to be distasteful. However, when trained for an extended period (ten trials) a concurrent decline in responding to the US was observed. In contrast, bees rewarded with pure sucrose showed a consistently high level of response throughout. When bees were moderately satiated, a decline in response to stimulation with the US, amounting to *ca.* 20% was observed over a period of six trials, but only when the sucrose reward had been supplemented with pollen. These findings suggest that bees are able to pre-ingestively detect distasteful compounds originating from the addition of pollen to sucrose, and that such compounds subsequently inhibit learning in an olfactory conditioning paradigm. The fact that when bees are hungry, a decline in response to the US does not become apparent until bees have been stimulated over an extended number of trials, suggests that when the motivation to feed is high, bees may be more inclined to respond to the sucrose component of the reward. Wright et al. (2010) found that the extent to which bees are willing to ingest aversive substances such as quinine is also dependent on the degree of satiation.

Whilst it is difficult to compare the responses of bees tested in separate experiments, given that external factors such as the time of year may influence responsiveness, it is interesting to note that moderately satiated bees showed much lower levels of spontaneous response to the CS on the first trial compared to starved bees (*ca.* 7% *vs.* 20%) lending weight to the suggestion that they are less motivated to respond to stimuli in general.

An attempt was made to reproduce the acquisition curve of pollen-sucrose rewarded bees by stimulating the antennae with a mixture of sucrose and salt (NaCl), a compound known to be distasteful to bees and is known to be detected at the antennae (de Brito Sanchez, et al., 2005). When stimulated at the antennae with a mixture of sucrose and salt the learning performance of bees was equal to that of bees rewarded with sucrose alone. In fact, bees rewarded with sucrose and salt appeared to slightly outperform sucrose-rewarded bees, possibly due to the slightly lower sucrose molarity in the pure

solution. This is surprising given that nectars containing salts are known to be less attractive to free-flying bees (von Frisch, 1942; Waller, 1972), and previous studies have shown weaker concentrations of NaCl than those used here (1.5 M compared to 2.5 M used here) are effective in negatively conditioning bees (Linander, et al., 2012; G. A. Wright, et al., 2009). However in these studies salt was added to water, and the effect of rewarding bees at the antennae with a mixture of sugar and salt has not been tested previously. Possibly the appetitive effect of the sugar outweighed the aversive effect of salt. Liu et al. (2007), for example, have shown that the repellent effect of secondary metabolites is dependent on the concentration of sucrose solution in which they are found. It is also important to note that bees in this experiment had been starved prior to conditioning, and thus, as hypothesised for pollen-sucrose rewarded bees, their level of response to the CS and US may have reflected a high motivation to respond to food stimuli, even those perceived as distasteful. Thus it would be of interest to repeat this experiment with bees which have been moderately satiated.

Taken collectively, the current results suggest that pollen-rewarded bees are not impaired in their ability to perceive the CS but that the decline in response results from the pre-ingestive detection of distasteful compounds present in the pollen and sucrose mixture. A similar effect is likely to account for the more rapid decline in response to the US seen in pollen and water rewarded bees. The pollen used in the current experiments is from multiple species, and so until the chemical analysis of the samples is complete it is not possible to say exactly which compounds are present in the pollen solutions, and at what concentrations. As a first step, I measured the pH of the pollen solutions used in this and the previous chapter. Waller (1972) has shown that free flying bees show no preference between artificial nectars ranging from pH 3.2 to 8.8, but that solutions outside this range would be rejected by foragers. Adding pollen lowered the pH of solutions considerably, from *ca.* pH 8 (ranging from pH 7.85 for pure water to 8.41 for 15% sucrose) to between pH 4.6-4.8. Though acidic, this is within the range accepted by free-flying bees at least.

All the pollen used in the current experiments was collected by honeybees themselves, and so it may seem suprisingly that bees would choose species containing compounds which they perceive to be distasteful. Two factors may explain this apparent paradox. Firstly, when pollen is added to liquid, it is placed under osmotic shock causing grains to burst and release their contents. Therefore the majority of distasteful compounds

responsible for the current pattern of responding may be locked within the intine of the grains and thus would be imperceptible to foragers. Nevertheless it is interesting to note that certain age classes of bees may regularly experience such compounds. For example, nurse bees ingest pollen in order to regurgitate it and feed it to the larvae, and young bees ingest pollen to support their own development (Crailsheim, et al., 1992).

Whilst it has been shown that the supplementation of nectar with amino acids can have a positive influence of feeding preferences, this is known to be concentration dependent, with a similar effect having been shown for the presence of secondary compounds in nectar. A second explanation could be that the volume of pollen added to the sucrose solution led to excessive concentrations of amino acids or secondary metabolites, which at more natural concentrations are typically perceived as neutral, but at such high concentrations may potentially account for the repellence of bees.

When the concentration of pollen present in the sucrose solution was reduced from 30% to 10% (w/w) an inhibition of learning was still observed. The antennal sensitivity of bees to varying concentrations of pollen in sucrose was tested, to determine if there is a concentration at which pollen has a positive, appetitive effect on PER, as has been shown for specific amino acids such as proline (G. A. Wright, et al., 2009). However, a generally negative effect on PER was observed, though interestingly this appeared to affect non-pollen foragers more than pollen foragers. It seems unlikely that non-pollen foragers were better able to detect pollen compounds, since when presented in solution with water, little difference between forager types in terms of responsiveness was observed. Reported differences in sucrose sensitivity between pollen and nectar foragers are also unlikely to drive this pattern of responding, given that the initial response to 15% sucrose was identical between forager groups. Possibly pollen foragers are more tolerant to distasteful compounds present in pollen, given that they may encounter these more frequently during collection. Further investigations might consider using much smaller volumes of pollen, in order to see if the inhibition of learning is still observed under more naturalistic changes in nectar biochemistry, and whether there is any concentration at which the addition of pollen is observed to improve learning in bees.

Chapter 7: General Discussion

7.1 Overview of main findings

Learning in sucrose-rewarded bees has been well studied, with the neural pathways involved in associative learning in honeybees quite well understood. Bees have been shown capable of forming associations between sucrose and visual, olfactory and tactile stimuli (Bitterman, et al., 1983; Heinrich, 1976; Heinrich, et al., 1977; Riveros & Gronenberg, 2009; Scheiner, et al., 1999; von Frisch, 1914, 1923), and are able to retain such associations over extended time periods (Menzel, 1999). The sucrose reward resembles nectar naturally encountered by pollinating insects whilst visiting flowers, but the question of what pollen-collecting bees learn has been little addressed. Pollen is collected as a food resource for developing larvae but is not ingested by foragers themselves, thus raising the question as to whether pollen can be characterised as an appetitive reward in the traditional sense. The central aim of this thesis therefore was to determine how pollen reinforces learning and memory formation in two well studied insect pollinators, *Apis mellifera* and *Bombus terrestris*.

First I established, under controlled conditions, that colour-naive free flying bees are indeed able to form an association between the availability of pollen and the presence of a coloured stimulus, and that following multiple training trials memory for the rewarded colour typically persists for at least 24 hours. As well as discriminating between coloured stimuli on the basis of differences in pollen availability, bees were also able to shift their preference towards a coloured stimulus associated with a more concentrated pollen and alpha cellulose mixture, though only if the difference in pollen concentration between samples was sufficiently large.

The reinforcing properties of pollen were observed to be dependent on behavioural context. Having established that pollen can reinforce learning in free flying bees, the proboscis extension response (PER) paradigm was employed in an attempt to determine those features of the pollen reward involved in the formation of learned associations. When bees were restrained, a prerequisite for controlled application of the stimulus and reward, multiple pairings of hexanol (conditioned stimulus) and the stimulation of the proboscis and/or antennae with pollen (unconditioned stimulus) did not lead to a

conditioned response (PER). This contrasts with previous studies in which sucrose serves as the US, where bees have been conditioned to olfactory stimuli under restrained conditions.

Not only did pollen fail to support olfactory learning in the PER paradigm, when added to sucrose it appears to devalue this reward, as evidenced by an inhibition of the conditioned response relative to bees rewarded with sucrose alone. This strongly contrasts with previous studies, where the addition of specific amino acids to sucrose have been shown to lead to an improvement in learning (Kim & Smith, 2000; G. A. Wright, et al., 2009). Whilst for bees pollen represents an important source of both protein and amino acids, this food reward also contains a variety of other nutrients and secondary compounds (Stanley & Linskens, 1974), some of which have been shown to be distasteful to bees (Inouye & Waller, 1984; Reinhard, et al., 2009). My results appear to suggest that such compounds may be responsible for the inhibition of learning through a devaluation of the sucrose US.

7.2 Implications for the study of learning in bees

The methods described here for studying pollen-rewarded learning in free-flying bees open up various opportunities to expand the study of learning in the honeybee and bumblebee model systems. I have shown that pollen-rewarded learning leads to the formation of memories which persist for at least 24 hours. In sucrose-rewarded bees, memories which are stable for this period of time are considered to have been consolidated to the long term memory, and are thought to stay with bees for life (Menzel, 1999). However, in terms of pollen-rewarded learning, much more remains to be explored, such as the number of training trials typically required for consolidation to long term memory, and the resistance of such memories to extinction. One can imagine replicating the original visual conditioning experiments of von Frisch, Menzel, Giurfa and colleagues (M. Giurfa et al., 1999; Menzel, 1967, 1968, 1969; von Frisch, 1923) with the ultimate goal of determining the properties of the neural substrate which mediate learning and memory formation with a reward which is encountered under the similar conditions to nectar, but not directly ingested. Furthermore, it is now possible to incorporate two nutritionally distinct rewards into a single paradigm, in order to

investigate whether bees can generalise learnt information across foraging contexts. For example it would be of interest to know whether pollen foraging bees recall cues learnt during nectar collection and *vice versa*, since this may have implications for the provision of food rewards by flowers.

One could also explore the notion of reward expectations and test whether, in addition to learning the contingency between the CS and US, bees also form memories of the particular properties of the reinforcer, or a 'representation' of the reward (Schulz, 2000). This has previously been addressed in both free-flying and restrained bees trained to 'expect' different reward values, in the form of either variable volumes or concentrations of sucrose solution (Gil, De Marco, & Menzel, 2007; Gil, Menzel, & De Marco, 2008). Using a dual-reward paradigm, one could train bees to associate each reward type with a different behavioural action (e.g. turning direction in a Y-maze), akin to the method used by Colwill and Rescorla (1985) to test for encoded representations of the reward during operant conditioning in hungry/thirsty rats.

The vertical presentation paradigm, in which the visual and olfactory environment is standardised for both forager types, enables further exploration of reported differences in the learning ability of pollen and nectar foragers. Previous comparative studies have used the restrained paradigm and rewarded both forager types with sucrose (e.g. Erber, Scheiner, & Page, 1998). However more recent evidence, alongside that presented here, suggests that behaviours displayed in the restrained paradigm do not always accurately reflect the behaviour of freely flying bees. Pollen foragers, when restrained, have been shown to have consistently lower gustatory response thresholds for sucrose detection resulting in faster acquisition and better memory retention in olfactory and tactile conditioning tasks, relative to nectar foragers (Scheiner, et al., 2003; Scheiner, et al., 1999; Scheiner, Kuritz-Kaiser, Menzel, & Erber, 2005; Scheiner, Page, & Erber, 2001a, 2001b). Mujagic and Erber (2009) observed only partial correlation between the sucrose acceptance thresholds of free-flying bees and gustatory response scores in the PER paradigm. Individuals that responded only to high sucrose concentrations when harnessed were immediately observed to collect much weaker solutions when released into the field. The vertical presentation method offers an opportunity to compare foragers under near identical conditions, and reinforce bees with their respective reward types (as in Chapter 3). The differences in memory recall observed following reversal colour training in Chapter 3 require further study. As yet it is not possible to fully

dissociate the effects that may be attributable to differences between forager types in terms of their ability to form and/or recall memories, from those that arise as a result of differences in the reinforcing properties of the two rewards. Thus it would be worthwhile to conduct further visual conditioning experiments, to compare memory recall and performance of bees rewarded with pollen against pollen foragers forced to switch to collecting sucrose solution instead.

7.3 The importance of behavioural context for the study of pollen-rewarded learning

Attempts to determine the sensory pathway involved in pollen-rewarded learning were hampered by the fact that pollen did not reinforce learning in the restrained paradigm. Conditioning visual stimuli using the PER paradigm has proven difficult, thus prompting the switch to an olfactory CS in Chapters 5 and 6 (Hori, et al., 2006; Niggebrügge, et al., 2009). However I deem it highly unlikely that the switch in modality is responsible for the lack of learning in harnessed bees. Honeybees are thought to learn odours more rapidly than visual stimuli (Masuhr & Menzel, 1972; Menzel, 1985), and are capable of distinguishing between pollen types on the basis of the signature scent emitted from the grain surface (H.E.M. Dobson, 1988). Nevertheless it is of interest to note that in free-flying tests of pollen preference (Chapter 4), bees appeared not to use olfactory cues to recognise the pre-trained pollen sample.

It seems most likely that the lack of learning stems from the inappropriate nature of the restrained conditioning method. As often discussed in the context of comparative studies of animal learning, difficulties can arise in the interpretation of such negative results. As stated by Kamil (1994) ‘it is theoretically impossible to prove there is no set of circumstances under which an animal can learn a particular task’. Both Kamil (1994) and before him, Bitterman (1965), promote the use of an approach in which environmental conditions are systematically varied in an attempt to identify those conditions under which learning might take place, thus enabling comparisons across species. Here I varied both the site (antennae and proboscis *vs.* antennae only) and method (dry *vs.* in solution) of pollen delivery, and in later experiments where pollen was delivered in solution with sucrose, the hunger state and strength of the CS and US

were also varied in order to demonstrate the robustness of the inhibitory effect that pollen has on learning.

Pollen collection involves a complex set of behaviours, and thus restraining bees may have precluded the performance of certain actions essential to the formation of an association between the CS and pollen. Though young hive bees consume pollen, it is rarely found in the gut of foraging bees (Crailsheim, et al., 1992), and so post-ingestive mechanisms were considered unlikely to be involved in processing the pollen reward. As a result, the antennae, which possess olfactory, gustatory and mechanical receptors, were targeted as the primary candidates for detection and processing of the pollen reward. Prior evidence for this stems from the finding that, when delivered to the antennae, both dry pollen grains and pollen in solution elicit the PER to a greater extent than alpha cellulose or water alone. However, given that multiple pairings of this 'reward' with the delivery of a neutral odour (CS) did not lead to an increase in response to the CS over time, it may be that such stimulation is not sufficient to reinforce learning. Hammer and Menzel (1995) suggest that stimuli which serve as the US are characterized by a releasing, modulating and reinforcing function, with the releasing and reinforcing functions of the sucrose reward having been shown to be dissociable (Menzel, et al., 1999). Therefore it is possible that, under restrained conditions, pollen may only perform the releasing function.

When reinforcing bees using the most naturalistic method of US delivery, dry pollen grains could only be applied to the antennae because of the tendency of grains to stick to the proboscis impairing extension. It may be that gustatory receptors found on the mouthparts, or possibly even on the tarsi, are more important in sensing pollen, and thus constitute a vital sensory input site for the pollen reward pathway. Sucrose has been shown to reinforce learning even when bees are prevented from ingesting the reward (Bitterman, et al., 1983; G. A. Wright, et al., 2007). When stimulated at the antennae only, acquisition is observed to be retarded compared to bees which are permitted to imbibe the reward. In contrast, bees stimulated at the proboscis but not permitted to drink show no difference in acquisition relative to bees which ingest the reward (Bitterman, et al., 1983). Sandoz et al. (2002) have suggested that whilst antennal input predicts reward delivery, proboscis input is more important and encodes more information about the nature of the reward. Mustard et al. (2012) found that the proboscis is more sensitive to caffeine (in solution with sucrose) than the antennae,

suggesting that the proboscis may also be important for the detection of bitter tastes. Indeed when sucrose and pollen were delivered to both the antennae and proboscis of starved bees, a larger decline in responding to the US was observed compare to bees stimulated at the antennae alone (Chapter 5, Figs. 5.3b, 5.4b). A paradigm for tarsal conditioning has been developed by de Brito Sanchez et al. (2008) therefore one could adapt this method, again substituting pollen for the sucrose reward, in order to test whether tarsal stimulation with pollen releases the PER and can reinforce the learning of olfactory stimuli in honeybees.

Though stimulation with pollen releases proboscis extension, this may not be an appropriate behavioural measure of learning in pollen-rewarded bees. The combined use of the free-flying and restrained paradigm may better serve to elucidate the sensory organs implicated in the pollen-reward pathway. Bees could be conditioned to odours in the PER paradigm (pollen US) and then released back to the flight cage where their foraging preferences for scented feeders could be compared against those bees which receive unpaired presentations of the CS and pollen US. One could stimulate sensory organs with pollen both individually and in combination to determine those gustatory receptors necessary for pollen reinforced learning. This design is a partial reversal of that described by Arenas and Farina (2012), in which they attempted to show that olfactory information learnt during pollen collection could be transferred to the laboratory setting. Individuals trained to collect pollen from scented feeders were not observed to show conditioned responding to the same odours in the restrained paradigm, providing yet further evidence that behavioural context is important in the study of learning in bees, and lending further weight to the notion that PER may not be an appropriate behavioural measure of learnt associations between the CS and pollen US.

Thus far pollen has been treated as a food reward and gustatory organs have been assumed to be implicated in assessment of this stimulus, although pollen is not ingested by the individuals that collect it. However, it may be that other receptors are involved, such as those sensitive to mechanical stimulation. In the honeybee, innervated setae (hairs) found on the corbiculae have been shown to be sensitive to the degree of mechanical displacement and have therefore been postulated to be involved in detecting changes in the size of the growing pollen load (Ford, Hepburn, Moseley, & Rigby, 1981). Bumblebees have been shown to adjust their handling time and grooming behaviour according to the availability of pollen at flowers (Buchmann & Cane, 1989;

Gori, 1989; Harder, 1990) suggesting they are sensitive to feedback in terms of their pollen foraging success at each inflorescence. For example, Buchmann and Cane (1989) found that when the apical pores of the anthers of nightshade flowers (*Solanum elaeagnifolium*) were sealed with glue, in order to prevent the release of pollen grains, bumblebees exhibited fewer buzzes, were less likely to groom themselves and had a reduced overall handling time, relative to control flowers which had been sham-glued. Results presented in Chapter 4 also provide further evidence that mechano-sensory cues may be important in determining foraging preferences in bumblebees, given that individuals showed a preference for pollen samples with which they had previous experience, even when an alternative sample containing a higher concentration of pollen was available. If mechano-sensory input, for example at the site of pollen-loading in the corbiculae, is necessary for the reinforcement of pollen collecting behaviour then this could also explain why pollen did not reinforce learning in the restrained paradigm.

7.4 Pre-ingestive sensitivity to distasteful compounds

Given that over time honeybees tended to withhold proboscis extension to antennal stimulation with a mixture of pollen and sucrose (Chapters 5 and 6), the inhibitory effect of pollen on acquisition of the conditioned response is thought to result from the release of distasteful compounds from pollen grains. Devaluation of the sucrose reward could stem from the release of poor-tasting compounds or toxins such as alkaloids and phenols and/or the presence of particular amino acids which are known to be less preferred by bees (e.g. serine Bertazzini, et al., 2010; Petanidou, et al., 2006). An alternative, not mutually exclusive, explanation is that the relatively large volumes of pollen added to the solution may have led to an unpalatable concentration of compounds or amino acids of the type typically perceived as neutral, or those that might even serve to improve feeding choice or rate when present at more naturalistic concentrations. Indeed, the deterrent effect of many secondary metabolites on honeybee feeding preferences has been shown to be true only at concentrations greater than those found naturally in nectar (Adler & Irwin, 2005; Hagler & Buchmann, 1993; Inouye & Waller, 1984; London-Shafir, et al., 2003; Reinhard, et al., 2009; Singaravelan, et al., 2005). Similarly the positive effect of amino acids on feeding preferences is also concentration dependent (Inouye & Waller, 1984).

The fact that pollen contains compounds perceived as distasteful by bees may, at first, seem somewhat paradoxical when one considers that all the pollen used in these experiments was collected by honeybees. However, there is still considerable debate as to whether bees possess the ability to detect nutritional differences between pollen species (see Chapter 4). Moreover, when added to water, pollen grains burst as a result of osmostic shock, releasing the contents of the cytoplasm into the solution. Since this may be where such distasteful compounds are typically stored, foraging bees, who do not ingest pollen grains, would not be in a position to detect these substances.

The gustatory repertoire of honeybees is often considered to be limited, given that relative to other invertebrates such as flies, few gustatory receptor genes have been identified in this species. Some have argued that this reflects the narrow breadth of the honeybee diet. Robertson and Wanner (2006)conclude that 'since plants have evolved mechanisms to attract and reward bees, bees have not required the ability to detect and discriminate between the numerous plant secondary chemicals and toxins usually deployed in the chemical ecological arms races between most plants and many insect herbivores'. In contrast, my findings suggest that bees are not only able to detect distasteful compounds, but that they may also impact on their ability to learn and recognise floral features. Current understanding of the gustatory system of bees remains sparse. Attempts to identify a dedicated receptor for bitter tastes has so far been unsuccessful (de Brito Sanchez, et al., 2005), but Wright et al. (2010) have shown that compounds such as quinine elicit a 'deterrent cell' pattern of responding in gustatory receptor neurons, in line with the 'across fibre pattern' theory of gustation, where each receptor neuron is broadly tuned to a wide range of stimuli and unique activation/inhibition patterns are responsible for the detection of gustatory compounds (de Brito Sanchez, 2011; de Brito Sanchez & Giurfa, 2011; R. P. Erickson, 1968, 2008). Whilst Wright et al. (2010) stimulated bees at the proboscis, my results suggest that bees are also able to detect distasteful compounds pre-ingestively at the antennae. A similar result has been shown for caffeine, found commonly in the nectar of citrus plants, though the proboscis was observed to be more sensitive to weaker concentrations of caffeine than the antennae (Mustard, et al., 2012).

7.5 The effect of distasteful compounds on learning in bees

Whilst many studies have looked at the effect of the bitter compounds and toxins present in both pollen and nectar on the feeding rates and survival of bees (Detzel & Wink, 1993; Reinhard, et al., 2009; Singaravelan, et al., 2006; Tan, et al., 2007), relatively few have tested how such compounds might affect the ability of insects to learn and recognise floral features, and thus impact on pollinator movements between flowers. Aside from deterring herbivory and nectar robbing, distasteful compounds in nectar have frequently been postulated to benefit plants by limiting the drinking time of individual visitors, meaning that pollinators visit more flowers, thus improving pollen transfer between individual plants. Indeed Kessler et al. (2008) found that when nicotine production in the nectar of *Nicotiana attenuata* flowers was limited, there was an increase in herbivory, nectar robbing and pollinator drinking times, resulting in a subsequent decline in both male and female fitness, relative to control plants. However, it is important also to consider the possibility that pollinators might learn to recognise such flowers on the basis of distasteful cues and begin to avoid these flowers altogether. Adler and Irwin (2005) found that artificially increasing the concentration of the alkaloid gelsemine in the nectar of *Gelsemium sempervirens* flowers did indeed reduce nectar consumption on individual visits. However pollinators also made fewer visits overall and so pollen transfer between flowers was observed to decline. Thus it seems that a subtle balance between attraction and repellence is necessary in the evolution of floral nectar chemistry.

Rewarding bees with a mixture of caffeine and sucrose has been shown to have an interesting effect on learning, blocking acquisition but not recall of the conditioned response following a delay of 24 hours. Though tests of antennal and proboscis sensitivity found that at high concentrations, the presence of caffeine would lead bees to withhold proboscis extension, inhibition of CS responding was observed even when bees were rewarded with sucrose, providing bees were fed caffeine prior to the experiment. This suggests that the aversive taste of caffeine alone is not solely responsible for inhibition of the conditioned response. In light of such findings, it may be worthwhile comparing memory retention in pollen-sucrose rewarded bees following such a delay, in order to see if the inhibited CS response persists over time.

Other secondary compounds such as amygdalin, common to both the nectar and pollen of almond flowers *(Amygdalus communis)*, may be more difficult for bees to sense and avoid prior to consumption, but may still be detected post-ingestively by bees as a result of general feelings of malaise. Wright et al. (2010) found that post-ingestive learning is mediated by a serotonin sensitive pathway, which results in slower acquisition than the dopamine-mediated pathway of pre-ingestive aversive learning. Thus Wright (2011) suggests that bees may still transfer pollen between amygdalin flowers, during the period before they become aware that a toxin has been ingested, which may have facilitated selection for such a trait in almond flowers.

Chemical analyses will be necessary to identify compounds present in the pollen solution potentially responsible for the inhibition of learning reported here. The concentration of substances is likely to be equally important in explaining their effects on behaviour and learning. Behavioural studies are somewhat limited in determining the mechanisms of detection and the manner in which such chemicals impact on learning, so both electrophysiological and pharmacological techniques will be necessary to determine the mechanisms of inhibition and, pending further behavioural study, may eventually assist in characterisation of the pollen reward pathway.

7.6 Pollen-rewarded learning and plant-pollinator relationships

The fact that pollen-collecting bees are able to learn not only the visual characteristics of pollen, but also additional coloured cues paired with this reward, may have important implications for current thinking regarding the emergence of plant-pollinator relationships. It has long been acknowledged that the sensory and learning capabilities of pollinating insects play a role in shaping the evolution of floral characteristics such as the colour and shape of petals. Galen (1989), for example, provided empirical support for this, by demonstrating that the morphology of alpine flowers is affected by the assemblage of insect pollinators present at a given altitude. Typically the ability of pollinators to recognise and preferentially visit highly rewarding flowers is characterised in terms of nectar-rewarded learning, despite the fact that insect pollination pre-dates the emergence of nectar producing organs. Since pollen, the original floral reward, can reinforce learning in bees, this raises the possibility that

cognitive processing in insects may have played a role in shaping the diversity of angiosperms from the very beginning of their radiation. The question remains as to whether it is appropriate to infer from studies of learning in pollen-collecting insects, the behaviour of ancestral pollinators, which most likely consumed pollen directly at the flower. Therefore it would be extremely interesting to test whether representatives of other groups of pollinating insects are also capable of forming pollen-rewarded associations with visual or olfactory stimuli. Of specific interest would be species from those groups which appeared prior to the emergence of bees i.e. those species considered to be the ancestral pollinators, such as flies, thrips and beetles.

In the first chapter, I proposed that the switch from pollen to nectar as the most common reward for pollinators, may have arisen from differences in the ease with which insects are able to form an association between each reward type and the features used to recognise particular flowers. The production of nectar, thought to have evolved as a by-product of solute excretion by flowers, may have led to the recruitment of novel pollinator groups which were better able to learn about those cues associated with highly rewarding flowers and thus may have eventually out-competed pollen consuming insects. From the plants' perspective, the more efficient the pollinator at locating and recognising flowers, the more likely it is that pollen will be transported between individuals of the same species, thus increasing the frequency of successful pollination events. More efficient learning of floral cues may also benefit plants through reducing the likelihood of receiving heterospecific pollen, which can adversely affect reproduction (Waser & Price, 1983). Comparing sucrose and pollen-rewarded bees trained under similar conditions is one way to test this theory. In the experiments presented here, pollen-rewarded honeybees were found to perform surprisingly well, especially when one considers that bees in this group did not consume their reward; a condition typically thought to limit acquisition in sucrose-rewarded bees (Bitterman, et al., 1983; G. A. Wright, et al., 2007). Following a reversal of the rewarded colour, pollen-rewarded bees did however exhibit longer search times relative to those rewarded with sucrose. Real-time observations of searching behaviour suggest that forager types exhibit different approach paths, with pollen-rewarded bees more likely to engage in zig-zagging flights, which may account for their increased latency in reaching the rewarding stimulus. However, before it is possible to draw any conclusions about the significance of such approach patterns, more in-depth video analysis is needed to confirm that consistent differences between forager types do indeed exist.

The major difference between sucrose and pollen-rewarded bees was in their recall of colours following several bouts of reversed-colour training. Whilst pollen-rewarded bees recalled both colours equally following a one hour delay, sucrose bees preferentially chose blue despite the fact that yellow was the last trained colour. Whilst pollen is able to support learning in bees, apparent variation in the strength and/or time course of memory formation for the two kinds of reward provides some tentative evidence for the notion that differences in the reinforcing properties of pollen and nectar may exist. Pending further characterisation of such differences in the strength of reinforcement, one may eventually conclude that the efficiency with which early pollinators learnt and recalled floral cues might have exerted a selective pressure on early angiosperms to vary the food reward provided to insect visitors.

Bees might be more sensitive to variations in reward quality when foraging for nectar as opposed to pollen, given that only the former is directly consumed by bees during the process of collection. Here bees were observed to display preferences for one pollen sample over another, though not always for the sample containing the highest concentration of pollen. Thus bees did not consistently choose the better quality resource, but were capable of selecting that sample with which they were more familiar, or in the case of novel samples, that which they encountered first during a foraging bout.

Under the current experimental conditions, where pollen was diluted with alpha cellulose, pre-exposure to a certain level of dilution appears to have an impact on foraging preferences. Even when offered pure pollen as an alternative some bees preferred to collect the sample which they had experienced during pre-training. Coupled with the findings of the matched cue test, in which bees were more likely to pay attention to the visual rather than olfactory characteristics of the pollen (Chapter 4), such findings suggest that bees paid less attention to the nutritional content of samples and were more guided by differences in their appearance.

Differences in the size of pollen grains and alpha cellulose particles may have also influenced bees preferences, by affecting the manner in which pollen samples packed into the corbiculae. Further investigations of pollen preferences using this method would benefit from attempts to control for such differences between pollen and alpha cellulose, in order to see if bees are still able to distinguish between samples. Through

this line of investigation it might be possible to resolve the debate as to whether bees are able to distinguish between pollen samples on the basis of nutritional cues alone.

Over the course of several exposures to different pollen types, bees became less selective in their choices and began to collect more diluted samples. Whilst the addition of a visual cue, in the form of a coloured disc, restored pollen foraging constancy this was true only when differences in the pollen concentration between samples was particularly large. Laboratory investigations of nectar-rewarded constancy have yielded similar results. For example Banschbach (1994) observed that bees did not shift their colour preference away from blue towards yellow when there was a 10% difference in sucrose concentration, but when flowers contained 10% and 30% sucrose respectively, preference for yellow increased. Grüter et al. (2011) argue that in order to observe flower constancy in the laboratory, it is necessary to use realistic rewards. Whilst the 90% and 60% pollen samples had a large difference in pollen concentration, the absolute volume of pollen present was still high, especially when one considers that bumblebees typically collect, on average, between 15-20 mg of pollen per foraging trip (Allen, Cameron, McGinley, & Heinrich, 1978). When foraging in the wild, additional factors may also be important in maintaining pollen foraging constancy. The manner in which pollen was presented to the bees, in an open dish, meant that there were few costs associated with visiting the less rewarding sample. Pollen-only flowers in particular, have evolved several morphological adaptations to reduce pollen wastage, often through increasing the difficulty with which pollen may be accessed by visiting insects (Buchmann, 1983; Harder & Thomson, 1989; Westerkamp, 1997; Westerkamp & Claßen-Bockhoff, 2007). Though the effect of handling effort on floral constancy has been considered in some detail in terms of nectar-rewarded learning (Laverty, 1980; Laverty & Plowright, 1988; Lewis, 1993), it has received little attention in pollen-rewarded bees. Raine and Chittka (2007b) have suggested that collecting pollen from poppy flowers (*Papaver rhoeas*) is a complex skill that benefits from learning and takes time to develop. Switching between flowers of different morphologies is considered to be costly since it requires bees to remember multiple handling methods (Laverty, 1980). Thus flowers which require specific behavioural adaptations on behalf of the pollinator, may better promote pollen foraging constancy and improve the degree of successful pollen transfer between individuals.

As well as fostering repeated visits by insects who have mastered the necessary handling skills, concealing pollen may also make it more difficult for insects to assess differences in the availability or quality of the reward prior to alighting (Harder, 1990). In experiments in which the visual properties of pollen were on display (horizontal paradigm, Chapter 3), bumblebees displayed a preference for the yellow stimulus over blue, in contrast to the blue preference typically reported in sucrose-rewarded bees (Chittka, et al., 2004; Gumbert, 2000; Ings, et al., 2009; Raine & Chittka, 2007a). This preference for yellow seemed to interfere with learning of the coloured cues predicting the availability of the pollen reward, presumably as a result of conditioning to the pollen colour during pre-training, thus suggesting that bees pay attention to the colour of pollen they collect. Manipulating the visual properties of the pollen reward and testing subsequent colour preferences could provide empirical evidence for this.

The fact that bees pay attention to the visual properties of pollen is of interest when one considers that in ancestral flowers, prior to the evolution of more complex flower morphologies and the differentiation of the calyx and corolla, the pollen producing organs were thought to serve as an attractant to pollinating insects (Crepet, et al., 1991). It would be interesting to know, in modern flowers, whether bees pay more attention to pollen-based cues or those of the perianth. In experiments in which the anthers and petals were switched between fresh and day-old, pollen depleted flowers, Dobson (1999) reported that androecial cues seemed to be more important in guiding landing decisions than petal cues. After training bees to associate coloured cues with the availability of strong versus weak dilutions of pollen and alpha cellulose, I reversed the colour cues between samples and found that in some cases bees appeared to be guided more by the learnt association with the coloured stimuli than the characteristics of pollen (Chapter 4). However the sample size was very small and so caution must be taken in drawing any firm conclusions from such observations. Nevertheless this is an area which could be worthy of further investigation, to determine the relative importance of the sensory cues and advertisements used by flowers in guiding pollinator behaviour, and their implications for the evolution of floral displays.

The findings presented in this thesis centre around the first demonstration, under controlled conditions, that pollen can reinforce the learning of visual cues in honeybees and bumblebees. I have proposed several methods to be used in future explorations of how bees sense and evaluate variations in the both the availability and quality of this

complex food resource. Studying pollen-rewarded learning could further our understanding of sensory and learning processes in an insect model and may influence current thinking regarding the early evolutionary relationship between angiosperms and their insect pollinators.

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