THE USE OF POLLEN CUES IN RESOURCE LOCATION BY A POLLINATOR AND A PEST

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To Darren
With love & hugs!

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Abstract

This thesis presents evidence that pollen colour and odour may be used as cues in resource-location by pollen feeding insects; namely, the pollen beetle, *Meligethes aeneus*, a pest of cruciferous crops such as oilseed rape, *Brassica napus* (OSR), and the honey bee, *Apis mellifera*, an economically important generalist pollinator.

Pollen beetle adults were attracted to both the colour and odour of OSR flowers in a wind tunnel, and linear track olfactometer studies showed that part of the attractive floral odour emanated from pollen. Beetles were attracted to OSR floral odour throughout their life cycle, indicating that 'innate' search images may be important in location of hosts upon which they can reproduce. Responses to floral odours from plants upon which the beetles do not reproduce varied according to the beetle's sex, life cycle phase and feeding history. There was some evidence for the use of pollen cues in oviposition, when oviposition incidence in male-fertile buds containing pollen and male-sterile buds without pollen were compared in the field. In feeding studies conducted in the laboratory, larvae displayed obligatory requirements for the resources from OSR flowers; they were unable to develop in field bean, *Vicia faba* (FB) flowers. Although pollen consumption was not obligatory for larval survival and development, it reduced developmental time, and improved survival and 'fitness'.

Restrained honey bees were able to learn the odours of OSR and FB pollens associatively, and could discriminate between them in the conditioned proboscis extension bioassay. This ability was confirmed in more natural conditions; free-flying bees discriminated between OSR and FB pollens on the basis of their odour, but showed a colour preference for the yellow OSR pollen over the grey pollen of FB.

The relative importance of pollen as an attractive signal in OSR and FB is discussed.

1. General Introduction

1.1. THE BIOLOGY OF POLLINATION AND THE ROLE OF POLLEN

Pollen is collectively a mass of individual microspores or pollen grains that each represent the male gametophyte of flowering plants. It is produced and released by the anthers of flowers (reviewed by Bedinger, 1992; Pacini, 2000) during the reproductive phase of plants.

Reproduction in flowering plants is achieved through pollination. This occurs when pollen is transferred from the anther of a stamen to the stigma of a pistil. On reaching the ovary, via the growth of the pollen tube, the male gametophyte fertilizes the ovule, which is followed by its growth into seed. Plants have evolved several mechanisms to achieve pollination. These include pollen dispersal systems that use wind or water, and systems effected by animal visitation, particularly insects (Fægri & van der Pijl, 1979; Procter, Yeo & Lack, 1996).

Pollination by insect visitation has evolved to become a generally mutual relationship between the pollinator and the plant (this is sometimes best thought of as mutual exploitation). To motivate visitation, plants provide rewards that satisfy an essential need of the insect. These include nutritive rewards, such as pollen and nectar, and non-nutritive rewards such as shelter, nest materials, sexual attractants, mating sites and egg-laying or brood sites (reviewed by Fægri & van der Pijl, 1979; Kevan & Baker, 1983; Dafni, 1992). The plant usually advertises these rewards by the production of flowers with species-specific colouration and odours. Flower-visiting insects use these advertisements as signals, or cues, in resource location, and their repeated visits to flowers for these resources bring about pollination of the plant.

The role of nectar as a reward, and the uses of flower colour and odour as cues in animal behaviour, have been the subjects of investigation for some time. However, the role of

pollen as a reward, and its importance in providing cues in resource location for flower-visiting insects, have been little studied, and are the subjects of this thesis.

1.2 POLLEN AS A REWARD FOR FLOWER-VISITING INSECTS

1.2.1 Pollen composition and nutrition

Pollen and nectar are the most common nutritive rewards often offered by plants within their flowers to entice insect-visitation. Nectar is an aqueous solution of sugars (usually glucose, fructose and sucrose) secreted by specialist glands (the nectaries) in the flower. Its exact composition, concentration and volume differs between species and the environment. Although it may contain other elements such as amino acids (Baker, 1977), it provides the major carbohydrate (energy) resource for flower-visitors. Its role as a reward in insect flower-visitation has been well studied in terms of pollinator energetics and choice behaviour (e.g von Frisch, 1967; Heinrich, 1983; Rathcke, 1992) and will not be considered in detail in this thesis.

Pollen is the major protein resource for flower-visiting insects. However, in addition to protein, it contains many other nutritious elements such as free amino acids, lipids, sterols, carbohydrates, starch, vitamins and minerals (Todd & Bretherick, 1942; Vivino & Palmer, 1944; Stanley & Linskens, 1974; Herbert & Shimanuki, 1978; Day *et al.*, 1990). The nutritive composition of pollens varies between species. They are all generally high in protein, but content ranges between 8-40% (Herbert, 1992). Lipid-rich pollen is found in species where pollen is the only nutritive reward and is particularly common in insect-collected pollens. Some lipids have a role in insect attraction (see section 1.4 below) and some include sterols that are important in insect hormone or pheromone production (Stanley & Linskens, 1974). The pollen of wind- and self-pollinated plants contains fewer lipids than insect-collected pollens and more starch, which is difficult to digest for some insects (Schmidt, Buchmann & Glaiim, 1989).

1.2.2 Pollen digestion

The pollen grain consists of four layers: 1) the pollenkitt - the lipid layer surrounding the exine 2) the exine, 3) the intine and 4) the protoplast. The pollenkitt can be partially or completely digested, but the exine is indigestible. The intine, a pectin and/or cellulose membrane is also largely indigestible. Utilization of the nutritious protoplast therefore

requires either puncturing or breaking open the exine by specialist jaws, or enzymatic action that may cause the pollen grain to burst open, or cause germination after which the protoplast may diffuse through the germination pores. The modes of pollen ingestion differ between pollen-feeding species and these have been reviewed by Roulston & Cane (2000).

1.2.3 Pollen-feeding insects

A wide variety of animals feed on pollen, including birds and mammals, but the majority are insects. Of the insects, pollen-feeding may be exhibited by the adults and/or the larvae. There are examples in many orders including Collembola (springtails), Thysanoptera (thrips), Plecoptera (stoneflies), Hemiptera (bugs such as Miridae), Coleoptera (beetles), Diptera (flies, especially syrphids, and bombyliids), some Lepidoptera (butterflies and moths) and Hymenoptera (including sawflies, some parasitoids, some wasps and the bees) (see Procter, Yeo & Lack (1996)). This thesis will focus on one example of the Coleoptera, the pollen beetle, *Meligethes aeneus* (Fabricius): Nitidulidae and one of the Hymenoptera, the honey bee, *Apis mellifera* L: Apidae.

Pollen-eating Coleoptera are considered to be the most primitive pollinators. Fossil records of the families of some flower-visiting beetles such as the Nitidulidae go back to the Mesozoic (Procter, Yeo & Lack, 1996). During this period, the beetles ate spores, and the pollen of primitive plants such as Cycads, and, at least some, would have visited the flowers of the earliest angiosperms of the late Jurassic and early Cretaceous periods (Fægri & van der Pijl, 1979). Beetles have few specialized adaptations for pollination. They have primitive mouthparts with simple mandibles able to bite and chew pollen outside the mouth. They are generally associated with open, bowl-shaped flowers from which they may imbibe the nectar, eat pollen and chew on floral parts. Both adults and larvae may depend on pollen for nourishment, and their feeding sometimes causes damage to plants that are cropped; many are considered to be destructive pests rather than useful pollinators (Procter, Yeo & Lack, 1996). The Hymenoptera, conversely, are generally considered to be the most important order of flower-visiting insects, and of them, the honey-storing social bees (Apidae) are the most highly adapted. Bees have mouthparts adapted for imbibing nectar, and specialist adaptations with which to collect pollen from flowers and transport it to their colony to provide nourishment for the developing brood. Furthermore, they have the ability to learn floral intricacies readily and are behaviorally adept at manipulating a wide range of flowers.

1.3 RESOURCE LOCATION BY FLOWER-VISITING INSECTS

1.3.1 Resource requirements

Insects visit flowers for pollen for several reasons: 1) to gain resources needed for their own development and/or maintenance, e.g. for sexual maturation; 2) to collect resources needed by their offspring, as is the case for bees; 3) to find oviposition sites if their offspring require pollen for development. These resources are gained from the insect's host plant, the occurrence of which may vary in time and space, necessitating mechanisms that enable the insect to locate them.

1.3.2 Plant location

Like phytophagous insects, which may feed on various plant parts, flower-visiting insects use plant cues that stimulate visual, olfactory, taste and tactile senses to locate their host plants. Host plant location is usually thought of as a succession of yes/no decisions operating at the habitat, patch and host plant levels with the insect responding to a different set of cues at each step (Hassell & Southwood, 1978; Miller & Strickler, 1984; Jones, 1991). Responses to host cues may be innate or learned; however, probably few responses are completely innate or completely learned. Responses to cues that are innately recognized are shown by completely inexperienced individuals, which respond spontaneously with a particular form of behaviour. Learning may be 'preprogrammed', whereby the insect is predisposed to pay more attention to certain signals than to others and can learn the former better and more quickly (Menzel, 1985). Otherwise, individuals learn by experience, forming associations between certain cues and the reward of the resource. Although taste and tactile cues are important, particularly during the steps of plant examination, and in the stimulation of feeding or oviposition (Miller & Strickler, 1984), only visual (namely colour) and olfactory (odour) cues are considered in detail in this thesis.

1.3.3 Colour cues

Plant height, the size and shape of the flowers and their colours against the background provide visual cues for flower-visiting insects (Fægri & van der Pijl, 1979; Prokopy & Owens, 1983; Procter, Yeo & Lack, 1996; Goulson, 2000). The colours of flowers and their patterns were first recognized as important visual cues in resource location by flower-visiting insects by Sprengel, (1793) and have been widely studied (see Kevan, 1983; Lunau, 1996). Floral colours have evolved to be related to the colour perception of

their pollinators (Chittka & Menzel, 1992; Menzel & Shmida, 1993). The compound eye is the organ of sight in adult insects that perceive these cues. Insect vision and flower recognition was recently discussed in a series of papers edited by Dafni, Giurfa & Menzel (1997). Unlike the human visual spectrum, which ranges from 400-700 nm (blue to red), the visual spectrum of insects generally extends within the shorter wavelengths of the daylight spectrum from ultraviolet (UV) to yellow-orange (300-650 nm). Within this range, many insects show peaks of sensitivity in the UV (300-400), blue-green (400-500) or yellow (500-600) wavelengths (Chittka, 1996). Most research has been conducted on the colour vision of honey bees which have trichromatic colour vision from three receptors in each of these regions (reviewed by Backhaus, 1992; Backhaus, 1993). Colourmetric analysis of floral colours, using a spectrophotometer to measure the light reflectance from the flower, has been one method used to assess the colour cues from flowers, and may lead to insights of how they may be perceived by insects (Dafni, 1992; Menzel & Shmida, 1993; Lunau, 1996).

Insects may orient towards colour cues from any direction. Colour may be important in functioning as a long-distance signal, enabling insects to locate flowers against the background, and to discriminate between flowers of different species at a distance. Floral patterns such as nectar guides probably act over a shorter distance to help the pollinator to position itself correctly on the flower to receive the reward and to maximize the effectiveness of the visit for pollination purposes. Colour may signal the availability of the reward; many plant species undergo post-pollination floral colour changes that signal the absence of the reward (Gori, 1983). Furthermore, colour may signal the quality of the reward; the colour preferences of bees for blue and yellow were correlated to an extent with the probability of a high concentration of nectar measured in flowers of this colour compared with flowers of other less preferred colours (Giurfa *et al.*, 1995). However, this is a relatively unexplored concept, and requires further investigation.

1.3.4 Odour cues

1.3.4.1 Floral odour as cues in insect behaviour

Floral odour has long been suspected to have a role in the attraction of insects to flowers. Darwin described insects flying to flowers covered with a muslin net and attributed this effect to flower odour (Darwin, 1878). The literature contains many reports describing systems in which a 'characteristic odour' is involved in orientation of some insects to

certain flowers: 'foul-smelling' flowers attract some species of flies, flowers smelling of 'fermenting fruit' are associated with some beetles, and bees are generally associated with 'sweet-smelling' flowers (see Kevan & Baker, 1983). Although some preliminary studies demonstrated that flower-visiting insects could be attracted by floral odours alone (von Frisch, 1919), it was not until the work of Kullenberg, on the attraction of male bees to *Ophrys* orchid flowers in the 1950's (see review by Williams, 1983), that the importance of floral odours as cues in insect behaviour began to be studied in detail. These, and subsequent studies, have been facilitated by the development of techniques to collect the odour released by plants and to separate and identify the volatile constituents. Collection generally involves trapping onto adsorbant polymers the volatiles released into the air 'headspace' surrounding a flower in an enclosed vessel. Separation of the odour into its components is achieved by gas chromatography (GC), and mass spectroscopy (MS) is used to identify and quantify the constituents. These techniques have been reviewed (e.g. Dobson, 1991a; Kaiser, 1991, Williams, 1983), and newer techniques have been described and compared by Agelopoulos & Pickett (1998).

Floral odour probably acts as a shorter range attraction cue than colour for some insects, although large stands of plants such as a flowering crop may be perceived down wind from several metres away. The more volatile floral compounds may be important in long-distance attraction and less volatile compounds are probably important in closer range attraction (Bergström, 1978). In addition to attraction, floral odours may also serve as releasers of behaviours such as feeding, mating and oviposition (see review by Dobson, 1994). The mechanisms by which insects orient and respond to odour cues vary between species and the distance over which the odour is operating. Distance orientation may occur by odour-induced anemotaxis (upwind), and chemotaxis may be used closer to the flower where steep gradients in odour concentration occur. Although not restricted to floral odour cues, Payne, Birch & Kennedy (1986) cover the subject of mechanisms in insect olfaction and Kennedy (1977) and Prokopy (1986) review insect orientation to odour sources.

1.4.3.2 Floral odour composition and perception by insects

The odour of a flower is composed of a mixture of many volatile compounds (Borg-Karlson, 1990; Knudsen, Tollsten & Bergström, 1993). These generally belong to three chemical classes: isoprenoids, fatty acid derivatives and benzenoids, although some flowers also contain sulphur- or nitrogen-containing compounds. The number and

composition of volatiles released by flowers varies widely. Variation in the chemical identities and the relative proportions of these volatiles brings about the great diversity in floral scent that results in different species having distinctive odours. This range of odours, combined with the highly developed olfactory systems insects have, may provide more finely-tuned identification labels than colours in resource location.

Recognition of the mixtures of volatile compounds that comprise floral odours is thought to be based on a few key compounds present in the total mixture (Waller, Loper & Berdel, 1974; Pham-Delègue *et al.*, 1986, 1993, 1997). The olfactory receptors principally responsible for the perception of odours are located on the antennae, and the electrical output of these receptors can be measured in response to stimulation by floral odours by electroantennography (EAG). Linked GC-EAG has been used to identify the components of floral odour from some species that are perceived by insects, such as the pollen beetle (Blight *et al.*, 1995) and the honey bee (Thiery *et al.*, 1990). The components perceived usually represent only a few of the total in the mixture and furthermore, behavioural responses are elicited by only some of those volatiles that are perceived (e.g. Pham-Delègue *et al.*, 1997).

1.4.3.3 Floral odour production and emission

Floral odour is emitted either actively from osmophores or it passively diffuses from certain floral regions (Vogel, 1963; Williams, 1983). The principal release sites may be determined by the human sense of smell (D'Arcy, D'Arcy & Keating, 1990), by using specific stains (Dafni, 1992), or by chemical methods (Dobson, Bergström & Groth, 1990; Bergström, Dobson & Groth, 1995). Using these methods, it has been found that each floral structure may produce a different blend of volatiles that contributes to the overall floral fragrance. In some species, the petals contribute most to volatile production and the distinctive floral odour (Gregg, 1983; Borg-Karlson, 1990; Dobson, Bergström & Groth, 1990). Petal odours may even differ between different regions of the petal, producing a gradient of increasing odour intensity towards the flower centre, providing flowers with 'odour guides' that may complement visual nectar guides (Bergström, Dobson & Groth, 1995). In other plants, the androecium (stamens, anthers and pollen) is responsible for production of most or the most distinctive floral volatiles (Coleman & Coleman, 1982; D'Arcy, D'Arcy & Keating, 1990; Pichersky *et al.*, 1994). Chemical studies have shown that of the androecium, the stamens emit odours that differ from the other floral parts (Dobson, Bergström & Groth, 1990; Knudsen & Tollsten,

1991). Pollen has also been shown to release distinctive odours (Dobson *et al.*, 1987; Dobson, Bergström & Groth, 1990; Bergström, Dobson & Groth, 1995) and in some studies, the volatile emissions from the androecium has been attributed entirely to the pollen itself (Lewis *et al.*, 1988). Pollen odours may, therefore, be used by pollen-seeking insects as cues to locate this resource.

1.4 POLLEN CUES FOR POLLEN-SEEKING INSECTS

1.4.1 The importance of pollenkitt

The pollen grains of insect-pollinated plants are typically coated with oily material known as pollenkitt (Knoll, 1930). Pollenkitt is deposited on the pollen grain by the tapetal cells of the anther just before dehiscence (Pacini & Franchi, 1993). It is composed of glycerides, sterols, various volatile terpenoid aromatic and aliphatic compounds, phospholipids, enzymes, glycoproteins, glycolipids, polysaccharides, phenolics as well as flavenoids and carotenoid pigments (Stanley & Linskens, 1974). Pollenkitt has many functions related to its constituents (reviewed by Dobson, 1989), including energy for germination, involvement in pollen-pistil interactions, and protection of the male gameophyte from ultra violet (UV) radiation, desiccation, predation and disease. The additional function of providing visual and olfactory cues to pollen-seeking flower visitors is becoming more recognized (Dobson & Bergström, 2000; Lunau, 2000).

1.4.2 Pollen colour

1.4.2.1 Pollen pigments and the evolution of pollen colour signals

Pollen from different plant species displays a wide range of colours, due to the presence of flavenoids and carotenoid pigments (Stanley & Linskens, 1974; see pollen colour guides of Hodges, 1952, 1984; Kirk, 1994). Flavenoids contained in the intine and cytoplasm produce the non-yellow and many of the pale-yellow colourations of pollen, whereas the vivid yellow-orange hues are due mainly to carotenoids (carotenes and xanthophylls) located in the pollenkitt (e.g Dobson, 1988). The more frequent occurrence of these carotenoid pigments in the pollens of insect-pollinated than wind-pollinated plants (Dobson, 1988) suggests they are an adaptation to pollination by insects by acting as a visual attractant.

The evolution of pollen signals as visual cues in resource location has been recently reviewed (Lunau, 2000). The early spermatophytes (seed-bearing plants) were wind-pollinated with exposed pollen that had UV-absorbant, pale yellow-coloured flavenoid pigments (Lunau, 2000). Pollen was therefore predestined to become an attractive signal to flower visitors, the earliest of which were apparently already capable of colour vision (Chittka, 1996). These visitors evolved behavioural responses corresponding to anther/pollen signals to find pollen, since pollen represented the primary reward at the onset of insect pollination systems. Later in the Cretaceous period, insect-pollinated plants developed enhancements of these signals, including additional yellow carotenoid pigments (spectrally pure, absorbing UV and blue light and reflecting at wavelengths >510 nm), and contrasting colour signals of the petals (UV reflective, less spectrally pure). Nectar rewards probably evolved later still. In some plants, structures that visually mimic anthers and pollen replaced the signaling function of pollen and anthers themselves (Osche, 1979; Osche, 1983; Magin, Classen & Gack, 1989; Cane, 1993).

1.4.2.2 The use of pollen colour cues by insects

Pollen-seeking insects are known to be able to orient to pollen and/or anthers by visual cues. Innate responses to these cues are particularly important in resource location by inexperienced, generalist flower visitors (Lunau, 1996). Newly emerged syrphids (hoverflies) land on yellow objects and extend their proboscis towards deep yellow areas (Lunau, 1988). The proboscis extension response of *Eristalis* spp. is wavelength-specific, elicited only between wavelengths of 520-600 nm and is inhibited by the addition of UV or blue wavelengths (Lunau & Wacht, 1994). This response corresponds with the reflection patterns of pollen, anthers and their mimics, and is related to pollen feeding. After the proboscis extension response, a gustatory stimulus, the free amino acid proline, triggered the ingestion of pollen (Hansen *et al.*, 1998). Bumble bees *Bombus terrestris* (L.) and *Bombus lucorum*, (L.) showed innate responses to visual flower patterns relating to pollen; they approached artificial flowers and touched the area of highest spectral purity with their antennae whilst still in flight (Lunau, 1990). This corresponds to the patterning of most flowers that have petals of lower spectral purity than anthers and pollen, or the centre of the flower, that have high spectral purity. Landing was triggered by visual cues that relate to the shape and size of anthers (Lunau, 1991) as well as pollen odour (Lunau, 1992). Thus innate responses to pollen signals may enable even inexperienced individuals to orient in such a way that they can make contact with pollen.

Some insects can learn to associate pollen signals with a reward, although established examples are few, and restricted to bumble bees (Gack, 1981) and hoverflies (Lunau, 1988). As described above, the pigmentation of pollen can contribute to the floral colour patterns by providing a contrast with the petal colour and other floral parts. Conversely, removal of pollen by one flower-visitor may serve as an avoidance signal to subsequent pollen-seeking visitors in the form of a post-pollination colour change (Gori, 1983). Foraging bumble bees were able to discriminate between recently visited and hence less rewarding flowers and unvisited, more rewarding, possibly by visually assessing the amount of pollen on flowers before landing (Zimmerman, 1982). However, this assessment could have also involved pollen odour cues.

1.4.3 Pollen odour

1.4.3.1 The evolution of pollen odours

Pollen odours probably evolved as defence against pathogens and pollen-feeding animals before the development of insect pollinated systems (Pellmyr & Thein, 1986), and may represent the oldest food attractants for flower visitors (van der Pijl, 1960; Fægri & van der Pijl, 1979; Crepet, 1983). A variety of functions have been proposed for pollen odour, but these are again related to the balance between pollinator attraction and pollen protection. The role of pollen volatiles in pollen defence has been reviewed recently by Dobson & Bergström, (2000), and will not be considered further here, since this thesis focuses on the use of pollen odour as (attractive) cues in resource location by flower-visiting insects.

1.4.3.2 Pollen odours are released from the pollenkitt

The lipid composition of pollen pollenkitt from 69 angiosperm species, representing 28 different families, was investigated by Dobson (1988). The lipids were almost exclusively of neutral classes, which are common constituents of essential oils, and it was suspected that these must be responsible for giving pollen its odour. It was later confirmed that volatiles contained in the pollenkitt are the same as those released into pollen headspace, and thus it was concluded that pollen odour emanates from the pollenkitt (Dobson *et al.*, 1987; Dobson, 1989; Szalai, 2000; reviewed by Dobson & Bergström, 2000).

The quantity of odour released by pollen is small compared with the emissions of the whole flower. Obtaining enough material for volatile analysis represents a challenge, and Dobson, (1991a) suggested some adaptations to collection and analytical techniques that meet some of the problems. Pollen odour is composed of several volatiles from the three major classes of compounds reported in floral odours (Knudsen, Tollsten & Bergström, 1993), namely fatty acid derivatives, isoprenoids and benzenoids (see Dobson & Bergström, 2000). Most of these volatiles originate from the pollenkitt i.e. are of tapetal origin (Pacini & Franchi, 1993). However, in some plants, the pollenkitt may adsorb volatiles from other odour-producing floral structures. The extent to which this occurs depends on the species, and on the length of time the pollen is within the flower before its removal (Dobson, Bergström & Groth, 1990; Dobson & Bergström, 2000).

1.4.3.3 Pollen odours are species-specific

In Dobson's (1988) study described above (1.4.3.2) in which the lipid composition of pollenkitt from a range of species was examined, their compositions varied widely between species, indicating that these lipids may provide pollen with species-specific odours. This was first confirmed by a study that compared the headspace volatile from pollens of two species of rose, *Rosa rugosa* (Thunb.) and *Rosa canina* (L.) and found them to be different (Dobson *et al.*, 1987). The pollen headspace odours of 15 species from 10 different families have been analysed to date, but only 12 species (representing 11 genera and 7 families) have provided sufficient quantities of volatiles to be able to identify the components (Dobson & Bergström, 2000). This highlights the difficulties associated with working with a material that produces volatiles only in small quantities. By comparing the volatile compounds present in the pollens of these 12 species, it is clear that species vary in the classes of compounds they contain as well as their amounts (Dobson & Bergström, 2000). Identification of the volatiles present in these 12 species demonstrates that each species has a specific volatile profile, representing a unique mixture of compounds. This mixture varies in the number of compounds present (between 5-30) and is usually dominated by certain volatiles belonging to one or two chemical classes; there is no common 'pollen volatile' (Dobson *et al.*, 1987; Dobson, Bergström & Groth, 1990; Bergström, Dobson & Groth, 1995; Dobson, Groth & Bergström, 1996).

Species-specific pollen odours may enhance the pollinators' ability to discriminate among pollen and flowers of different species very precisely, and therefore increase

flower-constancy and pollinator efficiency. For pollen-specialist insects, species-specific pollen odours could serve as key recognition cues, triggering innate behavioural or physiological responses. Species-specific cues could also be learned during foraging and used in flower-constant foraging.

1.4.3.4 Pollen odours are distinct

Pollen odour has been found to be distinct from that of the rest of the flower. Contrasts range from being subtle to strong, and may result from pollen and floral odours differing in one of three ways:

1. by differing only in the relative proportions of the constituent compounds present. For example, chicory (*Cichorium intybus*, L.)(Compositae)) (Dobson, 1991 b & c) and field poppy (*Papavar rhoeas* L.) (Papaveraceae)) (Dobson, Groth & Bergström, 1996)
2. by differing in both the relative proportion and the number (pollen usually has fewer) of volatiles, e.g. lupin (*Lupinus polyphyllus* (Lindley)) (Fabaceae) (Dobson, Groth & Bergström, 1996)
3. by pollen producing fewer volatiles, some of which are pollen-specific. These include rose (*R. rugosa* (Rosaceae)) (Dobson, Bergström & Groth, 1990), buttercup (*Ranunculus acris*, L.) (Ranunculaceae)) (Bergström, Dobson & Groth, 1995) and dropwort (*Filipendula vulgaris* (Moench) (Rosaceae)) (Dobson, Groth & Bergström, 1996).

A chemically distinct odour contrasting with odours from other floral parts may allow pollen odour to be used by pollen-seeking insects as a cue to locate this resource. Variations in the intensity of pollen odour may enable assessments of the availability of the reward whilst still in flight, and may guide the insect directly to the resource on alighting in the flower. This would benefit the plant through increasing pollination efficiency by attracting pollinators to flowers of reproductive maturity and by leading them to position themselves appropriately on the flower for effective pollination.

1.4.3.5 The use of pollen odour cues by insects

Pollen volatiles are quantitatively weak compared to the whole flower (Dobson *et al.*, 1987), so are probably used as short distance cues, just before and after landing on the flower. Pollen odour may be involved in the attraction of pollen-seeking insects to the pollen of their host plants, and in eliciting landing, feeding and oviposition behaviours.

Attraction Pollen odour has been implicated in the location of pollen resources from host plants by several specialist pollen-feeding insects. Host-specific thrips distinguished their host pollen from the pollens of non-host species without probing, and pollen odour was inferred in this recognition (Kirk, 1985b). Similarly, pollen beetle attraction to the anthers of its host by odour cues was implied (Charpentier, 1985) but in both these cases, orientation to odour cues was not specifically tested for. Most experimental evidence of the use of pollen cues in pollen-location is from studies involving bees. The species-specific pollen odours may be used in host plant recognition of some solitary bees. Naïve individuals of the oligolectic bee *Colletes fulgidus longiplumosus* (Stephen) were able to recognize their host plants from other species based on floral or pollen odours alone (Dobson, 1987). *Chelostoma florisomne* (L.) recognized their hosts (buttercup) better when offered pollen odours rather than floral odours, and this was found to be due to the presence of a key recognition compound, protoanemonin, in the pollen (Dobson, unpublished data in Dobson & Bergström, 2000).

Honey bees conditioned to pollen were able to discriminate between pollen and whole flowers of the same species, indicating that these odours are chemically distinct to bees (von Frisch, 1923; von Aufsess, 1960). Bees could, therefore learn to associate pollen odour with the pollen reward and use these cues to assess pollen availability during foraging. However, this possibility requires further investigation. Bees were attracted to, and discriminated between, pollens when these were offered as whole pollen (Levin & Bohart, 1955) or pollen extracts. Solvent extracts of pollen applied to non-pollen materials conferred attraction on these otherwise unattractive materials (Hügel, 1962; Taber, 1963; Lepage & Boch, 1968). In some cases attractive compounds have been isolated and identified as: sterols (Hügel, 1962), a carotenoid ester, and a free fatty acid (Lepage & Boch, 1968). However, these effects may be partially due to gustatory stimulation by non-volatile feeding stimulants, and require further investigation.

Landing Landing behaviour by bumble bees on artificial flowers as described above (1.4.2.2) was elicited by visual signals from the anthers but the combined presentation of visual signals and pollen odour were most effective at promoting landing (Lunau, 1992). Pollen odour was used as an evaluation of resource availability pre-landing by bumble bees: the addition of pollen volatiles (tetradecyl acetate and eugenol) to flowers without anthers increased landing and 'buzz' pollen-

collecting behaviour compared with flowers without their anthers (Dobson, Danielson & Van Wesep, 1999).

Feeding The presence of pollen-specific volatiles, possibly with defence-related functions, may be used by specialist pollen feeding insects as feeding stimulants. This may be the case for the solitary bee *C. florisomme* and protoanemonin in buttercup, as described above. Parthenin, an allelochemical present in the pollen of *Parthenium hysterophorous* (L.), was found to stimulate feeding in the chrysomelid beetle, *Zygogramma bicolorata* (Pallister), which specializes on feeding on this noxious plant (Jayanth *et al.*, 1993). Other, more general feeding stimulants may also be present in pollen (Schmidt, 1985; Lin & Mullin, 1999) and perhaps include free amino acids (Mullin *et al.*, 1994; Wacht, Lunau & Hansen, 1996, 2000; Hansen *et al.*, 1998).

Oviposition For insects whose larvae require pollen for development, pollen odours may act as oviposition stimulants. Sunflower (*Helianthus annuus* L.) pollen is required for the development of larvae of the sunflower moth (*Homoeosoma electum* (Hulst)). The odour of pollen contains a volatile thought to stimulate oviposition in opened flowers (Delisle *et al.*, 1989). Furthermore, sunflower pollen volatiles elicited earlier calling behaviour of virgin females and increased their rate of egg maturation, perhaps enabling females to match their reproduction with the availability of pollen in time and space (McNeil & Delisle, 1989). A similar effect of pollen volatiles was also reported for the European sunflower moth (*Homoeosoma nubulellum*) (Le Métayer *et al.*, 1993).

1.5 OILSEED RAPE

1.5.1 Agricultural importance

At present, oilseed rape (OSR) is the third most widely grown crop in the UK after wheat and barley (Winfield, 1992). The history of OSR production in the UK was reviewed by Bunting (1984) and the economic constraints of its cropping are discussed by Evans & Scarisbrick (1994). It is grown mainly for its oil, which is crushed from the seeds and used for human consumption as well as a variety of industrial purposes, and the residual meal is used as animal feed (Evans & Scarisbrick, 1994; Carruthers *et al.*, 1995).

Oilseed rape belongs to the genus *Brassica*, of the cabbage family, Brassicaceae (=Cruciferae), which contains many agriculturally important crops (Free, 1993). Oilseed rape is a general term that includes four species: *Brassica napus* L. (swede rape) *Brassica rapa* (ex. *campestris*) (L.), (turnip rape), *Brassica juncea* Coss, (mustard rape) and *Brassica carinata* L. (Bunting, 1984). It is cultivated almost worldwide, the major production areas being Europe, China, Canada and India. The species cultivated in a particular region depends mainly on its environmental conditions. In Europe, *B. napus* and *B. rapa* are most commonly grown, and their subspecies *oleifera* have annual and biennial varieties (spring and winter, respectively). Most OSR grown in the UK is *B. napus oleifera* and it is this sub-species of OSR that shall be considered in this thesis.

1.5.2 Floral biology

The floral biology of OSR has been described by several workers (Eisikowitch, 1981; Adegas & Couto, 1992; Free, 1993; Delapane & Mayer, 2000). The flowers are borne on racemes (Fig. 1.1). Flowers each have four sepals, four yellow petals, a central style with a two-lobed stigma surmounting the ovary, six stamens (four long ones that surround the style, and two shorter ones), and four nectaries at the base. Flowers begin to open early in the morning, and remain open for three to four days. When the flower opens, the stigma is already receptive, and is below the level of the short anthers, but style extension occurs over the life of the flower so that by the third day the stigma is close to the top of the anthers on the long stamens. The anthers begin to dehisce after the first day of opening; those on the two shorter stamens dehisce inwards, and those on the long stamens release pollen outwards. When the flower is about three days old, the long stamens bend towards the style, facilitating self-pollination. The flowering period of the plant lasts between 22-45 days.

1.5.3 Pollination requirements

The pollination requirements of OSR have been reviewed by Free (1993). *B. napus* is self-fertile, although it does not always self-pollinate (Eisikowitch, 1981) and benefits from cross-pollination. Both self- and cross-pollination are facilitated by wind and insect vectors (Free & Nuttall, 1968; Williams, Martin & White, 1987; Mesquida *et al.*, 1988). Eisikowitch (1981) found that many of the pollen grains remained adhered to the anthers even when exposed to high artificial wind velocities but when anthers were flicked upon insect visitation, a cloud of pollen was released. Insects may therefore aid the effectiveness of wind pollination. Insect pollination is reported to have many other

benefits, including increased production of pods and seeds, increased seed weight, and also an increase in the seed set of early flowers that causes evenness of ripening which facilitates harvest (Free & Nuttall, 1968; Williams, Martin & White, 1987; Adegas & Couto, 1992).

Figure 1.1 Oilseed rape flowering raceme

1.5.4 Insect visitors

The flowers of OSR display many of the typical 'general-insect-pollinated' criteria: bright yellow petals, pollenkitt-coated pollen grains, and the production of nectar (Fægri & van der Pijl, 1979) and a great diversity of insects, including bees, beetles, flies and butterflies can be found on the crop (Kirk, 1992). The most common bee-visitors to flowers are honey bees (Adegas & Couto, 1992; Delapane & Mayer, 2000). However, the most abundant insects on the crop are pollen beetles (Free & Williams, 1979; Attah & Lawton, 1984; Winfield, 1992). These are regarded as pests together with other insect visitors such as the cabbage seed weevil (*Ceutorhynchus assimilis* Payk.), the brassica pod midge (*Dassineura brassicae* Winn.), and the cabbage aphid *Brevicoryne brassicae*

L. (Alford, Cooper & Williams, 1991; Winfield, 1992; Evans & Scarisbrick, 1994), all of which have specialized to some degree on feeding on cruciferous plants such as OSR (Bartlet, 1996).

1.5.5 Attractive properties

Oilseed rape flowers secrete nectar at a concentration ranging from 10-30% (Pierre *et al.*, 1999). Nectar standing crops of 0 – 0.4 mg sugar per flower were found by Mesquida *et al.*, (1988). Pollen is presented open to the environment and may be easily accessed by pollen-seeking insects. It is nutritious, containing around 24-27% protein (Rayner & Langridge, 1985; Stace, 1996).

Flower colour has been shown to be important in attracting pollinators to plants of the Brassicaceae (Kay, 1976). The petals are coloured bright yellow (to which many of its pollinators have an innate attraction (Lunau, 1988; Lunau, 1991; Wäckers, 1994)), and the centre of the flowers also reflects in the near UV (350-400 nm) (Wäckers, 1994). UV-absorbing yellow pollen (Lunau, 1996) may provide a colour-contrast with the petals for efficient orientation of pollen-seeking insects.

Glucosinolates occur in all tissues of the Brassicaceae (Kjaer, 1976; Fenwick, Heaney & Mullin, 1983). Isothiocyanates are the volatile products of the degradation of glucosinolates by thioglucosidases when the tissues are damaged and are considered to be part of the plant's defence against fungal and insect attack (e.g. Feeney, 1977). However, they may be released in low levels by intact plants (Cole, 1980). For insects that are specialists on the Brassicaceae, isothiocyanates can serve as attractants (Free & Williams, 1978a; Blight & Smart, 1999; Smart & Blight, 2000) and may stimulate oviposition (Gupta & Thorsteinson, 1960; Nair & McEwen, 1976; Borg & Borg-Karlson, 1996). However, oilseed rape flowers release many other volatiles including terpenes, fatty acid derivatives, benzenoids and organic sulphides (Tollsten & Bergström, 1988; Evans & Allen-Williams, 1992; Robertson *et al.*, 1993). The role of these floral volatiles, the contribution of pollen to them, and their effects on insect attraction, have been relatively unexplored.

1.6 FIELD BEAN

1.6.1 Agricultural importance

Vicia faba L. (Leguminosae) the faba bean, is the sixth most important pulse crop after soybean, Phaseolus, peanut, pea, and chickpea (Stoddard & Bond, 1987). Two types are widely cropped in the UK; *V. faba* var. *major*, has large seeds and is commonly known as broad bean and *V. faba* var. *minor* has smaller seeds and is commonly known as field bean: these are grown for human consumption and as animal feed, respectively. This thesis will consider *V. faba* var. *minor* only.

1.6.2 Floral biology

A field bean plant has between 50-80 flowers, which are borne in the leaf axils on racemes of 8 or more flowers (Fig. 1.2). Flowers first open in the afternoon and reopen daily for 6-7 days after anthesis. The irregular-shaped corolla consists of an erect standard petal, two wing petals and two lower petals, united to form the keel. The keel encloses ten stamens and a single pistil. Nine of the staminal filaments are united in a sheath that surrounds the ovary, and the tenth is free. The ovary contains 2-4 ovules. The style is at right angles to the ovary and bears a brush of hairs below the stigma. The anthers dehisce so that a plug of sticky grey pollen is contained in a pocket in the keel petal above the stigma. The filaments then contract and the anthers are left near the neck of the style; some pollen is usually still available here and is harvested by pollen-foraging bees (Free, 1962). When a bee lands on the wing petal the flower is opened and the wing petal-keel petal complex is forced downward while the pistil stays in place, and the flower is 'tripped'. The brushes on the style brush some of the pollen from the keel pocket onto the stigma and the bee as it pushes into the flower to reach the nectar. In doing so, the bee breaks the stigmatic cuticle and its exudate, essential for pollination, is released, and either pollen already present on the bee from a previous visit or self-pollen brings about pollination. The field bean is therefore partially self- and cross-pollinated (Stoddard & Bond, 1987).

1.6.3 Pollination requirements

Field bean pollination is reviewed by Free (1993). Although self-pollination is possible, this is facilitated by insect visitation (Riedel & Wort, 1960; Scriven, Cooper & Allen, 1961). Only large bees are heavy enough to trip the flowers to effect pollination (Stoddard & Bond, 1987). The benefits of bee-pollination include greater seed set of the

lower flowers, resulting in earlier seed set, more simultaneous ripening and drying, and ease of harvest (Free, 1993; Manning, 1995). In addition, plants grown from highly outcrossed seed are more vigorous than those grown from inbred seed (Scriven, Cooper & Allen, 1961).

Figure 1.2 Field bean flowering raceme

1.6.4 Insect visitors

Bumble bees and honey bees are the most abundant visitors to field beans (Free, 1993). However, their value as pollinators differs, due to the nectar-robbing behaviour of short-tongued bumble bees; they bite holes in the base of the corolla to reach the nectar, failing to trip the flower and effect pollination. Nectar foraging bees can learn to gather nectar from these bite-holes, rather than tripping the flower, and are also of little value as pollinators. Long-tongued bumble bees and pollen-gathering honey bees are more consistent pollinators, since they trip the flowers upon their visits. Other insects that visit field bean flowers include pollen beetles (*Meligethes* spp.), pea thrips, (*Kakothrips pisivorus* (Westw.)), flower weevils (*Apion vorax* Herbst) and ants (Stoddard & Bond, 1987). Evidence suggests that these are not effective pollinators: male-sterile plants were not pollinated when caged with male-fertile ones in the presence of thrips and pollen beetles but without bees (Stoddard, 1986). Some of these insects can reduce pollination; thrips and pollen beetles through eating the pollen and flower weevils through damaging the pistil (Stoddard & Bond, 1987).

1.6.5 Attractive properties

Nectar standing crops from field bean flowers in the field range from 0 - 0.9 mg sugar per flower with a concentration ranging from 6-50% (Stoddard & Bond, 1987). Field beans also possess extrafloral nectaries which attract ants (Free, 1993). The pollen offers good nutrition, having around 23% protein (Stace, 1996). Field bean flowers advertise these rewards by their colour and odour. The flowers are presented in a group that makes a vivid display against the green background and the white corolla normally has complex coloured patterns; brown-purple marks are present on the standard petal and the two wing petals each bear a large dark brown-black spot (Fig. 1.2). These patterns are probably involved in eliciting the landing responses and positioning behaviour of visiting bees. The flowers produce a distinctive scent that is dominated by (*E*)-ß-ocimene (Sutton *et al.*, 1992). Bees may use its scent for flower-recognition, as has been shown for other legumes (Waller, Loper & Berdel, 1974). The petals completely enclose the pollen, therefore it is likely that the petals are responsible for the attraction of pollen-seeking insects to the flowers, although the role of pollen has not been investigated.

1.7 THE HONEY BEE

1.7.1 Classification

The order Hymenoptera contains the superfamily Apoidea: the bees, which contain 11 families and include species with varying degrees of solitary or social lifestyles (O'Toole & Raw, 1991). The family Apidae includes the stingless bees (Meliponinae), bumble bees (Bombinae), and the honey bees (Apinae of the genus *Apis*). The honey bee genus, *Apis* comprises seven species, *Apis laboriosa*, *Apis andreniformis* and four species of true honey bees (those which store considerable quantities of honey): *Apis dorsata,* the giant honey bee, *Apis florea*, the little honey bee, *Apis cerana*, the eastern honey bee and *Apis mellifera*, the western honey bee. The first three species occur wild in southern Asia, whilst *Apis mellifera* is indigenous to Europe and Africa, but has been introduced to almost every country in the world from Europe, and it is this species that is considered in this thesis. It is a highly evolved pollen-eating insect of great economic importance.

1.7.2 Life cycle

The honey bee *Apis mellifera* L. displays the highest degree of social development of all bees. It has three social castes: the queen (a fertile female) the worker (a non-reproductive female) and the drone (a male) (Fig. 1.3). A colony of honey bees normally consists of one queen, several thousand workers and a few hundred drones. The life of the colony is perennial, but the lives of individual workers and drones are relatively short, living for around one month in summer, and a few months over winter. A queen may live several years, and her main task is laying the eggs from which the other members of her colony develop. Under normal conditions, fertilized (diploid) eggs develop into workers or new queens, and unfertilised (haploid) eggs develop into drones. The diet of the diploid female larva determines whether the emerging adult will be a queen or a worker. All larvae are fed on 'royal jelly'; a protein-rich secretion from the mandibular and hypopharyngeal glands of nurse bees, derived from pollen. Larvae destined to become queens are fed greater quantities of royal jelly than workers or drones. The development of the eggs through the five larval instars and the pupal stage to eclosion of the adult takes place in the brood cells of comb (Fig. 1.3). The developmental times of each caste is different; on average, queens take 16 days to develop into adults, workers take 21 days and drones 24 days.

In Europe, the drones are present only during the early summer and their sole function is to mate with queens from other colonies. The worker bees' duties change with age. They are initially confined to the colony where they are involved with cleaning and comb-building duties and the nursing of developing brood (larvae). Queen-tending and food-handling are the next duties undertaken. When about 15 days old, workers become involved in outside tasks, such as colony ventilation, guarding, and foraging for water and food (namely nectar and pollen) for their colony. Pollen collected by foraging worker bees is the colony's main source of protein. Nectar is converted into honey by salivary enzymes and is used to feed the colony. Some pollen and honey is stored to provide food over winter and in periods of dearth.

Bees are highly efficient foragers, and learn to use both colour and odour as cues in locating rewarding flowers from which they collect pollen and nectar. An individual forager tends to remain constant to a particular species until they learn or are 'informed' that more highly rewarding species are available; their highly evolved communication system keeps bees informed of the most productive foraging sites, as well as coordinating

activities within the hive. These communications are beyond the scope of this thesis, but see e.g. von Frisch (1967) and Seeley (1985) for accounts of these processes.

The mechanisms by which foraging honey bees collect pollen from flowers and pack it into their corbiculae (commonly known as 'pollen baskets') for transportation back to the hive has been summarized by Hodges (1952, 1984). The importance of pollen in the life of the colony for the development of adult bees (De Groot, 1953) and especially for the developing brood (Herbert, Shimanuki, & Shasha, 1980; Imdorf *et al.*, 1998) is well known, but little is known about the cues which bees use to find pollen in a flower, or what makes pollen attractive to them, or why they seem to show preferences for some types over others (e.g. Levin & Bohart, 1955; Percival, 1955; Free, 1963; Doull, 1966; Boch, 1982; Schmidt *et al.*,1995). Such information could be used to increase the efficiency of bees' pollination of crops.

1.7.3 Importance as a pollinator

Due to their flower-constant foraging behaviour (Betts, 1935), whilst collecting food supplies for their colony, honey bees are responsible for the pollination of many wild and cultivated plants (Corbet, Williams & Osborne, 1991). The rental of honey bee colonies for commercial pollination is an important component of the bee-keeping industry (Williams, Corbet & Osborne, 1991; Carreck & Williams, 1998). The benefits of bee-pollination of plants that are cropped was recently reviewed by Delapane & Mayer (2000). In this review, it was quoted that one third of the human diet can be traced to bee pollination. In the UK, at least 39 crops are grown for fruit or seed that require insect pollination, and a further 32 need insects for propagative seed production, and bees make up the majority of insect visitors to these crops (Carreck & Williams, 1998). In a survey of 13 of the major field crops and two glasshouse crops, Carreck & Williams (1998) estimated that the annual value of insect pollination attributable to the activity of honey bees is £137.8 million.

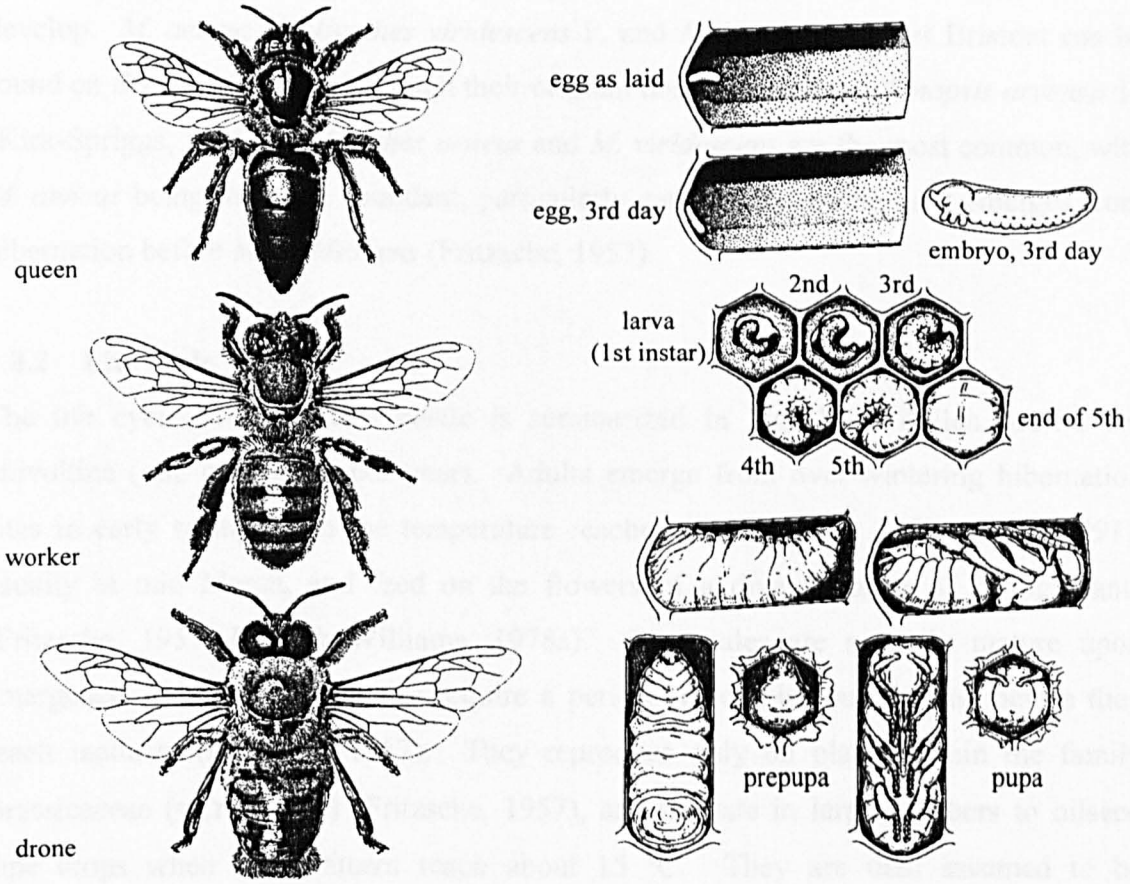


Figure 1.3. The castes and stages of development of the honey bee, *Apis mellifera*. Adult honey bees are shown on the left. The development of a worker honey bee from egg to pupa is shown on the right. Adapted from Dade (1977). Used with kind permission from the International Bee Research Association (IBRA).

1.8 THE POLLEN BEETLE

1.8.1 Classification

The bronzed blossom beetle, (*Meligethes aeneus* (Fabricius)), is commonly known as the pollen beetle, as it is often seen dusted with pollen. It is small (1.9-2.7 mm long and 1.3-1.5 mm wide), with foreshortened elytra and 11-segmented antennae with a compact 3-segmented club (Kirk-Spriggs, 1991, 1996). There are four subfamilies of British Nitidulidae (Kirk-Spriggs, 1991, 1996): Meligethinae, Carpophilinae, Nitidulinae, and Cryptarchinae. The latter three subfamilies have adults and larvae that develop in carrion and fungi, whilst the Meligethinae are associated with flowering plants, and are represented by two genera in Britain: *Pria*, which has one species (*Pria dulcamare* Scopoli) and *Meligethes,* which has 36 recorded British species (Kirk-Spriggs, 1996). Each species tends to be specific to an individual plant species on which the larvae

develop. *M. aeneus*, *Meligethes viridescens* F. and *Meligethes fulvipes* Bristout can be found on *Brassica* species, although their original host was probably *Sinapsis arvensis* L. (Kirk-Spriggs, 1996). *Meligethes aeneus* and *M. viridescens* are the most common, with *M. aeneus* being the most abundant, particularly early in the spring as it emerges from hibernation before *M. viridescens* (Fritzsche, 1957).

1.8.2 Life cycle

The life cycle of the pollen beetle is summarized in Fig. 1.4. Pollen beetles are univoltine (one generation per year). Adults emerge from over-wintering hibernation sites in early spring when the temperature reaches 10 °C (Láska & Kocourek, 1991), usually in mid March, and feed on the flowers of a diverse range of spring plants (Fritzsche, 1957; Free & Williams, 1978a). The males are sexually mature upon emergence, although the females require a period of polyphagous feeding before they reach maturity (Fritzsche, 1957). They reproduce only on plants within the family Brassicaceae (=Cruciferae) (Fritzsche, 1957), and migrate in large numbers to oilseed rape crops when temperatures reach about 15 °C. They are then assumed to be oligophagous on these plants, upon which they mate (Fritzsche, 1957; Free & Williams, 1978a). Females prefer to lay their eggs in flower buds 2-3 mm in size, ovipositing through an oviposition hole that they bite into the base of the bud (Scherney, 1953; Fritzsche, 1957; Nilsson, 1988b; Ekbom & Borg, 1996). Generally, 2-3 eggs are laid per bud, although up to 10 have been found (Ekbom & Borg, 1996). There are two larval instars (Osborne, 1964): the first instars develop within the bud for around 5-10 days (Nilsson, 1988c) after which they moult into the second and final instar, usually around the time that the bud opens. Second instar larvae feed on pollen within open flowers, and move up the raceme to feed on new flowers (Williams & Free, 1978) until they complete their development, in approximately 14 days (Bromand, 1983). They then drop to the ground where they pupate in the soil. The new generation adults emerges 1-5 weeks later (Bromand, 1983), and have a period of polyphagous feeding on the flowers of a diverse range of late summer-flowering plants before migrating to hibernation sites in moist debris in woodland areas (Fritzsche, 1957; Blazejewska, 1960).

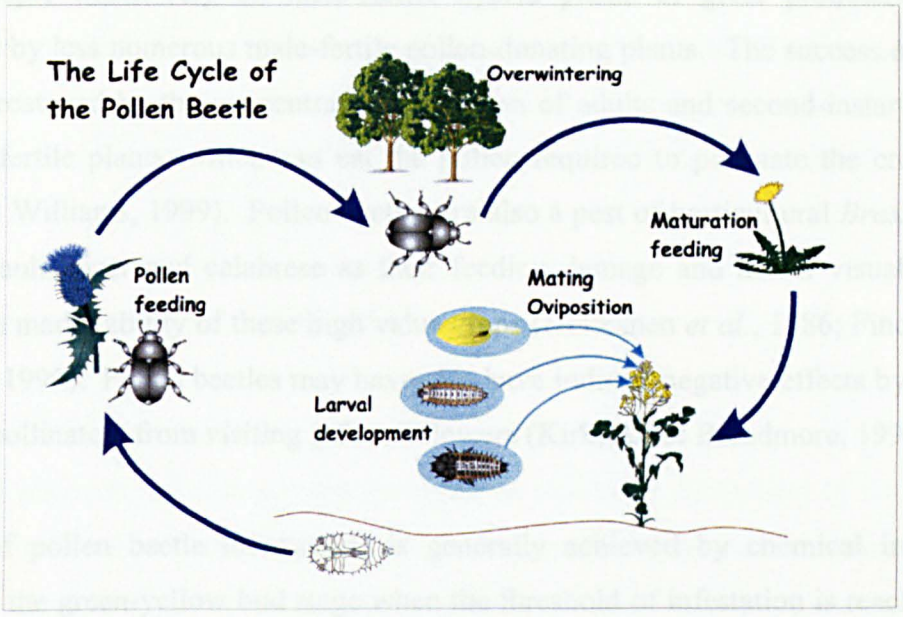


Figure 1.4 Pollen beetle *Meligethes aeneus* life cycle

1.8.3 Pest status and control

The mandibles of pollen beetles contain cavities with large openings (dorsally) to the exterior with a hollow that is possibly adapted to the transport of pollen grains (Crowson, 1981). The pollen beetle has thus been implicated in the pollination of its hosts (Faber, Fischer & Kalt, 1920; Scherney, 1953; Crowson, 1988; Williams, unpublished data in Alford, Cooper & Williams, 1991). However, this contribution is not considered to be economically important, and this is reflected in the thresholds for its control.

Feeding and oviposition damage by adults to the buds <2 mm in size, as well as feeding damage of many larvae inside a bud may cause bud abscission, resulting in podless stalks (Gould, 1975; Free & Williams, 1978b; Nilsson, 1988d). The crop is able to compensate for such damage to an extent: injury to the terminal raceme results in compensation through an increase in the number of side racemes, but seeds from pods on these racemes are generally smaller and contain less oil (e.g. Sylven & Svensson, 1976; Nilsson, 1987; Axelsen & Nielsen, 1990). The pollen beetle is a particular threat to the spring-sown crop (Alford & Gould, 1975), which flowers later than the winter one, and thus has more of the vulnerable small buds when the main migration of beetles occurs than winter-sown crops, and losses may reach 70% if left uncontrolled (Nilsson, 1987).

A new threat of pollen beetle infestation is posed to recently-introduced composite hybrid crops, which rely on male-sterile hybrid plants of great productivity being pollinated by less numerous male-fertile pollen-donating plants. The success of the crop can be threatened by the concentrated population of adults and second-instar larvae on the male-fertile plants, which can eat the pollen required to pollinate the crop (Cook, Murray & Williams, 1999). Pollen beetles are also a pest of horticultural *Brassica* crops such as cauliflower and calabrese as their feeding damage and actual visual presence reduce the marketability of these high value crops (Hokkanen *et al.*, 1986; Finch, Collier & Elliott, 1990). Pollen beetles may have also have indirect negative effects by deterring foraging pollinators from visiting infested flowers (Kirk, Ali & Breadmore, 1995).

Control of pollen beetle infestations is generally achieved by chemical insecticides applied at the green-yellow bud stage when the threshold of infestation is reached. This is currently an average of five beetles per plant on conventional cultivars or an average of one beetle in every five plants inspected for composite hybrid varieties (CPB Twyford, 1996). However, the development of integrated pest management and 'push-pull' strategies continue to be important, due to insecticide resistance and public pressure for a reduction in the applications of synthetic agrochemicals. Such strategies are based on augmenting chemical control by use of the natural enemies of pollen beetles together with deterring the colonization of the crop and trap-cropping. A degree of natural control can be exerted by larval parasitoids of the pollen beetle such as *Phradis morionellus* (Holmgren) and *Diospilus capito* Nees (Nilsson & Andreasson, 1987; Hokkanen, 1989), and entomopathogens such as *Metarhizium* (Butt *et al.*, 1998). Deterring the pollen beetle's colonization of the crop involves making the crop less attractive through the use of resistant cultivars that are less preferred by the pollen beetle, or though the use of pheromones, or repellent chemicals. Trap cropping (Hokkanen *et al.*, 1986; Buechi, 1990) involves the use of an area of attractive plants in which to concentrate the population where they can be destroyed (either chemically, but preferably with natural enemies). The area may consist of more preferred varieties of OSR or its relatives, and involve the use of attractive plant volatiles and sex /aggregation pheromones. The development and success of these strategies rely on an understanding of the cues that the beetles use in resource location so that their behaviour may be manipulated.

1.9 OBJECTIVES

The main objectives of this thesis were to:

1. Investigate the use of pollen cues, in particular pollen odour, in resource location by two pollen-eating insects: the pollen beetle, which specializes on oilseed rape for its reproduction, and is a pest of this crop; and the honey bee, an economically important generalist pollinator with a highly evolved ability to learn.

2. Compare the importance of pollen as an attractive signal in two important UK crop species, oilseed rape and field bean.

More specifically, to investigate:

- The relative roles of oilseed rape floral colour and floral odour cues in the attraction of the pollen beetle to oilseed rape racemes, and to determine the importance of pollen odour in this attraction (Chapters 3 & 4)
- How the responses of pollen beetles to floral odour cues from plants upon which they feed and plants upon which they can reproduce vary according to their physiological state (Chapter 5)
- The use of pollen cues in oviposition by pollen beetle females and the requirements of the larvae for oilseed rape pollen (Chapter 6)
- The ability of honey bees to associatively learn pollen odours and use them to discriminate between pollens of different species (Chapter 7)
- The use of pollen colour and odour cues by honey bees to discriminate between pollens of different species when foraging (Chapter 8)
- The comparative nutritional value of oilseed rape and field bean pollens (Chapter 9)

2. General Materials and Methods

2.1 INTRODUCTION

This chapter describes general materials and methods that are used in more than one chapter in this thesis. Other chapters contain materials and methods that are chapter-specific. All work described in this thesis was conducted between October 1996-1999 at IACR-Rothamsted, Harpenden, Hertfordshire, England.

2.2 FLOWERS

Flowers from oilseed rape (*Brassica napus* var. *oleifera* L.) and field bean (*Vicia faba* var. *minor* L.) plants were used in several experiments. Plants were grown from seed and maintained throughout their growth and flowering phases in a glasshouse maintained at 20 °C (day) and 10 °C (night), with a photoperiod of 16:8 light:dark. They were watered daily. Flowers were picked individually from plants by excision at the base of the sepals so that samples consisted of flowers only, with no pedicel or stem material. They were placed in clean glass containers immediately before use in experiments.

2.2.1 Oilseed rape

Two cultivars of oilseed rape (*B. napus*) were used: a spring cultivar, Aries, and a winter cultivar, Synergy. Flowers were usually picked at stage 2-3 of Charpentier's floral stage key (Charpentier, 1985) when pollen is free on the anthers.

2.2.1.1 cv. Aries

Flowers of spring oilseed rape were obtained from flowering plants of *B. napus* cv. Aries (Fig. 2.1). These were grown from seed with two seedlings transferred to 20-cm diameter pots filled with compost then maintained in the glasshouse (2.2.). The average time from planting to flowering was approximately six weeks. These plants are moderately tall, have a moderate glucosinolate content and were first recommended by the National institute of agricultural botany (NIAB) in 1993. The seed is distributed by Semundo (Cambridge, UK).

Figure 2.1 Oilseed rape cv. Aries with pollen beetle visitor

Figure 2.2 Synergy oilseed rape flowers male-fertile (left); male-sterile (right)

2.2.1.2 cv. Synergy

Synergy (distributed by CPB Twyford Ltd., Herts., UK) is a composite hybrid winter oilseed rape varietal association, consisting of 80% male-sterile hybrid plants (cvs. Falcon x Samouri) which produce no pollen, and 20% male-fertile pollinator plants (cv. Falcon) (see Figure 2.2). It was first recommended by NIAB in 1995. The seed is supplied having the 80:20 male-sterile:male-fertile ratio. However, seeds had 'colour coded' dressing; male-fertile and male-sterile lines having blue and grey dressing respectively. This enabled appropriate numbers of each line to be grown in the glasshouse when required. As Synergy is a winter cultivar, seedlings were vernalized to ensure that flowering would take place without a period of dormancy; when seedlings reached the stage of having three or four true leaves, they were transferred to a controlled temperature room, maintained at 4 °C with an 8:16 light:dark photoperiod and kept there for five weeks. Seedlings were then transferred individually into 20-cm pots and returned to the glass-house (2.2).

2.2.2 Field bean

Field bean flowers were obtained from flowering plants of *V. faba* cv. Alfred (Fig. 2.3). Four seedlings were transferred into each 20-cm diameter pot and plants were grown and maintained throughout growth and flowering in the glasshouse (2.2). Average time to flowering was approximately eight weeks and flowers were usually picked on the second or third day after opening. Alfred is a spring variety, relatively tall, and was first recommended by NIAB in 1986. The seed is distributed by Seed Innovations (Norfolk, UK).

Figure 2.3 Field bean cv. Alfred with honey bee visitor

2.3 POLLEN

Pollen used in experiments was either hand-collected or bee-collected.

2.3.1 Hand-collected pollen

Pollen was hand-collected from oilseed rape flowers by peeling back the petals to expose the anthers, and carefully scraping the pollen from the anthers using the blunt edge of a scalpel blade. All scalpel blades used were new and were dipped in ethanol and flamed before use to avoid contamination of the sample. Approximately 400 flowers were required to collect 1 g pollen by this method. Field bean pollen was collected in a similar manner. Bean flowers were held at the calyx between thumb and forefinger and the standard petal was pulled backwards and held within this grasp. This action extruded the stamens, and the pollen was gently scraped off the anthers with the blunt edge of the scalpel blade. The pollen plug situated between the keel petals was also added to the sample by splitting the keel petals apart. This allowed the pollen plug to drop into the sample. Approximately 250 flowers were required to collect 1 g pollen by this method.

2.3.2 Bee-collected pollen

In most experiments, honey bee-collected pollen loads were used as the pollen source, since it was easier to collect in large quantities than collecting by hand. Honey bees collect pollen from flowers, pack it into loads held within the corbiculae of their hind legs, and transport it back to the hive to feed to the larvae. During one foraging trip, honey bees usually collect pollen from only one plant species at a time as they are flower-constant (Betts, 1935). Therefore, a bee-collected pollen load represents a relatively pure source of pollen from just one plant species (Percival, 1947; Free, 1963). Freshly collected, sorted bee-collected pollen of the required species was stored sealed in clean glass jars (washed in hot soapy water, rinsed with diethyl ether, then distilled water, and oven-baked at 200 °C for 12 hours) in the freezer at –20 °C until use. All pollen used in experiments had been stored for no longer than 12 months. The effect on the pollen odour of the 'bee-derived' components added, or altered due to the pollen-packing process, and the effect of freezing was investigated (Appendix 1.2 and 1.4, respectively).

2.3.2.1 Collection of bee-collected pollen loads

Two hives of honey bees were placed in a flowering crop of the pollen species required. Each hive was fitted with a pollen trap at its entrance (Fig. 2.4). The trap consisted of strip of plastic sheet with small holes (5 mm) drilled through, held vertically above a wooden collecting tray (Fig. 2.5). Bees returning from foraging trips had to squeeze through the holes in the plastic sheet to enter the hive. This knocked the pollen loads from their corbiculae into the collecting tray. Trays were emptied daily, or if very fresh pollen was required (e.g. for volatile analysis), trays were put into position in early morning and emptied at midday.

2.3.2.2 Separation of bee-collected pollen loads

Most of the pollen loads collected in the trays were from the plant species in which the hive was placed, although pollen loads collected from other species were also invariably present. Pollen loads collected from the species required were separated individually from the others by hand, using a metal dissecting needle that had been dipped in ethanol and flamed before use to reduce contamination. The pollen loads were sorted firstly by separating the different loads into piles of same-coloured loads (Fig. 2.6). The source identities of the pollen loads were then confirmed by comparisons with colour charts and by examination of the pollen grain morphological characteristics.

Figure 2.4 Bee hive fitted with pollen trap

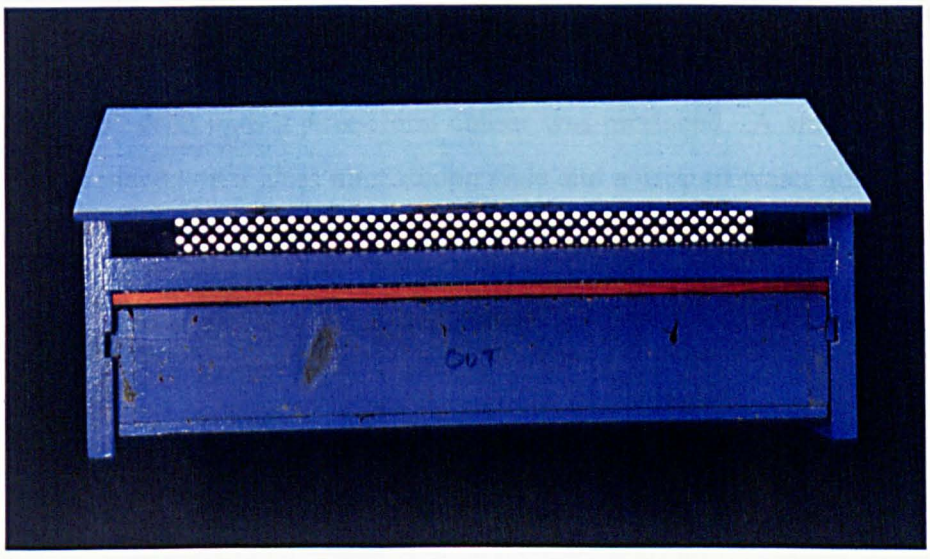


Figure 2.5 Pollen trap to collect bee-collected pollen loads from honey bee hives

2.3.2.3 Confirming the source identity of bee-collected pollen loads

Colour Pollen contains coloured pigments such as carotenoids and flavonoids (Stanley & Linskens, 1974). As different plant species contain different pigments in their pollen, they are coloured differently. Therefore, the colour of pollen loads can be used to help identify the source of the pollen from which it was collected. For example, oilseed rape pollen loads are yellow-brown, and those of field bean are grey-green (Fig. 2.6). Pollen loads from pollen traps were initially sorted by colour and compared with published pollen colour guides (Hodges, 1952, 1984; Kirk, 1994).

Pollen grain morphology Examination of the morphological characteristics of the pollen grain itself, such as size, shape and features of the exine (the outer grain covering) offers a more precise identification than colour characteristics alone. Therefore, the species source of bee-collected pollen loads was confirmed by microscopic analysis of the pollen grains from a few of the pollen loads of each colour-sorted sample. Samples were discarded if microscopic analysis proved the pollen loads to be of another species than the one required. The pollen grains were mounted on a microscope slide in Basic-Fuchsin stained glycerine jelly (e.g. Hodges, 1952, 1984; Sawyer, 1981; Kirk, 1994). This was prepared by melting 5 g glycerine jelly (Glycerol jelly, BDH Ltd, Poole, UK) in a petri dish on a warming plate, and adding the stain (0.1 g Basic Fuchsin in 10 ml methanol) drop by drop until a pink-claret colour was produced. A small amount of the pollen load was placed on a glass microscope slide and a drop of water added to separate the pollen grains. The suspension was dried by placing the slide on the warming plate. Then, a couple of drops of the stained glycerine jelly were added on top of the pollen grains and a glass cover slip was placed over the top. The slide was left on the warming plate for a further ten minutes to allow the stain to take effect, then removed and left to cool before examination under the microscope.

The pollen grains were examined using oil-immersion light microscopy (x 400) (Watson Microsystem 70). The shape of the grain, features of the exine and approximate size- as measured with an eyepiece graticule- were noted. The sample was identified using a combination of comparisons with drawings from Hodges (1952, 1984), photographs from Sawyer (1981), and Sawyer's pollen identification key (1981). The sizes of pollen grains were compared with those given by Kirk (1994a).

Oilseed rape pollen grains are small (around 27 μm diameter), round in shape with three furrowed apertures. The surface is net-like (pitted) and the exine is of medium thickness with regularly-spaced rods (Fig. 2.7a).

Field bean pollen grains are of medium size (around 44 μm diameter), long oval in shape, about 1½ times as long as they are broad. They have three apertures, which are furrowed with pores, and a smooth-pitted surface with a thin exine. The cell contents have a granular appearance (Fig. 2.7b).

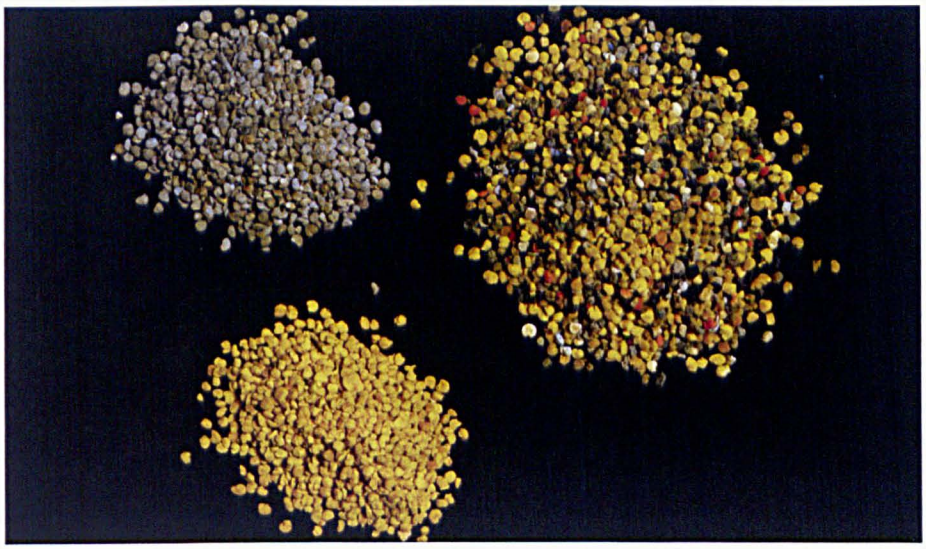
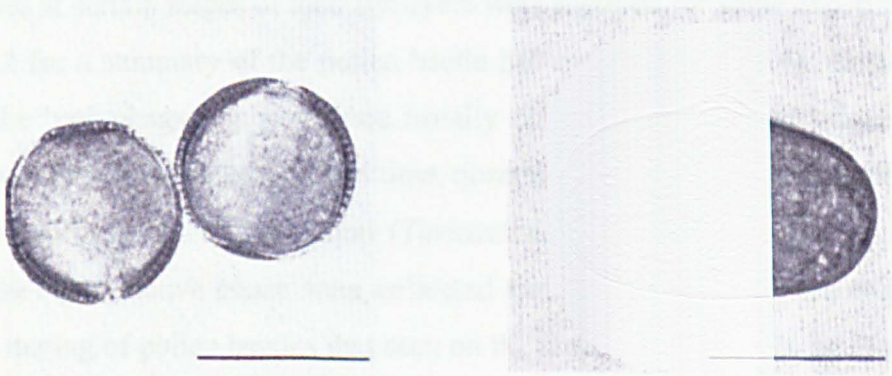


Figure 2.6 Bee-collected pollen loads from different plant species including oilseed rape (yellow) and field bean (grey)



(a) **(b)**

Figure 2.7 Optical section of pollen grains (x400): (a) oilseed rape (b) field bean Scale bars represent 25 μm. Used with kind permission from Sawyer (1981), Cardiff University

2.4 HONEY BEES

IACR-Rothamsted maintains about 80 colonies of honey bees (*Apis mellifera* L.) (Figure 2.3) in several apiaries within 5 km of Rothamsted. Honey bee colonies of appropriate sizes were selected from apiaries and transferred to the experimental site whenever they were required for experiments, and to fields of oilseed rape and field bean for pollen-collection.

2.5 POLLEN BEETLES

The pollen beetle (*Meligethes aeneus* (Fabricius)) (Fig. 2.1) is difficult to rear and maintain in long-term laboratory cultures (Bromand, 1983). Most experiments involving adult pollen beetles therefore used subjects that had been collected from the field and kept in short-term cultures. This sub-section describes how pollen beetles were collected from the field, maintained in culture and how their sex and species were determined.

2.5.1 Collection

Pollen beetles were collected from the buds and flowers of oilseed rape crops on Rothamsted farm. The beetles infesting a raceme were first disturbed by blowing on them and the raceme then tapped over a plastic box to dislodge them. Beetles were gathered up from the box using a battery-driven electric pooter and transported back to the laboratory in ventilated 60-ml plastic containers (Bibby Steralin Ltd, Stone, Staffs, UK).

Pollen beetles at certain stages of their life cycle were required for some experiments (see section 1.8.2 for a summary of the pollen beetle life cycle and Fig. 1.4). Post-diapause beetles in the 'polyphagous phase' were usually collected from winter oilseed rape as soon as they colonised the crop. At this time, normally in late March /April, other pollen beetles were still present on dandelion (*Taraxacum officinale* Weber). Post-diapause beetles in the reproductive phase were collected from winter oilseed rape crops during May, when mating of pollen beetles was seen on the crop and eggs could be found within buds. By this time, it was rare to find pollen beetles on dandelion. Pre-diapause pollen beetles were collected towards the end of flowering of spring oilseed rape (late July-August). At this time, new generation beetles could be found on most flowering plants around the farm and in gardens. Life stages overlap throughout the spring and summer,

so experiments that required distinct life stages were conducted at the extremes of these sub-seasons.

2.5.2 Culture

Field-collected pollen beetles were maintained until required for experiments, on flowering racemes cut from glasshouse-grown spring oilseed rape cv. Aries (2.2.1.1). Cultures were confined inside transparent plastic boxes (174 x 115 x 60 mm; Stewart Plastics, Waddon Marsh, Croydon, UK) that were ventilated and lined with filter paper (Whatman 1, Maidstone, Kent, UK) moistened with distilled water. Approximately 250 beetles were maintained in each box, on five flowering racemes. Boxes were kept in a controlled temperature room, which was maintained at 10 °C with a photoperiod of 16:8 h light:dark. Beetles were kept in culture for up to two weeks, and racemes were renewed every two days.

2.5.3 Sex determination

The determination of the sex of live adult beetles was carried out prior to experiments so that the behavioural responses of male and female beetles could be compared. Beetles to be sexed were placed in a ventilated plastic container (60-ml; Bibby Steralin Ltd, Stone, Staffs, UK), and maintained on ice to slow their movement. Each beetle was picked up with a moistened Sable hair paintbrush and placed on its dorsal side on a glass microscope slide under view of a binocular dissecting microscope (Wild MSA, Wild Heerbrugg, Switzerland) (x 25). A glass coverslip was placed over the beetle and gentle pressure was applied to its edge using the blunt wood end of the paintbrush. The pressure caused the genitalia to be exerted between the last visible sternite and last tergite. In females, the triangular-shaped ovipositor (Fig. 2.8 f) was exerted, whilst in males it was the U-shaped tegmen (Fig. 2.8 g). Figure 2.9 shows a photograph of the differences seen. The accurate sex determination of live adult pollen beetles has previously been deemed impossible (Fritzsche, 1955; Charpentier & Weibull, 1994; Borg & Ekbom, 1996). However, Ruther & Thiemann (1997) have recently published a method similar to the one described above.

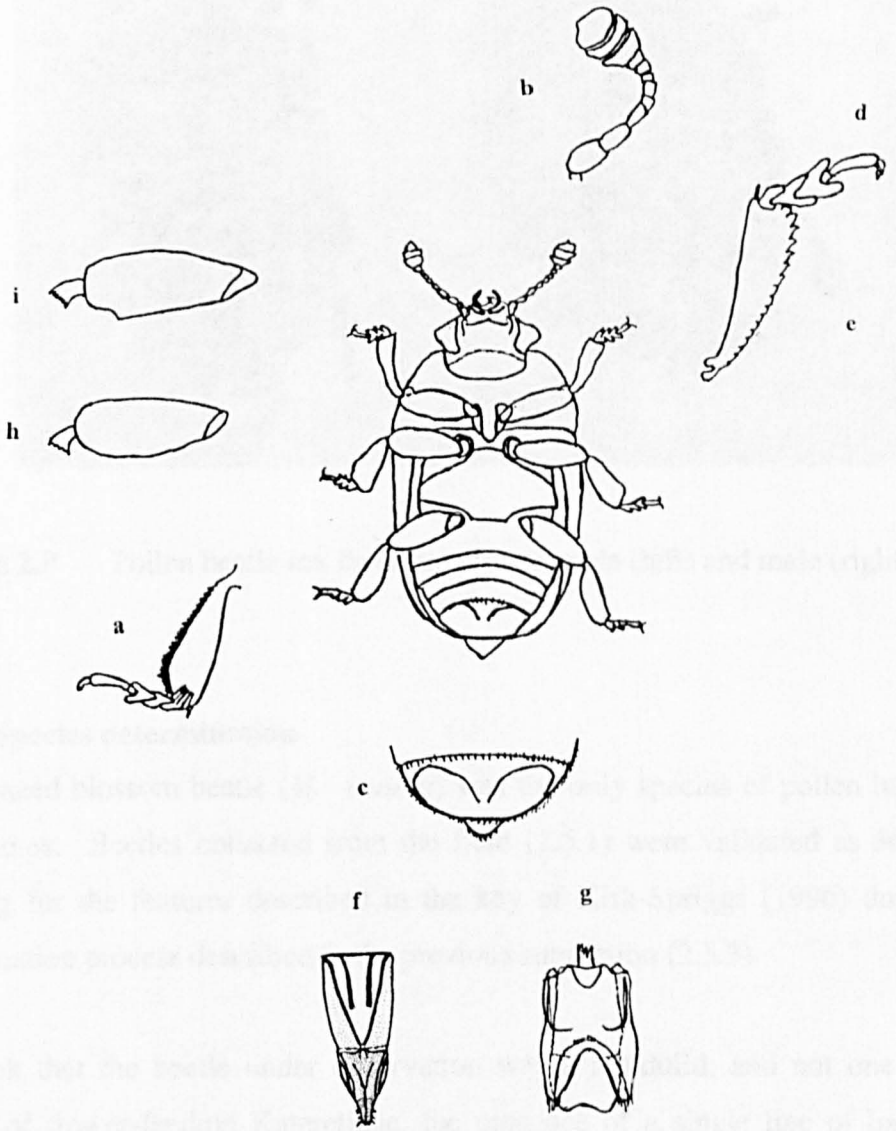


Figure 2.8 Characteristics used in the identification of *Meligethes aeneus*. Adapted from Kirk-Spriggs (1996) (a) hind tibiae showing hairs (carinae) on outer edge; (b) antenna showing 11 segments with compact 3-segmented club; (c) last abdominal sternite showing semi-circular lines; (d) tarsal claws (simple); (e) anterior tibiae showing crenulation; (f) female ovipositor showing styli; (g) male tegmen and aedeagus; (h) intermediate femora of *M. aeneus* showing simple lower edge (i) intermediate femora of *M. viridescens* showing downward- pointing projection on lower edge.

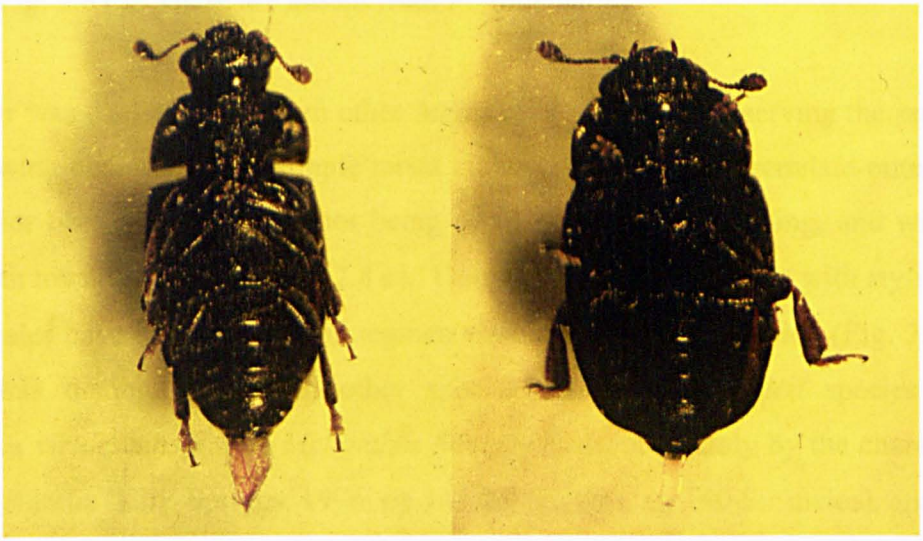


Figure 2.9 Pollen beetle sex determination: female (left) and male (right) genitalia

2.5.4 Species determination

The bronzed blossom beetle (*M. aeneus*) was the only species of pollen beetle used in experiments. Beetles collected from the field (2.5.1) were validated as *M. aeneus* by checking for the features described in the key of Kirk-Spriggs (1996) during the sex determination process described in the previous subsection (2.5.3).

To check that the beetle under observation was a Nitidulid, and not one of the nine species of flower-feeding Kateretidae, the presence of a single line of hairs (carinae) were sought on the outer edge of the intermediate and hind tibiae (Fig. 2.8 a). These are absent in the Kateretidae. Also, the antenna in Kateretidae has loosely connected antennal segments of the club, rather than a distinct club, clearly separated from the flagellum as seen in the Nitidulids (Fig. 2.8 b).

Nitidulid beetles collected from flowering plants are very unlikely to be of any other subfamily other than Meligethinae, as the other subfamilies have adults and larvae that develop in carrion and fungi, and are not normally associated with flowering plants (Kirk-Spriggs, 1996). The subfamily Meligethinae is represented by two genera in the UK; *Pria*, with one species, *Pria dulcamare*, and *Meligethes* which has 36 recorded British species (Kirk-Spriggs, 1996). *Meligethes* species were distinguished from *P.*

dulcamare by the presence of impressed semi-circular lines on the last visible abdominal sternite (Fig. 2.8 c). These are absent from *P. dulcamare*.

M. aeneus was distinguished from other *Meligethes* species by observing the presence of the following characteristics: simple tarsal claws (Fig. 2.8 d), a crenulate outer edge of the anterior tibiae, with the notches being regular in size and spacing, and with small, sharp teeth towards the apex (Fig. 2.8 e). Females have an ovipositor with styli (Fig. 2.8 f), and males have an aedeagus and tegmen visible with apical excision (Fig. 2.8 g). *M. aeneus* was distinguished from other species found on *Brassica* species (namely *Meligethes viridescens* F. and *Meligethes fulvipes* Bristout) mainly by the characteristics of their genitalia (Kirk-Spriggs, 1996; pp 145 for females, pp 140 for males), and by their colouring. *M. aeneus* and *M. viridescens* (blossom beetles) tend to be metallic in colouration (green or blue, rarely bronze), and have red-brown ovipositors. *M. fulvipes* is usually black, with yellow or red colouration to their antennae and legs and yellow ovipositors.

M. aeneus was distinguished from *M. viridescens* by its generally smaller size (1.9-2.7 mm), duller colour, and darker legs; *M. viridescens* is slightly larger (2.4-2.9 mm), brighter and has lighter brown-yellow legs. In addition, female ovipositors of *M. aeneus* were narrower than those of *M. viridescens*. Kirk-Spriggs (1996) also points out that *M. aeneus* has a simple lower edge to its intermediate femora (Fig. 2.8 h), whilst *M. viridescens* has a downwardly-directed projection at outer third lower edge of the intermediate femora (Fig. 2.8 i). However, this characteristic was difficult to see on a live (moving!) beetle at the magnification used for sex determination.

2.6 STATISTICS

All statistical data analysis in this thesis was conducted using the GenStat statistical package and procedures (GenStat, 1993, 1998). Results were regarded as significant at the 5% test level, where $P<0.05$. Where results are significant they are recorded as being either $P<0.05$, $P<0.01$ or $P<0.001$. In cases where results are not significant, the probability is recorded as $P>0.05$.

3. The role of floral colour, floral odour and pollen odour in the attraction of pollen beetles to oilseed rape in a wind tunnel

3.1 ABSTRACT

Phytophagous insects are known to respond to both chemical and visual stimuli during the host finding process. The pollen beetle, which is regarded as a pest of oilseed rape crops, is attracted to oilseed rape by both its yellow floral colour and the odour of its tissues. This study investigated the relative importance of floral colour and odour cues in the attraction of pollen beetles to oilseed rape in a wind tunnel, and explored the role of pollen odour in attraction. Pollen beetles were attracted upwind by both colour and odour cues from oilseed rape flowers. There was no statistical interaction between these two cues, and floral odour was found to be more important in attraction than colour. There was evidence that part of the attractive odour emanates from the anthers – possibly from the pollen itself.

3.2 INTRODUCTION

3.2.1 Floral advertisements: cues in resource location by phytophagous insects

Advertisement is an important function of flowers for the purpose of attracting pollinators (e.g. Fægri & van der Pijl, 1979; Dafni, 1992). Insect visitation to flowers is positively related to the size, symmetry, colour, and odour of flowers (e.g Williams, 1983; Procter, Yeo & Lack, 1996). Insects use these advertisements as signals, or cues, to help them find floral nectar and/or pollen resources (e.g. Kevan & Baker, 1983).

The pollen beetle (*Meligethes aeneus* Fabricius) is regarded as a pest of oilseed rape (*Brassica napus* L.) (OSR) crops (Alford & Gould, 1975; Free & Williams, 1979; Winfield, 1992). After over-wintering in the soil, and a period of polyphagous feeding on the pollen from many plant species (Fritzsche, 1957; Free & Williams, 1978a), the beetles migrate to the OSR crop where they mate, and females lay their eggs in the buds. Both adults and larvae eat pollen from buds and flowers. Like other phytophagous

insects, pollen beetles use visual and olfactory cues in host location (Görnitz, 1956; Tamir *et al.*, 1967; Free & Williams, 1978a).

3.2.2 Pollen beetle attraction to colour

Pollen beetles, like many other flower-visiting insects, have an innate attraction to yellow (Wäckers, 1994). This attraction has long been recognised. Wasman (1926) first noted that *M. aeneus* is attracted to 'rape yellow' and yellow water traps were first used in Germany in the 1950s to capture and monitor these beetles (Moericke, 1953; Nolte, 1955; Görnitz, 1956; Fritzsche, 1957). More recent studies investigating the attraction of pollen beetles to different coloured traps, have shown yellow ones to be most attractive (Láska, Zelenková & Bicík, 1986; Buechi, 1990; Ekbom & Borg, 1996; Blight & Smart, 1999). An investigation into the attractiveness of genetically similar lines of OSR, differing in petal colour, found that pollen beetles were more abundant on plants with yellow than white or cream flowers (Giamoustaris & Mithen, 1996).

3.2.3 Pollen beetle attraction to odour

Several studies have shown that pollen beetles are attracted to the odours of cruciferous plant extracts (Görnitz, 1956; Nolte, 1959; Free & Williams, 1978a; Evans & Allen-Williams, 1994). Trapping studies (Free & Williams, 1978a; Lerin, 1984; Kostal, 1992; Smart, Blight & Hick, 1993; Smart, Blight & Ryan, 1995; Blight & Smart, 1999) have shown attraction to baits releasing isothiocyanates, the volatile catabolites of glucosinolates, typical of the Brassicaceae (= Cruciferae) (Fenwick, Heaney & Mullin, 1983). However, these volatiles emanate mainly from the green parts of the plant. Although pollen beetles infest crops at the green bud stage and can locate hosts at this growth stage by odour alone (Ruther & Thiemann, 1997), numbers in the crop are low until yellow buds and flowers are present (Winfield, 1961; Buechi, 1990; Smart, Blight & Lane, 1996). Until then, beetles congregate on 'volunteer' or forward plants in flower (Free & Williams, 1978a; personal observation). This attraction could be due to floral odour as well as colour. However, few studies have directly examined the role of OSR flower volatiles in attraction. Evans & Allen-Williams (1994) demonstrated that both OSR leaf and flower odour were attractive to pollen beetles in an olfactometer, and in the field, beetles dispersed upwind and were recaptured in yellow water traps baited with extracts of OSR leaves and flowers. Recapture in traps baited with extracts of flowers was greater than those baited with extracts of OSR leaves (Evans & Allen-Williams, 1994), suggesting that floral odour is important in attraction.

3.2.4 Pollen as an attractant for pollen-seeking insects?

Insects that visit flowers primarily to feed on pollen may use cues from the pollen itself to help them locate it. Pollen odours are species-specific (Dobson *et al.*, 1987; Dobson, 1988; Dobson, Groth & Bergström, 1996) and are often distinct from those of the whole flower (Dobson *et al.*, 1987; Dobson, Bergström & Groth, 1990; Bergström, Dobson & Groth, 1995; Dobson, Groth & Bergström, 1996). They are thought to represent evolutionarily ancient attractants to flower-visiting insects (van der Pijl, 1960). However, few studies have investigated insect responses to pollen odours. Most have investigated the responses of various species of bees to pollen odour (Lepage & Boch, 1968; Dobson, 1987; Dobson, Danielson & Van Wesep, 1999; this thesis, chapters 7 & 8). Charpentier (1985) found that pollen beetles were attracted more to buds and anthers than to other parts of the oilseed rape plant, and that the stamens were preferred most when the quantity of mature pollen was at its maximum. Odours emanating from the anthers were thought to be responsible for this attraction, but were not specifically tested for.

3.3 AIM

This set of experiments had two aims:

1. To investigate the relative importance of floral colour and odour cues in location of oilseed rape plants by pollen beetles
2. To investigate the importance of pollen odour cues used in location of this floral resource by pollen beetles

Two separate experiments were conducted to address these aims (3.5.1 and 3.5.2, respectively); both were conducted using a wind tunnel.

3.4 MATERIALS AND METHODS

3.4.1 Wind tunnel

The wind tunnel (Fig. 3.1) used in these experiments has been described in detail by Du, Poppy & Powell (1996). It is a rectangular Plexiglass chamber (90 x 30 x 30 cm), through which air is pulled by an electronically operated fan, at a speed controlled by a variable voltage regulator. Incoming air was purified by passage through a charcoal and glass wool filter. A laminar flow was obtained by means of perforated mesh panels at

both ends of the tunnel; these created a uniform air stream. A black- and white-striped pattern occurred horizontally along the floor of the tunnel to provide optomotor cues for insects that need them in flight orientation (Fadamiro, Wyatt & Birch, 1998), and the sides and ceiling were transparent. The wind tunnel was lit by three U-shaped incandescent lights, suspended 35 cm from the ceiling of the tunnel. A sheet of tissue paper was placed between the lights and the tunnel roof to diffuse the light and deter the pollen beetles from flying directly upwards after release. The wind tunnel was housed in a controlled temperature room maintained at 25 °C ± 2 °C and painted non-reflective matt black to prevent visual distractions within the room.

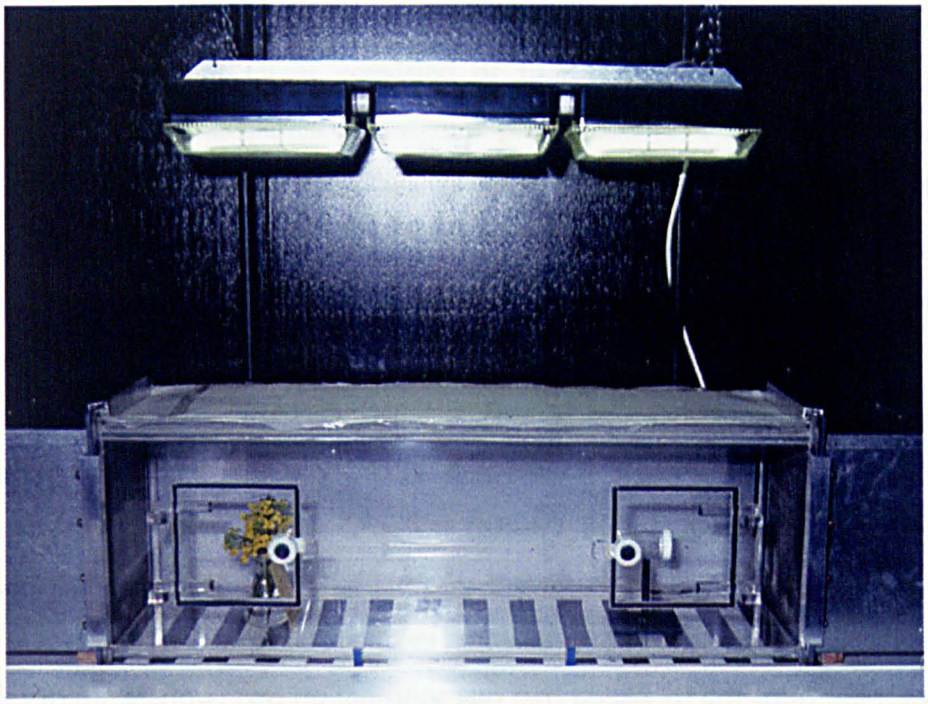


Figure 3.1 The wind tunnel. The target is placed in the upwind section (left hand side) and insects released from a platform located down wind of the target (right hand side)

3.4.2 Pollen beetles

Pollen beetles were collected from the field (2.5.1) and cultured on OSR racemes (2.5.2) until required. Two days before an experimental day, beetles to be used were starved for 48 h in clean culture boxes lined with moistened filter paper but devoid of flowers. At the beginning of an experimental day, groups of 50 starved beetles were transferred, using an electric pooter, from the culture box into ventilated plastic containers (60-ml;

Bibby Steralin Ltd., Stone, Staffs., UK). Two hours before the start of the experiment, groups of beetles were placed in the experimental room to acclimatize.

3.4.3 Flowers

Flowers and flowering racemes of oilseed rape (OSR) were cut from glasshouse-grown flowering plants of *Brassica napus* (2.2) for use in the targets.

3.4.4 Targets

The effect of the presence or absence of floral colour, floral odour, and pollen odour cues on upwind attraction of pollen beetles was compared using combinations of a colour and an odour target from the following targets:

- + Colour The colour target consisted of five flowering OSR terminal racemes contained within a transparent polystyrene plastic box (174 x 115 x 60 mm; Stewart Plastics Croydon, Surrey, UK) sealed with Parafilm (American National Can., Grenwich, CT, USA)
- - Colour The no-colour target consisted of an empty transparent polystyrene plastic box sealed with Parafilm
- + Odour The odour target consisted of four glass odour tubes (10 cm long, 4 cm diameter) each filled with 1.5 g OSR flowers. The tubes were covered with black paper to eliminate visual cues from the flowers, and were sealed at either end with doubled fine mesh gauze. This allowed the air to pass through, eliminated visual cues and prevented the beetles from accessing the flowers. These were placed parallel to the airflow, two on the floor and two raised 10 cm from the floor on grey plastic stands
- - Odour The no-odour target consisted of four empty odour tubes

3.4.5 Experimental procedure

Fifty pollen beetles were transferred into a 60-ml plastic container which had muslin-covered holes in the lid and base to allow through air–flow. This container was placed on a 10-cm high platform, facing upwind in the middle of the downwind section of the wind tunnel. At this height, beetles were exposed to the odour plume from treatments, and were assumed to be close enough to the striped floor for use of optomotor cues if needed (Fadamiro, Wyatt & Birch, 1998). Wind speed was adjusted to 0.375 m sec^{-1}. The beetles were left to acclimatize in the wind tunnel for one hour. After this period, the targets were placed in the upwind section of the wind tunnel, and the lid taken from

the pot allowing the beetles to fly out. Responses of the beetles to the different targets were measured in two ways:

1. By assessing the proportion of beetles in the upwind section of the wind tunnel
2. By totalling the number of beetles landing on the targets.

3.4.6 Proportion of pollen beetles in the upwind section of the wind tunnel

After release, the number of beetles in each third section of the wind tunnel (upwind, mid, and downwind) was recorded, and the proportion present in the upwind section used as a measurement of the attractiveness of the target factors (Nottingham & Coaker, 1985). This method was adopted since preliminary tests had shown that few beetles exhibited direct, oriented flight; therefore recording the take-off, flight behaviours and landing responses of individual beetles would have been too time consuming. The proportion of beetles present in the upwind section 25 minutes after release (this time period determined from experiment 3.5.1) was analysed by fitting a logistic regression model. In the model, the number of beetles in the upwind section was assumed to follow a binomial distribution with sample size (n) equal to the total number of beetles observed in the wind tunnel.

3.4.7 Number of landings

The number of beetles landing on the surfaces of the targets was recorded throughout the first 15 minutes of the experiment, and the total number of landings occurring for each combination of target factors was analysed by an analysis of variance.

3.4.8 Experimental protocol

Ten replicates of each of the four target combinations were conducted in a randomized order. Four replicates (one of each target combination) were conducted each day, to allow comparisons without bias due to day-effects. A different group of beetles was used for each replicate. The wind tunnel was cleaned with 70% ethanol between replicates.

3.5 EXPERIMENTAL METHODS

3.5.1 Relative importance of floral colour and floral odour cues

This experiment was designed in a factorial structure to compare the relative importance of the presence of OSR flower colour with that of OSR floral odour in the upwind attraction of the pollen beetle, and investigated any interactions between these two factors.

3.5.1.1 Targets

Combinations of colour and odour targets in the factorial design were as follows:

+ Colour **+ Odour** (= Colour & Odour)	**- Colour** **+ Odour** (= Odour)
+ Colour **- Odour** (= Colour)	**- Colour** **- Odour** (= no Colour & no Odour)

The factorial structure allows the comparison of the effect of:

- The presence of colour versus no colour:

by comparing the mean number of beetles upwind or number of landings in response to the following targets: mean [(Colour & Odour) + (Colour)] vs. mean [(Odour) + (no Colour & no Odour)]

- The presence of odour versus no odour:

by comparing the mean number of beetles upwind or number of landings in response to the following targets: mean [(Colour & Odour) + (Odour)] vs. mean [(Colour) + (no Colour & no Odour)]

- Interactions between colour and odour cues

Flowers used in the targets were oilseed rape cv. Aries (2.2.1.1)

3.5.1.2 Recording and analyzing the data

Proportion in the upwind section In this experiment, the numbers of beetles in each section of the wind tunnel were recorded at five-minute intervals after release for 30

minutes, then once again one hour after release. This was to compare the initial attraction of colour and odour factors of the targets and to measure changes over time to determine the most appropriate time interval after release to take upwind proportion measurements for target-response comparisons. This interval was determined to be 25 minutes (3.6.1.1). The logistic regression model for this experiment included terms for colour and odour and examined any interaction; i.e. examined the effect of presence of odour versus (vs.) no odour, presence of colour vs. no colour and the interaction.

Number of landings The total number of landings occurring for each target was transformed ($\log_{10}$ (n+1)) to normalise the data before the analysis of variance test.

3.5.2 The importance of pollen odour in the attraction of pollen beetles to the flowers of oilseed rape

This experiment compared the effects of OSR pollen odour on the attraction of pollen beetles upwind, by comparing beetles' responses to the odour of male-fertile and male-sterile flowers. In addition, the importance of colour and floral odour were also assessed.

3.5.2.1 Flowers

Flowers and terminal racemes from glass house–grown oilseed rape cv. Synergy (2.2.1.2) were used for this experiment. Synergy is a composite hybrid varietal association of male-sterile hybrid plants (cv.s Falcon x Samouri) which produce no pollen and male-fertile plants (cv. Falcon) which do produce pollen. This plant system enabled comparison of pollen beetle responses to the odour of flowers with and without pollen to be made without the need for cutting the anthers and therefore releasing damage volatiles (Bergström, Dobson & Groth, 1995).

3.5.2.2 Targets

Four target combinations were used:

1.	+ Colour	+ Odour	+ Pollen odour	(= Male-fertile)
2.	+ Colour	+ Odour	- Pollen odour	(=Male-sterile)
3.	+ Colour	- Odour	- Pollen odour	(=Colour)
4.	- Colour	- Odour	- Pollen odour	(=Control)

The + Colour cue consisted of six flowering OSR terminal racemes within the sealed box detailed in section 3.4.1.4. Three racemes were male-fertile and three were male-sterile.

The – Colour cue was an empty box (3.4.1.4). The + Odour cue consisted of 1.5 g of either male-fertile Synergy OSR flowers placed inside the odour tubes (3.4.1.4), in which case they also provided the + Pollen odour cue, or male-sterile Synergy OSR, in which case they also provided the – Pollen cue. The – Odour cue consisted of four empty odour tubes (3.4.1.4).

3.5.2.3 Recording and analyzing the data

Proportion in the upwind section The proportion of pollen beetles in the upwind section of the wind tunnel 25 minutes after release (this time point derived from results of the previous experiment, section 3.6.1.1) was analysed by fitting a logistic regression model with terms enabling comparisons of floral colour, floral odour and pollen odour effects. The following comparisons were decided *a priori*: Control vs. all other target combinations (i.e. effect of floral colour); Male-sterile and Male-fertile oilseed rape targets vs. Colour (i.e. effect of floral odour), and Male-sterile vs. Male-fertile targets (i.e. effect of pollen odour).

Number of landings The total numbers of landings on targets within these comparisons over the first 15 minutes of the experiment were analysed by an analysis of variance. No transformation was necessary.

3.6 RESULTS

3.6.1 Relative importance of floral colour and odour cues in attraction

3.6.1.1 Proportion of pollen beetles in the upwind section of the wind tunnel

Low numbers of beetles (<50%) were found in the upwind section of the wind tunnel at any one time. Figure 3.2 shows the mean proportions of beetles in the upwind section at the five-minute intervals after the beetles' release in response to the presence or absence of floral colour, and the presence or absence of floral odour of the targets. There was no difference in the initial attraction (5 minutes after release) to the floral colour or floral odour cues when these were present compared with no floral colour or no floral colour cues, respectively. However, the numbers of beetles present upwind gradually increased from 5 to 20 minutes and then remained relatively constant until the measurement taken 30 minutes after release. Therefore, analysis of any differences was conducted midway between these points at 25 minutes. Twenty-five minutes after the release of the beetles,

the only significant term in the logistic regression model was floral odour (χ^2_1 =7.99; P=0.005). Floral colour was not a significant factor of the treatments, and there was no interaction between floral colour and floral odour factors.

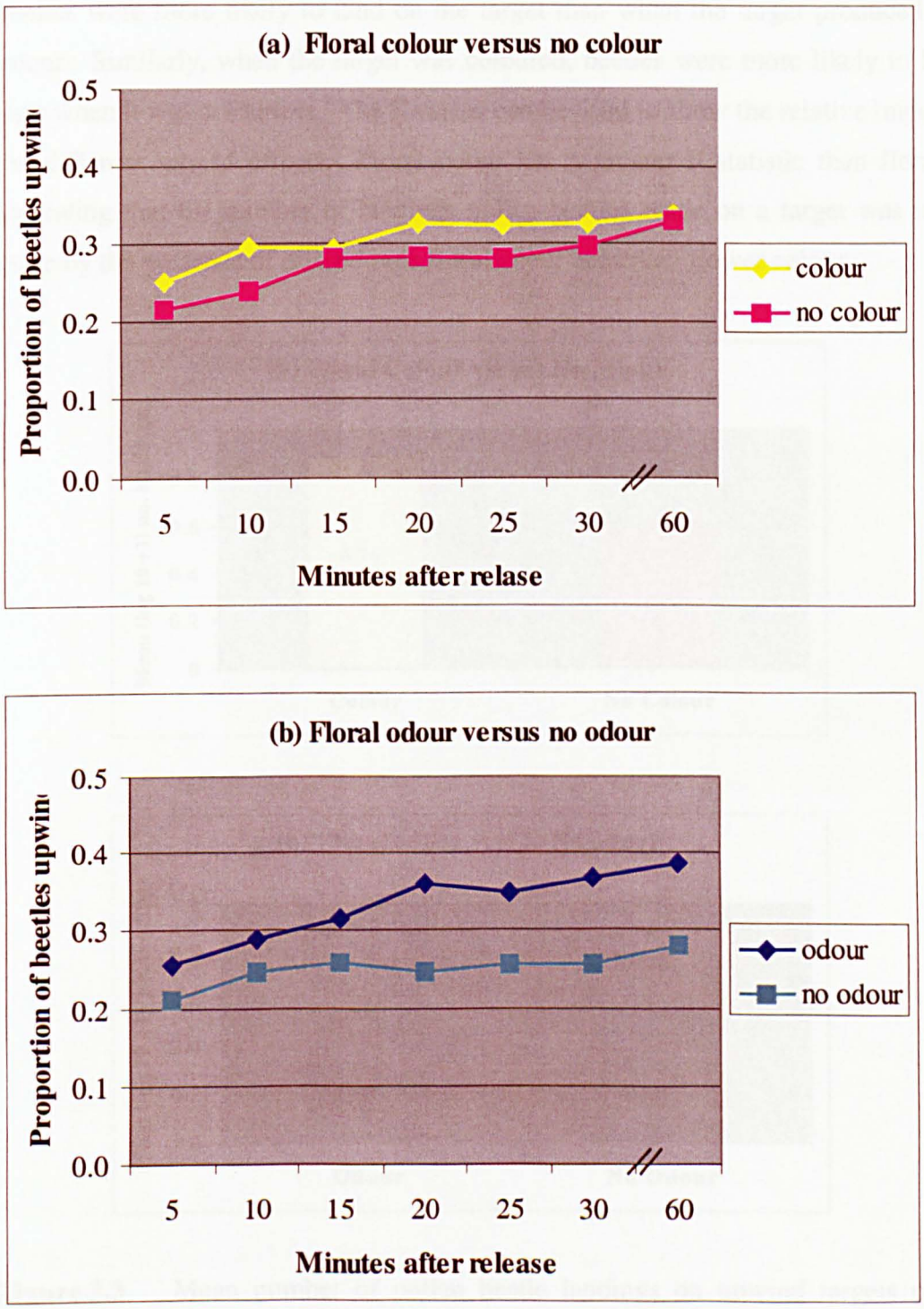


Figure 3.2 Proportion of pollen beetles present in the upwind section of a wind tunnel in response to the presence or absence of oilseed rape (a) floral colour and (b) floral odour cues of targets placed in the upwind section at 5-minute intervals after release of beetles in the down wind section

3.6.1.2 Number of beetles landing on the targets

There was a significant difference in the number of landings made on the targets when there was odour vs. no odour ($F_{1,36}$ = 28.43; P<0.001), and when there was colour vs no colour ($F_{1,36}$ = 15.76; P<0. 001) (Fig. 3.3). Thus, when OSR floral odour was upwind, beetles were more likely to land on the target than when the target produced no floral odour. Similarly, when the target was coloured, beetles were more likely to land on it than when it was colourless. The F values can be used to show the relative importance of the different sets of effects. Floral odour has a greater F statistic than floral colour, indicating that the number of landings pollen beetles made on a target was influenced more by the presence of oilseed rape floral odour cues than flower colour.

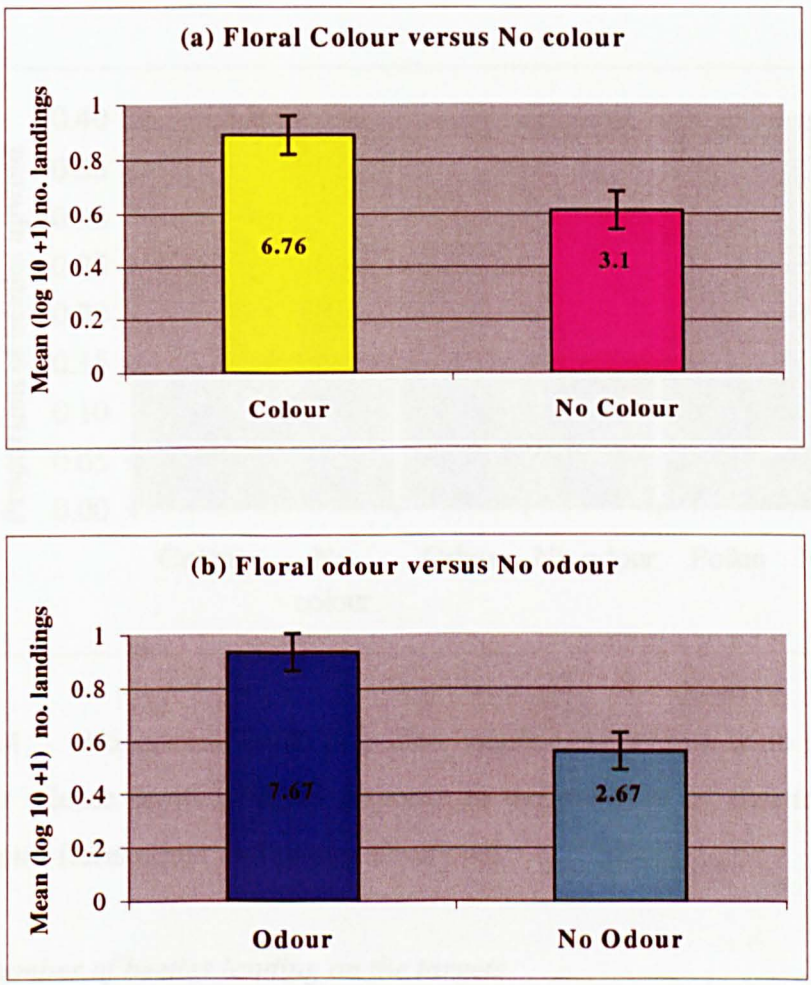


Figure 3.3 Mean number of pollen beetle landings on upwind targets over a 15-minute period after release of the beetles in the down wind section of a wind tunnel: (a) with and without oilseed rape floral colour cues (b) with and without oilseed rape floral odour cues. Means were transformed using $\log_{10}$ (n+1). Bars show back-transformed means within and s.e.d. error bars.

3.6.2 The importance of pollen odour in the attraction of pollen beetles to the flowers of oilseed rape

3.6.2.1 Proportion of pollen beetles in the upwind section of the wind tunnel

Twenty-five minutes after release of the beetles, floral odour was the only significant term in the logistic regression model analysing the effects of the floral colour, odour and pollen factors of the treatments on the proportion of beetles upwind ($\chi^2_1 = 6.17$; P=0.013) (Figure 3.4). However, there was evidence to suggest an effect of pollen; there was a larger proportion of beetles upwind 25 minutes after release in the presence of male-fertile OSR than when male-sterile flowers were present (figure 3.4); ($\chi^2_1 = 3.04$; P=0.08).

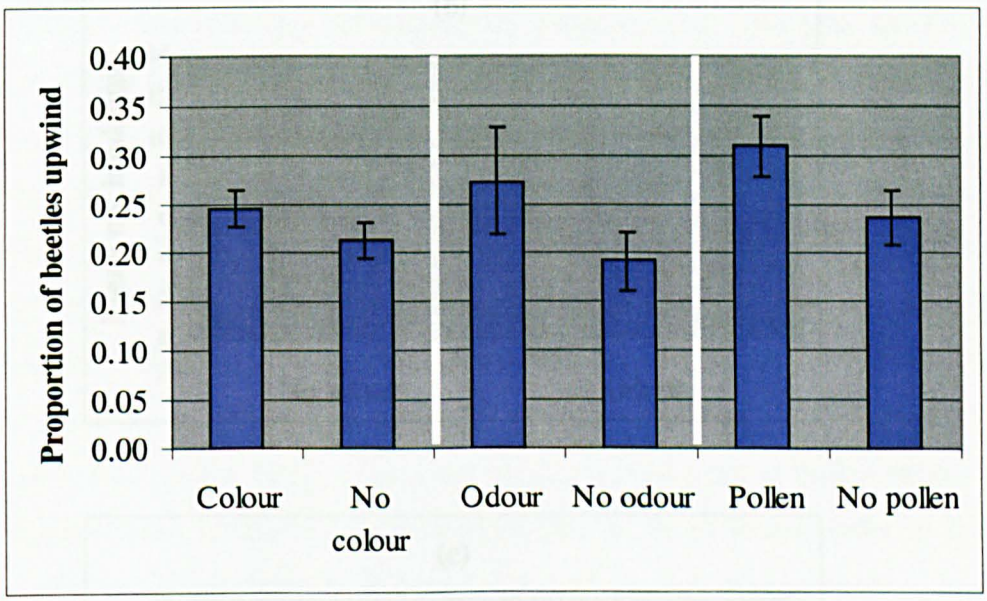


Figure 3.4 Proportion (± SE) of pollen beetles upwind in a wind tunnel 25 minutes after their release down wind, in response to the presence or absence of oilseed rape floral colour, floral odour and pollen odour cues

3.6.2.2 Number of beetles landing on the targets

Floral colour ($F_{1,36} = 19.51$, P<0.001), floral odour ($F_{1,36} = 15.35$, P <0.001) and pollen odour ($F_{1,36} = 6.56$, P <0.05) were all significant factors influencing the number of landings made by the beetles on the targets within the wind tunnel (Fig. 3.5). Thus, floral colour, floral odour and pollen odour were all attractive to pollen beetles.

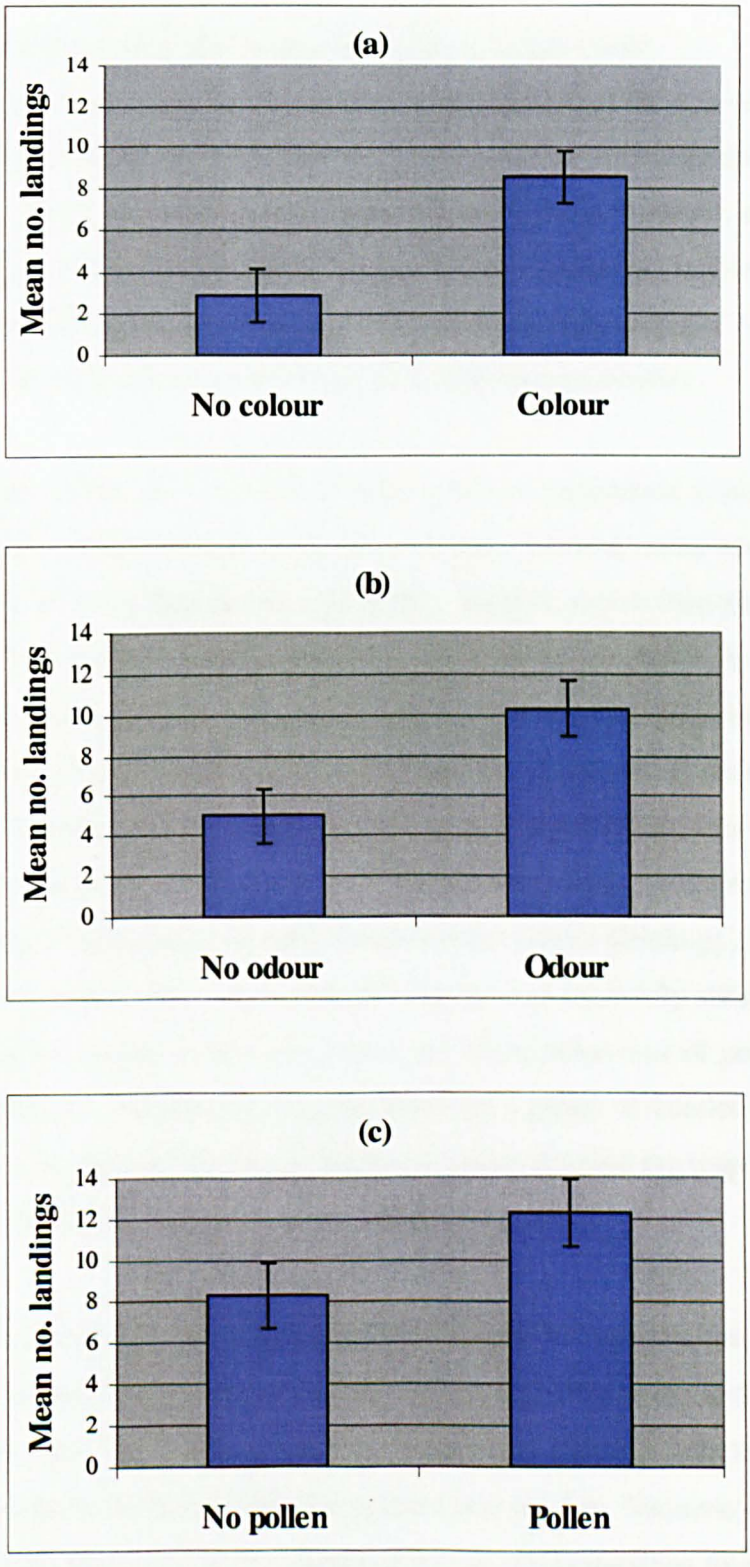


Figure 3.5 Mean number of pollen beetle landings on upwind targets over a 15-minute period after release of the beetles in the down wind section of a wind tunnel on targets with or without: (a) floral colour, (b) floral odour (c) pollen odour. Error bars represent s.e.d.

3.7 DISCUSSION

3.7.1 Measuring pollen beetle behaviour in a wind tunnel

This is the first study of pollen beetle orientation to host plant cues using a wind tunnel. The use of the wind tunnel in these experiments enabled the responses of pollen beetles to the major floral cues of their host plant to be compared in detail, and the functions of colour and odour teased apart in a precise fashion under controlled conditions. This compares with field studies, which are important to help explain functions under real conditions, but which do not allow such controlled manipulations.

Pollen beetles, unlike other insect species such as parasitoids (Du, Poppy & Powell, 1996) made few directed flights in the wind tunnel. Instead, many beetles dropped rather than flew out of the releasing pot and mostly walked, rather than flew upwind towards the target. This appears to be common behaviour of Nitidulids in wind tunnels; in a study on the attraction to host plant odour of the dried fruit beetle (*Carpophilius lugubris*), upwind orientation in a wind tunnel by walking was more common than by flying (Lin & Phelan, 1991). Some insects such as the cabbage root fly (*Delia radicum* (L.)) and the onion fly (*Delia antiqua* (Meigen)) when close to their host plants perform short 'hopping' flights and orientate between these flights (Prokopy, 1986). If this is the case for pollen beetles, then such directed flights over relatively short distances may not be part of normal pollen beetle behaviour; the flight behaviour of pollen beetles has not been fully studied. Measuring the proportion of a group of beetles released downwind that moved to the upwind portion of the wind tunnel enabled the responses of beetles that did not fly to and land on targets to be evaluated.

Very specific conditions are often required in order to achieve the flight of beetles in wind tunnels, therefore the low frequency of upwind orientation and landings on targets in this study may have been a result of inadequate lighting, temperature or humidity. However, Salom & McLean (1991) suggested that the low frequency of upwind flight in a study of flight behaviour of the ambrosia beetle (*Trypodendron lineatum* (Olivier)) in a wind tunnel was due to the variability in response behaviour within any insect population. Variation in the physiological state of the beetles may also be responsible; the beetles in this experiment were of mixed sex, and were field-collected, so variation was inevitable. Physiological state was considered an important factor in influencing the orientation of bark beetles to olfactory stimuli (Wood, 1982) and pollen beetles to visual

stimuli (Blight & Smart, 1999). Furthermore, beetles removed from their habitats have been found to perform migratory flights upon release and thus may not always respond to host plant cues (Whalon & Croft, 1985; Caprio & Grafius, 1990).

3.7.2 Hierarchy in the use of floral colour and floral odour cues by the pollen beetle

Both floral colour and floral odour cues were important factors in the attraction of pollen beetles to targets upwind in the wind tunnel when the number of landings on targets was considered. These results confirm the importance of both floral colour and floral odour in host location by the pollen beetle (Görnitz, 1956; Tamir *et al.*, 1967; Free & Williams, 1978a; Blight & Smart, 1999). Beetles were more likely to land in the presence of floral odour than colour, implying the greater importance of floral odour cues in this attraction. Conversely, Blight & Smart (1999), suggested from results of field trapping experiments, that visual cues are more important than odour cues in the orientation of pollen beetles. However, the relative importance of colour and odour cues may vary with the distance over which they are operating, as observed with the onion fly (*D. antiqua*) (Judd & Borden, 1991) and the apple maggot fly (*Rhagoletis pomonella* (Walsh)) (Green, Prokopy & Hosmer, 1994). In trapping experiments, colour may act as a long-range cue whilst odour may act over the shorter ranges. These experiments were conducted over a relatively short (<1 m) range, where odour cues may be more important.

When the proportions of pollen beetles upwind over time were measured, there was little initial difference in the number of beetles upwind in response to the presence of floral colour or floral odour in the treatments. This was probably due to the low proportion of beetles upwind after only five minutes. However, the proportion of beetles upwind gradually increased, particularly between 5-20 minutes, and this was more obvious in the presence of floral odour. This suggests that beetles were attracted upwind by the OSR floral colour and floral odour cues and that a proportion of them may have remained there to search for the resources that the presence of cues in the vicinity signalled. Odour may increase the motivation of insects to search for resources (Brantjes, 1978; this thesis, chapter 8). Thus, the measurement of the proportion of beetles upwind after 25 minutes could indicate the retaining qualities of the targets. Therefore, although both OSR floral colour and floral odour attracted pollen beetles upwind, only the presence of odour retained them there. This supports the conclusion that floral odour cues are more important than colour cues in pollen beetle orientation, particularly over a relatively short

range. The retaining properties of floral odour compared with floral colour could be further investigated in future wind tunnel studies, utilising a factorial design similar to that described in this chapter, and observing upwind-downwind movements of individual beetles, or small groups, over a period of time.

One more reason for the low attractiveness of floral colour cues, particularly when the proportions of beetles upwind was considered, could be that pollen beetles were not always able to see the yellow colour of the flowering racemes through the transparent plastic box. When light shines on plastic in a vertical position, the horizontal view is often of the reflectance of the light spectrum, rather than the object behind. Alternatively, the plastic may have altered the spectral reflectance of the yellow petals behind it, and, since the degree of pollen beetle attraction to colour is influenced by spectral reflectance and reflection intensity (Blight & Smart, 1999), pollen beetles could have been less attracted to the yellow flowers behind plastic within the wind tunnel than they would in the field. Recordings of spectral reflectance and intensity of OSR racemes in and outside the plastic box could be conducted using a spectrophotometer to investigate this further.

A clear attraction to colour cues was demonstrated in experiments that recorded the number of landings on the treatment targets; significantly more beetles landed on targets when yellow colour cues were present than when they were absent. The landing measurement is possibly a more direct measurement of attractiveness than proportions of beetles upwind after a period of time, although in these experiments it was not possible to determine whether the landings were made by the same individual or by many.

3.7.3 The role of pollen odour

More pollen beetles landed on the targets when there was the odour of male-fertile Synergy oilseed rape flowers (that possess normal, pollen-producing anthers) than when there was the odour of male-sterile flowers (which possess reduced anthers that produce no pollen) present. This suggests that pollen beetles were attracted by the odour of the male-fertile anthers - possibly from the pollen itself. However, the differences between pollen beetle attraction to male-fertile and male-sterile Synergy flowers could have been due to differences in their floral odours other than the presence/absence of pollen, as these lines are not isogenic. A chemical analysis of the volatiles from male-fertile and male-sterile Synergy flowers and Synergy pollen (Appendix 1.1) showed that there were

both quantitative and qualitative differences between the volatile components of the two lines, and there was evidence that some of these differences were due to the pollen present in the male-fertile flowers. Even so, this does not prove that the greater attraction of pollen beetles to male-fertile flowers is due specifically to volatiles from fertile anthers and not due to any of the other 'non-pollen' differences. The role of odour from fertile anthers in attraction could be further investigated using methods based on those described in this chapter; responses of pollen beetles to targets containing male-fertile anthers added to male-sterile flowers could be compared with responses to male-fertile flowers. If the degree of attraction were the same for both samples, this would support the presence of an attractive component in the anthers.

Charpentier (1985) suggested that odour cues were responsible for the attraction of pollen beetles to the anthers of OSR, and the seed weevil was found to be attracted to the odour of OSR stamens (Bartlet *et al.*, 1993). The odour of pollen itself (removed from anthers) has been found to be attractive to pollen-seeking insects; particularly bees (Dobson, 1987; Dobson, Danielson & Van Wesep, 1999; this thesis, chapter 8).

3.7.4 General discussion

These experiments demonstrate that both oilseed rape floral colour and floral odour cues are important in the attraction of pollen beetles upwind in a wind tunnel. The presence of floral odour cues was relatively more important than floral colour (yellow) in this attraction, and there was some evidence that floral odour may be involved in retaining resource-seeking beetles in the vicinity. There was evidence that part of the attractive odour emanates from the anthers of male-fertile flowers, and this raises the possibility that part of the attractive odour of oilseed rape emanates from the pollen itself. This question was investigated by assessing pollen beetle orientation responses to the odour of oilseed rape pollen and pollen-containing floral parts, and is described in the next chapter (4).

4. The role of pollen odour in the attraction of pollen beetles to oilseed rape flowers

4.1 ABSTRACT

A linear track olfactometer bioassay was used to investigate the role of pollen odour in attracting pollen beetles to the flowers of oilseed rape. The bioassay was first modified to eliminate all visual cues while testing the responses of pollen beetles to floral odours. The method and apparatus were also thoroughly tested for bias; that caused by the use of multiple beetles was reduced by assaying males and females separately. Beetles were attracted to oilseed rape flowers by their odour, as well as to the odour from anthers and bee-collected oilseed rape pollen. This indicates that at least part of the attractive odour of flowers emanates from pollen. The possibility of pollen specific odours as pollinator attractants is discussed.

4.2 INTRODUCTION

The use of host-plant volatiles is important in resource location for many phytophagous insects, including the Coleoptera (Metcalf, 1987). The pollen beetle (*Meligethes aeneus* (Fabricius)), is regarded as a pest of oilseed rape (*Brassica napus* (L.)) crops (Alford & Gould, 1975; Free & Williams, 1978a; Winfield, 1992). It is well known to be attracted by odours of oilseed rape (OSR) that emanate from the leaves, stems and buds (Free & Williams, 1978a; Evans & Allen-Williams, 1994; Ruther & Thiemann, 1997). However, numbers are generally low in the crop until the first flowers begin to open (Winfield, 1961; Buechi, 1990; Smart, Blight & Lane, 1996), and until then, beetles tend to congregate on 'volunteer' or forward plants in flower (Free & Williams, 1978a; personal observation). Many studies have demonstrated the attraction of pollen beetles to the yellow colour of the flowers of OSR (Wasman, 1926; Free & Williams, 1978a; Giamoustaris & Mithen, 1996; this thesis, chapter 3) but few have investigated the attractiveness of floral odour (Evans & Allen-Williams, 1994; this thesis, chapter 3).

The odour of a flower is composed of a blend of many volatile compounds (Borg-Karlson, 1990; Knudsen, Tollsten & Bergström, 1993). Variation in the chemical identities and the relative proportions of these volatiles brings about the great diversity in floral scent that results in different species having distinctive fragrances. Each floral structure may produce a different blend of volatiles that contributes to the whole-flower odour (Dobson *et al.*, 1987; Pichersky *et al.*, 1994; Bergström, Dobson & Groth, 1995; Dobson, Groth & Bergström, 1996). In some species, the petals contribute most to volatile production and the distinctive floral odour (e.g. Gregg, 1983; Borg-Karlson, 1990; Dobson, Bergström & Groth, 1990), whereas in others it is the androecium – the stamens and the anthers and pollen (e.g. Coleman & Coleman, 1982; D'Arcy, D'Arcy & Keating, 1990).

Anthers and the pollen they produce have evolved in some flowers to become a major floral signal, important in attracting pollen-seeking pollinators to flowers. This is evident from the common occurrence of floral structures which visually mimic them (Osche, 1979; Osche, 1983; Magin, Classen & Gack, 1989; Cane, 1993). In the Cretaceous period, pollen was probably the main reward of angiosperms to insects, before the evolution of nectar as a reward and petals as advertisements (Osche, 1979; 1983; Crepet, Friis & Nixon, 1991). Visual and olfactory cues from anthers and pollen probably served as the advertisements at that time. Chemical studies have shown that the stamens emit odours that differ from other floral parts (Dobson, Bergström & Groth, 1990; Knudsen & Tollsten, 1991; Bergström, Dobson & Groth, 1995). Furthermore, although the contribution to the whole-flower odour of pollen is quantitatively small, pollen volatiles are chemically distinct from those of the other floral structures (Dobson *et al.*, 1987; Dobson, Bergström & Groth, 1990; Bergström, Dobson & Groth, 1995). Pollen odour may therefore be used by pollen-seeking insects as cues to locate this resource.

Pollen beetle adults eat the pollen from many plant species (Fritzsche, 1957; Free & Williams, 1978a), and both adults and their larvae eat OSR pollen and that of other cruciferous hosts. Charpentier (1985) showed that pollen beetles were attracted to OSR anthers, and inferred that odour was important in this attraction. However, in this experiment no attempt was made to remove visual cues, and attraction was measured by the number of beetles that stopped to feed on the material; this variable was therefore more a measure of arrestment or retention than attraction. In the wind tunnel studies of the previous chapter of this thesis (3), pollen beetles landed more often on targets

emitting the odour of pollen-bearing male-fertile OSR flowers than on male-sterile ones without pollen. However, these differences may have been due to general differences between the flower volatiles of the two OSR varieties, or due to anther volatiles, rather than being due specifically to pollen. This chapter therefore aimed to determine whether the odours from pollen itself play a role in attracting pollen beetles to flowers of OSR. Since pollen beetle behaviour in the laboratory favours measurement of their walking responses, rather than flying (this thesis, chapter 3), experiments were conducted using a linear track olfactometer.

The linear track olfactometer (LTO) was designed by Sakuma & Fukami (1985) to examine the taxes of the German cockroach (*Blattella germanica* (L.)), towards its aggregation pheromone in an air current. Hardie *et al.* (1990) used a modified version to assess responses of male aphids to components of the female-produced sex pheromone and Bartlet *et al.* (1993) used a similar design to study the responses of the cabbage seed weevil, (*Ceutorhynchus assimilis* (Payk.)), to odours of oilseed rape. Preliminary tests using the LTO and method of Bartlet *et al.* (1993) confirmed that it was a suitable bioassay to assess the walking orientation responses of pollen beetles to the odours of OSR flowers. However, there was some concern that beetles may have been partially responding to floral colour visible in the olfactometer chambers, rather than to floral odour alone. Therefore, before the role of pollen odour in the attraction of pollen beetles to flowers could be investigated, the LTO apparatus and bioassay method had to be modified for use with pollen beetles.

4.3 AIMS

The aims of this series of experiments were to:

1. Develop a reliable bioassay utilising a linear track olfactometer (LTO) to investigate the responses of pollen beetles to floral odours by:
    - Modifying the design of the LTO set-up to eliminate colour cues during experiments, and to find an appropriate method of scoring the responses of pollen beetles in this bioassay
    - Testing the apparatus and method for bias
    - As a result of the bias tests, to reduce the interactions between beetles within the apparatus

2. Investigate the role of pollen odour in attracting pollen beetles to oilseed rape (OSR) flowers using the modified LTO bioassay from (1). Two experiments were designed to achieve this:
   - To investigate the attraction of pollen beetles to whole OSR flowers, flowers with and without their anthers, to anthers alone and finally to pollen alone
   - To directly compare the attractiveness of OSR flowers with and without anthers

4.4 MATERIALS AND METHODS

4.4.1 The modified linear track olfactometer apparatus

The linear track olfactometer (LTO) was made of transparent acrylic tubing (Perspex) and two stainless steel rods (1 mm width). One of the rods was suspended along the axis of a horizontal olfactometer tube and beneath this, the other was positioned vertically within a chamber to meet the horizontal wire, to form a T-junction. Test insects were introduced into a holding pot located at the base of the vertical wire. On leaving the pot, they climbed this to meet the horizontal wire at the T-junction where they chose to turn left or right into one of two equal flow airstreams. The holding pot was lined with fluon paint and was covered with a wire mesh lid to prevent test beetles from escaping and to allow odours and light into the pot. At either end of the olfactometer tube were side-arms consisting of a vertical chamber sectioned into two halves by a Perspex plate into which holes (5 mm diameter) had been drilled to allow through air flow. The bottom half of the side-arm chambers were plugged with silicone rubber bungs, as was the chamber that housed the vertical wire to the T-junction. By locating the holding pot in this bung, the apparatus was able to stand free on the bench, rather than being suspended from a clamp stand as in Bartlet *et al.* (1993). A vertical wire led from the horizontal wire to the top half of the side-arm chamber from which beetles were collected at the end of the experiment. Bartlet *et al.* (1993) used the top half of these side-arm chambers masked with filter paper to place host plants and other test materials. However, in experiments described in this chapter, the odour from test materials was introduced into the chambers through modified bungs from vessels located outside the olfactometer to completely remove all visual cues from the bioassay. The olfactometer apparatus, and the air movement within it is shown in Figure 4.1, and the experimental set-up is shown in Figure 4.2.

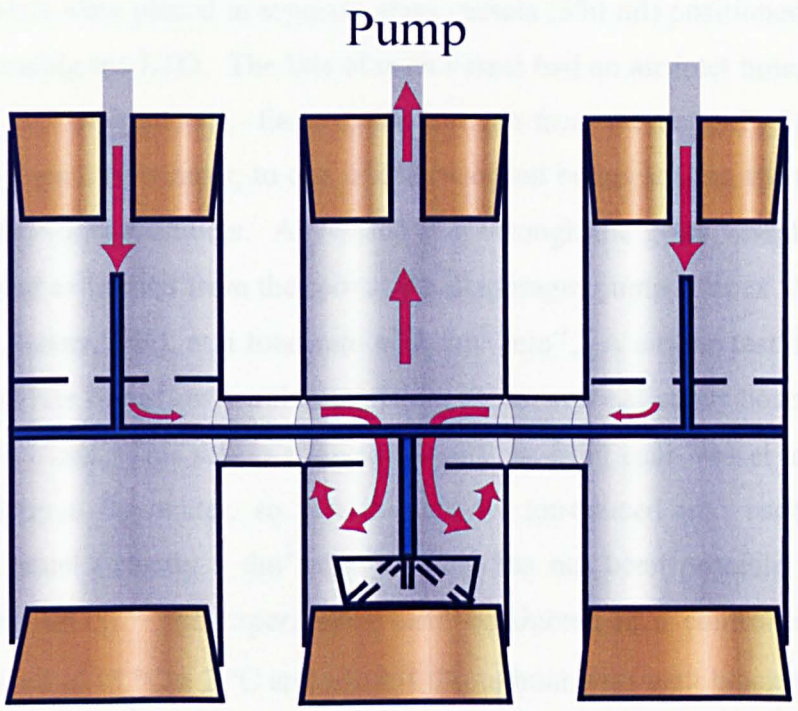


Figure 4.1 Schematic diagram of the Linear Track Olfactometer (LTO) showing air movement

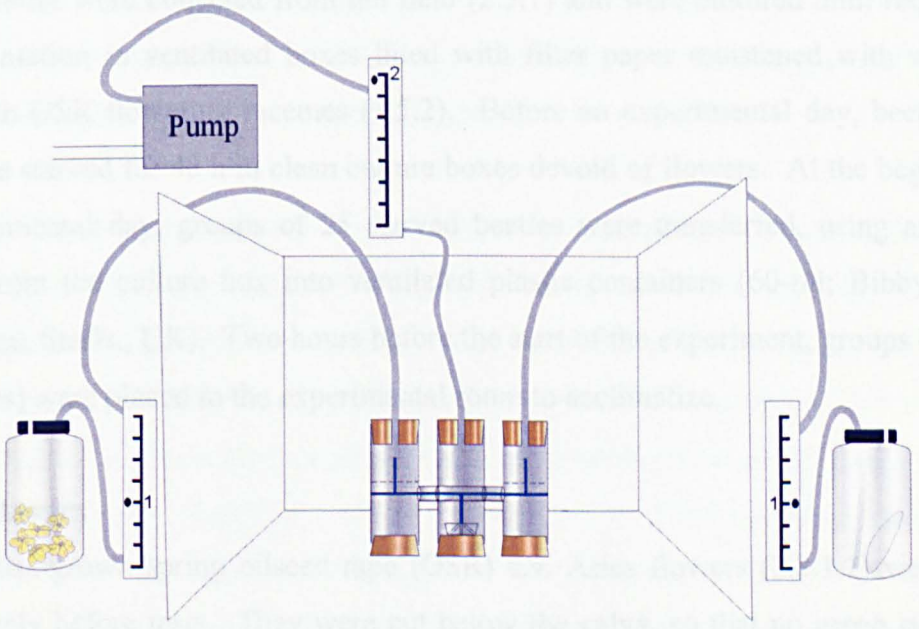


Figure 4.2 The linear track olfactometer experimental set-up

4.4.2 The experimental set-up

The test materials were placed in separate glass vessels (550 ml) positioned behind white screens surrounding the LTO. The lids of each vessel had an air inlet hole, and an outlet tube (Teflon, 50 mm diameter). Each outlet tube ran from the bottom of the vessel and was connected, via a flowmeter, to one of the modified bungs sealing the top half of the olfactometer side-arm chambers. Air was drawn through the glass vessels and into the olfactometer and exhausted from the room by a diaphragm pump (Capex V2 SE, Charles Austen LTD, Surrey, UK), at a total rate of 2 dm^3 min^{-1}. A smoke test had previously shown that this rate of airflow would maintain a sharp, vertical odour boundary at the T-junction of the wires. This set-up allowed the airflow from each vessel to be measured and regulated by a flowmeter, so that the airflow introduced into each olfactometer chamber was equal (exactly 1 dm^3 min^{-1}). This has not been possible with previous versions of the set-up. The experiments were conducted in a controlled temperature room, maintained at 15 °C ± 2 °C and painted throughout with matt black paint to reduce visual distractions. The apparatus was lit by an overhead fluorescent light that emitted daylight wavelengths, and was fitted with a plane-polarizing prismatic refractive cover to reflect the light evenly.

4.4.3 Pollen beetles

Pollen beetles were collected from the field (2.5.1) and were cultured until required for experimentation in ventilated boxes lined with filter paper moistened with water and filled with OSR flowering racemes (2.5.2). Before an experimental day, beetles to be used were starved for 48 h in clean culture boxes devoid of flowers. At the beginning of an experimental day, groups of 25 starved beetles were transferred, using an electric pooter, from the culture box into ventilated plastic containers (60-ml; Bibby Steralin Ltd., Stone, Staffs., UK). Two hours before the start of the experiment, groups of beetles (replicates) were placed in the experimental room to acclimatize.

4.4.4 Flowers

Glass-house-grown spring oilseed rape (OSR) c.v. Aries flowers (2.2.1.1) were picked immediately before tests. They were cut below the calyx, so that no green stems were present in samples. These flowers were between stages 2 and 3 of Charpentier's (1985) flower stage key, with free pollen around the apical third or over most of the anthers. The flowers (5 g) were weighed out on an electric balance before use.

4.4.5 General method

Test materials were placed in the glass vessels (550 ml) which were then connected to the LTO, and air was drawn through the apparatus for five minutes, during which time airflow through each vessel was regulated at 1 dm^3 min^{-1}. A group of 25 beetles was then transferred from their plastic container into the LTO holding pot and their behaviour observed for 15-minutes.

After each test, the beetles were removed from the apparatus using an electric pooter. The apparatus was then dismantled, soaked in a 5 % solution of Decon 75 cleaning fluid for 15 minutes, rinsed in distilled water and dried in an incubator at 50 °C. A new group of beetles was used for each test, and floral material was replaced after every four replicates. At the end of each day, the apparatus was soaked overnight in the cleaning fluid.

4.5 EXPERIMENTAL METHODS

4.5.1 Developing the bioassay

4.5.1.1 Scoring the responses

This experiment was designed to find an appropriate method of scoring beetles responses to host odour in the modified apparatus and set-up.

The experimental procedure followed that described in section (4.4.5). OSR flowers (5g) were placed in one glass vessel (550 ml) and filter paper (Whatman, Maidstone, Kent, UK) dampened with distilled water was placed in the other vessel (550 ml) as a control. To determine which behavioural response would be the most appropriate as a measure of beetles' responses to host plant odours, three scoring methods were compared:

1. *Total turns at the T-junction* The total number of beetles turning left or right at the T-junction of the odour boundary towards the odour of flowers or control was noted as in Hardie *et al.* (1990, 1994); Bartlet *et al.* (1993); Isaacs *et al.* (1993). A turn was recorded after the beetle had moved at least 10 mm along the wire after the T-junction. These responses were recorded in sequential order.

2. *Final responses* At the end of the 15-minute experimental period, the number of beetles present in each side-arm chamber was recorded as in Hori (1998).
3. *First responses* As the beetles' responses at the T-junction were recorded in sequential order, this allowed the response of the first beetle out of the pot in each replicate to be analyzed separately.

Sixteen replicates of pre-diapause beetles were conducted, using two LTO's, alternated between replicates. For both olfactometers, the test odour was introduced into each chamber of the olfactometer (chamber 1 & 2), in each direction (left & right), twice to reduce the effects of any bias. Each method of response scoring compared the number of beetles responding to the test odour (OSR flowers) or the control. Differences between these were analysed as follows: differences in the total number of turns at the T-junction were analysed by a Pearson's chi-square test, using a 2x2 contingency table to compare the proportion of beetles that turned left or right at the T-junction when the test odour was on the left or right. Pearson's chi-square test was also used to compare the numbers of beetles present in each chamber at the end of the experiment (final responses). Similarly, the response of the first beetle at the T-junction in each replicate (first responses) were analysed by a Fisher's exact test. In each case, the null hypothesis was that there was no difference in the number of beetles turning towards the test odour or control. The results of the three response scoring methods were compared to see if they supported each other.

4.5.1.2 Testing for bias

Despite alternating the direction of the olfactometer between replicates, and the introduction of the test odour into alternate chambers every other day, analysis of the data by Pearson's chi-square involves the assumption that there is no directional (left or right), or chamber (chamber 1 or 2) bias within olfactometers, and no difference between olfactometers. It also assumes, since 25 beetles are used per replicate, that each responding beetle is making an independent choice at the T-junction which is not influenced by visual or chemical cues from previous beetles.

Tests to investigate possible directional, chamber or olfactometer bias were conducted, and are described in Appendix 2; no such biases were evident. This series of experiments was designed to test the assumption that beetles make independent choices

at the T-junction, and are not influenced by visual or chemical cues from preceding beetles.

To eliminate factors connected with beetles' preferences for different odours, the same odour was introduced into both chambers of the olfactometer in these experiments, which were conducted as described in section 4.4.5. Two experiments were carried out, one in which no odour was introduced into either chamber (2 x controls of clean air) and one in which the odour of 5 g oilseed rape flowers was introduced into both chambers (2 x OSR). Pre-diapause beetles were used, and three linear track olfactometers were tested in both experiments, with 20 replicates per olfactometer in each experiment. The responses of beetles at the T-junction were recorded in their sequential order of turning so that the effect of the choices of preceding beetles could be evaluated on each beetle's choice. Beetle response was scored with an 'X' to indicate T-junction choice in the same direction as that of a beetle already present on the wire, and with a 'Y' if choice was in the opposite direction. If beetles were present on both sides of the wire, the beetle was scored with 'XY'. If there were no beetles on the wire, no score was made, other than the direction of turning.

Visual cues To examine whether a beetle's choice was influenced by the visual presence of a preceding beetle on the horizontal wire, the sequential responses of beetles turning at the T-junction were examined in each replicate. The numbers of beetles which 'followed' ('X') or 'did not follow' ('Y') a preceding beetle present on the horizontal wire were compared against their total in a binomial test.

Chemical cues To examine whether beetle choice was influenced by chemical trails on the wire left by preceding beetles, the sequential responses of beetles turning at the T-junction were examined in each replicate. The total number of beetles that turned in the direction of the preceding beetle was compared with the number that turned in the opposite direction by a binomial test. These counts were irrespective of whether the preceding beetle was present on the wire at the time of choice or not.

4.5.1.3 Testing for interactions: 'following'

In bias tests that used mixed-sex beetles (4.5.1.2), it was found that in the presence of host odour, a beetle was more likely to turn in the direction of a preceding beetle present on the horizontal wire than away from it (see visual cues, section 4.6.1.2 and Table 4.1).

This experiment was designed to investigate whether these 'following' responses were due to interactions between the sexes. Sexual interactions might be expected at the post-diapause stage of the life cycle when beetles mate, but perhaps not in the pre-diapause stage. Therefore, post-diapause and pre-diapause male and female beetles were tested separately to determine whether separation of the sexes would eliminate the following behaviour of beetles during their reproductive- and non-reproductive life phases.

Experiments on post-diapause pollen beetles were conducted in spring (March – April) and those on pre-diapause beetles were conducted in summer (July) of the same year. Pollen beetles were collected from the field (2.5.1) and their sex determined in the laboratory (2.5.3). Males and females were cultured separately (2.5.2). The experimental method was as described for the bias testing experiment (4.5.1.2) except that eight replicates of each test were conducted using two LTOs in each experiment. To enable comparison between males, females and both test odours (2 x control and 2 x odour) within life stages, one replicate of each was conducted daily. The total turns that 'followed' or 'did not follow' a preceding beetle present on the wire were compared against their total in a binomial test. Responses of male and female beetles to the two test odours were compared at the two different stages in their life cycle.

4.5.2 The role of pollen odour in the attraction of pollen beetles to oilseed rape flowers

The linear track olfactometer bioassay developed in the previous section (4.5.1) was used to investigate the responses of pollen beetles to the odour of samples of flowers with and without their anthers and to anthers and pollen alone. The general method followed that of 4.4.5. Pollen beetles were collected from a winter oilseed rape crop between May and July (2.5.1). Their sex was determined in the laboratory (2.5.3) and males and females were cultured (2.5.2) separately as described above until required for experiments (4.4.3). Male and female beetles were tested separately, with 16 replicates conducted in each test, using two LTO's as described in section 4.5.1.1. The total number of turns at the T-junction was recorded, as was the number of beetles present in the side-arm chambers after the 15-minute duration of each replicate.

Experiments involved testing treatments consisting of samples of flowers with and without their anthers. All six anthers were cut from flowers with surgical scissors, and flowers were blown to remove any pollen from the petals. The anthers were discarded from samples for treatments to be tested without anthers (-anthers), and were returned to the sample for those treatments to be tested with anthers (-/+ anthers). This was conducted to reduce differences between the volatile profiles of samples caused by wounding that would otherwise occur if whole flowers with their anthers were compared with those with their anthers excised. Changes in the volatile profile of flowers that have been damaged has been shown to occur (Bergström, Dobson & Groth, 1995), and such damage (to green leaf material, at least) may affect the behavioural responses of phytophagous insects to their host plant volatiles (Nottingham *et al.*, 1991; Du, Poppy & Powell, 1996).

4.5.2.1 Floral samples versus control

In the first set of experiments, five OSR floral treatments were each tested against a blank air control:

1. 5 g OSR flowers (whole flowers)
2. 5 g OSR flowers with their anthers excised and returned to the sample (-/+ anthers)
3. 5 g OSR flowers with their anthers excised and discarded (-anthers)
4. The excised anthers from 5 g OSR flowers (anthers)
5. 5 g fresh bee-collected oilseed rape pollen loads (section 2.3.1.2) (pollen)

4.5.2.2 Flowers with anthers versus flowers without anthers

In the second experiment, two floral treatments were prepared: one containing OSR flowers with anthers (-/+ anthers) and one without them (-anthers). These were tested directly against each other.

4.5.2.3 Statistical analysis

Differences in orientation at the T-junction and final chamber responses were analyzed by Pearson's chi-square test for both sets of experiments.

4.6 RESULTS

4.6.1 Developing the bioassay

4.6.1.1 Scoring the responses

Pollen beetles generally performed well in the LTO. Beetles rarely flew in the apparatus, indicating that the room temperature was maintained below their flight threshold. Of the 25 beetles placed in the holding pot, an average of 14.5 (SE=0.57) (58%) climbed the vertical wire out of the pot and made a choice at the T-junction. This sometimes occurred in quick succession. A few beetles managed to escape from the pot without using the wire, and crawled around the mid-tube of the olfactometer; lost to the experiment. The addition of a curled wire extension leading to the vertical wire on the holding pot lid minimized this occurrence.

Total turns at the T-junction Significantly more beetles turned towards the odour of the OSR flowers than towards the control at the T-junction ($\chi^2_1 = 36.08$; $P<0.001$) (Fig. 4.3 a). The positive orientation of pollen beetles towards floral odours can therefore be detected using this scoring method with this experimental apparatus and set-up.

Final responses At the end of the experiment, significantly more beetles were found in the chamber into which the odour of OSR flowers was introduced than in the chamber into which clean air was introduced ($\chi^2_1 = 44.92$; $P<0.001$) (Fig. 4.3 b). This result supports that of the total responses at the T-junction, and can therefore be used to detect attraction of pollen beetles to floral odour with this experimental apparatus and set-up. However, this scoring method may reflect the retaining qualities of the odour, rather than its qualities of initial attraction.

First responses The responses of the first beetle in each replicate at the T-junction did not reflect those of the total number of turns at the T-junction or final chamber responses; there was no difference between the number of first turns towards the floral odour and those turns away (Fisher's Exact, 2 tail probability = 1.0) (Fig. 4.3 c). The failure of this method to detect attraction to host odour was probably due to the small sample size (n=16), compared to the two previous tests where the maximum sample size was 400 (25 beetles per rep x 16 reps). The experimental design could be altered so that an increased number of replicates were conducted to measure first

responses; these would increase the sample size. However, this may be more time consuming than the method described using multiple beetles in each replicate, but would ensure the choice of each beetle was independent, and not influenced by the choice of others. To determine whether this was necessary, experiments were conducted to investigate whether multiple beetles made independent choices in the apparatus. These are described in sections 4.6.1.2 and 4.6.1.3.

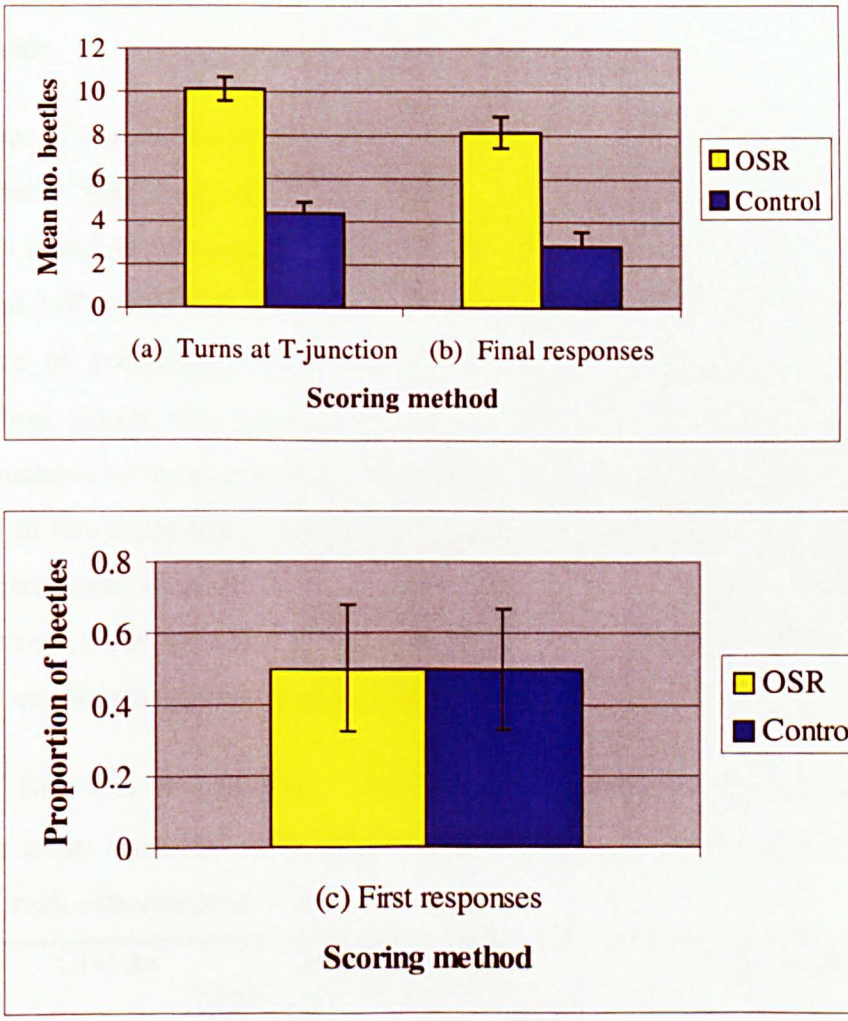


Figure 4.3. Beetles' responses to the odour of oilseed rape (OSR) flowers or clean air control in a linear track olfactometer using three methods of scoring the beetle's responses: (a) mean number of total turns at the T-junction (b) mean number of beetles in the chambers after 15-minutes (final responses) (c) proportion of first turns at the T-junction

4.6.1.2 Bias tests

Visual cues There was no significant difference between the number of beetles which turned in the same or opposite direction as a preceding beetle visible on the horizontal wire, when presented with two clean air controls as test odours. Therefore this experiment provides evidence to support the independence of choice at the T-junction when clean air (control) is introduced into both chambers. The test for beetles in LTO 1 reported a P-value close to the 5% test level (P=0.059) which suggests beetles could be 'following' (Table 4.1). However, this result was caused by one heavily influential outlying replicate.

When the odour of oilseed rape flowers was introduced into both chambers, more beetles orientated towards than away from the preceding beetle on the horizontal wire. The difference was found to be significant in LTO 2 (P=0.004) and close to the 5% test level in LTO's 1 and 3 (P=0.067 and P=0.064 respectively) (Table 4.1). This does not support the assumption of independent decision-making. These results suggest that, in the presence of host odour, the orientation responses of beetles at the T-junction are positively influenced by the presence of a preceding beetle on the horizontal wire. As the sex of beetles in this experiment were not determined before testing, these events could be due to interactions between the sexes that occur in the presence of host odour. Therefore, the next experiment (4.4.3.4) was performed to investigate whether incidences of 'following' could be removed by separating the sexes.

Table 4.1 Bias test: Visual cues. The number of pollen beetles turning towards (following) or away from (not following) a preceding beetle present on the horizontal wire in linear track olfactometers (LTO)

Test	LTO no.	Total no. beetles		Binomial Test		
		Following	Not following	K	N	P
2 x Control	1	67	46	46	113	0.059
	2	30	24	24	54	0.497
	3	47	47	47	94	1.000
2 x OSR	1	84	61	61	145	0.067
	2	97	60	60	157	0.004
	3	69	48	48	117	0.064

Chemical cues There were no significant differences between the total number of beetles that turned in the same direction as the preceding beetle compared with those which turned in the opposite direction (Table 4.2) when OSR or control odour was

introduced into both chambers. A beetle's choice at the T-junction is therefore not affected by the presence of any chemical trail left by preceding beetles.

Table 4.2 Bias test: Chemical cues. The number of pollen beetles turning the same way (following) or the opposite way (not following) to the previous beetle

Test	LTO no.	Total no. beetles		Binomial Test		
		Following	Not following	K	N	P
2 x Control	1	128	125	125	253	0.9
	2	101	98	98	199	0.887
	3	109	123	109	234	0.393
2 x OSR	1	164	130	130	294	1.00
	2	146	136	136	282	0.592
	3	137	114	114	251	0.165

4.6.1.3 Interactions between beetles: 'following'

When the sexes were tested separately, there was evidence to suggest that more post-diapause males turned towards than away from another male present on the wire in the presence of host odour (P= 0.057). Otherwise, there was no difference between the number of beetles turning towards or away from a preceding beetle present on the horizontal wire, regardless of the sex of the beetle or its stage in the life cycle. (Table 4.3).

Table 4.3 Bias test: Sex. The number of male and female pollen beetles orientating towards (following) or away from (not following) a preceding beetle on the horizontal wire of a linear track olfactometer

Experiment			Total no. beetles		Binomial Test		
Sex	Life Stage	Test	Following	Not following	K	N	P
F	Post-D	2 x Control	22	12	12	34	0.121
F	Post-D	2 x OSR	37	31	31	68	0.545
M	Post-D	2 x Control	29	18	18	47	0.144
M	Post-D	2 x OSR	44	27	27	71	0.057
F	Pre-D	2 x Control	24	23	23	47	1.000
F	Pre-D	2 x OSR	42	29	29	71	0.154
M	Pre-D	2 x Control	30	26	26	56	0.689
M	Pre-D	2 x OSR	44	35	35	79	0.368

Where F = Female; M = male; Post-D = Post Diapause; Pre-D = pre-diapause

4.6.1.4 Summary of results from developing the bioassay

A method of assessing the responses of pollen beetles to floral odour in the linear track olfactometer was developed using a set-up which eliminates all floral visual cues. The number of beetles turning left or right at the T-junction towards or away from host odour was found to be the most appropriate scoring method to assess the degree of attraction to host odour, although the number of beetles present in the olfactometer side-arm chambers at the end of the experiment may indicate the retention qualities of the odour. Although the experimental method was designed to reduce the effects of any directional and olfactometer chamber bias, as well as differences between olfactometers, experiments showed that these were statistically undetectable. There was some evidence for interactions between the sexes in tests that used multiple beetles of both sexes. However, beetles made independent choices at the T-junction when the sexes were separated. Therefore, the method can utilize multiple beetles per replicate as long as they are of single sex. This reduces the number of replicates that need to be conducted to show differences in responses to test odours that would otherwise be required if only one individual were tested per replicate.

4.6.2 The role of pollen in the attraction of pollen beetles to oilseed rape flowers

4.6.2.1 Floral treatments versus control

In the first experiment, in which each of five floral treatments were tested against a clean air control, both male and female pollen beetles turned at the T-junction towards all five OSR floral treatments significantly more times than they did the clean air control ($P<0.001$ for each treatment); (Table 4.4; and the responses of female beetles may be seen in Figure 4.4).

Similarly, at the end of the experiments, more beetles were found in the chambers into which the odour of the OSR floral samples were introduced than were found in the chamber into which the clean air control was introduced ($P<0.001$ for each sample) (Table 4.5).

Table 4.4 Responses of pollen beetles to the odour of oilseed rape floral samples and a blank air control at the T-junction of the linear track olfactometer

Test	Female		Male	
	X^2_1	P (and direction of significance)	χ^2_1	P (and direction of significance)
OSR flowers (5g) vs. C	46.03	P<0.001 (OSR)	61.54	P<0.001 (OSR)
OSR flowers (5g) -/+ anthers vs. C	21.38	P<0.001 (OSR)	41.85	P<0.001 (OSR)
OSR flowers (5g) - anthers vs. C	20.92	P<0.001 (OSR)	39.3	P<0.001 (OSR)
Anthers from 5g OSR flowers vs. C	27.78	P<0.001 (OSR)	84.53	P<0.001 (OSR)
OSR pollen loads (fresh; 5g) vs. C	25.41	P<0.001 (OSR)	39.14	P<0.001 (OSR)

Table 4.5 Final chamber responses of pollen beetles in response to the odour of oilseed rape (OSR) floral samples and blank air control (C) in a linear track olfactometer

Test	Female		Male	
	χ^2_1	P (and direction of significance)	χ^2_1	P (and direction of significance)
OSR flowers (5g) vs. C	43.47	P<0.001 (OSR)	35.64	P<0.001 (OSR)
OSR flowers (5g) -/+ anthers vs. C	12.64	P<0.001 (OSR)	17.01	P<0.001 (OSR)
OSR flowers (5g) - anthers vs. C	35.2	P<0.001 (OSR)	23.18	P<0.001 (OSR)
Anthers from 5g OSR flowers vs. C	43.88	P<0.001 (OSR)	44.26	P<0.001 (OSR)
OSR pollen loads (fresh; 5g) vs. C	32.69	P<0.001 (OSR)	23.56	P<0.001 (OSR)

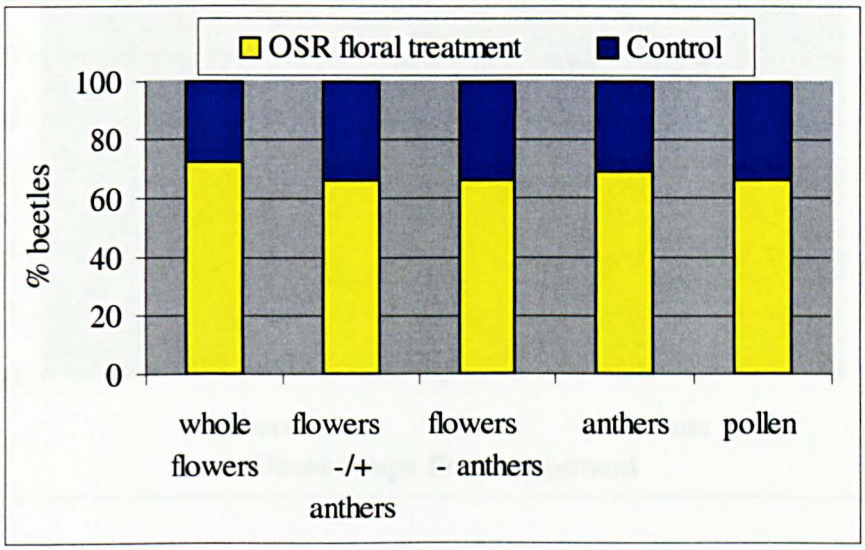


Figure 4.4 Proportion of female pollen beetles turning towards the odour of oilseed rape (OSR) floral samples or a blank air control at the T-junction of a linear track olfactometer

4.6.2.2 Flowers with anthers versus flowers without anthers

In the second experiment, pollen beetle responses to OSR flowers with their anthers excised were directly compared with responses to flowers with their excised anthers returned to the sample. Both male and female beetles orientated more towards the odour of OSR flowers with anthers than without (males $\chi^2_1 = 3.88$; $P<0.05$; females $\chi^2_1 = 5.32$; $P<0.05$). This can be seen for female beetles in Figure 4.5. More males were found at the end of the experiment in chambers into which the odour of OSR flowers with anthers was introduced, than those into which the odour of flowers without anthers was introduced ($\chi^2_1 = 9.79$; $P<0.001$). However, no such difference was seen in females' responses ($\chi^2_1 = 0$; $P = 0.997$).

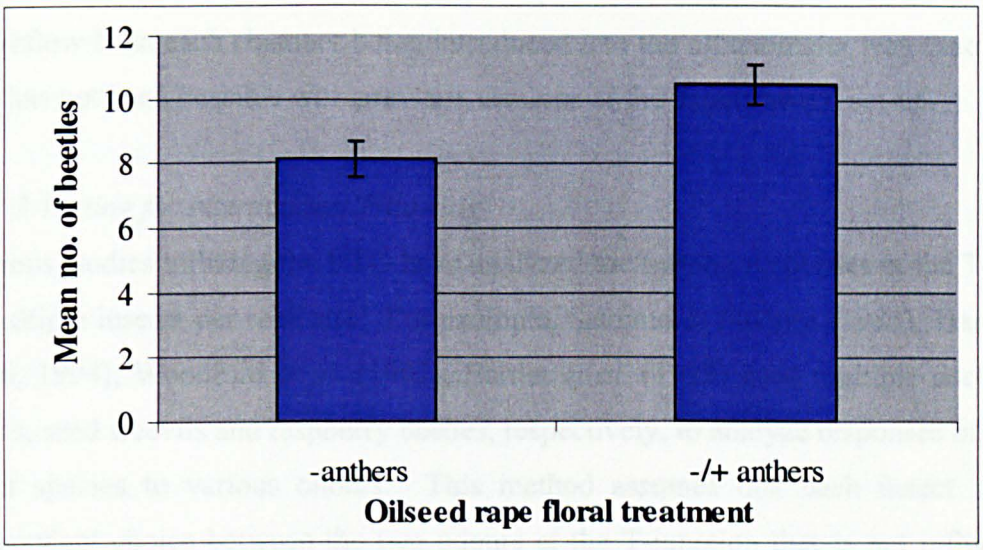


Figure 4.5 Mean number of female pollen beetles turning towards the odour of oilseed rape flowers with and without their anthers

4.7 DISCUSSION

4.7.1 Development of the bioassay

4.7.1.1 Removal of visual cues

These experiments describe a method of assessing the responses of insects to floral odour in the linear track olfactometer that completely eliminates visual cues. When investigating the effects of host plant odour on the attraction of phytophagous insects to their host, it is often necessary to remove visual cues to be confident that any responses observed are solely due to odour. In previous LTO studies to test the responses of insects to host plant odour, samples have been placed in the top half of side-arm chambers, and visual cues from the host material reduced by placing white filter paper around the sides of the chamber (Bartlet *et al.*, 1993; Hardie *et al.*, 1994). However, this may not completely eliminate the visual cues. The pollen beetle is attracted by visual cues from OSR flowers (Wasman, 1926; Free & Williams, 1978a; Giamoustaris & Mithen, 1996; this thesis, chapter 3) and yellow colours generally (e.g. Láska & Kocourek, 1991; Blight & Smart, 1999). It is therefore likely to be attracted by any yellow hue, and thus complete elimination of visual cues is desirable when studying the effects of OSR floral odour on pollen beetle behaviour. The method of introducing host plant odour into the

olfactometer from chambers outside the apparatus made this possible. It also enabled the airflow from each chamber to be precisely regulated through a flowmeter, ensuring that the airflow from each chamber being introduced into the olfactometer was exactly equal. This has not been possible with previous versions of the experimental set-up.

4.7.1.2 Testing for interactions 'following'

Previous studies utilizing the LTO have analyzed the turning responses at the T-junction of multiple insects per replicate. For example, Sakuma & Fukami (1985); Hardie *et al.* (1990, 1994); Woodford *et al.* (1992); Bartlet *et al.* (1993) used multiple cockroaches, aphids, seed weevils and raspberry beetles, respectively, to analyze responses of their test insect species to various odours. This method assumes that each insect makes an independent choice between the two odours at the T-junction that is not influenced by interactions with preceding insects (or those that follow after). Such interactions might occur between the sexes if multiple subjects of mixed-sex are used, or even within a sex, if single-sex subjects are used, and should be tested for, if appropriate. The cockroaches in Sakuma & Fukami's (1985) test were nymphs and therefore interactions between the sexes were unlikely at this stage. Studies on aphids (Hardie *et al.*, 1990, 1994; Nottingham *et al.*, 1991) used either males or virginoparae (females), and thus eliminated the possibility of sexual interactions. However, Bartlet *et al.* (1993) and Woodford *et al.* (1992) used mixed-sex adults and did not report tests for interactions between the sexes.

Even if sexual interactions are eliminated, interactions between multiple individuals used in a test may still occur. Sakuma & Fukami (1985) showed that the direction of turning of the cockroaches was not influenced by any chemical trail left by preceding subjects, but none of the above authors reported tests to ensure that individuals made independent choices at the T-junction in cases where preceding insects were visible on the horizontal wire. Therefore, these experiments ran the risk that interactions may have led to false results when data are interpreted in terms of odour cues acting on individuals making independent choices. The bias tests reported here showed that beetles' choices at the T-junction were not affected by the passage of the previous beetle (chemical cues), as did Sakuma & Fukami (1985). However, there was evidence to suggest that in the presence of host odour (but not in its absence), pollen beetles of mixed-sex were more likely to turn towards a beetle present on the horizontal wire than away from it. In experiments that test responses to a host plant odour versus a control, any such interactions might increase the proportion of beetles responding towards the host odour, but should not lead

to incorrect conclusions. However, such interactions should be avoided if possible. Reasons for the occurrence of this effect may include that, in the presence of odours from the plant upon which they reproduce, pollen beetles are attracted by visual or olfactory cues of conspecifics. Since these effects were not evident in tests in which the sexes were separated, male-female interactions could have been a likely cause. Host plant odour-conspecific odour interactions have been demonstrated in other insect species. For example, host–derived volatiles and pheromones interact to produce synergised attraction in bark beetles (Jones, 1985). Other possibilities for this result include some form of host-odour-induced visual attraction (Kennedy, 1977) of males by females (or vice-versa), although there have been few studies on this possibility. Male-female interactions are likely between pollen beetles at the reproductive, post-diapause stage, and although the experiment conducted on mixed-sexes (in which the following behaviour was observed) was done in summer, when many of the field population would be pre-diapause, a proportion would still be post-diapause.

When the sexes were tested separately, no 'following' was statistically evident for either males or females of either post-diapause or pre-diapause stage. This supports the conclusion that male-female interactions were mainly responsible for the earlier 'following' observations in experiments using multiple beetles of mixed-sex. However, there was an indication of male 'following' in the presence of host odour, but not in its absence. This could just be a statistical anomaly, since only eight replicates per test were conducted in these experiments, but it could be a manifestation of male aggregation. Aggregation pheromones released by males in the presence of host odour have been described for other species of Nitidulid beetles (Bartelt *et al.*, 1990; Dowd & Bartelt, 1991; Petroski, Bartelt & Vetter, 1994), and aggregation of pollen beetles has been observed by several authors (Free & Williams, 1978a; Nielsen & Axelsen, 1988b). Such a phenomenon could explain why, at the end of the experiment that directly tested flowers with and without anthers, more male pollen beetles were found in the chamber into which the odour of flowers with odour was introduced, an effect not seen in females. These sexual interactions and the possibility of post-diapause male aggregation are currently under further investigation (by the author) at Rothamsted.

4.7.1.3 Scoring methods

When the sexes were tested separately, the 'following' behaviour observed in the bias tests conducted with mixed-sex beetles was no longer evident. Therefore, there is evidence to support the assumption that beetles make independent choices at the T-junction when the sexes are separated. If the sexes are separated, scoring the number of turns at the T-junction of multiple beetles per replicate is therefore a valid method for assessing the responses of pollen beetles to host odour in the linear track olfactometer; scoring the first responses of one beetle per replicate to avoid interactions between multiple beetles is not necessary. One previous study utilizing the LTO scored the number of insects in the side arm chambers at the end of the experiment as a measure of attractiveness/repellency of the test odour (Hori, 1998). However, since this method of scoring allows insects to change their choice, and turn round and investigate the other side arm, it probably represents more a measure of the retaining qualities of the odour than initial attractiveness (Brantjes, 1978).

4.7.2 The role of pollen odour in attracting pollen beetles to oilseed rape flowers

4.7.2.1 The role of anthers

Pollen beetles were attracted to the odour of OSR flowers, suggesting that the beetles could locate their host plants on the basis of their floral odour alone. This supports earlier studies reporting similar findings (Evans & Allen-Williams, 1994; this thesis, chapter 3). Furthermore, these experiments show that pollen beetles are attracted to the odours of OSR anthers. Charpentier (1985) inferred that the attraction of pollen beetle to stamens was due to their odour, although this was not specifically tested for.

Pollen beetles were more attracted to the odour of flowers with anthers than those without. This indicates that there was a detectable difference between them. Since the excised anthers were added back to one of the samples, this was possibly merely due to a quantitative difference; if the anthers smell similar to the rest of the flower, the quantity of odour in the sample containing anthers would have been greater than in that without. However, the difference could also be qualitative, due to chemical differences between the volatiles emitted by anthers and the rest of the flower. Investigations comparing the volatile emissions of the OSR flower, its petals, anthers and pollen, suggest that pollen contains far fewer volatiles than the whole flower or its petals, and that those present in the pollen may be different from those in the petals and vice-versa (Appendix 1.3).

Furthermore, comparisons between male-fertile (with pollen) and male-sterile (without pollen) Synergy OSR flowering racemes and the pollen from fertile flowers showed that some volatiles present in the male-fertile flower, but absent in the male-sterile flower, could have been pollen-derived (Appendix 1.1). These studies suggest that there are qualitative differences between odours that are released by OSR pollen and its petals. However, these studies were preliminary, and further work is required to clarify the nature of these differences. There is good evidence that qualitative differences between volatiles produced by anthers and the rest of the flower exist, at least in some species, and that these are detectable by pollen-seeking insects. Behavioural discrimination between anthers and other floral parts by conditioned honey bees have been reported (von Frisch, 1923; von Aufsess, 1960; Coleman & Coleman, 1982). These are further supported by chemical evidence (Dobson, Bergström & Groth, 1990; Knudsen & Tollsten, 1991; Pichersky *et al.*, 1994; Bergström, Dobson & Groth, 1995).

4.7.2.2 The role of pollen odour

Although the odours from stamens and anthers do not always arise from pollen (Dudai *et al.*, 1988), the odour from their pollen was probably at least partially responsible for the difference in attraction caused by the presence of the anthers in the floral sample that the pollen beetles responded more to. Pollen beetles in this study were attracted to the odour of OSR pollen when this was presented alone. This suggests that some of the attractive odours of OSR flowers emanate from pollen. Similarly, the solitary bee *Colletes fulgidus longiplumosus* was attracted to both the odour of flowers and the pollen of its host plant, (Dobson, 1987). The presence of pollen or pollen volatiles also increased landing responses of bumble bees on flowers (Dobson, Danielson & Van Wesep, 1999). These studies suggest that pollen-seeking insects many use pollen odour to locate this resource.

4.7.2.3 Distinctive pollen odours as pollinator attractants

Pollen odour emanates from the pollenkitt – the oily coating of (angiosperm) pollen grains (Dobson *et al.*, 1987; Dobson, 1989; Szalai, 2000). The volatiles are thought to originate from the pollenkitt, which is deposited onto the grain by the tapetum during development (Pacini & Franchi, 1993), rather than being adsorbed onto the pollenkitt from other odour-releasing floral organs before re-release (Dobson, Bergström & Groth, 1990). Pollen odour has been found to be distinct from that of the rest of the flower, differing either in: 1) the relative proportions of the constituent compounds present 2) the relative proportion and the number (pollen has fewer) of volatiles, or 3) the

production of fewer volatiles, some of which are pollen-specific (Dobson, Bergström & Groth, 1990; Dobson, 1991 b & c; Bergström, Dobson & Groth, 1995; Dobson, Groth & Bergström, 1996). A chemically-distinct odour from the rest of the flower may allow pollen odour to be used by pollen-seeking insects as a cue to locate this resource. This may occur by enabling an assessment of pollen reward in individual flowers before landing (Ribbands, 1949; Dobson, Danielson & Van Wesep, 1999) or by aiding visitors in locating food rewards once they have alighted on the flower. Both these functions would benefit the plant through increasing pollination efficiency by attracting pollinators to flowers of reproductive maturity and by leading them to position themselves appropriately on the flower for effective pollination.

B. napus is self-compatible and is largely self-pollinated (Free & Nuttall, 1968; Free, 1993). Therefore, the selection pressure for the evolution of anther or pollen–specific volatiles to aid in insect-pollination may not have been great. However, the plant benefits from cross-pollination (Williams, 1978), and insect-pollination is thought to result in earlier and more uniform seed set and increased yield of the crop (Williams, 1978; Manning, 1995). Furthermore, the flowers exhibit many of the features of 'insect-pollinated plants', including bright yellow petals and nectar as a reward, and are visited by a great variety of insects (Kirk, 1992), some of which have been reported to be attracted to the anthers. Bartlet *et al.* (1993) found that seed weevils were attracted to the odour from OSR stamens, and anthers are an important cue for syrphid visitors (Brunel *et al.*, 1994; Golding, Sullivan & Sutherland, 1999). Therefore, distinctive volatile cues emanating from OSR anthers or pollen may attract these visitors, ultimately benefiting the plant, and remain an interesting possibility worthy of further investigation.

5. Temporal changes in the responses of pollen beetles to floral odours

5.1 ABSTRACT

Host plant choices of pollen beetles appear to vary during the three major phases of their life cycle: post-diapause, reproductive and pre-diapause. The responses of pollen beetles collected from oilseed rape crops to the floral odour of oilseed rape (a reproductive-phase host upon which they both feed and reproduce), and field bean (a non-reproductive-phase host, upon which they feed but do not reproduce) were assessed at the three main phases of the beetle's life cycle using a linear track olfactometer. The effect of conditioning on the responses of pollen beetles to floral odours was also investigated by conditioning post-diapause beetles collected from dandelion (a non-reproductive phase host) to either oilseed rape or dandelion and assessing their responses to the floral odours of these two plants. These experiments suggest that post-diapause pollen beetles that have recently emerged from over-wintering are polyphagous and respond to the odours of host flowers upon which they can feed and mature. However, beetles at this phase do not become conditioned to non-reproductive-phase hosts upon which they feed, and show a preference for odours from their reproductive-phase hosts onto which they must migrate. Once post-diapause beetles have experienced a reproductive-phase host plant, they become conditioned to it. This conditioning seems to have the effect of 'switching off' or reducing the attraction to floral odours of non-reproductive-phase host plants. Such behaviour might facilitate the migration of beetles to cruciferous plants in time for their reproductive phase. Starved beetles in their reproductive phase unexpectedly responded to the odour of field bean, indicating that conditioning can be overcome in hungry reproductive beetles – possibly due to high motivation to feed to meet the high energy demands of reproduction. Assumptions in the literature that reproductive pollen beetles are oligophagous on cruciferous plants are therefore incorrect: pollen beetles are in fact polyphagous throughout their life cycle.

5.2 INTRODUCTION

Phytophagous insects differ in their host-plant selectivity. Generalists (polyphagous insects) are least selective, and accept plants from many different unrelated families. Specialists are more selective: oligophagous species feed on a few species from related genera, and monophagous species accept only plants of a single genus –or even species (Schoonhoven, Jermy & van Loon, 1998).

Host-finding by phytophagous insects may be considered as a series of steps of encountering and responding to cues from the habitat, the patch and the host plant (Hassell & Southwood, 1978; Miller & Strickler, 1984; Jones, 1991). Insects may use visual and olfactory cues in locating their host plant from a distance; once on the plant, in addition they may use gustatory and tactile cues for final acceptance or rejection. Generalist insects respond to cues common to many plants such as green and yellow colours and a wide range of plant volatiles (Lance, 1983), and may be deterred from feeding only by certain feeding inhibitors (Bernays & Chapman, 1986). Specialists, conversely, may respond to only a few plant volatiles associated with a narrow range of plants, and may require more specialized feeding or oviposition stimulants – often those chemicals that deter generalists (Schoonhoven, Jermy & van Loon, 1998). There are relatively few studies on the cues used by phytophagous insects in initial host location (Visser, 1986) compared to those studies on subsequent host acceptance and suitability (Courtney & Kibota, 1990). In addition, there are even fewer studies investigating the many factors, such as phase in the life cycle, which might affect an insect's response to host cues during the host-location process (Zhang & McEvoy, 1996). However, knowing how insect phytophages locate their hosts, and how their responses may vary, is important for understanding and predicting the spatial and temporal dynamics of insect-plant interactions which are often required in the control of those phytophagous insects that are crop pests.

The pollen beetle (*Meligethes aeneus* (Fabricius)) is a pest of oilseed rape (*Brassica napus* L.) and other cruciferous (Brassicaceae = Cruciferae) crops. It is both a generalist and a specialist over its life cycle. Its life cycle has been described in early works such as Heeger (1855); Heikertinger (1920); Scherney (1953); Fritzsche (1957). According to such descriptions, after emergence from diapause, and while they mature reproductively, pollen beetles undergo a period of polyphagous feeding on the pollen of spring-flowering

plants of many different families. They then migrate to cruciferous plants such as oilseed rape upon which they are assumed to be oligophagous since they mate and lay eggs only on these plants. Upon emergence from pupation, the adult pre-diapause beetles undergo a phase of polyphagous feeding on the pollen from many summer/autumn-flowering plants to build reserves for their diapause over winter.

Pollen beetles are attracted to yellow colours (Wasman, 1926; Láska, Zelenková & Bicík, 1986; Blight & Smart, 1999; this thesis, chapter 3) - the colour of many of their cruciferous and non-cruciferous food host plants; visual cues therefore play an important role in host-location of the pollen beetle. Odour is undoubtedly also important in host-location. It has been demonstrated several times that pollen beetles are attracted to odours from plant parts of crucifers – plants upon which they can reproduce (their 'reproductive-phase' hosts) (e.g. Görnitz, 1956; Evans & Allen-Williams, 1994; this thesis, chapters 3 & 4). However, few studies have investigated their responses to the odours of non-cruciferous host plants (upon which adults feed, but do not reproduce: 'non-reproductive-phase' hosts) (Free & Williams, 1978a; Ruther & Thiemann, 1997). Furthermore, responses of hungry beetles to their food sources (flowers) throughout their active season have not been previously investigated. Therefore it is not known whether pollen beetles use both visual and olfactory cues to help them to locate non-reproductive-phase host plants for feeding, as they do in the location of their reproductive-phase host plants (Free & Williams, 1978a; Blight & Smart, 1999; this thesis chapter 3). In addition, it is not understood how pollen beetles switch from polyphagous behaviour to oligophagy, and whether host odour plays a role in this phenomenon.

5.3 AIMS

The aim of this series of experiments was to compare the responses of pollen beetles to the odour of flowers from oilseed rape (OSR), a reproductive-phase host plant, with those of field bean (FB) (*Vicia faba* L.), a non-reproductive-phase host plant, at each of the three main phases of the pollen beetle's life cycle, and to investigate the effect of host plant conditioning on these responses.

5.4 MATERIALS & METHODS

5.4.1 Responses of pollen beetles at different phases of their life cycle to the odours of flowers from their reproductive-phase and non-reproductive- phase host plants

5.4.1.1 Pollen beetles

Pollen beetles were collected from crops of OSR (2.5.1). Collections were made in:

- March-April to capture newly emerged beetles at the post-diapause 'polyphagous' phase of their life cycle,
- May-June to collect those in the post-diapause 'oligophagous' (reproductive) phase,
- late August for those of the pre-diapause 'polyphagous' phase.

The sex and species of the beetles were determined in the laboratory (2.5.3 and 2.5.4, respectively) and male and female *M. aeneus* were cultured (2.5.2) separately in ventilated plastic boxes lined with moistened filter paper that contained OSR flowering racemes. Beetles were starved for 48 hours before experiments in culture boxes devoid of food.

5.4.1.2 Flowers

OSR and FB flowers were cut from glass-house grown plants (sections 2.2.1.1 and 2.2.2, respectively) before experiments and weighed into samples of 5 g using an electric balance.

5.4.1.3 Linear Track Olfactometer

The responses of male and female beetles to reproductive- and non-reproductive-phase host plant odours were examined using a linear track olfactometer (LTO). The LTO is described fully in sections (4.4.1-4.4.2). It was made of transparent Perspex and two stainless steel rods that formed a T-junction at the point where two equal airstreams met. Air was drawn through two glass vessels (500 ml) containing test materials at a rate of 1 dm^3 min^{-1} through each vessel. These vessels were located behind screens surrounding the LTO apparatus to eliminate visual cues from the test materials and were connected, via a flowmeter, to the side-arm chambers of the LTO by Teflon tubing (see Fig. 4.2). Pollen beetles initially located in a holding pot at the base of the equipment, climbed the vertical wire to meet the T-junction where they chose to turn left or right into one of the two airstreams laden with the odour from the test materials.

Experiments were conducted in a controlled temperature room that was maintained at 15 °C ± 2 °C and painted matt black inside to reduce visual distractions. The apparatus was lit by an overhead fluorescent light that was fitted with a plane-polarizing prismatic refractive cover to reflect the light evenly.

5.4.1.4 Experimental method

Pollen beetle responses to the odour of flowers from their reproductive-phase host plant (OSR) and non-reproductive-phase host plant (FB) were assessed in three experiments. Test materials placed in the glass vessels for comparisons in these experiments were as follows:

1. Oilseed rape (5 g) flowers vs. clean air control
2. Field bean flowers (5 g) vs. clean air control
3. Oilseed rape flowers (5 g) vs. field bean flowers (5 g)

Test material for clean air controls consisted of filter paper (Whatman, Maidstone, Kent, UK) moistened with distilled water. Twenty-five beetles were used per replicate and their choices at the T-junction were recorded over a 15-minute period. After each replicate, the beetles were removed from the apparatus, which was then soaked in a 5% solution of Decon 75 cleaning fluid for 15 minutes, rinsed in distilled water and oven-dried. A new group of beetles was used for each replicate, and the test materials were replaced after every four replicates. Sixteen replicates were conducted per experiment, using two LTOs. Although previous tests had demonstrated that there was no chamber or direction bias (Appendix 2), each odour was introduced into both chambers (chamber 1 and 2) of the olfactometer in both directions (left and right) twice in both olfactometers. The total number of beetles turning left or right towards or away from a given test odour was analysed by Pearson's chi-square test.

The three experiments were conducted at each of the three main phases of the pollen beetle life cycle. Experiments on post-diapause polyphagous beetles were conducted in April, those on reproductive beetles were conducted in June, and those on pre-diapause beetles were conducted in August. Since independent data were collected for each comparison, each test in Table 5.1 is independent, despite the appearance of 'round-robin' testing.

5.4.2 The effect of conditioning on the responses of 'polyphagous' post-diapause pollen beetles to the odours of flowers from their reproductive-phase and non-reproductive-phase host plants

5.4.2.1 Pollen beetles

Post-diapause pollen beetles that had recently emerged from over-wintering were collected using an electric pooter during April and May from the flowers of dandelion (*Taraxacum officinale* (Weber)) growing wild in the field. Their sex and species were determined in the laboratory (2.5.3 and 2.5.4, respectively). Male and female *M. aeneus* were separated and each sex was split into two groups that received conditioning to either oilseed rape or dandelion by maintaining them in cultures (2.5.2) on these flowers for two days before tests in the LTO.

5.4.2.2 Flowers

Flowers of oilseed rape (OSR) were excised from glasshouse-grown plants (2.2.2). Dandelion (DL) flowers were cut from wild plants growing locally and any insects on them were removed before they were brought into the laboratory. Both floral samples were weighed before use into samples of 5 g using an electric balance.

5.4.2.3 Experimental procedure

The responses of male and female pollen beetles conditioned to either DL or OSR were assessed by recording the number of turns towards test odours at the T-junction of the LTO using the method described above (5.4.1.5). Test odours compared in the experiment were as follows:

1) Oilseed rape flowers (5g) vs. clean air control
2) Dandelion flowers (5g) vs. clean air control
3) Oilseed rape flowers (5g) vs. dandelion flowers (5g)

The first two tests were conducted simultaneously. Each day, the responses of males and females from both conditioning groups were assessed in both tests to enable comparisons of responses to both odours between the sexes and between the conditioning treatments with day-effects removed. Sixteen replicates were conducted for each test. Only eight replicates were completed for test 3, because pollen beetles on dandelions became more difficult to find as the season progressed. The total number of beetles turning left or right towards or away from a given test odour was analysed by Pearson's chi-square test.

5.5 RESULTS

5.5.1 The responses of pollen beetles at different phases of their life cycle to the odour of flowers from their reproductive-phase and non-reproductive-phase host plants

5.5.1.1 Post-diapause beetles (March-April, 'polyphagous')

Both sexes of 'polyphagous' post-diapause beetles turned significantly more times towards the odour of OSR flowers than the control ($P<0.001$). There was no difference between the number of beetles of either sex turning towards the odour of FB flowers or the control ($P>0.05$). When the floral odours of OSR and FB were tested directly, both sexes turned significantly more times towards the OSR than the FB (Table 5.1). These results suggest that post-diapause pollen beetles in this experiment were attracted to the odour of OSR flowers, but not those of FB.

5.5.1.2 Reproductive beetles (May-June, 'oligophagous')

Both sexes of post-diapause reproductive beetles turned significantly more times towards the odour of OSR flowers than the control ($P<0.001$). Similarly, both sexes turned significantly more times towards the odour of FB than that of the control ($P<0.001$). Female beetles turned significantly more times towards the odour of OSR than FB ($P<0.001$) when these two odours were directly compared, whereas males showed no difference ($P>0.05$) (Table 5.1). These results suggest that post-diapause pollen beetles in the reproductive phase are attracted to the floral odours of both OSR and FB, and indicate that females are able to discriminate between these two odours and have a strong preference for OSR, whilst males show no such preference.

5.5.1.3 Pre-diapause beetles (August, 'polyphagous')

As with the other stages, both sexes of pre-diapause beetles turned towards the odour of OSR more than the control ($P<0.001$). Neither sex differed between the number of turns towards FB and the control ($P>0.05$) nor between OSR and FB when these were directly compared ($P>0.05$) (Table 5.1). These results suggest that the pre-diapause beetles used in these experiments were attracted to the odour of OSR flowers, but were not attracted to the odour of FB flowers and did not prefer OSR over FB in the choice test.

Table 5.1 Responses of pollen beetles at three different phases of their life cycle to the floral odours of oilseed rape (OSR), a reproductive-phase host, and field bean (FB), a non-reproductive-phase host in a linear track olfactometer

Pollen beetle		Odours presented in the test χ^2_1 value; associated P value and (odour of preference)		
Life Phase	Sex	OSR vs. C	FB vs. C	OSR vs. FB
Post-diapause	Female	33.47; P<0.001 (OSR)	0.62; P=0.430	83.9; P<0.001 (OSR)
	Male	43.44; P<0.001 (OSR)	2.68; P=0.102	42.82; P<0.001 (OSR)
Reproductive	Female	46.03; P<0.001 (OSR)	18.84; P<0.001 (FB)	27.31; P<0.001 (OSR)
	Male	61.54; P<0.001 (OSR)	8.54; P<0.01 (FB)	0.16; P=0.685
Pre-diapause	Female	44.89; P<0.001 (OSR)	0.4; P=0.525	0.35; P=0.556
	Male	67.10; P<0.001 (OSR)	1.99; P=0.158	0.12; P=0.728

5.5.2 The effect of conditioning on responses of 'polyphagous' post-diapause pollen beetles to the odours of flowers from their reproductive-phase and non-reproductive-phase host plants

5.5.2.1 Responses of pollen beetles conditioned to dandelion

Both male and female pollen beetles conditioned to DL before the tests were significantly attracted to both DL and OSR odours when these were each presented against a clean air control (Table 5.2). When given the choice of OSR and DL floral odours, significantly more beetles orientated towards OSR than DL (P<0.001 for males; P<0.05 for females) (Table 5.2). Therefore 'conditioning' to dandelion had no effect on the beetles' preference for oilseed rape.

5.5.2.2 Responses of pollen beetles conditioned to oilseed rape

Males conditioned to OSR were significantly more attracted to OSR odour than the clean air control ($P<0.05$), although they showed no preference for DL odour over the control ($P>0.05$). Males were significantly more attracted to the odour of OSR than DL when these two odours were presented together ($P<0.05$) (Table 5.2).

Females conditioned to oilseed rape showed no differences in the numbers turning to either OSR or DL when these were offered along with a clean air control ($P>0.05$). Lack of attraction to DL was not surprising, given that males were also unattracted, but it would have been expected that beetles would at least have responded to OSR floral odour over the clean air control. More females turned towards OSR than DL when these two floral odours were directly compared, although the difference was only significant at the 6% level ($p=0.063$) (Table 5.2).

Table 5.2 Effect of conditioning on post-diapause 'polyphagous' pollen beetles responses to floral odours in a linear track olfactometer

Host plant conditioning	Sex of beetles	Test	Statistics		Odour of preference
			χ^2_1	P	
Dandelion	F	OSR vs. C	12.87	< 0.001	OSR
Dandelion	F	DL vs. C	3.87	=0.049	DL
Dandelion	M	OSR vs. C	23.1	< 0.001	OSR
Dandelion	M	DL vs. C	20.47	< 0.001	DL
Oilseed rape	F	OSR vs. C	2.06	0.152	NS
Oilseed rape	F	DL vs. C	0.39	0.531	NS
Oilseed rape	M	OSR vs. C	6.69	< 0.01	OSR
Oilseed rape	M	DL vs. C	1.08	0.299	NS
Dandelion	F	OSR vs. DL	4.88	=0.027	OSR
Oilseed rape	F	OSR vs. DL	3.45	0.063	NS (OSR at 6%)
Dandelion	M	OSR vs. DL	12.07	< 0.001	OSR
Oilseed rape	M	OSR vs. DL	5.1	=0.024	OSR

Where OSR = oilseed rape; DL = dandelion, NS = not significant at the 5% level

5.6 DISCUSSION

5.6.1 Responses of pollen beetles at different stages in the life cycle to floral odours from their reproductive and non-reproductive – phase host plants

Currently, pollen beetles are considered to be polyphagous only for a short period after emergence from diapause, quickly becoming oligophagous on cruciferous plants for their reproductive phase. The new-generation beetles in their pre-diapause state are also thought to be polyphagous whilst they build-up body reserves for diapause over-winter. Analysis of the pollen from the guts of pollen beetles captured throughout their active season (Free & Williams, 1978a; personal observations) and plant-visitation records of pollen beetles (Knuth, 1908-1909; Prüffer, 1958; Kirk-Spriggs, 1996; personal observations) support these assumptions.

In accordance with the polyphagy/oligophagy theory, when OSR and FB floral odours were presented against a clean air control, it was expected that post- and pre-diapause 'polyphagous' beetles would be attracted to both OSR and FB odours, (since these would indicate a food source), whereas beetles in the reproductive 'oligophagous' stage would be attracted only to the odour of OSR flowers. However, the reverse result was obtained; reproductive beetles responded to both OSR and FB floral odours, whilst post- and pre-diapause 'polyphagous' beetles were only attracted to the odour of OSR flowers and showed little attraction to FB floral odour even when tested alongside a clean air control. One reason why polyphagous beetles did not respond to FB when expected could be that beetles had become conditioned to OSR. Beetles used in these experiments were collected from an OSR crop and maintained on OSR prior to starvation and testing, and there is evidence that some insects may become conditioned to plants from which they were collected or to plants upon which they have been kept (Jermy, Hanson & Dethier, 1968; Phillips, 1977; Cassidy, 1978). Similar effects have been found for other coleopteran pests of cruciferous crops; the seed weevil *Ceuthorhynchus assimilis* (Payk.) became conditioned to plants upon which they had been feeding and subsequently preferred them in a study conducted by Free & Williams (1978a). Experiments that investigated the effects of conditioning on responses of pollen beetles to floral odours of reproductive-phase and non-reproductive-phase host plants later confirmed conditioning as a probable explanation, at least for post diapause beetles (see 5.6.2 below). Reproductive beetles may have been the only life phase attracted to FB odour because

the increased energy demands of their reproductive state increased their motivation to feed enough to overcome such conditioning. The nutritional demands of insects in their reproductive-phase are often increased (Wheeler, 1996; Irvin *et al.*, 1999) and hunger may reduce selectivity (Miller & Strickler, 1984).

When OSR and FB floral odours were presented together, post-diapause beetles preferred OSR. This preference was expected since it is important that beetles of this life phase are successful in finding a cruciferous plant in order to reproduce. However, conditioning to OSR may have accentuated this preference. Reproductive females discriminated between these two odours when they were presented together, and strongly preferred OSR, whilst males showed no preference. A preference for OSR was expected in both sexes, since reproduction occurs on this plant. However, males may have no great preference for OSR except to find females (and they may have other mechanisms for doing this), whereas females must lay eggs in the flower buds of these plants. The new generation pre-diapause beetles must feed before diapause. Gaining adequate nutrition before diapause is thought to be vital for over-wintering survival (Hokkanen, 1993). Therefore, it was surprising that beetles did not respond to the odour of FB flowers when this was presented against a blank air control. It is less likely that these beetles were conditioned to OSR than the post-diapause stage beetles, since such conditioning would be maladaptive at the pre-diapause stage in the life cycle when the only need is to feed and build energy reserves for hibernation. In addition, pre-diapause beetles did not prefer OSR over FB in the choice test, which would have been expected if conditioning had occurred. The last two tests (FB vs. control and OSR vs. FB) conducted at this stage in the life cycle were possibly carried out after the majority of pollen beetles in the field had fed sufficiently to build reserves for over-wintering. Bartlet *et al.* (1993) found that pre-diapause seed weevil responses to host odours diminished after less than seven days of post-eclosion feeding, and suggested the lack of attraction was due to adequate feeding and readiness for winter diapause.

5.6.2 Effects of conditioning

Pollen beetles collected from and maintained on dandelion responded to both DL and OSR floral odours when these were presented against a clean air control. Hungry beetles would be expected to respond to the odour of food sources if odour is used in host location. These experiments therefore support the theory that post-diapause pollen

beetles that have recently emerged from over-wintering are polyphagous and suggest that they are able to locate host flowers upon which they can feed and mature by their odour. When floral odours of OSR and DL were presented together, these beetles preferred OSR over DL. The beetles collected from dandelions had probably not yet (in the season) experienced any cruciferous plants (otherwise they would probably have remained on them). This therefore supports the existence of a life-long (probably innate) preference for and importance of OSR odour, unaffected by maintenance ('conditioning') on dandelion. Conversely, pollen beetles collected from DL and then maintained on OSR were no longer attracted to the odour of DL – even when this was presented against a blank air control. This suggests that these pollen beetles had become conditioned to OSR, indicating that once post-diapause pollen beetles have experienced a reproductive host plant, they become conditioned to it, and this conditioning has the effect of 'switching off', or much-reducing, the attraction to floral odours of non-reproductive-phase host plants.

Dandelion was chosen as the non-reproductive-phase host in 'conditioning' tests, since post-diapause pollen beetles can readily be found on their flowers in early spring and field bean crops are not yet in flower at this time. However, to prove that post-diapause beetles are attracted to FB floral odour, and to support the tests that imply that beetles conditioned to OSR cease to be responsive to other floral odours, a similar experiment is required in which post-diapause beetles are collected from dandelions, maintained on DL, OSR and FB, and then tested for their responses to these three odours. If pollen beetles are indeed polyphagous, and use odour to locate hosts, then those maintained on DL would respond to FB; if beetles do become conditioned to OSR once they have experienced it, those maintained on OSR would subsequently not respond to DL or FB. Further studies involving testing a wider range of cruciferous 'reproductive-phase' hosts and non-cruciferous food plants need to be conducted to fully test this hypothesis.

That females cultured on OSR were not attracted to the odour of DL flowers when this was presented against a clean air control is not surprising, given that males behaved similarly; this is consistent with the conditioning theory. However, it was expected that females would respond to the odour of OSR flowers, but they did not. Previous tests have all demonstrated the attraction of female pollen beetles to the odour of this quantity of OSR flowers when presented against a clean air control (see chapter 4 -Fig 4.3a and Table 4.4-; this chapter section 5.5.1). Other than the possibility of a statistical fluke, this

result is hard to explain. It is unlikely that the sample of pollen beetles or the culturing processes contributed to this result, since several collections of beetles were made for these tests, all cultures were maintained in the same environment, and those maintained on OSR used material from the same plants. Since these experiments were conducted at the same time as males conditioned to OSR, and both males and females conditioned to dandelion, experimental conditions are also unlikely to be responsible for this result. However, it may have been due to a general decrease in the response of beetles in this particular set of experiments. For example, when tested with OSR vs. Control, males in this experiment prefered OSR with a level of significance of <0.01 (Table 5.2), whereas in previous experiments, the level of significance for the same test was always greater ($P<0.001$); (see Table 5.1; and Table 4.4).

5.6.3 General discussion

Pollen beetles orientated towards the odours of oilseed rape, dandelion and field bean flowers during these experiments. Attraction to the floral odour of OSR, a cruciferous host upon which the beetles can reproduce (a 'reproductive-phase' host), was generally ubiquitous across the three life phases. Smart & Blight (2000) similarly reported that catches of pollen beetles to traps baited with volatiles commonly found in oilseed rape were found to be phase-independent. Conversely, responses to FB and DL flowers, food hosts upon which beetles do not reproduce ('non-reproductive-phase' host plants), varied according to the beetles' physiological state; in particular their phase in the life cycle, sex, and past feeding history. There have been no previously reported studies on pollen beetle responses to non-reproductive-phase host plant odours throughout their active season.

These experiments support the literature that proposes the polyphagous nature of post-diapause pollen beetles, and furthermore they suggest that these beetles are able to locate floral resources upon which they can feed and mature at least partially by odour. Ruther & Thiemann (1997) also reported attraction of post-diapause beetles to the odours of plants from non-cruciferous families (Solanaceae, Graminaceae and Asteraceae). The pollen beetle antenna was found to detect 26 compounds present in the volatile blend of oilseed rape racemes (Blight *et al.*, 1995). Smart & Blight (2000) reported behavioural attraction of pollen beetles to 17 of these compounds, and pointed out that many of them occur in the flower volatiles of other plant families. Response to a wide variety of plant

derived volatiles is typical of polyphagous insects (Lance, 1983; Kirk, 1985a; Smart & Blight, 2000).

Although pollen beetles are able to respond to a wide variety of volatile compounds, they were still able to discriminate between floral odours of their reproductive-phase and non-reproductive-phase hosts, and oriented towards odours of the former in most cases. This indicates that there are perceptible differences between the odours of these flowers. It is likely that when pollen beetles respond to the odour of non-reproductive-phase plants they respond to volatiles common to many plants (including oilseed rape). However, they seem to have an innate attraction for oilseed rape odour, and may respond to more specialised volatiles in this host recognition. These may include the isothiocyanates, known to attract pollen beetles (Free & Williams, 1978a; Blight *et al.*, 1995; Smart & Blight, 2000), or specialised floral volatiles. Further work is required to investigate this. Once post-diapause pollen beetles have experienced a reproductive-phase host plant, they appear to become conditioned to its odour. This conditioning seems to have the effect of 'switching-off' or reducing responses to the general odours of non-reproductive-phase hosts. Such behaviour might facilitate the migration of pollen beetles to cruciferous plants and maintain them there, enabling successful reproduction.

Reproductive beetles unexpectedly responded to the floral odour of field bean, a non-cruciferous plant, indicating that conditioning can be overcome in reproductive beetles that have been starved, possibly due to high motivation to feed to meet the high energy demands of reproduction. This result does not support the assumptions in the literature that pollen beetles in the reproductive phase are oligophagous on cruciferous plants. It suggests that in the absence of cruciferous hosts, pollen beetles in the reproductive phase are polyphagous, and are therefore polyphagous throughout their life cycle. However, since pollen beetles are able to reproduce on cruciferous plants, which due to their widespread cultivation are no longer scarce, the beetles are commonly found only on crucifers during this phase in their life cycle. They are also likely to spend longer on a plant to feed and reproduce than on a non-reproductive-phase host plant upon which they just feed, and may therefore be more likely to be found on the former than the latter. Perhaps as a consequence of this, they have been mistakenly thought to be oligophagous during this phase.

Experiments to investigate the effects of conditioning on the responses of pollen beetles to floral odours were conducted on pollen beetles in their post-diapause phase only, and therefore can only support findings of the first experiment at this same stage. To fully understand the temporal changes in the responses of pollen beetles to floral odours, their responses to the odours of several more non-reproductive-phase host flowers need to be compared with reproductive-phase host flowers of several more species over all three phases of their active season, and their volatile profiles compared and contrasted.

6. Effects of pollen on pollen beetle oviposition and the survival, development and fitness of larvae

6.1 ABSTRACT

Pollen is thought to be a significant dietary component for pollen beetles at both adult and larval stages. However, the role of pollen in the survival and development of larvae has not previously been studied. Furthermore, larval specificity for resources of cruciferous flowers has not been investigated. Two experiments were conducted to examine the role of a pollen diet in larval survival and the use of pollen cues by adults in choice of oviposition site. The effects of pollen presence and absence were compared through the use of plants and flowers of Synergy, a composite hybrid oilseed rape variety comprising a mixture of male-fertile and male-sterile plants. In the field, more adults were found on flowering male-fertile plants than male-sterile ones, indicating that these plants are more attractive. There was no difference between the number of male-fertile and male-sterile buds with oviposition damage, although more sterile buds were found devoid of eggs and larvae, indicating that these were less preferred than male-fertile ones for oviposition. However, in buds that were accepted for oviposition, there was no difference in clutch size between male-sterile and fertile plants. There was no difference between the number of first instar larvae on the two lines, although more second instars were found on male-fertile flowers than male-sterile ones. This could indicate a greater survival of larvae on male-fertile plants, possibly due to better nutrition afforded to larvae by a pollen-containing diet. This was supported by laboratory experiments, which compared the survival and development of larvae maintained on male-fertile and male-sterile oilseed rape flowers as well as on field bean flowers. These demonstrated that although pollen consumption is not obligatory for larval survival and development, it reduced developmental time, improved survival to adulthood and resulted in heavier pupae and adults. Larvae maintained on oilseed rape flowers survived and developed well. Conversely those kept on field bean flowers were unable to develop beyond the second instar, suggesting that larvae have requirements for factors of cruciferous flowers.

The evolutionary aspects of larval requirements for cruciferous plants, the benefits they gain from pollen–feeding and oviposition specialization of females on the buds of cruciferous plants are discussed.

6.2 INTRODUCTION

6.2.1 The importance of pollen in the diet of larvae of flower-visiting insects

Pollen and nectar represent the major food sources for flower-visiting insects. Nectar provides carbohydrate (Kevan & Baker, 1983) whilst pollen is the main source of protein and other nutrients such as vitamins, minerals, etc (Barbier, 1970). Some adult insects visit flowers to consume pollen as a protein source required for their sexual maturation and/or body maintenance; whilst others visit flowers to lay eggs, providing their offspring with a ready supply of proteinaceous food required for larval growth and development. Therefore, adult flower-visitors or their offspring, or both, consume pollen.

Whether pollen-consumption in flower-visiting insects is obligatory or facultative (or, indeed, beneficial at all) differs between species. For example, pollen-feeding seems to be obligatory for the development of adult seed weevil (*Ceutorhynchus assimilis* (Payk.)) ovaries (Free & Williams, 1978a) and for the brood-rearing of honey bees (*Apis mellifera* L.) (Herbert, Shimanuki & Caron, 1977; Imdorf *et al.*, 1998). A diet of pollen is important in achieving the maximal reproduction and fastest development of many flower thrips (Trichilo & Leigh, 1988; Teulon & Penman, 1991). However, Milne *et al.* (1996) concluded that a pollen-containing diet was no more beneficial to *Frankliniella schultzei* thrips than diets containing other floral parts. The pollen specificity (the species of pollen required) by pollen-feeding insects also differs between species. Some insects are highly pollen-specific, such as some solitary bees (Linsley, 1961). Other flower-visitors feed on the pollens of a few related species belonging to one or only a few plant families such as some flower thrips (Kirk, 1985b). Polyphagous flower-visitors, for example many syrphids, will eat pollen from plants of many different species (Gilbert, 1986). Likewise, there are reports that larvae differ in their pollen-host specificity, but whether this is due to an absolute requirement of the larvae for a particular pollen-type or (for less mobile larvae) due to the female's oviposition preferences is often difficult to determine.

6.2.2 The importance of pollen in the diet of the pollen beetle

There are examples of pollen-eating insects in almost every insect order (Procter, Yeo & Lack, 1996). Of the Coleoptera, 23 families are known to feed on pollen (Stanley & Linskens, 1974). One of these families is the Nitidulidae, which contains the genus *Meligethes* (Meligethinae), of which there are 35 pollen-feeding species occurring in the UK, most with host-specific larvae (Kirk-Spriggs, 1996). The pollen beetle (*Meligethes aeneus* (Fabricius)) is the most commonly occurring species of this genus in the UK, and is an important pest of oilseed rape (*Brassica napus* L.) crops throughout Europe (Winfield, 1992). Like others in its family, both adult pollen beetles and their larvae eat pollen. The larval host plants are those of the Brassicaceae (=Cruciferae). Adults emerge from over-wintering sites in the spring, and after a period of polyphagous feeding on the pollens of many different families of flowering plants, they migrate to cruciferous plants, such as oilseed rape, for reproduction. Females lay their eggs in the flower buds of their reproductive-phase hosts through an oviposition hole, which they chew into the base of the bud. The larvae develop within the bud, feeding on the anthers, and at about the time the flower opens, they moult to the second and final instar. Second instar larvae complete their development in the flowers, moving up the raceme to feed on pollen from new flowers as they open. On maturity, they drop to the ground and pupate in the soil. Upon eclosion, pre-diapause beetles feed on the pollen from many autumn-flowering plant species, and then seek over-wintering sites.

Pollen is therefore a significant component in pollen beetle diet –or is it? Pollen- feeding in adults has been frequently reported (Knuth, 1908-1909; Prüffer, 1958; Free & Williams, 1978a; personal observations), and their need for pollen is implied by their use of olfactory cues that may aid in its location (Charpentier, 1985; this thesis, chapters 3 & 4). However, for insects whose offspring develop on flowers, pollen is usually assumed to be a vital resource, but its effects on the survival and development of larvae have been little studied (Pesho & van Houten, 1982; Kirk, 1985a; Tulisalo & Wuori, 1986; Teulon & Penman, 1991; Milne *et al.*, 1996). This is true of the pollen beetle. Although larvae have been observed to feed on pollen (Scherney, 1953; Fritzsche, 1957; Blazejewska, 1960; personal observations of larval gut contents) it is not known if this feeding is obligatory or facultative since its effect on their survival and development has never been investigated. Furthermore, studies on larval specificity for cruciferous pollen for survival and development has not been reported. Such studies may improve the understanding of the evolution of reproductive host specificity in the pollen beetle.

6.2.3 Host selection process –oviposition of the pollen beetle

Pollen beetle females only lay their eggs in the flower buds of cruciferous plants (Fritzsche, 1957). It is not known if this host-specificity is driven by larval dietary requirements or other factors such as female feeding preference or requirements for enemy-free space (Bernays & Graham, 1988; Thompson, 1988). If the nutritional requirements of larvae were a major issue influencing reproductive host-specificity, it would be expected that the offspring perform best on the females' preferred choice, and that females would use cues which signal the presence of the required resources in determining oviposition preference. Previous chapters of this thesis have discussed the cues used by pollen beetles in finding food resources for themselves, whilst this one focuses on the cues used in providing for their offspring.

Pollen beetles probably use many different plant cues, visual, olfactory, tactile and gustatory ones, in making host quality assessments for oviposition. These cues are only beginning to be investigated, and focus mainly on those used to determine preferences between different cruciferous plants (Borg & Borg-Karlson, 1996; Borg & Ekbom, 1996; Ekbom & Borg, 1996), rather than between crucifers and non-crucifers.

6.2.4 Synergy

Synergy is a composite hybrid winter oilseed rape; a varietal association of 80% male-sterile hybrid plants (cvs. Falcon x Samouroi) which produce no pollen, and 20% male-fertile pollinator plants (cv. Falcon). It therefore allows assessment of pollen beetle adult and larval responses to pollen in both the field and the laboratory without the need to manipulate anthers artificially. In the UK, commercial crops of Synergy have regularly yielded over 5 t/ha; an increased yield compared with the average of around 4 t/ha from conventional cultivars such as Apex (CPB Twyford, 1996). These yield improvements result from the hybrid vigour of the male-sterile plants and their saving in energy otherwise spent on pollen production. The success of the crop, however, depends upon the pollinator plants producing enough pollen to fertilize the whole crop. Infestations of pollen-feeding insects such as the pollen beetle could pose an increased threat to the pollinator plants, particularly if they are able to discriminate and favour these over the male-sterile plants.

6.3 AIMS

Two experiments were conducted to investigate the effects of pollen on adult pollen beetle incidence and oviposition, and on the survival, development and fitness of the larvae. In addition, the specificity of pollen beetles for reproduction on cruciferous plants was investigated by comparing the effects of rearing larvae on a diet of flowers from their reproductive-phase host plant, oilseed rape, *Brassica napus* (OSR), with those of a non-reproductive-phase host, field bean *Vicia faba* (FB).

6.3.1 Specific aims

1. The first experiment aimed to compare, in a field situation, the effect of OSR pollen on the incidence of adults, oviposition and larvae, and on larval fitness. The following questions were posed:
   - Are adults more abundant on oilseed rape plants with pollen than those without?
   - Do females use pollen cues to assess host quality: are there differences in the incidence of oviposition holes in buds that contain pollen and those that do not, and are buds lacking pollen less favoured for oviposition?
   - If eggs are laid in buds lacking pollen, do larvae within them survive, and is there a difference between the weight of larvae born on plants with pollen and those without?

2. The second experiment aimed to investigate further the effects of a diet containing pollen on the survival, development and weight (fitness) of pollen beetle larvae and furthermore, to investigate their requirement for flowers of the reproductive-phase host plant. The following questions were addressed:
   - Is pollen necessary for the survival and development of pollen beetle larvae: do pollen beetle larvae survive better and develop faster when reared on a diet containing pollen than when reared on a diet with no pollen?
   - Does a diet containing pollen result in heavier (fitter) larvae and adults than one without, and is there a difference in the sex ratio of adults reared on these two diets?
   - Can pollen beetle larvae survive and develop normally when reared on flowers from a non-reproductive-phase host? Is the survival, development and weight (as a measure of fitness) of pollen beetle larvae reared on a diet of OSR flowers better than that of larvae reared on FB flowers?

6.4 MATERIALS & METHODS

6.4.1 Effects of pollen on pollen beetle adult incidence and oviposition, and the incidence and fitness of larvae in the field

6.4.1.1 General method

Oilseed rape plants with pollen (male-fertile Synergy) and those without pollen (male-sterile Synergy) were sampled throughout the growth of the crop. The abundance of adult pollen beetles was recorded on selected plants from both lines in the field. The plants were then taken to the laboratory for assessments of the occurrence of oviposition holes in buds, eggs and larvae. Assessments were then compared between the two plant lines.

6.4.1.2 Sampling

Sampling was performed on five occasions from a Synergy OSR crop growing on Rothamsted farm. Assessments were taken from parts of the plant as described below. Growth stages (GS) refer to those of Sylvester-Bradley & Makepeace (1984). On each sampling occasion, five male-fertile and five male-sterile plants were selected at random from each of the four sides of the crop.

1. Green bud, GS 3,6 - 3,7, from the terminal raceme
2. Yellow bud, GS 3,9, from the terminal raceme
3. Flowering 1, GS 4,2 – 4,8, from the terminal, primary and secondary racemes
4. Flowering 2, GS 5,5 – 5,9 (pod development of the terminal raceme), from the primary, secondary and tertiary racemes (these in GS 4,2 – 4,8)
5. Flowering 3, GS 6,2 – 6,7 (seed development of the terminal raceme), from the first three flowering (GS 4,2 – 4,8) tertiary branches from the secondary racemes

At the green bud stage, it was difficult to distinguish a male-fertile plant from a male-sterile one in the field. Therefore, 25 plants were sampled from each side, and taken back to the laboratory for identification. Several buds from each plant were taken, and their anthers dissected under a microscope. Male-fertile plants were identified as those producing anthers containing pollen, and male-sterile plants were identified as those producing anthers devoid of pollen. The intention was to retain the first five male-fertile and the first five male-sterile plants identified for examination of oviposition and larval incidence. However, fewer than five male-fertile plants were obtained from each side's

sample. Therefore, an equal number of male-sterile and male-fertile plants were retained for examination from each side, and a second sampling occasion was performed in the same manner until five plants of each type at the green bud stage were obtained from each side for examination.

6.4.1.3 Adult incidence

To determine whether pollen beetles were more abundant on plants with pollen than those without, the number of adults present on male-fertile and male-sterile plants selected at random in the field for assessment were recorded and compared. The data were normalised by transformation ($\log_{10}$ (no. adults +1)) on all sampling occasions except yellow bud and analysed by an analysis of variance with a blocking factor included to account for any variation between the sides of the field. To eliminate any differences between the first and second sampling occasion performed at the green bud stage, the sampling occasion was also incorporated into the blocking for this stage.

6.4.1.4 Incidence of oviposition holes, eggs and larvae

Racemes sampled for adult incidence were cut, bagged, and taken to the laboratory, where the top 50 buds >1 mm were examined for oviposition holes. These were identified by a small neat hole in the base of the bud (Fig. 6.1). Buds with oviposition holes were dissected and the number of eggs (Fig. 6.2) and larvae within were recorded. Larvae were recorded as being in the 1st or 2nd instar, determined by characteristics described by Blazejewska (1960), Osborne (1964) and Kirk-Spriggs (1996). Simply, 1st instar larvae are smaller than those of the 2nd instar and have two bold black marks on the first thoracic segment, whilst those of the 2nd have more complicated 'diffuse' markings on this segment (Fig. 6.3 b and c, respectively).

Incidence of oviposition holes

To ascertain whether female beetles assess host quality at oviposition, the proportion of buds with an oviposition hole from those sampled was compared between male-fertile and male-sterile plants by a logistic regression. In addition, the proportion of buds with an oviposition hole but devoid of eggs or larvae was compared, also using a logistic regression. Both models included terms to remove sample and side effects. In some of the analyses, the data exhibited overdispersion (variance greater than expected under the binomial assumptions) as indicated by a residual mean greater than unity. Williams'

procedure was used to account for the over-dispersion before treatment differences were tested.

Incidence of eggs

Number of eggs per plant To determine if one plant line was preferred over the other for oviposition, the total number of eggs laid in buds sampled from each plant was recorded. The mean number of eggs found on each plant line was compared by an analysis of variance with blocking (6.4.1.3). The mean numbers of eggs per plant at the green bud stage and at flowering 3 were transformed ($\log_{10}$ (n+1)) to normalise the data.

Clutch Size To determine whether clutch size varied between buds with and without pollen, the average number of eggs laid in accepted buds (those buds with oviposition holes with eggs/larvae inside) were compared between male-sterile and male-fertile plants using a log-linear model. The model allowed for the differing numbers of accepted buds on each plant. Larvae found inside buds were treated as an egg in this analysis, as it was assumed that they would have hatched from one laid within the bud.

Incidence of larvae

In buds For both 1st and 2nd instar larvae, the total number found within the sample size of 50 buds was transformed ($\log_{10}$ (n+1)) and compared between male-sterile and male-fertile plants by an analysis of variance.

In flowers During the flowering period, the first 20 flowers were examined for larvae and the number of 1st and 2nd instars found in them was recorded. These totals were transformed ($\log_{10}$ (n+1)) and compared between male-sterile and male-fertile plants by an analysis of variance.

6.4.1.5 Larval fitness

On the final sampling date, larvae were grouped according to their developmental stage (1st or 2nd instar) and their location on the plant (in buds or in flowers) to give four groups: 1st instar from buds, 1st instar from flowers, 2nd instar from buds and 2nd instar from flowers. Each group of larvae was weighed (Cahn C-33 Microbalance, distributed by Scientific & Medical Products Ltd., Shirley Institute, Didsbury, Manchester, UK). The mean weight of larvae in each group was compared between male-fertile and male-sterile plants using a weighted least squares regression where the weighting was equal to the number of larvae observed. As there were very few 2nd instar larvae found within buds on this sampling occasion, no analysis was performed on this group.

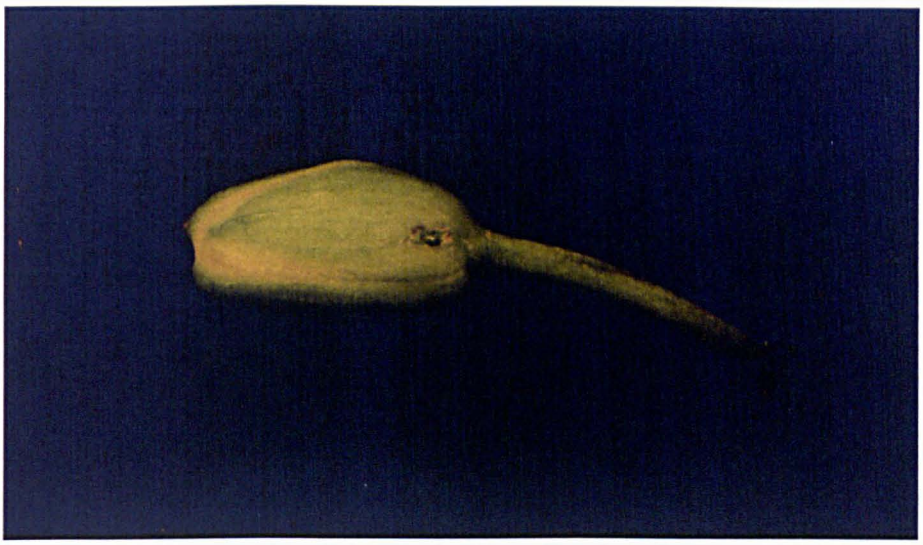


Figure 6.1 Oviposition hole made by a pollen beetle in the base of an oilseed rape bud

Figure 6.2 Pollen beetle eggs within an oilseed rape bud

6.4.2 Effect of pollen on development, survival and fitness of pollen beetle larvae and their requirement for flowers of reproductive-phase hosts

6.4.2.1 General method

Pollen beetle larvae were reared on diets of oilseed rape flowers with and without pollen and on field bean flowers. Their developmental stage and weight were recorded daily in order to compare survival, developmental rate and fitness between diets.

6.4.2.2 Pollen beetle larvae

Pollen beetle larvae were hatched from eggs collected from buds of field-grown male-fertile Synergy oilseed rape. Buds with oviposition damage (Fig. 6.1) were dissected in the laboratory, and eggs found within were removed with a moistened sable hair paintbrush. The eggs were placed in petri dishes lined with filter paper moistened with distilled water. These were stored in a controlled temperature room maintained at 23 °C ± 2 °C, with a light:dark photo-period of 16.5 : 7.5 h. The eggs were observed daily, and the filter paper kept moist, until they hatched.

6.4.2.3 Rearing larvae on diet treatments

Upon hatching, a larva was assigned to one of three diet treatments:

1. Oilseed rape flowers with pollen (male-fertile Synergy)
2. Oilseed rape flowers with no pollen (male-sterile Synergy)
3. Field bean flowers (these were male-fertile)

Synergy OSR flowers and FB flowers were excised from glasshouse-grown plants (sections 2.2.1.2 and 2.2.2, respectively). The larvae were placed individually into a single flower, placed within a small, ventilated transparent polystyrene plastic box (79 x 47 x 22 mm; Stewart Plastics, Surrey, UK) lined with filter paper moistened with distilled water. Larvae were reared in these boxes in a controlled environment room maintained at 23 °C, with a light:dark photo-period of 16.5 : 7.5 h. The flowers were changed every second day to keep them fresh and larvae were examined daily to determine their survival rate, developmental times and weight. One hundred pollen beetle eggs were collected for each treatment in total. As time constraints prevented comparisons being conducted on 100 eggs or larvae in each treatment at one time, two replicates of 50 eggs per treatment were conducted, spaced one week apart.

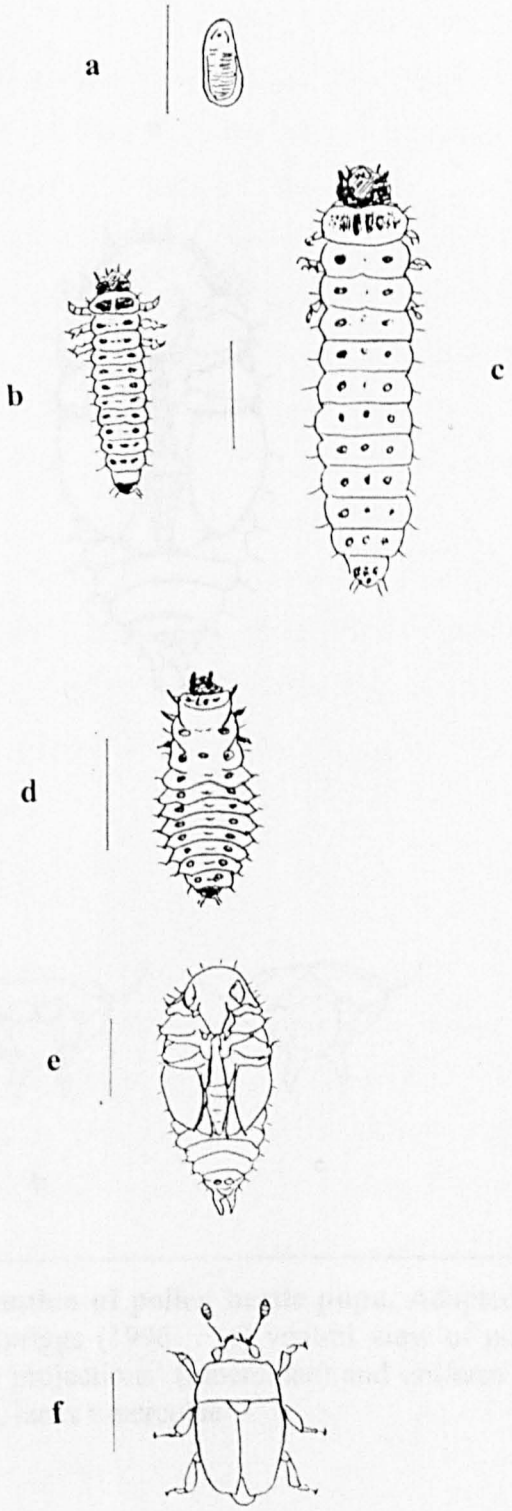


Figure 6.3 Developmental stages of the pollen beetle. Adapted from Blazejewska (1960) and Kirk-Spriggs (1996). (a) Egg; (b) 1st instar larva showing two bold markings on first thoracic segment - dorsal view; (c) 2nd instar larva showing two diffuse markings on first thoracic segment - dorsal view; (d) Prepupa - dorsal view (e) Pupa - ventral view; (f) Adult - dorsal view. Scale bars represent 1 mm.

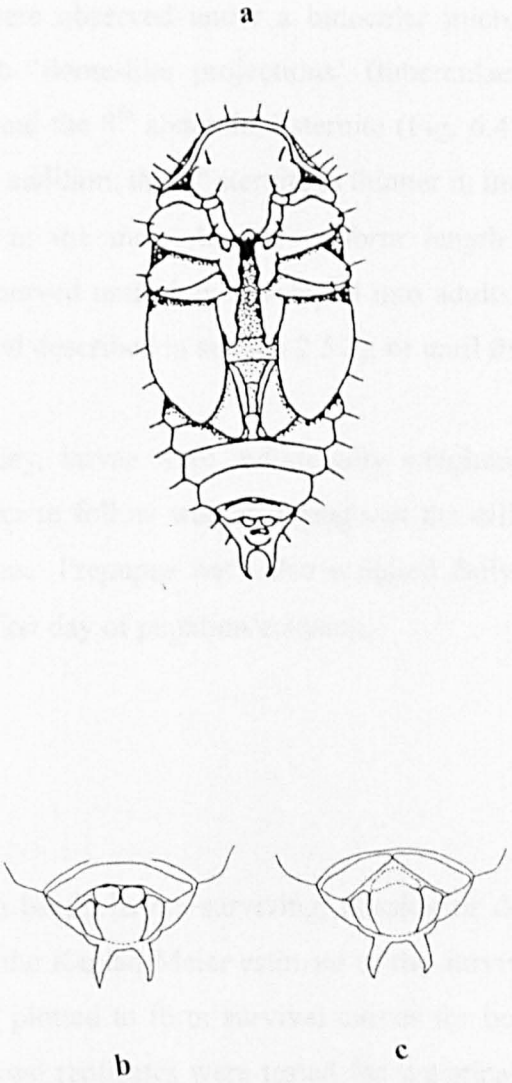


Figure 6.4 Sex determination of pollen beetle pupa. Adapted from Charpentier & Weibull, (1994) and Kirk-Spriggs (1996). (a) ventral view of pollen beetle pupa: (b) female, showing 'dome-like projections' (tuberculae) and uniform 8th sternite; (c) male, showing biparate 8th sternite, lacks tuberculae

Survival rate and developmental time

The developmental stage (1st instar, 2nd instar, prepupa or pupa) of each larva was recorded daily to determine their survival and development times. First and second instar larvae were distinguished, as described in section 6.4.1.4. (and see Fig 6.3 b & c). The prepupal stage was determined when the extremely mobile 2nd instar larvae dropped from the flowers, ceased to move and became slightly shorter and more rounded (Fig. 6.3 d). The pupal stage was easily distinguishable from both larval stages as it was entirely ivory

white in colour (Fig. 6.3 e). The sex of a pupa was determined using characteristics described by Charpentier & Weibull (1994). Pupae were placed on their dorsal sides and their ventral sides were observed under a binocular microscope (x 50) (Fig. 6.4 a). Females possess two 'dome-like projections' (tuberculae), visible between the 9th segment (pygidium) and the 8th abdominal sternite (Fig. 6.4 b), which are absent in the males (Fig. 6.4 c). In addition, the 8th sternite is thinner in the middle and more or less in two parts (biparate) in the male, but of uniform length and width in the female. Larvae/pupa were observed until they developed into adults (Fig. 6.3 f) when their sex was confirmed (method described in section 2.5.3), or until they died.

Weight Each day, larvae were individually weighed on a balance (Cahn C-33 microbalance), in order to follow weight throughout the different developmental stages as a measure of fitness. Prepupae were also weighed daily, but pupa and adults were only weighed on the first day of pupation/eclosion.

6.4.2.4 Data analysis

Survival

The number of pollen beetle larvae surviving, missing or dead on each treatment each day was totalled, and the Kaplan-Meier estimate of the survivor function was calculated. These estimates were plotted to form survival curves for both replicates of 50 eggs on each treatment. The two replicates were tested for statistical differences by the logrank test (Peto & Peto, 1972). Since the null hypothesis that the two replicates within treatments had the same survival rate failed to be rejected, the two replicates of 50 eggs were combined together in each treatment. Differences between survival on male-fertile and male-sterile OSR flowers and the OSR (male-fertile Synergy) and FB flowers were analysed by logrank tests (Peto & Peto, 1972).

Development

Time taken to reach each developmental stage from hatching The mean number of days since hatching (into a 1st instar larva) taken for larvae to reach the 2nd larval instar, prepupa, pupa and adult developmental stages were compared by a one-way analysis of variance between male-fertile and male-sterile OSR and between OSR (male-fertile Synergy) and FB flowers.

Time spent within developmental stage The mean number of days spent in each developmental stage (1st instar, 2nd instar, prepupa, pupa) was compared by a one-way analysis of variance between male-fertile and male-sterile OSR flowers and between OSR (male-fertile Synergy) and FB flowers.

Fitness (weight at each developmental stage)

The mean weight of larvae on successive days since hatching was calculated for each treatment. The mean weight of subjects on the first day of each developmental stage (egg, 1st instar, 2nd instar, prepupa, pupa and adult) was compared between the male-fertile and male-sterile oilseed rape diet treatments by a one-way analysis of variance with randomized blocking (where blocks represented the two replicates of 50 eggs). Weights of larvae developing on FB and OSR flowers were compared in the same manner.

6.5 RESULTS

6.5.1 Effects of pollen on pollen beetle adult incidence and oviposition, and the incidence and fitness of larvae in the field

6.5.1.1 Adult incidence

There was no difference between the number of adult pollen beetles on male-sterile and male-fertile plants sampled at the green bud and the yellow bud stage ($F_{1,39}$=0.4; P>0.05 and $F_{1,39}$=2.49; P>0.05 respectively). However, significantly more beetles were found on male-fertile than on male-sterile plants during flowering ($F_{1,39}$=12.94; $F_{1,39}$=24.95; $F_{1,39}$=37.5 for flowering 1, 2 and 3 respectively; P<0.001 for all three samples). This can be seen from Figure 6.5.

6.5.1.2 Incidence of oviposition holes

Proportion of buds sampled with oviposition holes There was no difference in the proportion of buds sampled that had oviposition holes between male-fertile and male-sterile plants on any of the sampling occasions, except for the final flowering stage, in which more male-fertile than male-sterile buds sampled had oviposition holes (P<0.05) (Table 6.1).

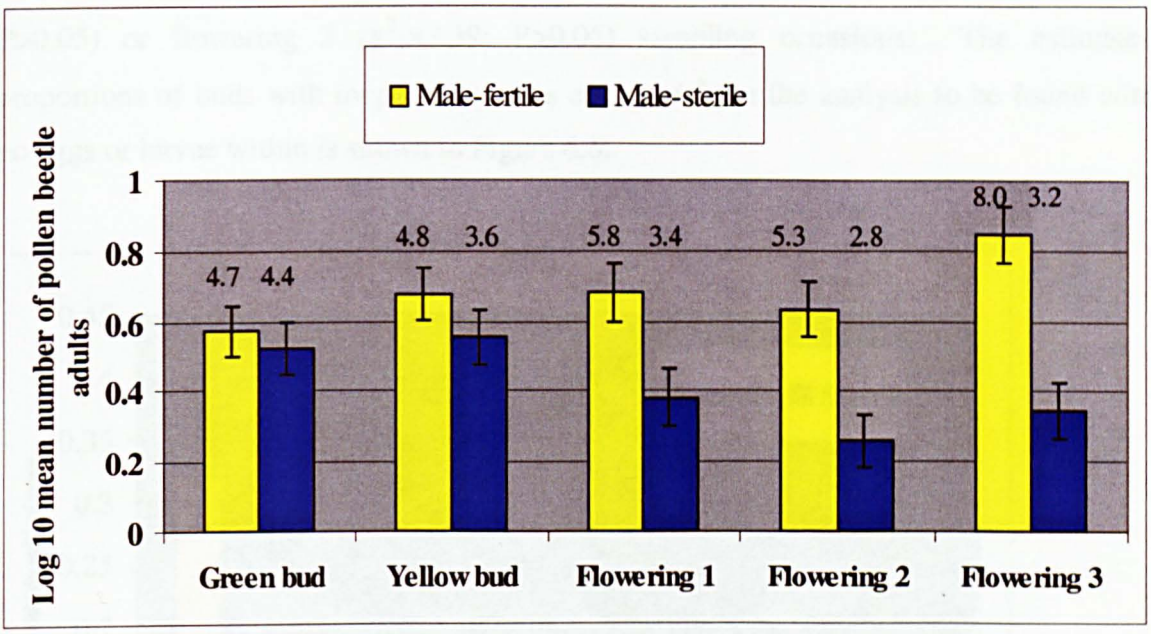


Figure 6.5 Mean number of adult pollen beetles found on male-fertile and male-sterile Synergy oilseed rape plants on different sampling occasions (showing SED error bars and back-transformed means)

Table 6.1 Proportion of male-fertile and male-sterile Synergy oilseed rape buds that had pollen beetle oviposition damage

Sampling occasion	Mean proportion of buds sampled with oviposition damage				χ^2_1	P
	♂ Fertile		♂ Sterile			
	Mean	SE	Mean	SE		
Green bud	0.138	0.022	0.130	0.021	0.09	>0.05
Yellow bud	0.197	0.025	0.174	0.024	0.46	>0.05
Flowering 1	0.225	0.013	0.203	0.013	1.39	>0.05
Flowering 2	0.174	0.022	0.160	0.020	0.25	>0.05
Flowering 3	0.398	0.045	0.265	0.039	5.01	<0.05

Proportion of buds with oviposition holes devoid of eggs and larvae The proportion of buds with oviposition holes that were devoid of eggs and larvae from male-sterile plants was significantly greater than that from male-fertile plants at the yellow bud (χ_1^2=19.61; P<0.001), flowering 1 (χ^2_1=32.81; P<0.001) and flowering 2 (χ^2_1=4.6; P<0.05) sampling

occasions. However, there were no such differences at the green bud (χ^2_1=0.001; P>0.05) or flowering 3 (χ^2_1=3.39; P>0.05) sampling occasions. The estimated proportions of buds with oviposition holes expected from the analysis to be found with no eggs or larvae within is shown in Figure 6.6.

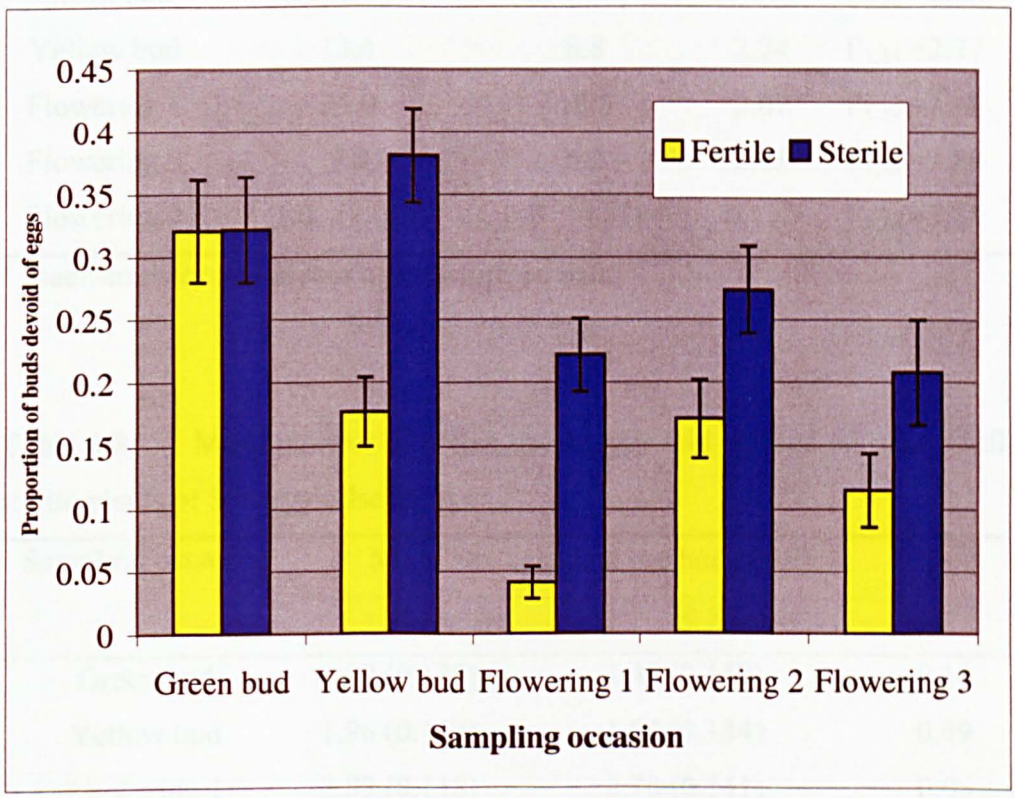


Figure 6.6 Expected proportion (± SE) of buds with oviposition damage devoid of pollen beetle eggs and larvae on male-fertile and male-sterile Synergy oilseed rape plants at different sampling occasions.

6.5.1.3 Incidence of eggs

Number of eggs laid per plant The mean number of eggs laid per plant did not differ between male-fertile and male-sterile Synergy plants (Table 6.2).

Clutch size At each sampling occasion, the number of eggs laid per accepted bud did not differ significantly between male-fertile and male-sterile plants (Table 6.3).

Table 6.2 Mean number of pollen beetle eggs laid per plant on male-fertile and male-sterile Synergy oilseed rape

Sampling occasion	Mean of the total no. eggs per 50 buds		SED	F	P
	♂ Fertile	♂ Sterile			
Green bud	0.8 [5.3] *	0.7 [4.0] *	0.112	$F_{1,26}$ =0.08	>0.05
Yellow bud	13.4	8.8	2.74	$F_{1,34}$ =2.77	>0.05
Flowering 1	24.0	18.5	3.07	$F_{1,34}$ =3.16	>0.05
Flowering 2	9.0	6.2	2.03	$F_{1,33}$ =1.89	>0.05
Flowering 3	1.0 [9.0] *	0.7 [4.01] *	0.152	$F_{1,28}$ =3.88	>0.05

* [back-transformed means of transformed data]

Table 6.3 Mean number of pollen beetle eggs laid per bud on male-fertile and male-sterile plants of Synergy oilseed rape

Sampling occasion	Mean No. eggs laid per bud (±SE)		χ_1^2	P
	♂ Fertile	♂ Sterile		
Green bud	1.39 (0.137)	1.45 (0.159)	0.14	>0.05
Yellow bud	1.96 (0.112)	1.84 (0.134)	0.49	>0.05
Flowering 1	2.83 (0.118)	2.78 (0.141)	0.06	>0.05
Flowering 2	3.12 (1.162)	2.91 (0.158)	0.80	>0.05
Flowering 3	2.89 (0.164)	2.54 (0.186)	1.97	>0.05

6.5.1.4 Incidence of larvae

Incidence of larvae in buds There were no larvae in any of the buds dissected during the green bud stage. Therefore, the number of 1st instar larvae found in male-fertile and male-sterile buds were compared at the yellow bud, and the three flowering stages only. There was no difference between the number of 1st instar larvae present in male-fertile or male-sterile buds at any of these sampling occasions (Table 6.4).

There were no 2nd instar larvae found within buds sampled at the yellow bud stage. Therefore, the numbers of 2nd instars in buds were compared between male-fertile and male-sterile buds during the three flowering phases only. There was no difference

between the number of 2nd instar larvae present in male-fertile or male-sterile buds at any of these sampling occasions (Table 6.4). This result was probably due to the very low numbers of 2nd instar larvae found in buds.

Table 6.4 Mean number of pollen beetle larvae found in male-fertile and male-sterile buds of Synergy oilseed rape plants.

Sampling occasion	Larval instar	Mean of the total no. larvae per 50 buds [back-transformed totals]		SED	F	P
		♂ Fertile	♂ Sterile			
Yellow bud	1st	0.39 [3.46]	0.19 [2.55]	0.119	$F_{1,34}$=2.85	>0.05
Flowering 1	1st	0.67 [5.70]	0.40 [3.51]	0.146	$F_{1,34}$ =3.5	>0.05
Flowering 2	1st	0.96 [10.1]	0.84 [7.97]	0.120	$F_{1,34}$ =0.89	>0.05
Flowering 3	1st	0.34 [3.17]	0.23 [2.68]	0.099	$F_{1,32}$ =1.21	>0.05
Flowering 1	2nd	0.02 [2.04]	0.07 [2.17]	0.040	$F_{1,34}$ =1.73	>0.05
Flowering 2	2nd	0.18 [2.52]	0.28 [2.91]	0.90	$F_{1,34}$ =1.20	>0.05
Flowering 3	2nd	0.27 [2.87]	0.29 [2.94]	0.119	$F_{1,32}$ =0.02	>0.05

Incidence of larvae on flowers The numbers of 1st and 2nd instar larvae present on male-fertile and male-sterile Synergy oilseed rape flowers during the three flowering phases were compared (there were no flowers to assess during green and yellow bud stages of the crop!). There was no difference in the number of 1st instars present on male-fertile or male-sterile flowers. However, over the first two sampling occasions during flowering, more 2nd instar larvae were found on male-fertile than on male-sterile flowers (Table 6.5).

6.5.1.5 Larval fitness

The weights of larvae from the two plant types did not differ significantly (Table 6.6).

Table 6.5 Total number of pollen beetle larvae present on male-fertile and male-sterile flowers of Synergy oilseed rape

Sampling occasion	Larval instar	Total number of larvae per 20 flowers [back-transformed totals]		SED	F	P
		♂ Fertile	♂ Sterile			
Flowering 1	1st	1.10 [11.7]	0.93 [7.7]	0.080	$F_{1,34}$ =2.63	>0.05
	2nd	0.80 [5.3]	0.47 [2.2]	0.123	$F_{1,34}$ =7.08	<0.05
Flowering 2	1st	1.33 [19.8]	1.22 [15.6]	0.084	$F_{1,35}$ =1.66	>0.05
	2nd	1.10 [11.4]	0.75 [4.7]	0.118	$F_{1,35}$ =8.62	<0.01
Flowering 3	1st	0.83 [5.8]	0.72 [4.3]	0.095	$F_{1,35}$ =1.31	>0.05
	2nd	0.88 [6.7]	0.77 [4.9]	0.120	$F_{1,35}$ =0.92	>0.05

Table 6.6 Mean weight of pollen beetle larvae from buds and flowers of male-fertile and male-sterile Synergy oilseed rape plants

Larval group	Mean weight (mg) of larvae		F	P
	♂ Fertile	♂ Sterile		
1st instars from buds	0.113	0.117	$F_{1,30}$ = 0.41	> 0.05
1st instars from flowers	0.178	0.160	$F_{1,34}$ = 2.20	> 0.05
2nd instars from flowers	0.854	0.748	$F_{1,33}$ = 0.78	> 0.05

6.5.2 Effect of pollen on survival, development and fitness of pollen beetle larvae and their requirement for flowers of reproductive hosts

The raw data for the survival/development/weight of larvae reared on the three diets in this experiment are presented in full in Appendix 3.

6.5.2.1 Survival

The number of subjects that survived through each developmental stage is shown in Table 6.7 along with percentage mortality at each developmental stage. More subjects survived through all developmental stages on male-fertile Synergy oilseed rape than the other two diet treatments. Survival was better on male-sterile Synergy oilseed rape than on field bean flowers. Mortality was greatest on each diet in the first larval instar.

Table 6.7 Number of pollen beetle immatures surviving through each developmental stage, and percentage mortality within stages for those reared on a diet of male-fertile or sterile Synergy oilseed rape flowers or field bean flowers

Developmental stage	Diet treatment					
	♂ fertile OSR		♂ sterile OSR		Field bean	
	No. survived	% mortality	No. survived	% mortality	No. survived	% mortality
Eggs (start)	100		100		100	
Eggs	83	17	82	18	92	8
1st instar larvae	52	37.4	27	67.1	2	97.8
2nd instar	42	19.2	18	33.3	0	100
Prepupa	31	26.2	11	38.9	/	/
Pupa	25	19.4	6	45.5	/	/
	Total survival = 25%		Total survival = 6%		Total survival = 0%	

Survival of larvae on male-fertile and male-sterile oilseed rape flowers There was a difference in the survival rate for the two treatments (log rank $\chi^2_1 = 9.53$; $P < 0.01$), with survival of larvae reared on a diet of male-fertile OSR flowers being greater than that on male-sterile flowers (Fig. 6.7).

Survival of larvae on oilseed rape and field bean flowers. There was a difference in the survival rate for the two treatments (log rank $\chi^2_1 = 15.34$; $P < 0.001$), with survival of larvae reared on a diet of OSR flowers being greater than that on FB flowers (Fig. 6.8).

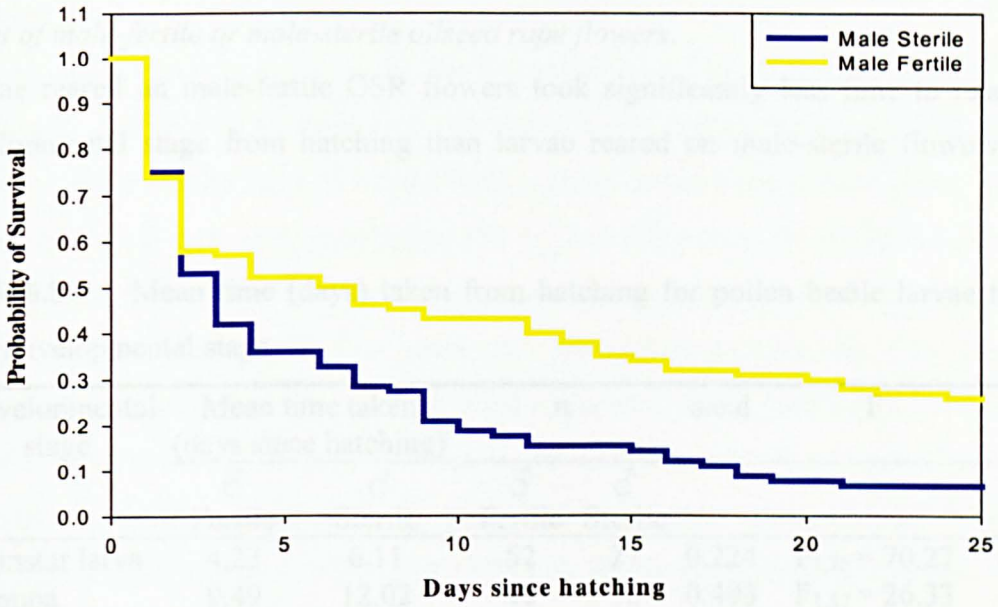


Figure 6.7 Kaplan-Meier estimates of survival of pollen beetle larvae reared on male-fertile and male-sterile Synergy oilseed rape flowers on successive days since hatching

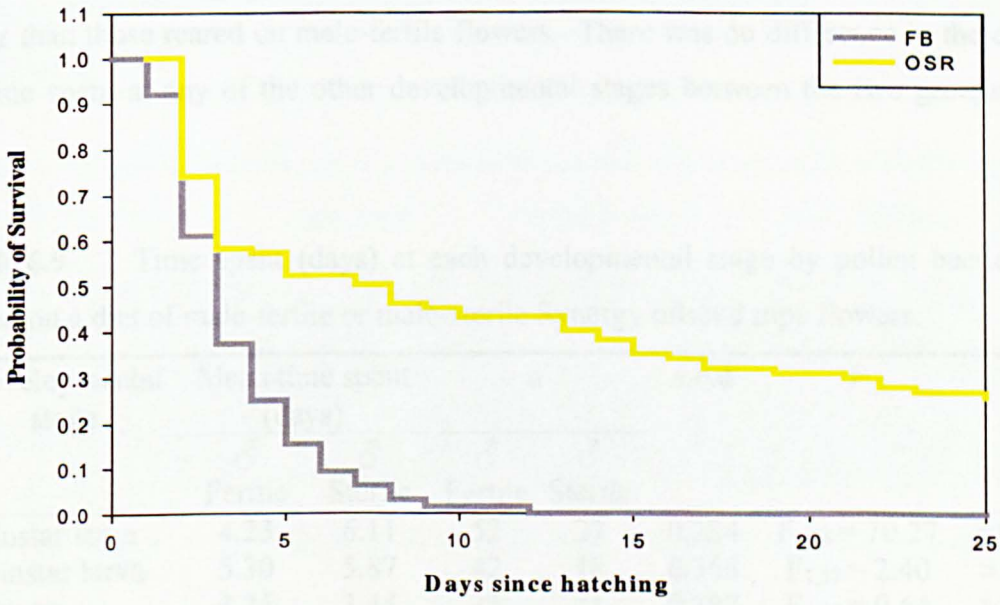


Figure 6.8 Kaplan-Meier estimates of survival of pollen beetle larvae reared on oilseed rape or field bean flowers on successive days since hatching

6.5.2.2 Development

Time taken for larvae to reach each developmental stage from hatching when reared on a diet of male-fertile or male-sterile oilseed rape flowers.

Larvae reared on male-fertile OSR flowers took significantly less time to reach each developmental stage from hatching than larvae reared on male-sterile flowers (Table 6.8).

Table 6.8 Mean time (days) taken from hatching for pollen beetle larvae to reach each developmental stage

Developmental stage	Mean time taken (days since hatching)		n		s.e.d	F	P
	♂ Fertile	♂ Sterile	♂ Fertile	♂ Sterile			
2nd instar larva	4.23	6.11	52	27	0.224	$F_{1,76} = 70.27$	< 0.001
Prepupa	9.49	12.02	42	18	0.493	$F_{1,57} = 26.33$	< 0.001
Pupa	13.03	16.18	31	11	0.526	$F_{1,39} = 35.90$	< 0.001
Adult	19.27	22.39	25	6	0.694	$F_{1,28} = 20.26$	< 0.001

Time spent at each developmental stage by larvae reared on a diet of male-fertile or male-sterile oilseed rape flowers.

Larvae reared on male-fertile oilseed rape flowers spent significantly less time in the first instar than those reared on male-fertile flowers. There was no difference in the duration of time spent at any of the other developmental stages between the two groups (Table 6.9).

Table 6.9 Time spent (days) at each developmental stage by pollen beetle larvae reared on a diet of male-fertile or male-sterile Synergy oilseed rape flowers.

Developmental stage	Mean time spent (days)		n		s.e.d	F	P
	♂ Fertile	♂ Sterile	♂ Fertile	♂ Sterile			
1st instar larva	4.23	6.11	52	27	0.224	$F_{1,76} = 70.27$	< 0.001
2nd instar larva	5.30	5.87	42	18	0.368	$F_{1,57} = 2.40$	> 0.05
Prepupa	3.75	3.44	31	11	0.387	$F_{1,39} = 0.64$	> 0.05
Pupa	6.46	6.57	25	6	0.239	$F_{1,28} = 0.21$	> 0.05

Development of larvae reared on a diet of oilseed rape or field bean flowers.

Only two larvae reared on field bean flowers developed to the 2nd instar, and neither of these survived to the prepupal stage. Therefore only the time taken to reach the 2nd instar from hatching, (the time spent within the first instar) could be compared between the two treatments. There was no difference between the two treatments ($F_{1,51}$ =2.86; P>0.05). However, these results must be treated with caution, as the sample sizes being compared in each treatment were very unbalanced (52 vs. 2 in this case). Of the larvae developing on field bean that did not survive to the second instar, many survived for up to 6 days, and in one case 9 days, in the first instar stage before dying (Appendix 3.3). Since they died before moulting, they were not included in the analysis of 'time spent in first instar'. However, had these larvae been included in the analysis, there would have been a clear difference in the time spent in the 1st instar between larvae raised on OSR flowers and those raised on FB flowers, with those raised on FB flowers spending a longer duration of time in the 1st instar before dying.

6.5.2.3 Weight of larvae throughout development (Fitness)

The mean weight of larvae on successive days since hatching for each treatment

The weight of larvae throughout their development was greatest on male-fertile OSR flowers, intermediate on male-sterile OSR flowers and least for larvae reared on FB flowers (Fig. 6.9).

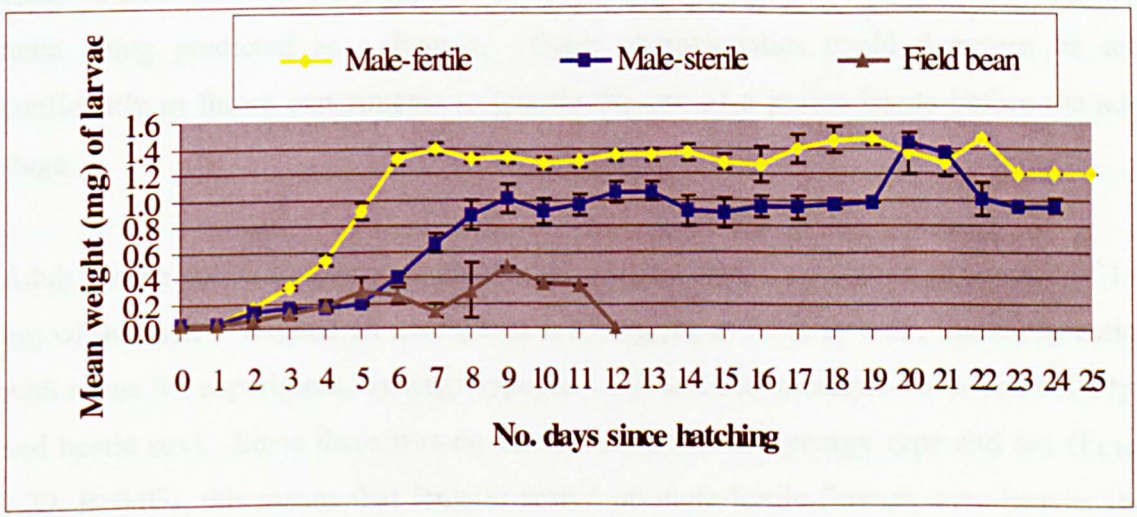


Figure 6.9 Mean weight (mg) of pollen beetle larvae reared on male-fertile or male-sterile Synergy oilseed rape flowers or field bean flowers on successive days since hatching

Weight of larvae at each developmental stage reared on a diet of male-fertile or male-sterile oilseed rape flowers.

Pollen beetle 2nd instar larvae, prepupae, pupae and adults were significantly heavier when reared on a diet of male-fertile than male-sterile OSR flowers. The weights of pollen beetle eggs at the beginning of the experiment did not differ, as would be expected, and neither did the weights of first instar larvae hatching from these eggs (Table 6.10).

Table 6.10 Mean weight (mg) of pollen beetle larvae reared on a diet of male-fertile or male-sterile oilseed rape flowers on the first day of each developmental stage.

Developmental stage	Mean weight (mg)		n		s.e.d	F	P
	♂ Fertile	♂ Sterile	♂ Fertile	♂ Sterile			
Egg	0.037	0.037	100	100	0.001	$F_{1,197}$= 0.04	> 0.05
1st instar larva	0.036	0.035	77	75	0.001	$F_{1,149}$= 2.00	> 0.05
2nd instar larva	0.516	0.369	43	26	0.050	$F_{1,66}$ = 8.62	< 0.01
Prepupa	1.183	0.875	42	18	0.080	$F_{1,57}$=14.69	< 0.001
Pupa	1.431	1.134	31	11	0.078	$F_{1,39}$=14.57	< 0.001
Adult	1.429	1.114	25	6	0.934	$F_{1,28}$=11.33	< 0.01

The predictions of the sex of adult beetles using the characteristics described by Charpentier & Weibull (1994) were largely accurate, with 90% of the predictions at the pupal stage being correct. Of the 41 predictions at the pupal stage, 31 developed into adults for confirmation. Only three of these predictions were proved wrong, with two cases of an adult female being predicted at the pupal stage as a male, and one case of a male being predicted as a female. These characteristics could therefore be used confidently in future experiments to predict the sex of a pollen beetle before the adult stage.

Adult female pollen beetles were heavier than males; females weighed an average of 1.45 mg whilst males weighed an average of 1.27 mg ($F_{1,24}$=5.74; P<0.05; linear regression; with terms for experiment, Synergy type, and sex and the interaction between OSR type and beetle sex). Since there was no interaction between Synergy type and sex ($F_{1,24}$ = 1.29; P>0.05), this means that females reared on male-fertile flowers were heavier than those reared on male-sterile flowers and likewise for males (Fig. 6.10).

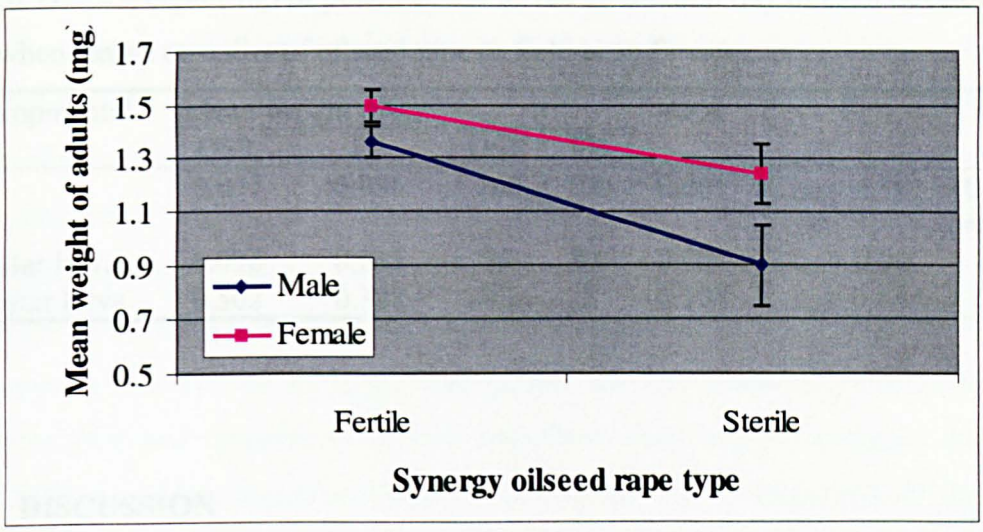


Figure 6.10 Mean weight (mg) of male and female adult pollen beetles reared on male-fertile or male-sterile Synergy oilseed rape flowers (±SE)

The sex ratio was close to 1:1 with a slight bias for more females than males on both OSR diets. The ratio of males:females produced on male-fertile Synergy was 11 males : 12 females, whilst on male-sterile flowers, 2 males: 4 females was produced. The difference between the ratios of males:females produced on each Synergy type was not significant (Fisher's Exact P=0.663).

Weight of larvae at each developmental stage reared on a diet of oilseed rape or field bean flowers.

Since no larvae reared on field bean flowers developed as far as the prepupal stage, only the weights of eggs and those of the 1st and 2nd larval instars were compared (Table 6.11). There was no difference in the weights of larvae reared on the two treatments. However, since only two larvae survived to the 2nd instar on field bean, these results must be treated with caution. Surprisingly, there was a difference found in the weights of the eggs at the start of the experiment; eggs used in the OSR treatment were heavier than those used in the FB treatments. Since the eggs were taken from the same plants at the same time and distributed randomly between all three diet treatments, this result is probably due only to chance.

Table 6.11 Weight (mg) of pollen beetle larvae on the first day of each developmental stage when reared on a diet of oilseed rape or field bean flowers.

Developmental stage	Mean weight (mg)		n		s.e.d	F	P
	OSR	FB	OSR	FB			
Egg	0.037	0.036	100	100	0.001	$F_{1,197} = 4.10$	<0.05 (=0.044)
1st instar larva	0.036	0.035	77	92	0.001	$F_{1,166} = 0.22$	> 0.05
2nd instar larva	0.502	0.388	43	2	0.124	$F_{1,42} = 0.84$	> 0.05

6.6 DISCUSSION

6.6.1 Effect of pollen on adult occurrence

More adult pollen beetles were found on male-fertile Synergy plants than male-sterile ones throughout flowering. This difference was probably caused by the presence of pollen in the former and not the latter, and could be due to attraction to pollen and/or retention to feed on it. The presence of pollen in male-fertile flowers may have retained beetles on them to feed. Pollen may be accessed by beetles from plants pre-flowering by chewing into the buds and feeding on the developing anthers within. However, there was no difference between the numbers of adults on the two plant lines pre-flowering, i.e. the presence of pollen within buds did not have the effect of retaining beetles on them as was observed in the flowering stages. This suggests that in addition to retaining feeding beetles, exposed pollen in flowers may have some attractive properties. Pollen (UV absorbing) may form an attractive visual contrast with the petals (UV reflecting) (Lunau, 2000). Pollen odour may also be attractive; there is supporting evidence to suggest that pollen cues, in particular pollen odours, are used in resource-location by pollen beetles (Charpentier, 1985; this thesis, chapters 3 & 4) and other pollen-seeking insects such as solitary bees (Dobson, 1987), and bumble bees (Dobson, Danielson & Van Wesep, 1999).

6.6.2 Effect of pollen on oviposition

There was no difference in the proportion of buds with oviposition damage between male-fertile and male-sterile Synergy lines. However, as generally fewer adults were found on male-sterile than male-fertile plants, this indicates a greater number of oviposition holes made per female on male-sterile plants than male-fertile ones. The

reason for this increased number could be due to females' unfulfilled search for suitable oviposition sites; more male-sterile buds were found devoid of eggs than male-fertile ones. This suggests that fewer females completed oviposition into male-sterile buds than male-fertile ones, possibly because they were less preferred due to their lack of pollen, which may have been perceived after chewing the oviposition hole, and buds were subsequently rejected. Ekbom & Borg (1996) similarly observed that some buds from the field with oviposition damage were devoid of eggs, and indicated that the proportion of damaged buds devoid of eggs was greater in less preferred species, (*Crambe abyssinica* (L.) and *Sinapsis alba* (L.)) on which they laid fewer eggs, than more preferred species (*B. napus*, *Brassica juncea* (L.) and *Brassica campestris* (L.)), although this comparison was not specifically tested. Factors such as oviposition stimulants may have contributed to these preference.

After chewing an oviposition hole into a bud, females place their abdomens over the hole several times before ovipositing (Borg & Ekbom, 1996). The styli on the ovipositor tip of pollen beetles and many other insects bear various sensillae. These are likely to be important in sensory discrimination during oviposition (Crowson, 1981). In a study of the seed beetle (*Acanthoscelides obtectus*, (Say)) which oviposits inside haricot beans (*Phaseolus vulgaris* L.) the ovipositor tip was found to be used in discriminating degrees of humidity within pods, and there was also evidence that it had tactile and chemosensory functions (Pouzat, 1975 in Crowson, 1981). The behaviour of female pollen beetles placing their abdomen over the oviposition hole could therefore represent further host quality assessment using the ovipositor. Borg & Borg-Karlson (1996) suggested that females could be detecting plant volatiles such as isothiocyanates from the damaged tissue surrounding the oviposition hole, which may then act as oviposition stimulants. In the case of Synergy, females may have rejected more male-sterile buds than male-fertile ones due to an assessment of poor host quality - derived through a lack of pollen/anther cues from male-sterile buds or due to the absence of oviposition stimuli from pollen/anthers emanating from within the buds. Oviposition stimulants present in pollen have been previously reported: sunflower (*Helianthus annuus* L.) pollen was found to contain an oviposition stimulant active on the sunflower moth (*Homoeosoma electellum* (Hulst)) (Delisle *et al.*, 1989; Le Métayer *et al.*, 1993). Ekbom & Borg (1996) suggested that the occurrence of buds with oviposition holes devoid of eggs was due to interrupted oviposition, and this could also be a possible explanation in this case. Female pollen beetles rested more and placed their abdomen over the oviposition hole more often

on buds of a less preferred host (*S. alba*) than those of preferred hosts (e.g. *B. napus*) (Borg & Ekbom, 1996). Thus if cues derived from the bud are not optimal, there seems to be an increased likelihood of behaviours other than egg-deposition that could increase the chances of oviposition being interrupted before it is completed. More detailed observations of oviposition behaviour on male-fertile and male-sterile lines of Synergy are required to further investigate this.

Once a female had accepted a bud for oviposition, there was no difference between the average number of eggs laid per bud on male-fertile and male-sterile plants. This indicates that females did not adjust clutch size according to host quality. It might have been expected that clutch size would be reduced in male-sterile buds if these were perceived by females to be of lower quality compared to male-fertile buds. Pollen beetle females have been shown to alter clutch size depending on the quality of the host; in general they lay fewer eggs in the buds of less preferred hosts (Borg & Ekbom, 1996; Ekbom & Borg, 1996; Hopkins & Ekbom, 1999; Börjesdotter, 2000). Exposure to low quality hosts has been shown to reduce egg production by pollen beetles (Hopkins & Ekbom, 1996). However, in the field, it is likely that females were frequently exposed to both male-fertile and male-sterile plants: this may have reduced any effect of exposure to low quality hosts that might otherwise have occurred if females had been confined to male-sterile plants. Laboratory tests could be easily conducted to resolve the importance of this factor in the Synergy case. Bud size has also been implicated as an important factor in determining clutch size. Ekbom (1998) suggested that pollen beetles modify clutch size according to resource availability as measured by bud size, in order to reduce effects of larval competition. Since there was little difference between the bud sizes of male-fertile and male-sterile lines (personal observations), a difference in clutch size may not after all have been expected.

6.6.3 Effect of pollen on the incidence of larvae

There was no difference in the number of 1st instar larvae in male-fertile or male-sterile buds. Given that there was no difference in the number of eggs laid in these buds, this is not surprising. That these larvae were still alive despite the absence of pollen, given that this developmental stage lasts between 2-6 days (Bromand, 1983; Nielsen & Axelsen, 1988a; this chapter, section 6.5.2.2), suggests that pollen consumption is not obligatory for survival at this stage. This conclusion is supported by results from diet experiments; pollen beetles reared on Synergy flowers without pollen still moulted into the 2nd instar

(this chapter, section 6.5.2.1). The number of 2nd instar larvae in male-fertile and male-sterile buds did not differ. However, the absence of any statistical difference was mainly due to the numbers of 2nd instars found in buds being too low to compare. Low numbers of this larval stage in buds was expected, since by the time larvae have moulted into the 2nd instar, the bud has usually opened. For the same reason, few 1st instar larvae were expected to be found on flowers, and subsequently no statistical difference was evident between numbers present on either line. Interestingly, there were generally more 2nd instar larvae on flowers of male-fertile plants than male-sterile ones. This could be due to movement of the very mobile 2nd instar larvae from male-sterile flowers to male-fertile ones. Alternatively, this could indicate a greater survival of larvae from the first instar into the 2nd on plants with better food resources (pollen). This was investigated further, and supported, by the diet experiments conducted in controlled laboratory conditions (this chapter, section 6.5.2.1).

6.6.4 Effect of pollen on the fitness of larvae and the adults they become

Weight is often used as one measure of fitness; bigger/heavier insects tend to be fitter, producing more offspring during adulthood than smaller/lighter ones (Dixon & Wratten, 1971) and the size an insect achieves is largely dependent on the nutrition it receives as an immature (Leather, 1994). There was no difference in the weight of larvae collected from either plant line in the field. These results could indicate that pollen in the diet has no beneficial effect on the weight of larvae and therefore may have no benefit on the fitness of the adults they become. However, movement of larvae born on male-sterile plants to male-fertile flowers (and vice versa) may have occurred. Such movement would reduce diet quality differences between larvae found on plants with pollen and those without that might otherwise be evident in the absence of movement.

When movement of larvae between male-fertile and male-sterile flowers was prevented by conducting diet experiments in the laboratory, the weight of all pollen beetle developmental stages except for the egg and first instar larvae was greater when reared on male-fertile oilseed rape flowers than on male-sterile ones. Since eggs and first-day 1st instar larvae would not have had the benefit of feeding, these differences are most likely due to the better food resources (pollen) available in male-fertile flowers. Thus, a diet containing pollen results in heavier larvae and pupae from which heavier adults develop: pollen therefore improves both survival and fitness of the pollen beetle. Adult body weight of pre-diapause pollen beetles was found to be a major factor influencing

over-wintering survival, with heavier beetles having greater survival than lighter ones (Hokkanen, 1993). Adult weight in pollen beetles has also been found to be correlated with reproductive fitness, as shown for other insects, such as aphids (Dixon & Wratten, 1971) and carabid beetles (Juliano, 1985). Smaller female pollen beetles eclosing from lighter pupae raised on *S. alba* laid fewer eggs than larger females reared on *B. napus* (Ekbom, 1997). Since females reared on male-fertile flowers were heavier than ones reared on male-sterile flowers, they may be more fecund.

Egg load and the number of eggs laid is a more direct measure of fecundity than adult body weight. A pollen-containing diet has been shown to be obligatory for the normal development of the ovaries of some insects, such as the seed weevil (Free & Williams, 1978a), and beneficial in increasing the egg load of other insects including flower thrips (Teulon & Penman, 1991) and pea weevils (Pesho & van Houten, 1982). However, the egg load of the females that developed on these diets was not measured since it would have meant maintaining them over their diapause. The high mortality involved in doing this effectively precluded it. Assessing the effect of dietary pollen on pollen beetle ovarian development and egg load could be assessed in the future using females over-wintered in the laboratory, which, after emergence, could be cultured on male-fertile and male-sterile Synergy for a period before assessment.

6.6.5 Effect of pollen on larval survival and development

Although pollen in the diet of pollen beetle larvae was not essential for their survival or development to adulthood, it increased survival through each developmental stage and resulted in faster development. Similar findings are quite frequent in the literature. For example, larval mortality was lowest and developmental time fastest on diets containing pollen compared with those without in studies of flower thrips (Trichilo & Leigh, 1988; Teulon & Penman, 1991). Such weight gain may be directly attributed to pollen nutrition; when provided with a pollen-containing diet, lacewing (*Chrysoperla carnea* (Stephens)) larvae grew more rapidly and developed into larger adults than those given prey alone. Analysis of their isotopic signatures showed that their increased growth was attributable to assimilation of pollen nutrients (Patt *et al.*, in prep.).

The faster developmental time of larvae raised on male-fertile Synergy flowers than on male-sterile ones was mainly due to the lower duration of time spent in the first instar. Ingestion of pollen at this stage also seems to be particularly important for other insect

species whose larvae develop in flowers. Larvae of the sunflower moth (*H. electellum*) were more prone to eat pollen in the first larval instar than subsequent instars (Rogers, 1978), indicating that it was required or beneficial at this stage. Carisey & Bauce (1997) found that pollen consumption during the early larval stages reduced the total developmental time of the spruce budworm (*Choristoneura fumiferana* (Clem.)). Larvae reared on male-sterile flowers probably gained the nitrogenous nutrition needed for survival and development from the small amounts of amino acids present in nectar (Baker & Baker, 1973) and other floral parts; feeding damage to the stigma, nectaries and petals was frequently observed in male-sterile buds and flowers. However, since these may be more difficult to digest than pollen, development occurred slower in the absence of pollen than in its presence. Prolonged developmental times can increase the duration of time that vulnerable larvae are open to attack from natural enemies, such as parasitoids (Benrey & Denno, 1997). Thus, a pollen diet confers many benefits upon a developing larva.

The sex ratios of pollen beetles tend to be in the region of 1:1, with any bias in favour of females (Nilsson, 1988a; Sedivy & Kocourek, 1994) as was found in this study. It is not known whether sex allocation of the pollen beetle is random, dependent on the food resources of developing larvae or determined by the mother –either by her nutritional status or by her assessment of a host plant. Although heavier pupae tended to be female, there was no difference in the ratios of males:females produced on each Synergy type. This indicates that pollen in the diet did not affect sex determination of developing larvae, and suggests that sex determination is not related to larval nutrition. In some insects such as sawflies (*Euura* spp.) females alter progeny sex in response to variation in host attributes, and lay fertilized eggs (female) on hosts perceived to be of better quality and unfertilized (male) eggs on plants of lower quality (Craig, Price & Itami, 1992). It would be interesting to compare the sex ratios of larvae reared from eggs laid in male-fertile and male-sterile buds to determine whether female pollen beetles alter progeny sex according to host quality assessment.

6.6.6 Larval requirements for cruciferous hosts

This is the first recorded attempt to rear pollen beetles on resources from a non-reproductive-phase host plant. In contrast to the good probability of larvae surviving to adulthood on OSR flowers, larvae did not survive beyond the second instar on FB flowers. Of the 1st instars that did not survive into the 2nd instar on FB, many perpetuated

in the 1st instar for about a week before eventually dying. This suggests that FB flowers afforded the larvae inappropriate conditions for development through to the 2nd instar. Field bean pollen contains a smaller proportion of the essential amino acids required for insect growth than oilseed rape, but still represents a good source of these nutrients (this thesis, chapter 9). Thus the fact that field bean contains nutritious pollen, yet supported larval development less well than male-sterile OSR flowers without pollen, is conclusive proof that pollen-feeding is not obligatory for pollen beetle larval development, and that oilseed rape pollen and the flower contain other important factors required for larval development.

In many cases the larvae moved off the field bean flowers and desiccated on the walls of the box. This could indicate the presence of chemical deterrents or absence of appropriate chemical stimulants in field bean flowers that may have prompted larvae to move off the flower –perhaps in search of a more suitable host. Charpentier & Charpentier (1986) reported that larvae were stimulated to feed by some glucosinolates, but did not give further details. Glucosinolates have been recorded in OSR pollen (Dungey *et al.*, 1988), and would be present in both male-fertile and male-sterile OSR floral tissue, but not in field bean, since they are specific to the Cruciferae (Kjaer, 1976; Fenwick, Heaney & Mullin, 1983). In addition, the floral structure did not seem to suit the larval microclimate requirements and behaviours for feeding on the nectaries. In oilseed rape flowers, larvae were often observed beside the filaments, with their heads down towards the nectaries. When larvae did this in field bean flowers, they ended up at the end of the thin passage of the stamen sheath, where they tended to get stuck, and many died in this way.

6.6.7 General discussion

The incidence of adults and 2nd instar larvae was higher on flowering male-fertile Synergy plants than male-sterile ones. These stages will eat the pollen from open flowers that is required to pollinate the crop. In addition, more male-fertile buds than male-sterile ones were likely to contain eggs and developing larvae (as the same proportion were found damaged, but more male-sterile buds were found devoid of eggs or larvae than male-fertile buds). Larval feeding damage may contribute to bud abscission and the subsequent loss of flowers that would produce the pollen necessary to pollinate the crop. Thus it is apparent that the smaller population of male-fertile plants within the Synergy crop will be at risk from increased infestation by the pollen beetle

compared with male-sterile plants, and severe attacks could result in inadequate pollination of the crop. The threshold for control of the pollen beetle in Synergy crops is one beetle per five plants inspected compared with 15 beetles per plant on conventional winter cultivars (CPB Twyford, 1996). This threshold is extremely low, but seems a sensible precaution in the light of the results of this study.

Any differences between male-fertile and male-sterile Synergy flowers in this study are assumed to be due to the presence on pollen in the former and not the latter. However, any differences could have been due to cultivar differences other than the presence or absence of pollen. For example, male-fertile flowers were slightly larger than male-sterile ones and this could have resulted in increased adult attraction. Plant chemistry including volatile production from both flowers and green tissues could also be different between these cultivars (Appendix 1.1). However, until isogenic lines of male-fertile and male-sterile plants are developed, this system provides useful material for assessing the effects of pollen without artificially manipulating the plants; an impossible task on a field scale.

These studies showed that whilst larval survival and development on cruciferous flowers were good, on field bean they were extremely poor. Larvae are often capable of surviving and developing on a broader range of host species than are used for ovipositing (e.g. Wiklund, 1973). However, the inability of pollen beetle larvae to develop on FB flowers demonstrate that to develop they require factors (perhaps feeding stimulants, nutrients, microclimate conditions or requirements for certain aspects of the floral structure) not offered by FB flowers. This suggests that it is these larval requirements that may have driven the oviposition-specificity of the pollen beetle on cruciferous plants. These experiments also demonstrate that in terms of larval requirements, pollen feeding is facultative rather than obligatory. Although pollen beetles do not need pollen to survive and develop, they benefit from it in their diet through faster development through the first larval instar, an increased probability of survival into adulthood and increased weight. Such benefits may have driven the female oviposition preference for flower buds, where there is both safety from attack from parasitoids and predators for the early developmental stages and a ready, long-term food supply.

It seems therefore that in general there is a good correlation between larval requirements for cruciferous hosts and benefit from a pollen diet, and female oviposition preference for

the flower buds of cruciferous plants. However, females still accepted male-sterile buds for oviposition, even though larvae perform less well on male-sterile than on male-fertile flowers. A correlation between larval performance and oviposition preference seems an obvious relationship, but it is not always displayed (see Berdegué, Reitz & Trumble, 1998). Females may oviposit on unsuitable novel hosts, for a variety of reasons, including insufficient evolutionary time for female oviposition preferences to shift away from the use of these hosts (Thompson, 1988). This may be the case with Synergy. Where a pollen diet has benefits for offspring development, females should evolve to use cues directly from pollen (or associated with it), to determine oviposition preferences. However, since pollen beetles have not evolved in an environment where natural male-fertile and male-sterile reproductive-phase hosts exist, selection pressure to develop the ability to detect and reject male-sterile buds is only recent. Although there was some evidence that pollen beetles seemed to reject male-sterile buds more often for oviposition than male-fertile ones, the reasons for this require further investigation. The criteria used by females in host-quality assessment are not fully understood, and in particular the cues perceived by females from within the damaged bud whilst they have their abdomens over the oviposition hole have not been investigated. Pollen cues may form part of the normal host cues perceived by females given that they do examine the bud interior through the oviposition hole before oviposition. However, it seems unlikely that these cues are currently used to determine oviposition preferences. The area of oilseed rape crops consisting of male-fertile and male-sterile varietal associations is increasing (Pinochet & Bertrand, 2000). If pollen cues are not already used by females in host plant quality assessment during oviposition, they may evolve to do so, given their apparent ability to adapt quickly and evolve to changing farming practices (Hokkanen, 2000).

7. Conditioned proboscis extension (CPE) responses of restrained honey bees to pollen odour

7.1. ABSTRACT

Honey bees learn to associate the colour and odour cues of rewarding flowers to help them forage efficiently and locate other rewarding flowers. The conditioned proboscis extension bioassay was used to investigate the ability of honey bees to learn associatively the odour of pollen, to examine generalization of odour-recognition between the pollens of two different species (oilseed rape and field bean) and to determine if bees could discriminate between the pollen odours of these two species. Bees efficiently learned to associate the odours of both oilseed rape and field bean bee-collected pollens with a reward. They generalized the recognition of the odours from these two pollens, indicating at least some of the volatiles involved in the recognition of one pollen type were present in the other. However, experiments using hand-collected pollens indicated that some of these similarities were due to 'bee-derived' components present in both bee-collected pollens. When bees were differentially conditioned, they discriminated between the odours of the oilseed rape and field bean pollens, indicating the existence of chemical differences between them that can be detected by bees. Such differences could be biologically important in species discrimination. Bees conditioned to oilseed rape pollen were tested to the odour of a whole oilseed rape flower in a generalization test. Although this test was inconclusive, the possibility that volatile components of pollen are associatively learned from rewarding flowers and used in location of other rewarding flowers is discussed.

7.2 INTRODUCTION

7.2.1 The importance of olfactory cues for foraging honey bees

Olfactory cues play very important roles in the lives of honey bees (*Apis mellifera* L.). Within the colony, pheromones emitted by the queen and workers communicate a variety of types of information which elicit responses that help to co-ordinate the activities in the colony (Seeley, 1985). Outside the colony, foraging worker bees visit flowers to collect pollen and nectar resources to provision the colony with nutrients vital for the rearing of young bees. Unlike the pollen beetle (*Meligethes aeneus* (Fabricius)), the focus of the previous chapters, which largely rely on innate or 'pre-programmed' responses to host plant cues in resource-location, honey bees show extremely plastic behaviour in foraging. They learn to associate floral cues from rewarding flowers to enable them to forage efficiently and find food resources to provide for their colony. Prior to learning, floral odours may not elicit such strong innate responses as do pheromones (von Frisch, 1967), but afterwards they are vital cues used in the future identification of rewarding flowers promoting floral constancy.

Floral odours are composed of mixtures of many volatile compounds from different chemical classes (Borg-Karlson, 1990; Knudsen, Tollsten & Bergström, 1993) and these vary over space and time due to factors such as plant species, variety and phenology (Pham-Delègue *et al.*, 1986, 1989), plant damage (Bergström, Dobson & Groth, 1995) and climatic conditions (Robaker *et al.*, 1982). Therefore, once a bee has learned the odour of a rewarding flower, the likelihood of the occurrence of an odour of exactly the same concentration and blend as in the conditioning odour would be low – even within the same floral species. Foraging honey bees must recognise odours that predict a reward reliably, despite the odour not being exactly the same, whilst 'filtering out' stimuli which, although similar, are not useful in predicting rewards. Bees therefore generalize recognition to non-identical presentations of learned stimuli on the one hand, but discriminate between similar but different stimuli on the other (Smith, 1993).

7.2.2 Olfactory learning in honey bees

Generalization of the recognition of two similar odours and discrimination between different odours depends on the ability of bees to learn components of the conditioning stimulus. Associative olfactory learning as a consequence of conditioning has been much studied in bees, and research has followed two main approaches. In pioneering

studies, von Frisch (1919) demonstrated that after experiencing an odour associated with a sucrose reward, bees responded to the odour alone. These experiments, and many that have followed (e.g. Menzel, Erber & Mashur, 1974; Waller, Loper & Berdel, 1974; Pham-Delègue *et al.*, 1986; Laska *et al.*, 1999; this thesis, chapter 8), used free-flying bees as their subjects, whilst others took the approach of using restrained subjects and employed bioassays involving conditioning of the proboscis extension reflex (e.g. Vareschi, 1971; Bitterman *et al.*, 1983; Smith & Menzel, 1989; this chapter).

7.2.3 CPE bioassay

The conditioned proboscis extension (CPE) bioassay is based upon the proboscis extension response (PER). The PER is a natural reflex elicited when a foraging fly, butterfly, moth or bee lands on a flower and contacts nectar with its tarsi or antennae. It leads to food uptake and the learning of flower cues associated with the nectar. It was first demonstrated by Minnich (1921, 1932), and Frings (1944) first conditioned the PER in honey bees by pairing the presentation of coumarin odour with a sucrose solution reward so that eventually presentation of coumarin alone elicited the PER. Bitterman *et al.* (1983) showed that such learning of an odour when paired with a reward was associative in the classical sense (Pavlovian learning; Pavlov, 1927). This involves establishing an association between two stimuli:

1. the unconditioned stimulus (US) which elicits an unconditioned (reflex) response (UR) in a motivated subject
2. the conditioned stimulus (CS) which is usually neutral, eliciting no response prior to conditioning.

Pairing of the US with the CS leads to an association between the two, and eventually to the ability of the CS alone to trigger the UR. In the case of odour conditioning of the PER in honey bees, the US is usually sucrose, which elicits the proboscis extension reflex (UR), and the CS is an odour.

Takeda (1961) and Vareschi (1971) showed that the response to the conditioning odour could be extended to some additional odours (generalized) but not others (discriminated): the PER of restrained bees to conditioned and unconditioned (novel) odours could therefore be used to evaluate the degree of odour similarity to the bee. Since then, the learning of odours, the generalization of their recognition and their discrimination by restrained honey bees has been widely studied (for reviews see Menzel, Greggers & Hammer, 1993; Smith, 1993; Smith & Getz, 1994). Most studies however, have

investigated the learning and recognition of single-compound odours. Such odours are not part of the normal environment of flower-visiting insects, since, as described above, plant odours are composed of spatially and temporally variable mixtures of compounds. How bees learn to recognize these mixtures and generalize or discriminate between them, has only begun to be studied relatively recently. Getz & Smith (1987) evaluated the quantitative discrimination of components in mixtures using binary combinations of compounds involved in kin-recognition of the honey bee, but so far there have been few studies involving floral volatiles.

7.2.4 Recognition of floral odours: mixtures of volatile compounds

Recognition of the mixtures of volatile compounds that comprise floral odours is thought to be based on a few key compounds present in the total mixture. Free-flying honey bees conditioned to the odour of alfalfa flowers used components predominant in the conditioning mixture as cues to locate rewarding feeders (Waller, Loper & Berdel, 1974). Similarly, recognition of sunflowers by foraging bees was cued by a subset of volatiles comprising less than 10% of the total volatile blend (Pham-Delègue *et al.*, 1986). Further studies utilizing the CPE bioassay to assess the responses of restrained honey bees to oilseed rape volatiles showed the existence of hierarchies, with some compounds cueing mixture recognition more effectively than others (Pham-Delègue *et al.*, 1993, 1997; Wadhams *et al.*, 1994; Le Métayer *et al.*, 1997). Behaviourally active components were later identified by coupling gas chromatography (GC) with CPE (Blight *et al.*, 1997) and three volatiles: phenylacetaldehyde, linalool and *(E, E)*-α -farnesene were found to play key roles in recognition of oilseed rape flowers by honey bees.

7.2.5 Pollen volatiles as cues for pollen-foraging bees?

To date, studies on mixture recognition have focused on whole-flower volatiles. In many species, each floral structure produces a distinctive blend of volatiles that contribute to the whole flower odour (Dobson *et al.*, 1987; Dobson, Bergström & Groth, 1990; Pichersky *et al.*, 1994), yet the origin of compounds with behavioural importance has never been investigated. Pollen volatiles are also composed of mixtures of different compounds, often distinct from the volatiles produced by other floral parts (Dobson, Bergström & Groth, 1990; Bergström, Dobson & Groth, 1995; Dobson *et al.*, 1997). The distinctive smell of pollen could help in its location by pollen-seeking insects, and some insects have been shown to be attracted to pollen odour (Dobson, 1987; Dobson, Danielson & Van Wesep, 1999; this thesis, chapters 4 & 8). It is therefore possible that

some of the behaviourally active components involved in flower recognition are present in pollen, and may be learned, particularly by pollen-seeking bees, to improve resource location. However, the ability of bees to learn pollen odour associatively has never been investigated. Furthermore, although it has been shown that pollens from different species have species-specific odours (Dobson *et al.*, 1987; Dobson *et al.*, 1997) it is not known whether bees can learn these differences and use them to discriminate between species.

7.3 AIM

The aim of this set of experiments was to investigate whether bees can learn to associate the odour of pollen with a reward, and to examine their ability to generalize and discriminate between pollen odours from different plant species. The effect of the method of pollen collection (bee-collected or hand-collected) on generalization was investigated, and the generalization of the odour from oilseed rape pollen with that of the whole flower was also considered. Experiments were conducted to answer the following questions:

1. Can honey bees learn to associate the odours from both oilseed rape (OSR) and field bean (FB) bee-collected pollen with a reward?
2. Do honey bees generalize the recognition of odour from bee-collected OSR pollen odour with that of FB and vice-versa; i.e. are these odours similar to honey bees?
3. Can honey bees discriminate between the odours of bee-collected OSR and FB pollens; i.e. do their odours have differences that are detectable by honey bees?
4. What is the effect of 'bee-derived' components in the generalization of OSR and FB bee-collected pollens?
5. Can honey bees learn to associate the odours from both OSR and FB hand-collected pollen with a reward?
6. Do honey bees generalize between OSR and FB hand-collected pollen?
7. Do bees generalize the recognition of odour from OSR pollen with that from the whole flower?

7.4 MATERIALS & METHODS

7.4.1 Restrained honey bees

Honey bees were collected from the hive entrance of their colony (2.4) using a 'Bee-Vac' (Insect Vac, BioQuip®, 17803 LaSalle Ave, Gardena CA, USA), a portable, hand-held insect vacuum, powered by a modified Black & Decker Dust-Buster™. It had a nose section with a clear acrylic tube that held a removable collecting chamber (12.7 cm long x 5.1 cm diameter). This had an aluminium mesh screen across one end for air flow and a flap valve at the other. Approximately 15 bees were collected in each chamber. Bees were taken into the laboratory and individually removed from the collecting tube, through the flap valve by grasping them on the dorsal side of the thorax with a pair of forceps. Each bee was then restrained according to the procedures described by Bitterman *et al.* (1983). Bees were harnessed individually in small glass tubes, leaving their forelegs and head free, protruding from the top of the tube (e.g. Fig. 7.1). Tape (Scotch fine line 3/32 inches) was placed over the bee's head to secure it in position, and electrical tape was secured around the back of the tube to prevent the bee from protruding its abdomen to sting and to help prevent its escape from the tube. When all the bees required for an experiment had been restrained in this manner, each was fed to satiation with 30% sucrose solution, then starved for three hours. This was to minimize differences in motivational states between bees. This period was found through preliminary trials to be sufficiently long to evoke the PER upon stimulation with sucrose.

Prior to the start of the experiment, each bee was tested for the PER by touching each of its antennae with 30% sucrose solution applied by a cotton wool bud. Only those bees that extended their proboscis in response to this stimulation (e.g. Fig. 7.2) were used in the experiment and non-responders were discarded since the bioassay involved recording these responses.

7.4.2 Pollen

Bees were conditioned and tested to oilseed rape (OSR) and field bean (FB) pollen which was either hand-collected from flowers (2.3.1) immediately prior to experiments, or from defrosted bee-collected pollen loads (2.3.2.).

7.4.3 The conditioned proboscis extension (CPE) bioassay

The odour conditioning of the honey bees' PER is based on the association of an odour (the conditioned stimulus, CS) and a sucrose solution reward (unconditioned stimulus, US). During conditioning, the CS is forward-paired with the US by three seconds, after which time the CS is simultaneously presented as the PER (unconditioned response, UR) is elicited by stimulating each of the bees' antennae with sucrose. As the reward, the bee is allowed to feed on the sucrose solution. After repeated paired presentations of these stimuli, the bee becomes conditioned, and the delivery of the odour alone induces the PER, which is then known as the conditioned proboscis extension (CPE) response. This response indicates that associative learning has taken place.

7.4.4 Odour-delivery device

A device based on that used at INRA Bures-sur-Yvette, Paris, France (Pham-Delègue *et al.*, 1993; Sandoz, Roger & Pham-Delègue, 1995; Laloi *et al.*, 1999) was constructed at Rothamsted to deliver the conditioning and test odours to the bees in these experiments (Fig. 7.4). A constant airflow from a compressed air cylinder, maintained by passage through flowmeters at 1.05 dm^3 min^{-1} was delivered through a 1 cm diameter glass tube. This airflow was composed of a 'clean air' main airflow of 1 dm^3 min^{-1} and a secondary airflow of 0.05 dm^3 min^{-1} that was used for the test odour delivery. The secondary flow was delivered continuously into the main airflow either through a pipette containing the test odour source or an identical empty 'control' pipette; the ends of both were each placed through holes leading into the airflow delivery tube. A solenoid valve was used to switch control and test secondary airflows. An electronic system (a computer chip) controlled the valve as well as visual and audible prompts to the operator conducting the bioassay. The operator pressed a button to activate the sequences controlled by the electronic system. The valve switched the secondary airflow delivery from the control to the test odour over a period of six seconds and a green light would flash, and a beep would sound three seconds after the start of the test odour delivery and again at its end. This was to alert the operator at time periods essential in the conditioning of the bees (see below). The test odour consisted of 0.025 g pollen (either OSR or FB), placed in the bottom end of a glass pipette. A small plug of glass wool, placed at the end of the pipette, prevented the pollen from falling out. The control pipette contained a similar plug but no pollen.

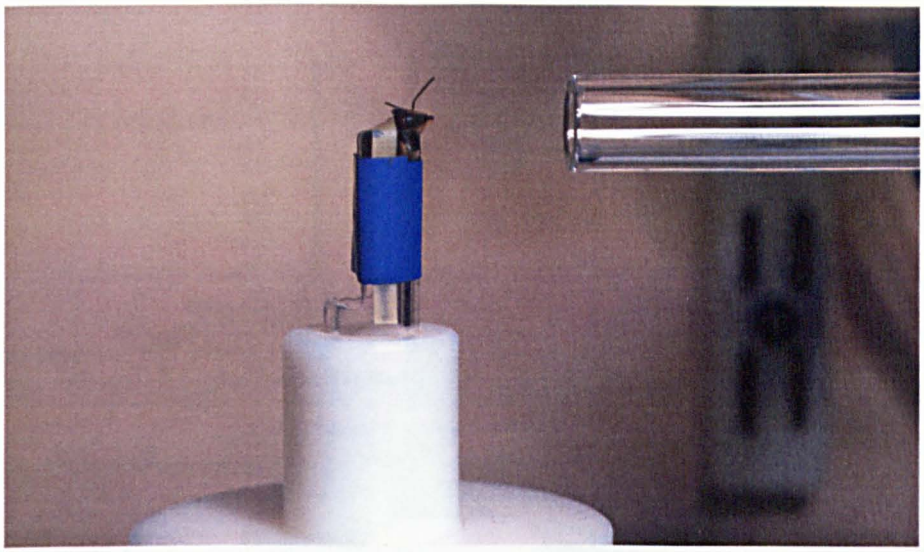


Figure 7.1 Restrained honey bee, showing no response

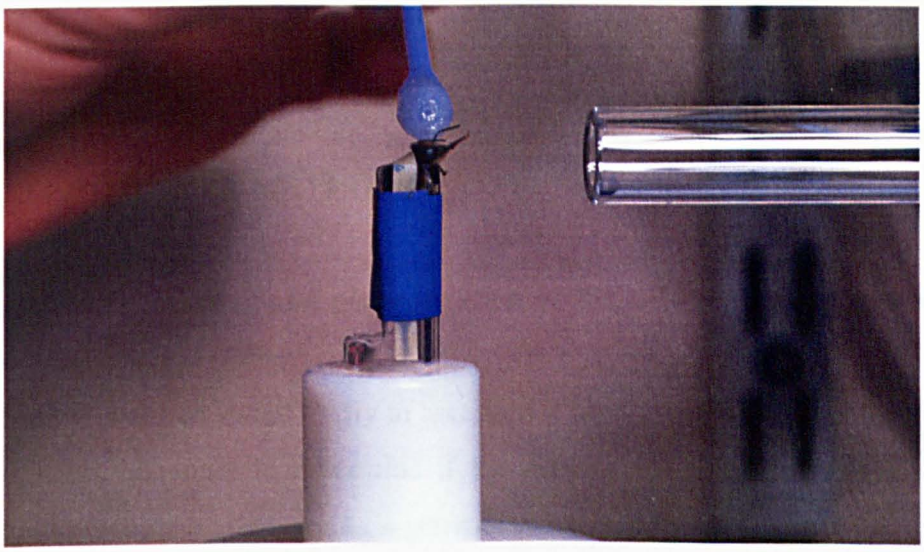


Figure 7.2 Induced proboscis extension response (paired with odour presentation)

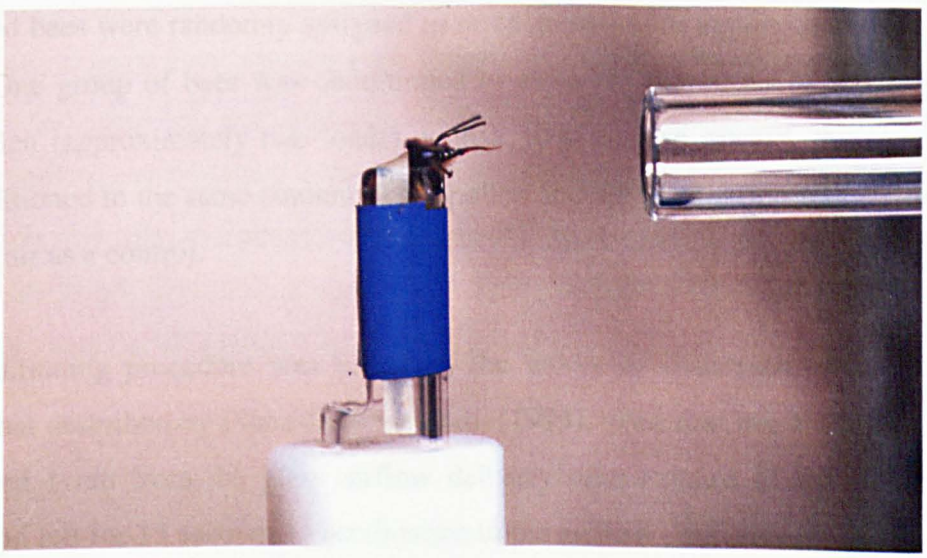


Figure 7.3 Conditioned proboscis extension (CPE) response

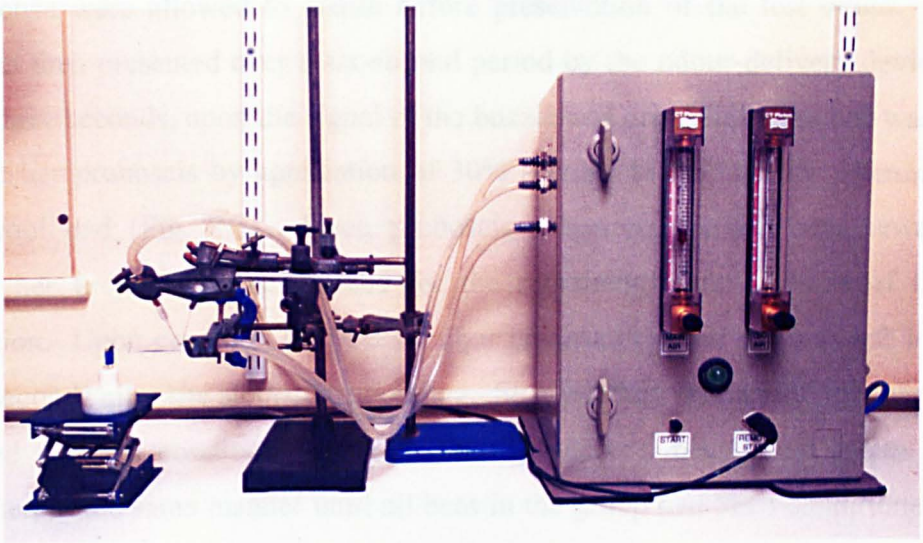


Figure 7.4 CPE experimental set up: showing odour delivery device (on right), and restrained bee in front of odour delivery tube (on left)

7.5 EXPERIMENTAL METHODS

7.5.1 Associative learning and generalization of the odours from oilseed rape and field bean bee-collected pollen

This experiment was conducted firstly to ascertain if bees could learn the odour of pollen associatively and secondly to determine if the odours are perceived to be similar or different to honey bees by performing a generalization test.

7.5.1.1 Conditioning bees to associate a test odour with a sucrose reward

Restrained bees were randomly assigned to three groups with approximately 14 bees per group. One group of bees was conditioned to associate the odour of 0.025 g crushed OSR pollen (approximately two loads), with a 30% sucrose reward, the second group was conditioned to the same amount of FB pollen and the third group was conditioned to the clean air as a control.

The conditioning procedure was based on the works of Bitterman *et al.* (1983) and follows that described by Pham-Delègue *et al.* (1993). The first bee from the test group was placed 1 cm from the glass airflow delivery tube exhaust of the odour-delivery device, and left for 15 seconds to acclimatize to the airflow (Fig. 7.1). If the bee showed a spontaneous PER in response to being positioned in front of the airflow she was

allowed time to stop responding. After the cessation of such a response, 15 seconds of non-response were allowed to elapse before presentation of the test odour. The test odour was then presented over a six-second period by the odour-delivery device. After the first three seconds, upon the signal of the buzzer and green light, the bee was induced to extend her proboscis by application of 30% sucrose to both of her antennae with a cotton wool bud (Fig. 7.2). Upon proboscis extension, the bee was rewarded, by allowing her to feed on the sucrose for the remaining three seconds of the odour presentation. Upon cessation of the test odour presentation, the bee was left in position for five seconds after she stopped responding. She was then returned to the group having completed the first conditioning trial (C1). The second bee of the group was then conditioned in the same manner until all bees in the group had been conditioned once in succession.

Two more conditioning trials, C2 and C3, were conducted, with 15 minute inter-trial periods. Between conditioning trials, bees were placed in a gentle airflow from a fan to help reduce the occurrence of spontaneous responses to the airflow.

7.5.1.2 Recording the data during conditioning

For every bee, during each conditioning trial (C1-C3), it was noted whether the bee showed a PER during the first three seconds of the test odour presentation - before stimulation of the antenna with sucrose. As described earlier, after the first paired presentation of the odour and the reward, a PER is known as CPE response, and is indicative that the bee has learned to associate the test odour with the reward (Fig 7.3). These responses were scored if the tip of the proboscis protruded beyond the open mandibles. Hence by examining these responses during conditioning, each conditioning procedure is in itself a trial for learning. It was also noted whether the bee extended her proboscis and received the sucrose reward in the final three seconds of the odour presentation - after stimulation of the antennae with sucrose. If a bee failed to extend her proboscis in response to sucrose stimulation, and therefore failed to receive the reward, she was discarded from further testing, since this bee would not have completed three rewarded paired presentations of the test odour and sucrose.

7.5.1.3 Presenting and recording the generalization test

After the conditioning process, each group of bees conditioned to pollen odour was randomly assigned to two sub-groups for the presentation of the test odour. Half the bees in the group conditioned to OSR pollen odour were tested for their responses to their conditioning odour, whilst the other half of the group was tested with the odour of FB pollen. Similarly, half the group conditioned to FB pollen odour received this odour in the test, whilst the other half were tested with the odour of OSR pollen. The test consisted of fresh samples of pollen (0.025 g in both cases) and was presented to the bee as in conditioning trials. A positive (CPE response) or negative (no CPE) response was recorded over the duration of the odour presentation for each bee. The test was unrewarded. Since the test only used bees that had learned their conditioning odours, it was unnecessary to conduct a test on the control group, which was used only to demonstrate that learning had occurred.

A second test was then performed on each bee that had received pollen odours in conditioning trials. The test odour in this instance was the clean air control. Bees showing a CPE response to this test were termed 'nonselective learners' (Laloi *et al.*, 1999) and were discarded from all analyses, since it was not certain whether they had been responding to the test odour or merely to the airflow or other mechanical or physical stimulation associated with the conditioning process. Bees were re-tested for the proboscis extension response at the end of the experiment by stimulating the antennae with sucrose solution: those that did not respond were subsequently excluded from all analyses.

Each day, two groups of about 10 bees were conditioned and tested, with each group being taken through the full procedure before the next group was used. The order of dealing with the groups was reversed each day. This procedure was followed until approximately 40 bees had been tested with each pollen type.

7.5.1.4 Analysis of the data

Associative learning A proboscis extension response exhibited during the first three seconds of the odour presentation (before stimulation of the antennae) in a conditioning trial showed that a bee had associatively-learned the test odour. In order to compare associative learning of each test odour, the proportion of the total number of bees exhibiting CPE responses during the first three seconds of the conditioning odour

presentation at each conditioning trial (C1-C3) was plotted to produce associative-learning (odour acquisition) curves.

A 'learning test' was performed which compared the proportion of bees 'learning' (exhibiting a CPE response) and 'not learning' (not exhibiting a CPE response) at C3 (after two paired presentations of the conditioning odour and the reward). Three analyses were conducted using a Fisher's Exact test to compare the following responses:

1. Bees conditioned to the odour of OSR pollen with bees conditioned to the control
2. Bees conditioned to the odour of FB pollen with those conditioned to the control
3. Bees conditioned to the odour of OSR pollen with those conditioned to the odour of FB pollen.

Generalization The generalization test compares the proportion of bees exhibiting a CPE response to the conditioning odour with the proportion of CPE responses to the unconditioned, novel odour at the test. It measures the perceptual similarity between two odours (Kalish, 1969; Smith & Getz, 1994). A difference in the proportions indicates that bees discriminate between these two odours; no difference indicates generalization. The ability to generalize or discriminate requires recognition of the conditioning odour, and therefore bees that did not become properly conditioned (those that did not show a single conditioned response during the conditioning period) were discarded from the analysis as 'nonlearners' (Laloi *et al.*, 1999). Bees responding to the blank air control in the second test were also discarded from the analysis as described previously. The proportion of positive and negative responses in the generalization test to the odour of OSR and FB pollens were compared in two Fisher's exact tests: one examined the effect of conditioning to OSR pollen odour on the responses, and the other the effects of conditioning to FB on these responses.

7.5.2 Discrimination between the odours of oilseed rape and field bean bee-collected pollens

This experiment was conducted to ascertain if the odours from bee-collected OSR and FB pollens contain some differences detectable by honey bees. To determine whether a bee can discriminate one odour from another may require differential (discriminative) conditioning. This involves presentations of a rewarded conditioning odour (CS+) and a non-rewarded or punished conditioning odour (CS-) (Bitterman *et al.*, 1983; Getz & Smith, 1987). Previous work (e.g Bitterman *et al.*, 1983; Gerber *et al.*, 1996) has shown

that non-rewarded CS- enables discrimination, as does punished CS- using negative stimuli such as electric shocks (Smith, Abramson & Tobin, 1991) and salt solutions (Getz & Smith, 1987; Bhagavan & Smith, 1997). However, Smith, Abramson & Tobin (1991) showed that the differentiation of the stimuli was quicker if the negative stimulus is truly aversive. Therefore the use of salt solution as a negative reinforcement of the CS- was adopted for this experiment.

7.5.2.1 Conditioning

Restrained bees were randomly assigned to two groups, with eight bees in each. For the first group (OSR^+/FB^-), during conditioning, the odour of 0.025 g OSR pollen (bee-collected) was paired with a positive reward of 30% sucrose and presentations of FB pollen odour were negatively conditioned by application of salt solution (3M sodium chloride) to the antennae and proboscis if it was extended. The reverse conditioning procedure was carried out for the second group (FB^+/OSR^-); FB pollen odour presentation was paired with the 30% sucrose positive reward, whilst bees were negatively rewarded with salt solution to the odour of OSR pollen. A control group was not necessary since each bee served as its own control (Stone, Abramson & Price, 1997). Conditioning was carried out and data recorded as described in the previous experiment, except that each bee was conditioned 16 times, receiving either positively or negatively rewarded odours in individual trials. Each pollen odour was presented eight times in total. There was an inter-trial time of eight minutes and conditioning odours were presented in a pseudo-randomized order (to reduce the chance of bees learning sequential information (Smith, Abramson & Tobin, 1991; Smith & Getz, 1994). Trials began with a negative stimulus so that spontaneous responses could be assessed. Thus, the order of conditioning odour presentations for each group were as follows:

For the OSR^+/FB^- group of bees: 2 x (FB^- OSR^+ OSR^+ FB^- OSR^+ FB^- FB^- OSR^+)

For the FB^+/OSR^- group of bees: 2 x (OSR^- FB^+ FB^+ OSR^- FB^+ OSR^- OSR^- FB^+)

7.5.2.2 Presenting the discrimination test

As in the generalization test, half the conditioned bees in each group were tested to a fresh sample of the odour associated during conditioning to a positive reward, whilst the other half were tested to a sample of the pollen odour that had been associated during conditioning with the negative reward. The number of positive (CPE) and negative (no CPE) responses during test odour presentations was recorded. All bees were then assessed during a second test for their responses to the blank air control. Bees were

tested for the PER response at the end of all testing, and those that did not respond were excluded from all analyses.

Each group was conditioned and tested separately, with bees conditioned to OSR^+/FB^- conditioned first and then tested before bees were conditioned to FB^+/OSR^- then tested. The order in which groups were conditioned and tested was reversed daily; the conditioning and test procedures were carried out each day until approximately 30 bees had been tested with each pollen type.

7.5.2.3 Analysis of the data

Associative learning The proportion of CPE responses made during the first three seconds of each conditioning odour presentation was calculated. The proportion of CPE responses exhibited by bees on the 1^{st}, 2^{nd}, 3^{rd}.... 8^{th} presentations of the positive and negative conditioning odour was plotted separately. An odour acquisition curve was plotted in this way for both groups of bees (OSR+/FB-) and (FB+/OSR-).

Discrimination tests The proportion of positive and negative CPE responses when tested to FB or OSR pollen odour was compared in two Fisher's exact tests for the two groups. Bees showing a conditioned response to the clean air control, bees that did not protrude their proboscis in response to sucrose stimulation (and therefore did not receive rewards) and bees that did not show any CPE responses during conditioning trials were excluded from this analysis, since recognition of the conditioning odour is required for discrimination (Laloi *et al.*,1999).

7.5.3 Learning and generalizing the recognition of the odours from hand-collected oilseed rape and field bean pollens

This experiment was conducted to assess the effect of 'bee-derived' components in the generalization of the odours from OSR and FB bee-collected pollens. It investigated the possibility that generalization occurred due to the learning of 'bee-derived' components, present in both pollens as a result of the collection process of the honey bee, rather than due to there being similarities between the pollen odours. It involved carrying out the same test using hand-collected pollens so that comparisons between their outcomes could be made. The experiment was carried out exactly as described in experiment 7.5.1, except that 0.025 g hand-collected pollen was used instead of bee-collected pollen.

7.5.4 Generalizing the recognition of the odour of oilseed rape pollen with that of the whole flower

This experiment was conducted to assess how similar bees find the odour of oilseed rape pollen and that of the whole flower. It investigated the possibility that pollen-seeking bees could learn pollen odours from amongst the flower during foraging to help them locate this resource in other flowers. It was conducted in collaboration with Dr. Jean-Christophe Sandoz (then a visiting scientist at BEC department, IACR-Rothamsted).

Restrained bees were randomly assigned to two groups with 10 bees per group. One group of bees was conditioned to the odour of OSR pollen (0.025 g, bee-collected) and the other was conditioned to the clean air control. Conditioning was conducted as previously described. After conditioning, both groups of bees were then tested to the odour of a single OSR flower (cv. Drakkar) as a generalization test. The flower was in stage 3 of the key described by Charpentier (1985), with free pollen covering most of the anther, and remained attached to the plant. It was enclosed in a glass air entrainment vessel (Agelopoulos *et al.*, 1999) and its headspace odour was passed via Teflon tubing at a rate of 1.05 dm^3 min^{-1} to the test pipette of the delivery tube. Experimental replicates were conducted over a period of four days, using a different flower each day. In total, 30 control bees (conditioned to clean air) and thirty-one test bees (conditioned to bee-collected OSR pollen odour) were tested to the odour of a single OSR flower. The number in each group responding positively (CPE response) and negatively (no CPE response) to the odour of the OSR flower was analysed by a Fisher's exact test.

7.6 RESULTS

7.6.1 Associative learning and generalization of the odours from oilseed rape and field bean bee-collected pollen

7.6.1.1 Associative learning of odours from bee-collected pollens

Bees learned to associate the odours of both OSR and FB bee-collected pollens with a reward, but not that of the clean air control. Figure 7.5 shows odour acquisition curves of control bees, being 'conditioned' to clean air, and bees being conditioned to either OSR or FB pollen odours. Data points represent the number of conditioned responses

exhibited during the first three seconds of the odour presentation during conditioning trials (C1-C3).

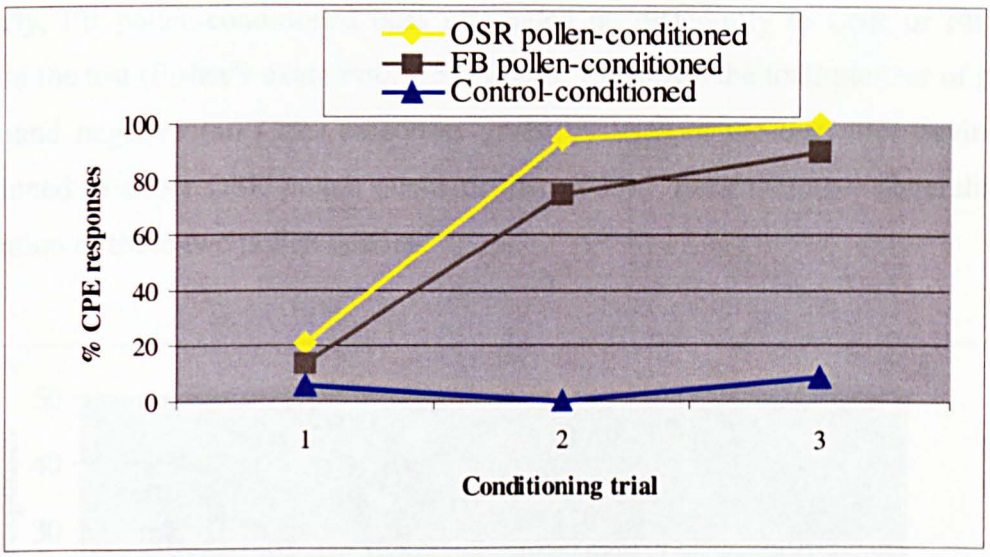


Figure 7.5 Associative learning of the odours from oilseed rape (OSR) and field bean (FB) bee-collected pollen by restrained honey bees: the proportion of conditioned proboscis extension (CPE) responses exhibited by bees during 3 associative conditioning trials to the odour OSR and FB pollens and a clean air control

A significantly larger proportion of OSR pollen-conditioned bees responded positively to this conditioning odour (i.e. were 'learners') during the final conditioning trial (C3) than the proportion of control-conditioned bees that responded positively to the control at C3 (Fisher's exact $P<0.001$). Similarly, FB pollen-conditioned bees exhibited a CPE response more often to the odour of FB at C3 than control-conditioned bees responded to the clean air (Fisher's exact $P<0.001$). The proportion of 'learners' at C3 was higher for OSR pollen-conditioned bees than FB pollen-conditioned bees (Fisher's exact $P=0.003$). The conditioning efficiency (Pham-Delègue *et al.*, 1993) of OSR and FB pollens can be found in table A.4.1 of Appendix 4 (this compares, for each conditioning odour, the: numbers of bees that learned the odour, 'nonlearners', 'nonselective learners', bees that did not respond at the end of testing with a PER and numbers finally used in the generalization test).

7.6.1.2 Generalization

OSR pollen-conditioned bees showed no difference in the number of CPE responses to the odour of OSR or FB pollen in the generalization test (Fisher's exact P=0.616). Similarly, FB pollen-conditioned bees responded no differently to OSR or FB pollen odour in the test (Fisher's exact P=0.115). Figure 7.6 shows the total number of positive (CPE) and negative (no CPE) responses given by bees in the test after having been conditioned to either OSR pollen odour or that of FB. Bees therefore generalized the recognition of these two pollen odours.

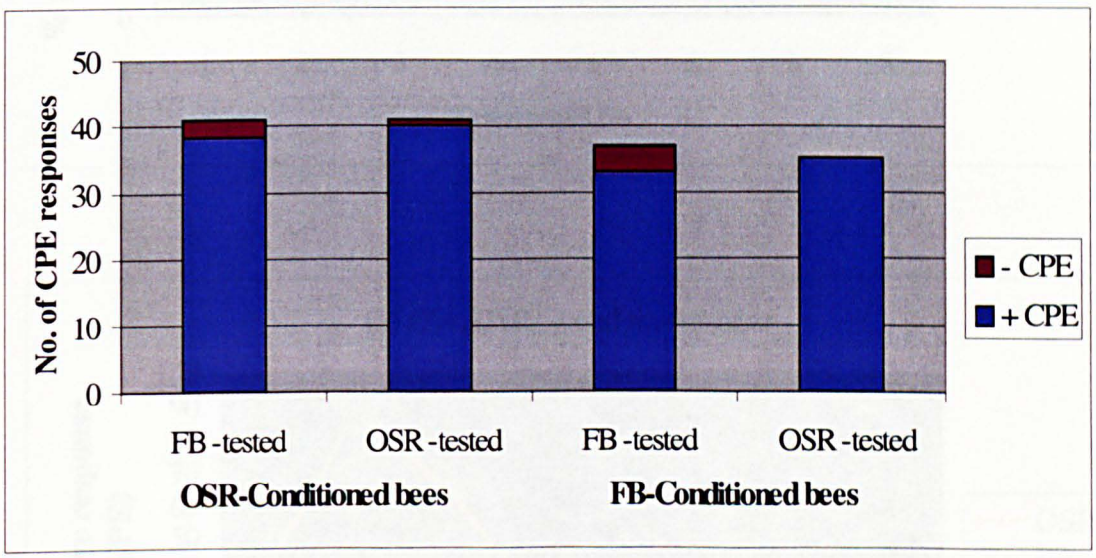
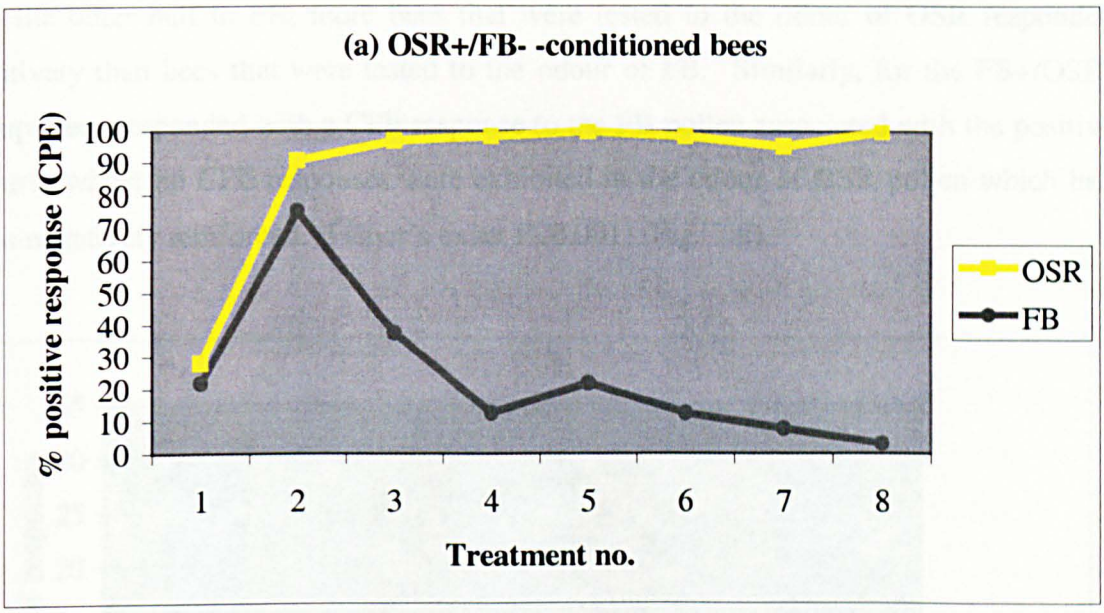


Figure 7.6 Generalization of the odours from oilseed rape (OSR) and field bean (FB) bee-collected pollen by restrained honey bees: the total number of positive conditioned proboscis extension (+CPE) and negative (-CPE) responses to a test odour of either OSR or FB after conditioning to either OSR or FB

7.6.3 Discrimination between the odours of oilseed rape and field bean bee-collected pollens

The bees' learning process throughout the discriminative conditioning trials is shown in Figures 7.7a and 7.7b for bees positively conditioned to OSR and negatively conditioned to FB (OSR+/FB-) and for bees positively conditioned to FB, negatively rewarded to OSR (FB+/OSR-), respectively. Bees quickly learned to discriminate the odour associated with the positive reward from that associated with the negative reward, with the proportion of bees responding to the negatively rewarded odours declining after just two presentations of each odour.



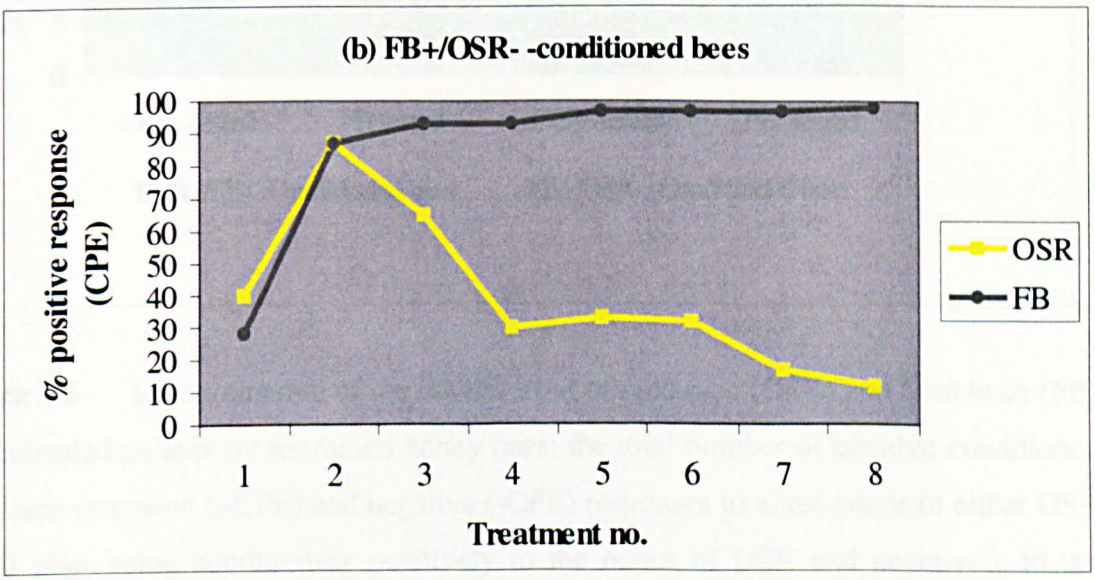


Figure 7.7 The proportion of conditioned proboscis extension (CPE) responses exhibited by restrained honey bees during successive paired presentations of a positively-rewarded (sucrose) and negatively-rewarded (salt) odour (either OSR or FB bee-collected pollen) during discriminative conditioning: (a) bees positively conditioned to OSR, negatively conditioned to FB (OSR+/FB-); (b) bees positively conditioned to FB, negatively conditioned to OSR (FB+/OSR-)

In the discrimination test, in which half the OSR+/FB- group of bees were tested to OSR, and the other half to FB, more bees that were tested to the odour of OSR responded positively than bees that were tested to the odour of FB. Similarly, for the FB+/OSR- group, bees responded with a CPE response to the FB pollen associated with the positive reward, whilst no CPE responses were exhibited to the odour of OSR pollen which had been negatively reinforced. (Fisher's exact $P<0.001$) (Fig. 7.8).

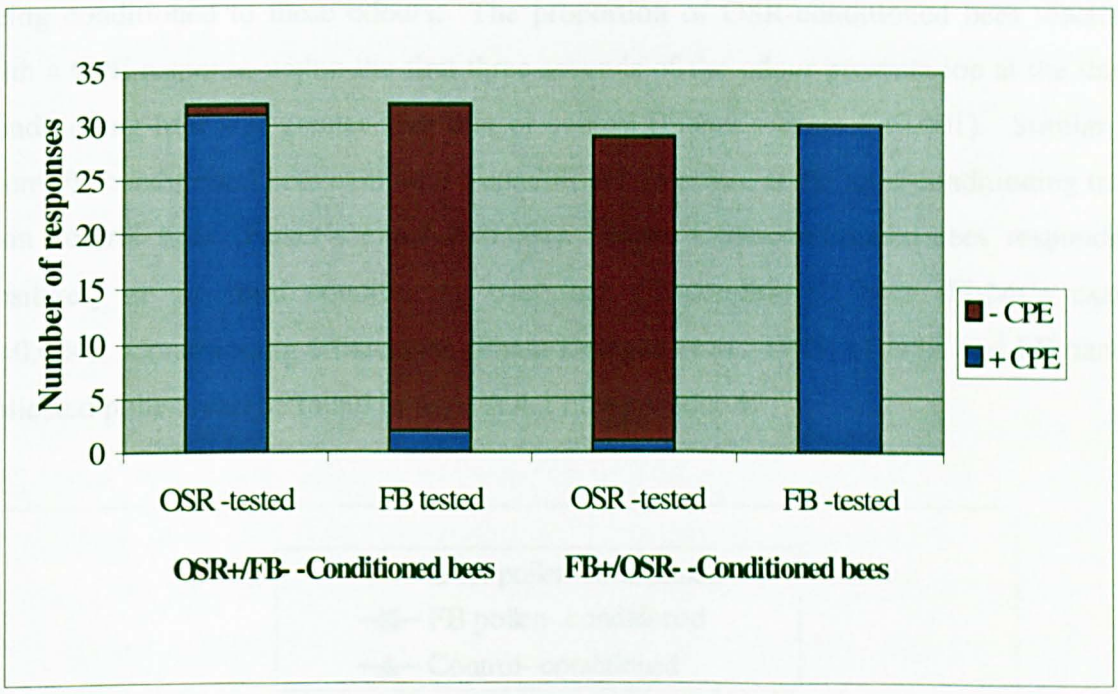


Figure 7.8 Discrimination of the odours from oilseed rape (OSR) and field bean (FB) bee-collected pollens by restrained honey bees: the total number of positive conditioned proboscis extension (+CPE) and negative (-CPE) responses to a test odour of either OSR or FB after being conditioning positively to the odour of OSR and negatively to the odour of FB (OSR+/FB-) or positively to the odour of FB and negatively to the odour of OSR (FB+/OSR-)

7.6.4 Learning and generalizing the recognition of the odours from hand-collected oilseed rape and field bean pollens

7.6.4.1 Associative learning of hand-collected pollens

Bees learned to associate the odour of both OSR and FB hand-collected pollens with a reward, but not the odour of the clean air control. Fig. 7.9 shows the odour-acquisition curves of hand-collected OSR and FB pollen odours against a clean air control by bees being conditioned to these odours. The proportion of OSR-conditioned bees reacting with a CPE response within the first three seconds of the odour presentation at the third conditioning trial was greater than that of control (Fisher's exact $P<0.001$). Similarly, more FB-conditioned bees exhibited a conditioned response at the third conditioning trial than control bees (Fisher's exact $P<0.001$). More OSR-conditioned-bees responded positively at the third conditioning trial than FB-conditioned bees (Fisher's exact $P=0.004$). Conditioning efficiencies (Pham-Delègue *et al.*, 1993) of OSR and FB hand-collected pollens can be found in table A.4.2 of Appendix 4.

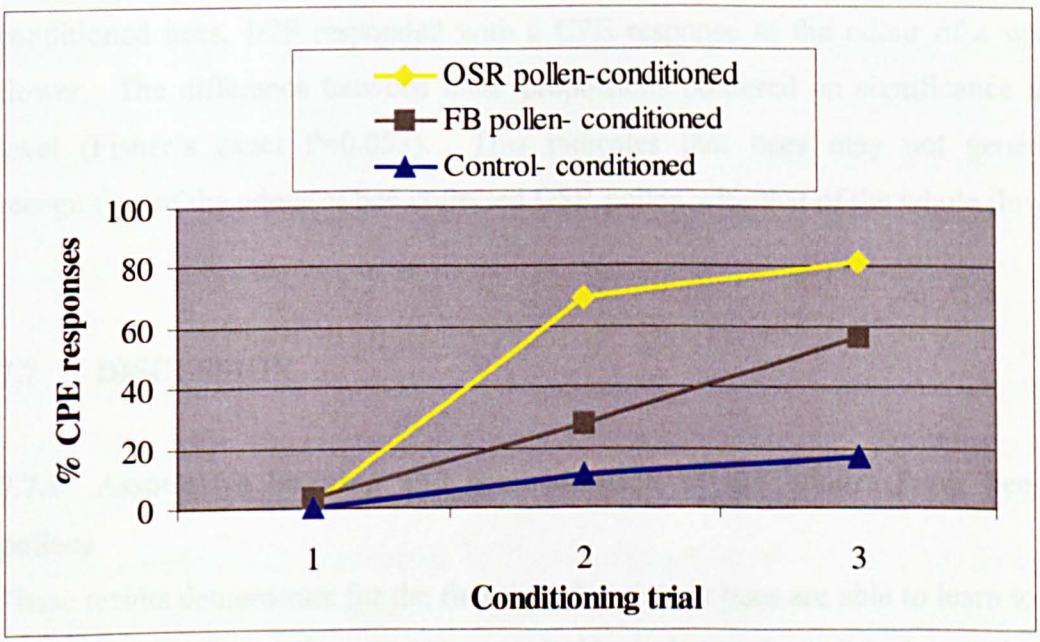


Figure 7.9 Associative-learning of the odours from oilseed rape (OSR) and field bean (FB) hand-collected pollen by restrained honey bees: the proportion of conditioned proboscis extension (CPE) responses exhibited by bees during 3 associative conditioning trials to the odour OSR and FB pollens and a clean air control

7.6.4.2 Generalization

In the generalization test, bees conditioned to the odour of hand-collected FB pollen showed no difference between the number of positive responses to OSR or FB odour in the test: 15/16 showing positive CPE responses to the presentation of FB pollen odour, and 12/16 responding to OSR pollen odour (Fisher's exact P=0.332). However, when conditioned to the odour of hand-collected OSR pollen, 24/25 bees responded positively to the presentation of OSR pollen odour in the test, whilst only 22/29 responded to the odour of FB pollen. The differences in these proportions borders on significance at the 5% level (Fisher's exact P=0.056). These results indicate that whilst bees conditioned to the odour of FB hand-collected pollen generalize the recognition of this odour to that of OSR pollen, bees conditioned to the odour of OSR hand-collected pollen may not generalize these two pollen odours (i.e. they may discriminate them).

7.6.5 Generalizing the recognition of the odour of oilseed rape pollen with that of the whole flower

Of the bees that were conditioned to the odour of OSR bee-collected pollen, 7/24 responded with a CPE response to the odour of a whole OSR flower. Of the control-conditioned bees, 1/29 responded with a CPE response to the odour of a whole OSR flower. The difference between these proportions bordered on significance at the 5% level (Fisher's exact P=0.053). This indicates that bees may not generalize the recognition of the odour of bee-collected OSR pollen with that of the whole flower.

7.7 DISCUSSION

7.7.1 Associative learning and generalization of the odours from bee-collected pollens

These results demonstrate for the first time that honey bees are able to learn to associate the odour of pollen with a sucrose reward. Upon the third conditioning trial, more bees had learned to associate the odours of bee-collected OSR and FB pollens with the reward than control bees conditioned to clean air.

Spontaneous responses to pollen odour shown at the first conditioning trial before any paired presentations of the conditioning odour and the reward had been made, were relatively low (below 20%). Such responses are either weak innate responses or

responses evoked by memory of past foraging experiences (Menzel, Greggers & Hammer, 1993; Gerber *et al.*, 1996). Associative conditioning elevated the proportion of bees responding to a test odour with a proboscis extension response, indicating learning. Both pollen odours were learned quickly, with about 80% bees showing a conditioned response after only one paired presentation of the pollen odours and the reward, rising close to 100% by the third conditioning trial. Such results are typical of classical associative odour conditioning of the PER. Menzel & Müller (1996) quote that 'a single pairing of the odour as the CS with sucrose as the US changes the PER probability from a spontaneous level of usually $\leq$10% to a level of $\geq$ 60% and multiple trials to an asymptotic level of $\geq$ 80%'. It is tempting to conclude that such high degrees of learning indicate that pollen odours are biologically meaningful to bees; indeed, bees often associatively learn floral odours better than other odours of the same concentration (Marfaing, Rouault & Laffort, 1989). However, learning does not prove importance, since bees will eventually learn to associate any odour, even repellent odours such as Lysol (von Frisch, 1967; Menzel, 1990) and propanol (Bitterman *et al.*, 1983). Currently, there is no evidence of any odour to which bees cannot be conditioned using a sucrose reward; even carbon dioxide, water vapour and the bees' own sting alarm pheromone, can be associatively-learned (Menzel, 1985). Resistance to extinction in trials where the learned odour is presented unrewarded over successive trials can be used as a measure of the extent and the magnitude of the association between the conditioning odour and reward (Rescorla, 1988; Smith & Getz, 1994) and such tests could be performed in future experiments.

There was evidence that bees learned the odour of OSR pollen more efficiently than that of FB. This difference occurred for both bee-collected and hand-collected pollens, and could be due to differences in salience of the components within the two odours, or could indicate differences in concentration. The salience of an odour refers to a constant and experience-independent feature of a conditioning odour, which determines the rate at which it can enter into associations with a reward (Pelz, Gerber & Menzel, 1997). Certain odours are more salient conditioning stimuli than others – for example aldehydes, ketones, and alcohols were more salient conditioning odours than acetate and monoterpene conditioning odours, evoking a higher level of associated response (Smith & Menzel, 1989). Thus the odour of OSR pollen may contain more salient components than that of FB. In addition, odours at high concentration also support stronger

associations (Pelz, Gerber & Menzel, 1997). If the concentration of odours of OSR pollen were higher than that of FB pollen, then this could also explain this difference. Bees generalized the recognition of the odours of bee-collected OSR and FB pollens, indicating that bees perceive these two odours as having some similarities. This suggests that they have similar components in their volatile profiles, that some (or all) of the key compounds learned in odour recognition of OSR bee-collected pollen as a conditioning odour were present in the odour of bee-collected field bean as a novel odour and vice-versa. These components could be the 'bee-derived' volatile components of the bee-collected pollen loads or 'pollen' components that are present in both pollen odours. A generalization test using hand-collected pollens (without 'bee-derived' components) was conducted to investigate this further, and is discussed below. Results indicated that generalization was partially, if not completely, based on 'bee-derived' components.

Generalization may also have occurred due to the components in the two pollen odours being chemically related (rather than identical as suggested above). Generalization by bees to related chemicals having similar structures has been demonstrated (Smith & Menzel, 1989; Laska *et al.*, 1999). Smith & Menzel (1989) used electromyogram quantification of the CPE response in which the potential evoked by one of the muscles (M17) that operate during proboscis extension was measured. In these studies it was found that although responses to novel odours occurred in generalization, the intensity of response was less strong than that to the conditioning odour, and the intensity of the generalized response differed according to structural similarity to the conditioning odour. In tests presented in this chapter, generalization was recorded according to the presence or absence of CPE responses. Measurement of the intensity of the response in future generalization tests involving pollen odours, either by electromyogram recordings (Rehder, 1987; Smith & Menzel, 1989) or by recording the duration of response (Smith & Cobey, 1994) may provide a more precise measure of the bees' perceptual similarity of the conditioning and test odours.

7.7.2 Discrimination between the odours of bee-collected pollens

A generalization procedure can describe perceptual similarity because it does not depend on a complete lack of an ability to discriminate two odours (Smith & Getz, 1994). Therefore, that bees generalized between the two pollen odours does not mean that they were not able to discriminate between them; they did not discriminate between them given that particular conditioning regime. When bees were differentially conditioned,

discrimination test results indicated that honey bees can discriminate between the odours of OSR and FB bee-collected pollens.

In the early stages of odour presentations during the discriminative conditioning process, initial generalization was observed: a large number of bees responded to the odours of both pollens upon their second presentation, despite having been negatively conditioned to one of them once. This phenomenon has been noted in other discriminative learning studies (Bitterman *et al.*, 1983) and is due to the generalization of the recognition of the components that are similar in both pollens which is initially high, but decreases as differential learning progresses (Menzel & Müller, 1996).

The ability of bees to discriminate OSR from FB odour suggests that OSR and FB pollens contain chemical differences which bees used to distinguish between them. This is further supported by GC analysis of the volatile profiles of these bee-collected pollens (Appendix 1.2). Assuming that the 'bee-derived' components of both these pollens are the same, since the two pollens were collected from a pollen trap on the same hive, these differences could be due to variations in the 'pollen' components between the two species. This is also supported by GC analysis of the volatile profiles of these hand-collected pollens (Appendix 1.2). These differences could be biologically important, and used for discrimination between species.

7.7.3 Learning and generalization of the odours of hand-collected pollens

When bees pack the pollen collected from flowers into their corbicular loads, they add honey from their stomach to help the grains stick together (Hodges, 1952, 1984). Pollen loads therefore do not consist of pure pollen as is found in flowers, and contain 'bee-derived' elements such as honey and salivary enzymes. Both these components may alter the volatile profile of pollen by the addition of some new components and altering others by enzymic action. The volatile profiles of bee-collected pollen and hand-collected pollens are therefore different (Appendix 1.2). To determine the extent to which generalization of the recognition of bee-collected OSR and FB pollens is attributed to the common 'bee-derived' components of the pollen loads rather than common 'pollen' components, the generalization experiment was repeated using hand-collected pollen from the flowers of OSR and FB.

Bees associatively learned the odours from OSR and FB hand-collected pollens. Interestingly, they did not learn the odours of hand-collected pollens as well as they learned the odour of bee-collected pollens. In particular, the odour of hand-collected FB was not learned as well as the odour from the same amount of bee-collected FB pollen; by the end of the third conditioning trial, 100% and 90% of bees had learned to associate OSR and FB bee-collected pollens, respectively, compared to 81% and 56% learning these hand-collected pollens. Although these experiments were not conducted simultaneously, this difference may be due to the differences in concentration between the odours of hand- and bee-collected pollens. There was little noticeable difference to the human sense of smell between the intensity of the odours of bee-collected OSR and FB pollens (personal observation of the effluent from the airflow delivery tube). Although they were noticeably different (OSR pollen smelled characteristically of the flower, whilst FB was more 'subtle', smelling of green-leaves rather than of FB flowers), they could both be smelled at similar intensity at the beginning and end of conditioning trials. Conversely, hand-collected OSR pollen did not smell as strong as bee-collected pollen, but its odour was noticeable, whilst the same amount of hand-collected FB pollen smelled so faint it was hardly perceptible. Since concentration is related to salience (Pelz, Gerber & Menzel, 1997) it would be expected that the lower concentration of hand-collected pollens would be less well associated than the higher odour concentrations of bee-collected pollens.

Von Frisch's experiments (1967) showed that the honey bee's sense of smell was similar to that of humans, although it has since been shown to be much more sensitive (e.g. Ribbands, 1954). Bees may therefore have perceived a concentration difference between the odours of hand-collected OSR and FB pollens. Such concentration differences may explain the differences in the generalization results using hand-collected pollens. Bees conditioned to the odour of hand-collected FB pollen generalized its recognition with that of hand-collected OSR pollen, whilst it is statistically doubtful that bees conditioned to the odour of hand-collected OSR generalized its recognition with the odour of hand-collected FB pollen. In experiments to determine the effects of odour intensity on generalization abilities of honey bees, Bhagavan & Smith (1997) found that when conditioning to odours of low concentrations, responses to a novel odour (or higher concentrations of the same odour) resulted in strong generalization, whilst at higher conditioning concentrations, significantly less generalization was observed to a novel odour or to a lower concentrations of the same odour. Odours at higher concentrations

are more salient, having a stronger capacity to act as discriminative stimuli (Pelz, Gerber & Menzel, 1997); hence the near-ability of bees conditioned to the odour of OSR pollen (of a high concentration) to discriminate between this and the novel FB odour (of lower concentration).

In the absence of 'bee-derived' compounds, concentration of 'pollen' components became an issue in the learning and generalization of the odours of hand-collected pollens. This suggests that the generalization of the recognition of bee-collected pollens was at least partially due to bees learning 'bee-derived' components during mixture recognition which were present in both species. However, since bees conditioned to the odour of hand-collected FB pollen generalized its recognition with that of OSR, there is some evidence of similarities between the 'pollen' component of pollens. Further work is required to investigate the effects of 'bee-derived' components and odour concentration in the generalization of pollen odours.

7.7.4 Generalizing the recognition of the odour of oilseed rape pollen with that of the whole flower

This experiment, to assess how similar bees find the odour of oilseed rape pollen and that of the whole flower, was conducted to investigate the possibility that pollen-seeking bees could learn pollen odours from amongst the flower during foraging to help them locate this resource in other flowers. Generalization tests between OSR bee-collected pollen and the whole flower odours were inconclusive. Although more bees conditioned to the odour of OSR pollen responded to the odour of a whole OSR flower than did bees conditioned to the clean air control, the difference only bordered on significance. Thus it is difficult to conclude whether or not bees conditioned to OSR pollen generalize the recognition of this odour with that of a whole flower. Bees were conditioned to a large amount of pollen, relative to that found in the flower, so quantitative differences may have contributed to this result. Bees conditioned to a high concentration of an odour did not respond to the same odour presented at a concentration 100 times lower; and absence of perception was not responsible for this, as bees were subsequently conditioned to respond to concentrations 1000 times lower than the first (Vareschi, 1971).

Had bees generalized in this test, the result would have implied that the compounds learned in pollen odour recognition were also present in the flower. This could have been due to pollen and floral odours being similar (e.g. due to the adsorption of floral

odours onto the pollenkitt), or could have implied the existence of pollen specific volatiles learned during conditioning to pollen odour and perceived amongst the whole-flower volatiles by bees. Such information could have provided evidence that pollen volatiles can be learned by foraging bees from rewarding flowers, and perceived amongst the volatiles of other rewarding flowers. Since no previous work has been conducted to investigate this possibility, this experiment is worth repeating, and a few changes to the method could easily improve it, with the hindsight of results presented in this chapter. As before, one group of bees could be conditioned to clean air as controls, and another group to the odour of OSR pollen. If bees were conditioned to pollen-presenting anthers from an OSR flower, this would remove differences occurring due to the presence of 'bee-derived' components in the pollen and not the flower, and remove differences due to the quantity or concentration of conditioning and test odours. A generalization test consisting of the odour from an OSR flower could then be presented to both groups as before. This would demonstrate whether or not compounds learned in the recognition of OSR pollen are perceived amongst the odour of an OSR flower. Simultaneous, reciprocated conditioning of a group of bees conditioned to the odour of an OSR flower and tested to the odour of OSR anthers would demonstrate whether or not key compounds learned in the recognition of OSR flowers were present in its pollen: this test is vital to address the question fully.

7.7.5 General discussion

The experiments described in this chapter were performed between the months of October and February. These months are amongst the best for odour conditioning of bees taken from outside colonies (Ray & Ferneyhough, 1997a). They were therefore conducted using 'winter bees', which had probably not in their lifetime foraged on either OSR or FB (since both these crops finish flowering around August), thus reducing pre-conditioning to either of these pollen types that may have influenced spontaneous responses and learning abilities (Gerber *et al.*, 1996). Use of bees kept in a flight room (see chapter 8) whose foraging histories are known could further decrease any possibility of pre-conditioning. Bees were taken from the hive entrance, a routine practice by most workers utilising the CPE bioassay. These bees are likely to be either foragers, cleaning bees or guards (Winston, 1987), and thus bees used in experiments may have differed in their colony duties and ages. The age of bees (Pham-Delègue, De Jong & Masson, 1990) as well as their role in the colony (Ray & Ferneyhough, 1997b) have been found to affect the ability of bees to learn, and may partially account for variation in bees' abilities to

discriminate odours (Smith, Abramson & Tobin, 1991). Use of bees of known age (Sandoz, Roger & Pham-Delègue, 1995) may reduce some of the variability in results, and should be considered for future experiments.

These experiments clearly demonstrate for the first time that honey bees can associatively-learn the odours of pollen. There was evidence that OSR pollen was more easily learned than FB pollen, either due to the presence of more salient components in OSR than FB or due to its greater odour intensity. Such differences may reflect the importance of pollen odour in these two species as attractants in facilitating the reproduction of the plant. OSR flowers expose their anthers so that pollen can be both seen and smelled by approaching flower-visiting insects. It is quite likely, therefore, that the pollen of OSR (or its wild ancestors) has evolved to have some attractive properties to pollinating insects. This is supported by evidence presented in chapters 3, 4 & 8: that OSR pollen odour is attractive to pollen beetles and honey bees. Conversely, the petals of FB flowers enclose the anthers within the flower, so the pollen is concealed. The pollen of this species is probably less likely to have evolved attractive or salient properties since only the petals are exposed to the environment.

The odours of OSR and FB pollens comprise mixtures of volatile compounds (Appendix 1.2). Bees probably used a few key components of the OSR and FB pollen odours for recognition, as in other floral mixtures (Waller, Loper & Berdel, 1974; Pham-Delègue *et al.*, 1986, 1993, 1997). Through perceptual similarities and differences, they then generalized and discriminated their recognition with novel pollen odours when these were presented. Further work using CPE coupled with gas chromatography (GC) (GC-CPE) techniques (Blight *et al.*, 1997) could be employed to identify which of the volatile components are used in the recognition of OSR and FB pollen odours. Using these methods, bees would be either conditioned to OSR or FB pollen odours. An air entrainment (Blight, 1990; Appendix 1) made of the conditioning odour would be separated into its constituent parts by GC and a conditioned bee placed in the effluent would be exposed to its component parts as they eluted from the GC column. Bees would exhibit CPE responses to the volatiles used in the conditioning odour recognition as they eluted. Such volatiles could be subsequently identified by GC-mass spectroscopy (GC-MS). In this way, the compounds involved in both OSR and FB pollen recognition could be identified. Furthermore, components used in generalization could be similarly

identified by observing the CPE responses of an OSR-conditioned bee to the separating components of FB pollen and vice-versa.

In conclusion, these experiments demonstrated that honey bees can associatively learn the odours of pollen. They suggest that although pollens have similarities in their chemistry they also contain differences that may be used in species discrimination. The CPE bioassay has great potential to identify the key components learned in pollen odour recognition and to assess the possibility that such components are associatively learned from rewarding flowers and used in location of other rewarding flowers by pollen-seeking bees. However, there has been concern that use of restrained bees in CPE bioassays does not relate to natural foraging conditions (Mauelshagen & Greggers, 1993); bees in restrained conditions are taught to associatively learn particular stimuli, and just because they can learn a particular stimulus and use its properties in discrimination tasks in these conditions, does not mean that they do so in the natural situation, where bees have a choice. The choice-behaviour of free-flying bees conditioned to pollen stimuli in more natural foraging conditions is addressed in the next chapter (8).

8. Conditioned responses of free-flying honey bees to pollen cues

8.1 ABSTRACT

The responses to the odour and colour of oilseed rape (OSR) and field bean (FB) pollens by free-flying honey bees were examined using an artificial flower. Bees were conditioned to one type of pollen (either OSR or FB; the other served as the novel pollen type) which served as both the reward and the conditioning stimuli (i.e. colour and odour). Bees were then given a rewarded choice test between the conditioning pollen type and the novel pollen type. The number of first landings, visits, and duration of visits to each of the pollens in the choice test were recorded. Bees discriminated between OSR and FB pollens when conditioned to OSR (i.e. they chose OSR more often than FB in the choice test). However, when conditioned to FB, an asymmetrical result was found, in which bees showed no more responses to FB than to OSR. This difference could be due to variation in the nutritional qualities between the two pollens, resulting in different strengths of association being formed between the two pollens and their stimuli. Alternatively, it could be due to a preference for the stimuli of OSR pollen that overrides conditioning to FB pollen stimuli. To investigate further any such preferences, experiments were conducted in which the reward was kept constant, and pollen colour and odour stimuli were separated. Since it is difficult to condition bees to pollen without either its colour or odour, bees were conditioned in reciprocal experiments to an equally rewarding sucrose solution associated, in one experiment, with the odour of OSR or FB pollen, and in a second experiment, with pollen colours. Bees were tested in unrewarded choice tests to the stimuli of the conditioned pollen and the novel pollen. When conditioned to pollen odour alone, bees discriminated between the odours from OSR and FB pollens, making more first landings, visits, and spending more time searching the feeder in the choice test that emitted the odour to which they had been conditioned. When bees were conditioned to the colour of the pollens (OSR yellow, or FB grey), they showed no difference in the mean time spent searching a coloured feeder, irrespective of conditioning. However, they made significantly more visits to OSR yellow when

conditioned to this, but showed no difference in the number of visits to either colour when conditioned to FB grey. The possibility of a preference for OSR yellow as the reason for the preference for OSR pollen over FB pollen is discussed.

8.2 INTRODUCTION

8.2.1 The use of floral cues by foraging honey bees

The diversity of floral colour and odour patterns in angiosperm plants is known to be an adaptation aimed at their pollinators' capacity for learning and discriminating these advertisement signals (Chittka & Menzel, 1992; Menzel & Shmida, 1993). This species-specific labelling improves pollen transfer between the flowers of the same species and is based on the flower constant foraging behaviour of the pollinators (Waser, 1986). When foraging for nectar and pollen resources, honey bees (*Apis mellifera* L.) associatively-learn these floral cues with the food rewards gained from their visit. The learning process is directed initially on by 'innate search images' which guide their orientation and shape their learning processes (Menzel, 1985). The learned cues are later used as reward-predicting stimuli that help bees to identify further rewarding flowers. Colour is probably most important in identifying flowers of the rewarding species from afar, whilst odour cues are used by the bee at closer range, to confirm that the flower is of the correct species (Backhaus, 1993).

8.2.2 Associative-learning of floral colour cues by foraging honey bees

The colour vision of honey bees was first demonstrated by von Frisch (1914). Bees were rewarded with sucrose solution upon visits to a feeder on blue-coloured cardboard. In an unrewarded choice test, in which the coloured board was placed amongst a range of others of various shades of grey, bees searched the blue-coloured board more often than any of the others. The decision of the bee in colour discrimination tests is assumed to be directly related to the perceptual colour difference/similarity measure of the bee. Honey bees have colour-vision enabled by three colour receptors, ultra-violet (UV), blue and green, with maxima of their spectral sensitivity functions at 350 nm, 440 nm and 540 nm, respectively (Daumer, 1956). The perception of colours by honey bees has been reviewed by Menzel & Backhaus (1991); Backhaus (1992, 1993), and insect vision and flower-recognition is discussed in a series of papers edited by Dafni, Giurfa & Menzel (1997).

Colour learning and discrimination by honey bees was studied in depth by Menzel (1967), who conditioned bees to monochromatic lights (spectral colours) under constant background conditions, and then tested them in a dual choice test. Violet (400-420 nm) was learned fastest and was chosen most accurately against a novel colour and blue-green was learned slowest, with least precision. Colour learning was dependent on the wavelength, rather than a function of brightness, and spectrally pure (saturated) colours were learned faster than unsaturated colours, although they could be easily discriminated from each other (Menzel, 1967). More recently, it was found that another important determinant of colour discrimination is the colour contrast against the background and to other flowers (Menzel & Shmida, 1993; Goulson, 2000).

8.2.3 Associative-learning of floral odour cues by foraging honey bees

Odours are learned faster and are chosen more accurately than colours, and flower-like odours are learned faster than other odours (Menzel, 1985). Bees may have an 'innate preparedness' to associate floral odours with a food source, but their general learning ability is powerful enough to enable them to learn any odour as a food signal. Olfactory conditioning of honey bees in a free-flying situation was first investigated by von Frisch, (1919). Bees were simultaneously offered several odours, one of which was presented with sucrose solution as a reward. Bees became strongly attracted to the odour associated with the sucrose, and this attraction remained after the suppression of the reward and the randomization of the position of the feeder. Bees discriminated 28 from 32 pairs of odours, and partially 'confused' the others, which smelled similar to humans. Since this study, honey bee learning of odours has progressed using two approaches: the restrained approach and the free-flying approach. In the former, bees are harnessed in holders and learning is studied by observing a stimulus-induced behavioural reflex (the PER- proboscis extension reflex) controlled by the operator. The free-flying approach is conducted in less controlled, but more natural situations, and learning is a consequence of bees' decisions during foraging. The restrained approach was the focus of the previous chapter (7), which demonstrated that bees <u>can</u> learn and discriminate pollen odours. In this chapter, the free-flying approach is used to investigate whether bees <u>do</u> learn these cues and use them in discrimination in more natural foraging situations.

8.2.4 Choice performance in foraging

Bees tend to maintain floral constancy within a species during successive visits, especially if the flowers are abundant, and if by doing so, foraging becomes more

effective and less risky (Heinrich, 1983; Shapiro, 2000). Foraging bees participate in information flow within the colony about alternative food sites (Seeley, 1994). Therefore each bee is informed about the effectiveness of its foraging performance. Bees focus their efforts on the most productive flowers, optimizing their foraging efforts by choosing flowers with high rewards as opposed to those with lower rewards (Heinrich, 1983). This has been well-demonstrated on nectar-foraging bees in experiments that have altered the quantity or concentration of sucrose solution delivered to feeders in choice tests (Couvillon & Bitterman, 1991; Loo & Bitterman, 1992; Greggers & Menzel, 1993). Some studies have directly addressed the effects of floral cues on foraging behaviour. When the amount of reward associated with a colour was reduced, honey bees used colour cues to shift their foraging choices to a new, more rewarding colour (Giurfa & Núñez, 1989). Banschbach (1994) assessed how interactions between floral stimuli and reward strength affected choice behaviour by varying colour association and sucrose concentration of the reward. Similar interactions have been studied using different odour cues (Couvillon & Bitterman, 1991). However, the effect of pollen cues on the choice behaviour of bees has not yet been determined.

8.3 AIMS

The aim of this set of experiments was to investigate the ability of free-flying honey bees to learn to associate oilseed rape (OSR) and field bean (FB) pollen odour and colour cues with a pollen reward, and to investigate the roles of these pollen cues in discriminating between species.

A set of three experiments was conducted, investigating bees' abilities to discriminate between these two pollens using:

1. Colour and odour cues from the pollen
2. Pollen colour cues alone
3. Pollen odour cues alone

8.4 MATERIALS AND METHODS

The conditioned responses of free-flying honey bees to pollen cues were investigated using artificial flowers in a bee-flight room.

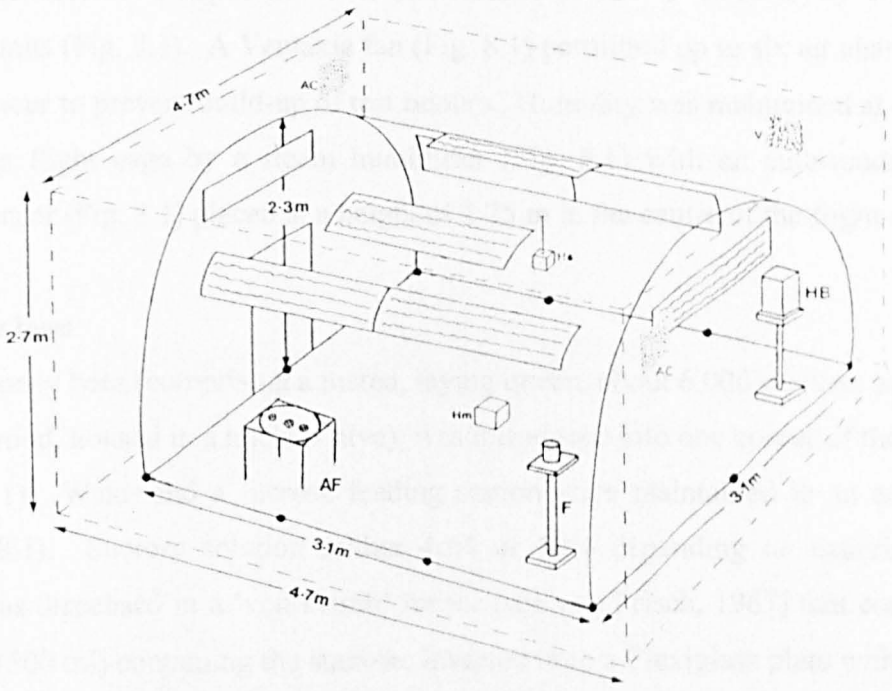


Figure 8.1 The bee flight room: showing flight cage within constant environment room and lighting panels around. AC = air conditioning unit; V = ventilation unit; Hm = humidifier; Hs = humidistat; AF = artificial flower; F = feeding station; HB = honey bee colony

8.4.1 The bee flight room

The bee flight room facility at Rothamsted has been described in detail by Poppy & Williams (1999). It consists principally of a flight cage contained within a windowless constant-environment room (Fig. 8.1). The flight cage (3.2 x 3.1 m x max. height 2.3 m) was made of netting (Clear Saran fabric, 0.78 x 0.38 mm mesh; Simpers Ropeworks Ltd, Cambridge, UK) supported by steel tubes. The cage was lit by eight luminaires (5062 PSN/2; Trilux lighting Ltd., Surrey, UK), fitted with white-light flourescent tubes (1500 mm Phillips 58 watt T8) and /or UV light fluorescent tubes (1500 mm Phillips 80 watt TL05), arranged around the room outside the cage (Fig. 8.1). Each was fitted with a prismatic cover to diffuse the light. The lights had high flicker frequencies (>300 Hz) to give the appearance of uninterrupted light to honey bees, which have a light flicker fusion frequency of 300 Hz (van Praagh, 1972). This was facilitated by an electricity supply to the room via an ultra-high frequency ballast (>25 kHz). The lights were controlled by a single phase, two-channel time switch providing a cyclic photoperiod of 10:12 h light/dark, with a half-hour dawn and dusk simulation.

The controlled environment room (4.7 m x 4.7 m x 2.7 m) was painted white throughout to aid light diffusion. Temperature was maintained at 23 °C ± 1 °C by two air-conditioning units (Fig. 8.1). A Ventaxia fan (Fig. 8.1) permitted up to six air changes in the room per hour to prevent build-up of test odours. Humidity was maintained at 60% ± 5% within the flight cage by a steam humidifier (Fig. 8.1) with an auto-modulating control and sensor (Fig. 8.1) placed at a height of 2.25 m in the centre of the flight cage.

8.4.2 Honey bees

A colony of honey bees (comprising a mated, laying queen, about 6,000 workers and five combs with brood, housed in a nucleus hive), was introduced into one corner of the flight room (Fig. 8.1). Water and a sucrose feeding station were maintained in an adjacent corner (Fig. 8.1). Sucrose solution (either 40% or 50% depending on experimental conditions) was dispensed in a 'von Frisch' feeder (see von Frisch, 1967) that consisted of a glass jar (500 ml) containing the sucrose, inverted onto a Plexiglass plate with radial grooves from which nectar-collecting bees could imbibe the solution. A pollen feeding station that consisted of two Petri dishes filled with honey bee-collected pollen (8.4.3) on a white turntable was maintained in the corner opposite the hive (Fig. 8.1) for pollen-foraging bees. The bees were left for two weeks to 'acclimatise' to their new conditions before experiments began (Poppy & Williams, 1999). This meant that bees participating in the experiments were flower-naïve, since they had emerged in the flight room.

8.4.3 Pollen

Oilseed rape (OSR) and field bean (FB) honey bee-collected pollen (2.3.2), that had been collected during the previous summer and stored frozen at –20 °C, was thawed and used in all experiments described in this chapter. When experiments were not in progress, bees were fed pollen of mixed species that excluded pollen from test species (OSR and FB) to eliminate non-experimental conditioning.

8.4.4 Test arena with artificial flowers

A test arena containing artificial flowers (e.g.Waller, 1972; Pham-Delègue *et al.*, 1986) was used in experiments in which the responses of honey bees to both pollen colour and odour were assessed (section 8.5) (see Fig. 8.2). The test arena was supported on a white platform (40 cm x 40 cm), 1 m above the floor. It consisted of a transparent Perspex box (40 cm x 40 cm x 30 cm) with plastic mesh-covered ventilation holes. The bees entered

the arena box through a diamond shaped aperture in one of its sides. The aperture was adjustable in size to restrict or permit bees' entry into the arena. A white plastic turntable (30 cm diameter) was placed inside the arena, upon which three artificial flowers could be positioned equidistant from each other, perpendicular to the bees' line of flight. An artificial flower consisted of a small glass Petri dish base (35 mm diameter) containing pollen.

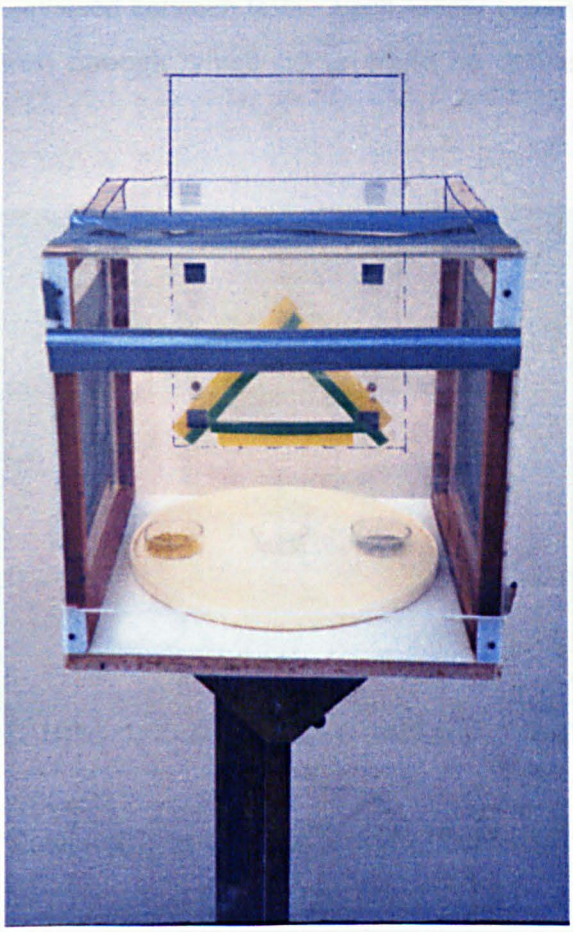


Figure 8.2 Test arena showing position of the conditioning artificial flower (centre) and test artificial flowers (outer dishes) containing oilseed rape (yellow) and field bean (grey) pollens

8.4.5 The 'floweroid'

The 'floweroid' (Fig. 8.3) (Poppy *et al.*, unpublished data) combines the principles of an artificial flower with those of electrophysiology, which involve the delivery of naturally realistic amounts of odour in an air stream. This apparatus allows for the first time the delivery of odours in quantifiable amounts to investigate bees' responses to floral volatiles. In the following experiments, it was used to assess the ability of the honey bee

to discriminate between pollens using pollen odour cues alone, and its use was modified to assess the importance of pollen colour in pollen discrimination.

The top platform of the floweroid comprised three artificial flowers (feeders) positioned in a row 5 cm apart on a white painted steel platform (40 cm x 40 cm). The central feeder was used as the conditioning feeder and the outer ones used as the choice test feeders. The platform could be rotated 180° so that the relative orientation of the choice test feeders could be reversed between tests. Each feeder consisted of a perforated steel plate (3.5 cm diameter) through which odour could be delivered via glass chambers beneath the platform.

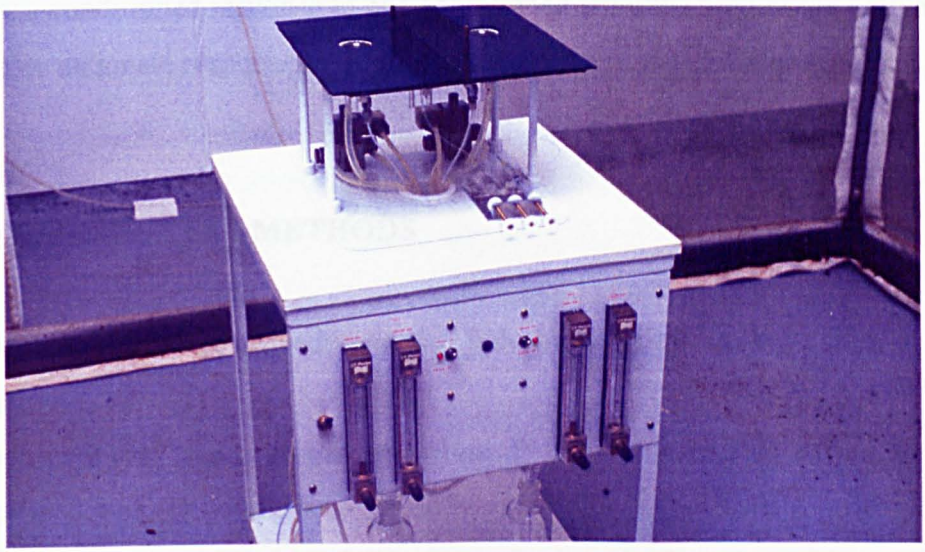


Figure 8.3 The 'floweroid': an artificial flower / odour delivery system: showing on the top platform, the two outer test feeders (the conditioning feeder in the centre of these is covered)

8.4.6 Conditioned choice-test bioassay

Free-flying bees were conditioned to associate pollen cues with a reward in Pavlovian (Pavlov, 1927) associative conditioning (see chapter 7). During repeated foraging visits to the centrally-located conditioning artificial flower or feeder in which a reward (the unconditioned stimulus, US; either pollen or sucrose solution) was presented along with pollen cues (the conditioned stimulus, CS; pollen colour and/or odour), bees learned to associate the reward with the conditioning stimulus. A choice test was then presented in the two outer artificial flowers/feeders: one presented the conditioned stimulus, and the

other presented the unconditioned (novel) stimulus. The behaviour of conditioned bees was recorded in the test. A reciprocated test, where the novel stimulus in the first test became the conditioning stimulus in the reciprocal test, and the conditioning stimulus from the first experiment became the novel stimulus in the reciprocated test was then carried out with a new group of bees. If bees choose the artificial flower/feeder associated with the conditioned stimulus in preference to the feeder associated with the novel stimulus, this represents a conditioned response, and means that bees have successfully learned the conditioning stimulus and can discriminate between it and novel ones. If bees show no preference in both choice tests, this implies that either bees have not successfully learned the conditioning stimulus, or that they generalize the recognition of the conditioning stimulus with the novel stimulus. Asymmetrical results, in which bees show a conditioned response to one conditioned stimulus but not the reciprocal one, may indicate an innate response to or preference for the novel stimulus (Menzel, 1967).

8.5 EXPERIMENTAL METHODS

8.5.1 Discrimination of oilseed rape and field bean pollens using pollen colour and odour cues

This experiment was conducted to determine if, when conditioned to one pollen type such as oilseed rape (OSR), bees could differentiate this pollen from a novel pollen type such as field bean (FB) and vice-versa using **colour and odour cues** from pollen. Two experiments were conducted to investigate this, which differed in the method of recording the choices of bees:

1. First choice: the first choice (visit) made by individual bees in the choice test
2. Entire foraging trips: the number and duration of visits to each pollen type during the choice test in which the pollen reward was continuously offered

8.5.1.1 Honey bees' provisions

Unlike previous versions of bee-flight rooms, colonies of bees maintained pollen-collection sufficient for brood-rearing without the need to add supplements to the hive (Poppy & Williams, 1999). Thus the pollen-collecting behaviour of bees could be confidently assumed to approach that under natural conditions. Pollen-collecting bees were used in this experiment and were able to obtain pollen (8.4.3) for feeding their colony only from within the test arena (8.4.4). In this way the bees became used to

foraging for pollen from the arena. Sucrose solution (50%) was supplied in the von Frisch feeders described above to satisfy nectar-collecting bees throughout this set of experiments.

8.5.1.2 Conditioning bees

The conditioning process involved placing 5 g of the conditioning pollen, either oilseed rape (OSR) or field bean (FB) pollen loads, in the central artificial flower in the test arena and allowing bees to forage upon this for two hours. This time period was chosen after preliminary experiments demonstrated that the mean time taken by a bee to collect a pollen load was 4.9 minutes (± 0.26; n=5) and the mean time spent in the hive between collections was 6.6 minutes (± 0.76; n=6). This would mean that each pollen-collecting bee would experience the conditioning pollen approximately ten times before experimentation, and in doing so would learn to associate cues (CS) from the conditioning pollen with its successful collection (US). During conditioning, foraging bees were captured and marked with a coloured and numbered disc (Opalithplättchen: 'Queen-marking kit' distributed by E. H. Thorne, Ltd., Wragby, Lincoln, UK) glued to the dorsal side of its thorax. After conditioning, the arena was cleared of bees by allowing those already within to complete their foraging trip and fly back to the hive as usual, but preventing any incoming bees from entering the arena by closing the entrance. When the arena was clear of bees, the turntable containing conditioning pollen was removed. The arena was cleaned with 70% ethanol in preparation for the presentation of the choice test.

8.5.1.3 Presenting the choice test

A second, clean turntable was placed inside the arena. Two artificial flowers were placed on it, equidistant from the previous position of the central conditioning artificial flower, and perpendicular to the direction of incoming flight through the entrance. One artificial flower contained a new sample of the conditioning (conditioned) pollen (0.25 g) and the other contained an equal amount of the other, unconditioned (novel) pollen type.

8.5.1.4 Recording and analyzing choices in the choice test

The entrance was opened, a single, marked bee was allowed into the arena (the test bee), and the entrance was then closed to prevent entry of further bees into the arena.

The choices of test bees were recorded in two ways. In the first experiment, the pollen type upon which the bee first landed was recorded. In the second experiment, the entire foraging trip of the bee was observed and its behaviour was recorded into a dictaphone, noting the number and duration of visits (a landing followed by a collecting bout) to each pollen type until the bee left to fly back to the hive.

After each test, the artificial test flowers were removed, the turntable was cleaned, and two clean test dishes were placed in the arena, OSR and FB each orientated in a pre-determined randomized position. The entrance was opened and another bee let in. Each time, a different bee was used; unmarked bees and those previously tested (identified by their coloured and numbered disk) were refused entry during test periods to prevent reuse of the same bee. Choice tests were presented over a 30-minute period, after which the conditioning pollen (5 g) was returned to the central position for one hour to allow reconditioning.

In the first experiment, in which only the first landing of test bees were recorded, bees were first conditioned to FB pollen, and the conditioning and testing sequence was repeated until 20 bees had been tested. Bees were then conditioned to OSR pollen and a further 20 bees were tested. The effect of conditioning on bees' first choices was examined by analysing the total number of first choice visits on the left or the right feeder when the conditioning pollen was placed on the left or right by a Fisher's Exact test. This test removed any bias of the direction of the feeders in the analysis.

In the second experiment, in which the entire foraging trip of individual bees was recorded, bees were first conditioned to FB pollen and conditioning and testing was continued as described until ten bees had been tested. The experiment was then repeated; this time, bees were conditioned to OSR pollen and a further ten bees were observed in the choice test. The data were later transcribed. The total number of visits to each pollen type over the entire foraging trip was compared using a Wilcoxon Matched-Pairs test. The mean duration of time spent foraging per visit on each pollen type for each bee observed was compared using a paired T-test.

8.5.2 The role of odour in the discrimination of oilseed rape and field bean pollens

The aim of this experiment was to determine whether bees could discriminate between oilseed rape (OSR) and field bean (FB) pollens by their **odours alone.**

To remove variation caused by possible nutritional differences between pollen, this experiment would have optimally required the provision of a 'standard' pollen type associated with either the odour of OSR or FB pollen. However, it is technically difficult (if not impossible) to separate pollen colour and odour cues and present whole pollen as a reward. Therefore artificial pollen was required, but again, it is technically impossible to produce a proteinaceous substance with no colour or odour of its own. Therefore sucrose was offered as an equal-strength reward, alongside the odour of real pollen. Although studying the effect of pollen cues on nectar-foraging bees is a different approach from studying the effects on pollen-collecting bees, as all bees can forage on nectar and pollen the mechanisms by which they make choices can still be studied.

8.5.2.1 Honey bees' provisions

The pollen foragers of the colony were provided with bee-collected pollen loads (8.4.3) at a feeding station adjacent to the hive. In the opposite corner, bees were supplied with 40% sucrose solution supplied in a von Frisch feeder. The sucrose concentration was lower than in the previous experiment conducted on pollen foragers, since it was necessary to motivate bees to visit the test site where a higher concentration of sucrose (60%) served as a reward (Williams & Poppy, 1997). The floweroid was placed in the corner opposite the hive where the artificial flower test arena had been in the previous experiment. The arena (8.4.4) was not needed with the floweroid to restrict bees' visits during tests, as the number of nectar-foragers at the test site was easier to manipulate than visits of pollen-foragers.

8.5.2.2 The 'floweroid'

The floweroid was set up to distribute the conditioning odour from the central feeder during conditioning periods and test odours from the outside feeders during tests. The odour emitted from each feeder was from the headspace of the conditioning and test pollens, each contained in a Dreschel flask (500 ml) placed underneath the unit supporting the top platform so that bees could not see the test material, and thus only olfactory cues from pollen were presented. Air from a pressurized cylinder passed via flow meters (located on the unit supporting the test platform) into each flask at 0.5 dm^3

min^{-1}. The displaced headspace was passed through Teflon tubing (3 mm diameter) to a glass tube (6 mm diameter) located at the base of the glass chamber beneath the feeder and was emitted through the perforations in the steel plate covering the feeder. The conditioning pollen (10 g of either OSR or FB pollen) was placed in a flask whose headspace odour was supplied to the glass chamber beneath the conditioning feeder. The same amount of the conditioning pollen was placed in a second flask connected to the glass chamber beneath one of the two choice-test feeders. The second choice-test feeder was connected to a third flask, containing 10 g of the unconditioned (novel) pollen type. During conditioning, the flask containing the conditioning pollen was connected to the airflow, and the flasks containing the test pollens were left unconnected. During the tests, these connections were reversed. This is shown schematically in Figure 8.4. The conditioning pollen and test pollen were taken from the same batch of frozen pollen loads, so their volatile compositions were identical.

In the centre of each feeder was a Teflon disc (5 mm diameter) on which a drop of sucrose could be maintained. This was only done in the central conditioning feeder and only during conditioning periods; the test was unrewarded to allow motivation to be measured. The sucrose was dispensed from a plastic syringe (5 ml), whose plunger was finely controlled by a screw thread, through a plastic tube (1 mm outer diameter) that led to the centre of the Teflon disc (Fig. 8.4).

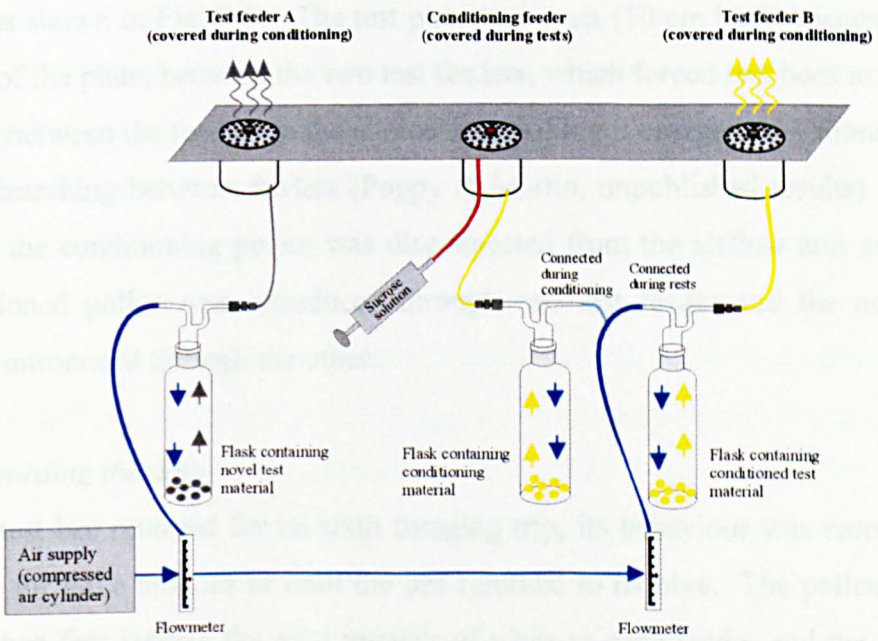


Figure 8.4 Schematic diagram of the distribution of conditioning and test odours from oilseed rape and field bean pollens to the feeders during conditioning and tests, and the sucrose dispensing system, used to condition bees.

8.5.2.3 Conditioning bees to pollen odour

During conditioning, the top of the floweroid was covered by a dark grey plastic plate with a hole exposing only the central conditioning feeder. Foraging bees were taken individually from the 40% von Frisch feeder using a cotton wool bud, and placed on the conditioning feeder to feed until a bee returned to this feeder of its own accord. Once this occurred, the bee was marked on the dorsal side of its thorax with water-based, coloured correction-fluid whilst feeding. All foraging trips to the conditioning feeder by marked bees were recorded. A foraging trip was defined as the round journey of a bee's flight from the hive to the floweroid for the collection of sucrose, followed by flight back to the hive to discharge its load. After a bee had completed five conditioning trips in succession, the test was set up so that its sixth foraging trip could be tested. Five conditioning trips were used by von Frisch (1919) and Laska *et al.* (1999) to ensure that free-flying bees formed robust associations between the food reward and the conditioning odour before tests.

8.5.2.4 Presenting the choice test

Whilst the bee was discharging its load from the fifth foraging trip, all other foraging bees were caught from the floweroid and caged in marking cages (E. H. Thorne Ltd., Wragby, Lincoln, UK) so that the bee could complete its choice test independently of other foragers. The plate covering the test feeders was removed and a second plate, which covered the conditioning feeder and exposed the two outer test feeders, was put in its place (as shown in Fig. 8.3). The test plate had a net (10 cm high) suspended across the centre of the plate, between the two test feeders, which forced test bees to fly, (rather than walk) between the feeders in the choice test, making it energetically more expensive to switch searching between feeders (Poppy & Martin, unpublished results). The flask containing the conditioning pollen was disconnected from the airflow and odour of the test-conditioned pollen was introduced through one test feeder and the novel pollen odour was introduced through the other.

8.5.2.5 Recording the data

When the test bee returned for its sixth foraging trip, its behaviour was recorded into a dictaphone for three minutes or until the bee returned to its hive. The pollen type upon which the bee first landed, the total number of visits to each feeder and the duration of time spent searching the feeder for food on each visit were recorded. After the test period, the bee was caught (either at the end of the three minute period, or upon its return

from the hive if it returned there before the end of the test period) and was permanently removed from the test area to prevent the same bee from being tested twice.

After each test, the conditioning set-up was re-assembled and any captured bees were released onto the conditioning feeder to recommence foraging. The conditioning process and tests were continued until 20 bees had been tested. The odour of each pollen type was emitted from each choice test feeder the same number of times (ten) and the left/right orientation of the feeders was rotated in a pseudorandomized order such that the odour of each pollen type was distributed on the left and right ten times each. Bias due to feeder or orientation was thus eliminated, although bias tests had previously shown that these were negligible (Appendix 5). OSR pollen was used as the conditioning pollen odour for the first experiment, which was then repeated using FB as the conditioning odour, testing a further 20 bees in the choice test.

8.5.2.6 Analyzing the data

For each experiment, the total number of first choice landings on the left or right feeder when the conditioning pollen odour was on the left or right was analysed by a Fisher's Exact test. A Wilcoxon Matched-Pairs test was used to analyse differences in the total number of visits made to each test feeder throughout the test. The mean duration of time spent searching each feeder per visit throughout each test was analysed by a paired T-test.

8.5.3 The role of colour in the discrimination of oilseed rape and field bean pollens

The aim of this experiment was to determine whether bees could discriminate between OSR and FB pollens by their **colour alone**.

8.5.3.1 Honey bees provisions

This experiment was conducted on 'nectar' foraging bees, rather than pollen foragers, since it was difficult to reward pollen foragers with an equally rewarding pollen with natural colour but no odour as described in section 8.5.2. Bees and the flight room were as described in the previous section (8.5.2.1).

8.5.3.2 The 'floweroid'

The floweroid set-up and mode of operation was modified, since this experiment did not require any of its odour-distributing facilities. The tubes used to bring odour into the

glass chambers beneath the feeders were removed and the holes sealed with tape. The perforated steel plates and Teflon discs were removed and the glass chambers beneath were filled to the top with pollen. The conditioning pollen type (either OSR or FB) was placed in the central conditioning feeder and OSR pollen loads were placed in one choice test feeder chamber, and FB pollen placed in the other. The platform was covered with a sheet of glass so that the bees could see the surface of the pollen in the feeders, but not smell (or touch) it. The effect of the glass plate on pollen colour cues as perceived by the bees was investigated (8.5.3.6).

8.5.3.3 Conditioning bees to pollen colour

During conditioning, the two choice test feeders were each covered with white filter paper disks (under the glass sheet) so they were not visible, leaving only the central conditioning feeder visible. As the glass plate prevented use of the sucrose dispensing system, a Teflon cap (inner diameter 5 mm) was placed on the glass in the centre of the feeder, and was filled with 60% sucrose solution from a pipette. Foraging bees were trained to come to the floweroid as described in section 8.5.2.3. Bees returning to the feeder of their own accord were marked on the dorsal side of the thorax with coloured correction fluid whilst feeding, and the conditioning trips of marked bees were recorded until a bee had completed ten conditioning foraging trips. Ten foraging trips ensured that bees had enough opportunity to learn the colour stimulus; previous tests on free-flying bees showed that over five rewarded trips were sometimes necessary to achieve 90% accuracy of correct responses for bees conditioned to colours that were poorly learned (Menzel, 1985). The bee's eleventh foraging trip was tested.

8.5.3.4 Presenting the choice test

After a marked bee's tenth foraging trip, while it was in the hive discharging its load, all the other foraging bees were captured in marking cages and the choice test was then set up. The glass sheet was lifted, the choice test feeders were uncovered, and the central conditioning feeder covered with filter paper so that the conditioning feeder was invisible, and the coloured test feeders were visible. The Teflon cap was removed from its central position on the glass sheet, and the glass cleaned with 70% ethanol and repositioned. A clean, empty Teflon cap was placed in the centre of each coloured choice test feeder and the positions of the OSR and FB pollens noted.

8.5.3.5 Recording and analyzing the data

When the bee returned for its eleventh foraging visit, its first landing, the number of visits to each feeder, and the duration of visits were recorded as described in the previous section (8.5.2.5). In total, 20 bees were conditioned to the colour of OSR pollen (yellow) and tested in the choice test. A further 20 bees were conditioned to the colour of FB pollen (grey) and then tested. The responses in the choice test of bees conditioned to each pollen colour were analysed as described in section 8.5.2.6.

8.5.3.6 Spectral emissions of oilseed rape and field bean pollens

The light spectra of OSR and FB pollens were measured in the flight room to gain information on how bees might perceive them, and how covering the pollen with glass may change its appearance. Recordings were conducted using a spectrophotometer (Licor LI-1800 scanning Spectroradiometer). Measurements were taken in the bee flight room of the two pollens uncovered as they would have been in experiment 8.5.1 and covered with glass as they were in this experiment.

8.6 RESULTS

8.6.1 Discrimination of oilseed rape and field bean pollens using pollen colour and odour cues

8.6.1.1 First choice

There was an effect of conditioning on the choices of bees in the choice test (FEXACT P=0.011). Bees conditioned to OSR were more likely to land on OSR in the choice test than on FB (FEXACT P=0.020), whereas bees conditioned to FB showed no preference (FEXACT P=1.0) (Fig. 8.5 a & b, respectively).

8.6.1.2 Entire foraging trip

Number of visits (landings) during a foraging trip Bees conditioned to OSR visited OSR pollen more times than FB during an entire foraging trip (Table 8.1). However, bees that had been conditioned to FB showed no difference in the number of visits to OSR or FB (Table 8.1).

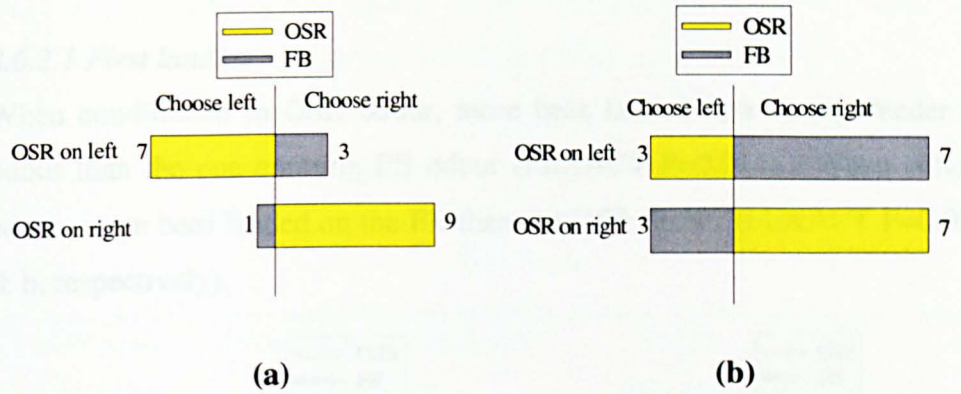


Figure 8.5 Total number of honey bees landing first on oilseed rape (OSR) or field bean (FB) pollens in a choice test after conditioning to either (a) OSR or (b) FB pollen

Table 8.1 Mean number of visits to test pollens made by honey bees in a choice test after conditioning to either oilseed rape (OSR) or field bean (FB) pollen

Conditioning pollen type	Mean no. of visits (±SE)		Wilcoxon Test statistic	Normal approximation	P
	OSR	FB			
OSR	6.80 (1.08)	1.10 (0.50)	4.5	2.344	<0.05
FB	4.92 (0.61)	4.25 (1.51)	21.0	1.412	>0.05

Mean time spent foraging. There was no difference in the mean time spent foraging per visit to OSR and FB pollens throughout entire foraging trips during choice tests, regardless of the bee's conditioning (Table 8.2).

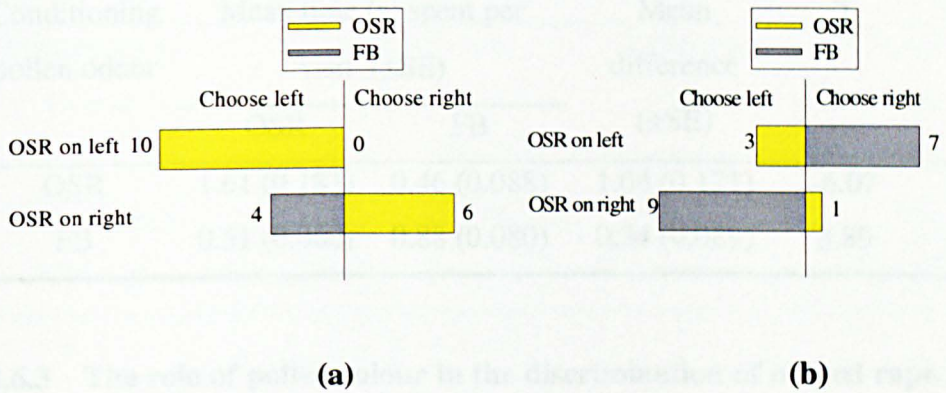
Table 8.2 Mean time (in seconds) spent foraging per visit on test pollens in a choice test by honey bees after conditioning to either oilseed rape (OSR) or field bean (FB) pollen

Conditioning pollen type	Mean time (s) spent per visit (±SE)		Mean difference (±SE)	T	df	P
	OSR	FB				
OSR	6.29 (0.85)	5.37 (2.47)	-0.916 (2.93)	-0.31	9	>0.05
FB	10.23 (2.59)	12.01 (2.63)	1.751 (0.38)	0.56	11	>0.05

8.6.2 The role of odour in the discrimination of oilseed rape and field bean pollens

8.6.2.1 First landing

When conditioned to OSR odour, more bees landed first on the feeder emitting OSR odour than the one emitting FB odour (FEXACT P=0.011). When conditioned to FB odour, more bees landed on the FB than the OSR feeder (FEXACT P=0.020) (Fig. 8.6 a & b, respectively).



(a) **(b)**

Figure 8.6 Total number of honey bees landing first on feeders emitting the odour of OSR or FB pollens in a choice test after conditioning to either (a) OSR or (b) FB pollen odour

8.6.2.2 Total number of visits throughout the test

Bees conditioned to OSR pollen odour made significantly more visits to the feeder emitting OSR odour than FB odour throughout the choice test (P<0.001) and similarly, bees conditioned to FB odour made more visits to the FB odorous feeder than the OSR one (P<0.001) (Table 8.3).

Table 8.3 Mean number of visits to test feeders emitting the odour of oilseed rape (OSR) or field bean (FB) pollen made by honey bees in a choice test after conditioning to either OSR or FB pollen odour

Conditioning odour	Mean no. of visits (±SE)		Wilcoxon Test statistic	Normal approximation	P
	OSR	FB			
OSR	13.53 (1.215)	3.9 (0.624)	0	3.92	<0.001
FB	2.9 (0.619)	7.7 (1.115)	21	3.316	<0.001

8.6.2.3 Mean duration of time spent searching

Bees spent longer searching the feeder emitting the odour to which they had been conditioned than the unconditioned odour during visits (Table 8.4).

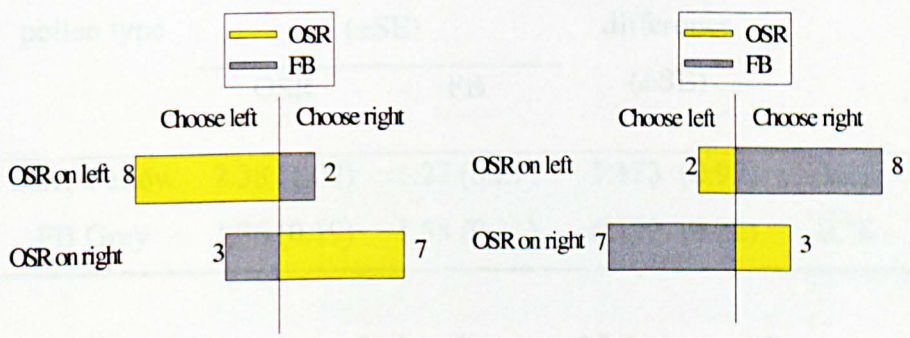
Table 8.4 Mean time (seconds) spent by honey bees searching feeders emitting oilseed rape (OSR) or field bean (FB) odour after conditioning to either OSR or FB pollen odour

Conditioning pollen odour	Mean time (s) spent per visit (±SE)		Mean difference (±SE)	T	df	P
	OSR	FB				
OSR	1.61 (0.183)	0.46 (0.088)	1.04 (0.171)	-6.07	18	<0.001
FB	0.51 (0.068)	0.88 (0.080)	0.34 (0.089)	3.89	16	<0.001

8.6.3 The role of pollen colour in the discrimination of oilseed rape and field bean pollens

8.6.3.1 First landing

When conditioned to OSR yellow, more bees landed first on OSR yellow than FB grey (Figure 8.7a). When conditioned to FB grey, more bees landed on FB grey than OSR yellow. However, these differences were not significant at the 5% level, although they were at the 7% level (FEXACT P=0.069 for both tests) (Figure 8.7b).



(a) **(b)**

Figure 8.7 Total number of honey bees landing first on feeders coloured oilseed rape (OSR) yellow or field bean (FB) grey in a choice test after conditioning to either (a) OSR yellow or (b) FB grey

8.6.3.2 Total number of visits throughout the test

Bees conditioned to OSR yellow made significantly (P<0.001) more visits to the OSR yellow test feeder than the FB grey (Table 8.5). However, bees conditioned to FB grey made a similar number of visits to both test feeders (P>0.05) (Table 8.5).

Table 8.5 Mean number of visits to oilseed rape pollen (OSR) yellow or field bean pollen (FB) grey – coloured feeders made by honey bees in a choice test after conditioning to either one of these pollen colours

Conditioning Colour	Mean no. of visits (±SE)		Wilcoxon Test statistic	Normal approximation	P
	OSR Yellow	FB Grey			
OSR Yellow	9.9 (1.194)	5.45 (1.198)	17	3.139	<0.001
FB Grey	5.15 (0.955)	7.0 (0.849)	49.5	1.831	= 0.07

8.6.3.3 Mean duration of time spent searching

There was no difference in the mean time spent by bees searching each feeder during visits throughout the choice tests, regardless of their conditioning (Table 8.6).

Table 8.6 Mean time per visit (in seconds) spent searching oilseed rape pollen (OSR) yellow or field bean pollen (FB) grey – coloured feeders made by honey bees in a choice test after conditioning to OSR yellow or FB grey pollen colours

Conditioning pollen type	Mean time (s) (±SE)		Mean difference (±SE)	T	df	P
	OSR	FB				
OSR Yellow	2.38 (1.02)	1.27 (0.27)	-1.173 (0.97)	-1.21	18	>0.05
FB Grey	1.36 (0.19)	1.58 (0.31)	-0.172 (0.22)	-0.78	17	>0.05

8.6.3.4 Spectral emissions of oilseed rape and field bean pollens

Spectral emission curves are shown in Figure 8.8. These show that oilseed rape reflects light around the yellow-green region (530-580 nm) and at the UV region (300-330 nm). Field bean reflects light in the UV region and little elsewhere in the spectrum, and has a uniform reflectance throughout the wavelengths. The two pollens are therefore similar in that they both reflect UV light under the UV lights of the bee-flight room, but are

otherwise different. Placing the pollens under glass reduced their reflectance of UV as would be expected, but otherwise the patterns of reflectance were similar to those of the pollens that were uncovered.

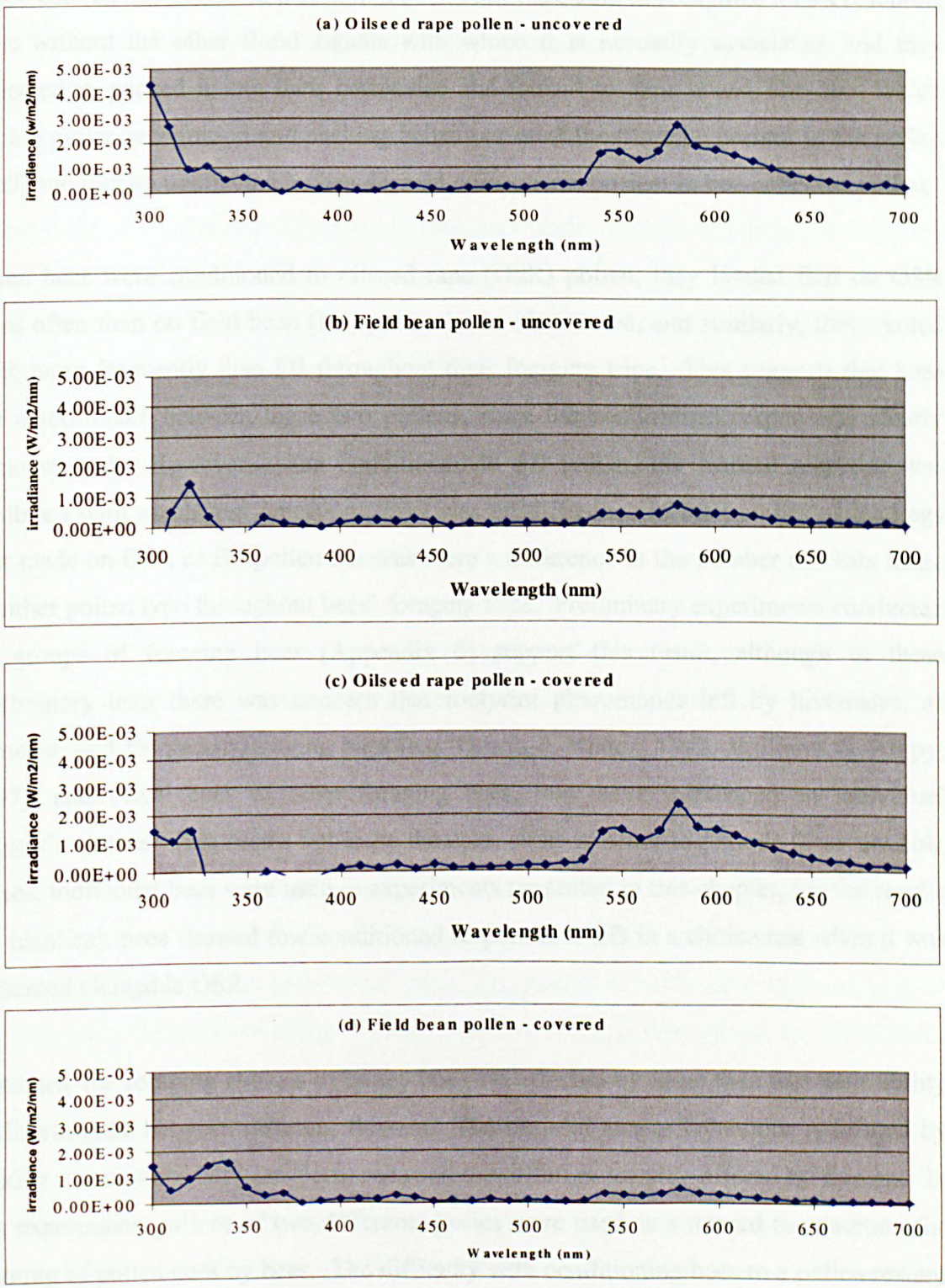


Figure 8.8 Spectral emission curves of oilseed rape (OSR) and field bean (FB) pollens in the bee flight room (a) OSR - uncovered; (b) FB – uncovered; (c) OSR under glass plate; (d) FB under glass plate

8.7 DISCUSSION

8.7.1 Pollen colour and odour

When offered bee-collected pollen in a dish, bees were able to recognize it as a resource, even without the other floral signals with which it is normally associated, and they collected it, packed it into their corbiculae and took it to their hive. The cues which initiate pollen recognition and packing behaviour must therefore be present in the pollen itself, and remain unaffected by bee-derived components present in bee-collected pollen.

When bees were conditioned to oilseed rape (OSR) pollen, they landed first on OSR more often than on field bean (FB) pollen in the choice test, and similarly, they visited OSR more frequently than FB throughout their foraging trips. This suggests that bees can discriminate between these two pollens, since the conditioned response is clearly demonstrated. However, when conditioned to FB pollen, the learned response was exhibited with much less precision: there was no difference in the number of landings first made on OSR or FB pollen nor was there a difference in the number of visits made to either pollen type throughout bees' foraging trips. Preliminary experiments conducted on groups of foraging bees (Appendix 6) support this result, although in these preliminary tests there was concern that footprint pheromones left by hivemates, as demonstrated for nectar-foraging bees (e.g. Giurfa & Núñez, 1992; Williams & Poppy, 1997), and visual cues of other foraging bees, may have influenced an individual forager's choice. This seems not to be the case, since in order to remove these possible biases, individual bees were used in experiments presented in this chapter, yet the results are identical: bees showed few conditioned responses to FB in a choice test when it was presented alongside OSR.

In nature, the foraging choices of honey bees are affected by more than just their ability to discriminate between different flowers. The basis of choice behaviour is formed by making associations between floral cues and the different rewards offered by flowers. In this experiment, pollens of two different species were used as a reward to condition the learning of pollen cues by bees. The difficulty with conditioning bees to a pollen reward is that the reward itself also functions as the stimulus with which the reward is associated. Unlike the equal reward of 30% sucrose offered in experiments on restrained honey bees (chapter 7), these pollens are not identical and probably differ in nutritional value (see chapter 9). Thus, they are probably unequal as unconditioned stimuli (US), in

which case conditioning was conducted using different rewards. This may have affected the strength of the association made between OSR and FB rewards and the stimuli associated with them. Therefore if FB pollen is of lower nutritional quality than OSR, when given the opportunity in a rewarded test, bees conditioned to FB were possibly more 'willing' to switch to another species, than were bees conditioned to OSR pollen.

The effects of varying rewards on pollen-collecting bees has never been studied, although there have been several studies into the effects of varying sucrose concentrations on choice behaviour. In one such study, sucrose concentration affected the strength of the association with colour stimuli, with low and high concentrations evoking low and high strength associations, respectively (Loo & Bitterman, 1992). In another study, when bees were conditioned to two sucrose concentrations, each associated with a different colour, in a choice test when differences in sucrose concentrations were large, colour stimuli did not affect choice behaviour and bees always chose the larger reward; but when the difference was small, bees made their choice based on colour preferences (Banschbach, 1994).

Another possibility that may explain these results is that the pollen stimuli learned in association with the pollen rewards could be more preferred in OSR than in FB and that this preference overrides FB conditioning effects. Thus OSR cues may be more salient (more strongly-learned) than FB cues, and /or learned cues in FB may be present in greater quantities in OSR, or these are innate responses to OSR cues. Such preferences would be best studied by conditioning pollen-foraging bees to associate either OSR or FB pollen colour or odour stimuli with an equally rewarding protein resource, and comparing their responses in a choice test. This would be difficult to achieve, since it would require either formulating a pollen substitute with neither colour nor odour to be presented as a reward alongside the stimulus under question, or presenting real pollens with manipulated (equal) nutritive values with only their colour or odour evident at one time (as discussed in section 8.5.2). Therefore a 'compromise' was made, and bees were rewarded with a fixed concentration of sucrose paired with either pollen colour or odour stimuli to further investigate preferences for OSR stimuli. Although studying the effect of pollen cues on nectar-foraging bees is a different approach from studying the effects on pollen-collecting bees, it has not been shown that pollen-foraging bees use different mechanisms to make choices than nectar foraging bees, and therefore those mechanisms can probably still be studied using pollen cues.

8.7.2 Pollen odour

When bees were conditioned to equally-rewarding feeders, emitting either the odour of OSR or FB pollen, they landed first on the feeder emitting the odour of the pollen type to which they had been conditioned more often than that emitting the odour of the novel pollen type in the choice test. They also made more visits throughout a foraging trip on the feeder emitting the conditioning odour than that emitting the novel pollen odour. These results suggest that bees can learn pollen odour and can use it to discriminate between these two pollens, and that they show no preferences for either odour.

Bees do not show innate preferences for particular floral odours (Waller, Loper & Berdel, 1974; Pham-Delègue *et al.*, 1986; Henning *et al.*, 1992). Although bees may be innately attracted to floral odour (Giurfa *et al.*, 1995), they learn to associate particular odours with rewards and use odour as a precise means of discriminating between species, learning and then preferring new odours when there is a benefit in switching floral constancy (Couvillon & Bitterman, 1991).

That free-flying bees are able to learn and discriminate between pollen odours confirms the results obtained by conditioned proboscis extension (CPE) studies using restrained bees (this thesis, chapter 7) which demonstrated that both OSR and FB pollen odours could be learned quickly and that bees had the ability to discriminate between them. However, bees in CPE tests only discriminated when they were differentially conditioned (conditioned simultaneously to both odours, one being positively rewarded, the other being negatively rewarded); when they were conditioned to the odour of one pollen type only and then offered the odour of a novel pollen in the generalization test, they generalized (responded to the novel pollen odour and the conditioning pollen odour). This highlights a second major difference between free-flying and restrained preparations. In both, bees are conditioned to one stimulus. However, in the free-flying test, bees are offered the choice of the conditioning stimulus along with a novel stimulus, and in the CPE generalization test, bees are not offered a choice; they are offered either the conditioning odour or the novel odour. Since generalization presumably serves an adaptive function, if the novel odour is at all similar to the conditioning odour, the bee has nothing to lose by responding to the novel odour. However, in the free-flying choice test situation, bees always have the choice of responding to the conditioning stimulus, and failure to do so in the foraging situation would lead to a mistake – either a missed

opportunity to gain a reward, or a wasted visit to a potentially unrewarding site (Smith, 1993).

These results suggest that, in the first experiment, bees could have discriminated between OSR and FB pollens on the basis of the pollen odour but they did not – either due to poorer nutritional factors of FB resulting in a weaker association as discussed above, or due to a preference for OSR yellow.

8.7.3 Pollen colour

When bees were conditioned to equally-rewarding feeders presenting the colours of either OSR pollen (yellow) or FB (grey), there was no difference in the number of first landings made on OSR yellow or FB grey feeders regardless of conditioning in the choice tests. However, a trend was evident, in which bees landed first on the pollen colour to which they were conditioned more often than the novel pollen type. Since sample sizes were relatively small (n=20) in this study, further replication may clarify this response. The measurement of the total number of visits per foraging trip increases the sample size, enabling a more confident comparison. When conditioned to OSR yellow, bees made significantly more visits to the feeder coloured OSR yellow than that coloured FB grey. When conditioned to FB grey, there was no difference in the number of landings made on each feeder. This result is identical to that of the first experiment, in which colour and odour cues of the pollen reward were used in conditioning and a rewarded test was given. These results suggest that bees can discriminate between OSR yellow & FB grey pollen colours, when conditioned to OSR pollen, but they do not discriminate when conditioned to FB pollen. This could be due to a 'generalization' of the stimuli learned during conditioning to FB grey with those present in OSR. Both pollens reflect light in the UV wavelengths; this is the main reflectance of FB, but it is also present in 'higher amounts' in OSR. Thus unless bees had learned to discriminate against yellow, they may generalize grey to yellow. However, OSR pollen also reflects with a peak at 530 nm; this could be learned when conditioned to OSR, and since this reflectance is not present in FB pollen, this may explain why these colours are not generalized in this case.

A second possibility is that conditioning to FB grey is over-ridden by a preference for OSR yellow. Yellow may have been more preferred to grey in these tests if this colour is

more detectable than grey against the background (white). However, colour 'distance' to the background was not found to be a factor in the colour preference study conducted by (Giurfa *et al.*, 1995). To humans, yellow appears brighter than grey. Thus, if bees prefer bright colours, this may explain why yellow was favoured. However, intensity differences have also been shown to be irrelevant for experienced foragers in colour discrimination (Menzel, 1967; Menzel, 1985; Giurfa, 1991). This leaves an innate preference for yellow hues as the most likely cause for this preference.

Some research has indicated that honey bees have an innate preference for yellow flowers (e.g. Real, 1981). An innate preference for yellow was demonstrated by Giurfa *et al.* (1995). Bees 'pretrained' to a grey disk were then offered a choice of 12 different colour filters in an unrewarded choice test. Bees displayed a preference for feeders reflecting light wavelengths at 530 mn. This corresponds to yellow (as perceived by humans) and was the dominant hue of OSR pollen measured in this experiment. That bees trained to grey subsequently preferred yellow lends support to the findings of the present study, although the conditioning feeder was not one of those offered in the choice test in Giurfa's study.

The possibility that conditioned responses to FB grey in the choice test of bees conditioned to this colour were not evident because bees did not properly learn to associate FB grey with the sucrose reward cannot be excluded from these experiments. To be confident that this is not the case, a simple test could be conducted in which bees are conditioned to associate FB grey with a reward as described in this experiment (8.5.3), and then tested in a choice test to the same conditioning colour and a feeder similar to the background colour (white). However, it is unlikely that bees did not associatively learn the FB grey stimulus with the sucrose reward. In early studies investigating colour learning in honey bees, all colours tested were associatively learned (Menzel, 1967), and as mentioned above, Giurfa *et al.* (1995) showed that bees could become conditioned to 'unchromatic' grey disks.

8.7.4 Mean time spent at each artificial flower/feeder during choice tests

During conditioning, bees develop memories that relate the feeder stimuli with a measure of its profitability. The amount of time insects spend searching for a reward associatively-learned with a conditioning stimulus may be related to how the learned cues affect motivation (anticipation of reward; Couvillon & Bitterman, 1984), and are

therefore very informative. In unrewarded tests, bees conditioned to pollen colour did not differ in the mean time spent searching each feeder, regardless of conditioning. Conversely, bees conditioned to pollen odour spent significantly longer per visit searching the feeder which emitted the conditioning odour than the novel odour. These results support the theory that colour is important in influencing landing responses, but odour is necessary for retention and motivated searching (Brantjes, 1978; this thesis, chapters 3 & 4). This may be because odours signal reward strength more reliably than colours (Buchanan & Bitterman, 1988).

8.7.5 General discussion

These experiments show that free-flying honey bees are able to learn cues from pollen and use them to discriminate between OSR and FB pollens. When pollens were used as both the reward and the conditioning stimulus, bees showed a preference for OSR pollen. This preference could have been due to nutritional differences between OSR and FB pollens affecting the strength of the associations made between reward and stimulus for the two pollens. Alternatively, the preference may have been due to bees' innate preference for yellow (reflected wavelength of 530 nm). The conclusive experiment in this case would be to condition bees to a combination of OSR or FB pollen colour and odour stimuli using a constant reward (such as the 60% sucrose used in these experiments) and then to repeat the choice tests. This may involve some re-design of the floweroid feeders, perhaps using ones constructed from glass (instead of stainless steel) with ventilation holes so that bees can see the pollen colour within the feeder, smell its odour and be rewarded via the sucrose dispensing system. In the proposed test, if bees again showed a preference for OSR over FB, this would indicate that differences were due to innate preference for OSR yellow. However, if bees showed no such differences in the proposed choice test, this experiment would demonstrate that nutritional differences between OSR and FB pollens as rewards were largely responsible for the preference of bees for OSR. In this case, colour may have been used as an indicator of reward quality. These responses could be further investigated using different concentrations of sucrose as differential rewards associated with combinations of OSR and FB pollen colours and odours.

Experiments were conducted to investigate nutritional quality differences between OSR and FB pollens, and these are described in the following chapter (9). These indicated that there were small differences between the quality of OSR and FB pollen, although

OSR was the better of the two since it contained a greater proportion of the essential amino acids required for bee growth than FB. Thus preferences for colour stimuli may have defined the responses between two unequal but similar US rewards (Banschbach, 1994). If this is the case, these results imply that if a bee foraging on field bean pollen sees a yellow–coloured flower, it will switch. However, in nature this is not the case, since bees are constant to FB (Free, 1962). These results could reflect the importance of OSR and FB pollens as signals in nature. OSR has open flowers, with exposed pollen so that its odour and colour can be detected by pollen-seeking insects upon their approach to the flower, and pollen colour may form a visual contrast with the petals (e.g Lunau, 2000). Therefore, OSR pollen cues may play a role in the floral constancy of bees by providing odour and colour cues which may aid in discrimination. Conversely, FB pollen is enclosed in the petals: bees probably do not smell the pollen until after it has been dispensed from the flower onto their bodies, and they are unlikely to ever see it. Thus the petals of FB play an all-important role in reward advertisement. Bees will learn to associate the petal cues with the floral rewards, and these cues are therefore likely to be more salient than those of its pollen, which probably does not serve an attractive function itself.

In conclusion, these results demonstrate that pollen colour and odour cues can both be learned by free-flying honey bees in a semi-natural foraging situation. However, the role of pollen stimuli in aiding the bee in discrimination between flowers may depend on the floral structure of the species. The role of pollen stimuli in signalling the quality of the reward and thus their effect on the strength of associations made between the bee and the US reward require further investigations, and are briefly discussed in the next chapter (9).

9. Pollen nutritional quality: the free amino acid composition of oilseed rape and field bean pollens

9.1 ABSTRACT

The free amino acid content of pollen from oilseed rape and field bean was assessed by high performance liquid chromatography (HPLC) as a measure of their total protein amino acid composition. Amino acid compositions were then related to the nutritional requirements of the honey bee and the nutritional quality of these two pollens compared. There were few qualitative differences in the free amino acids present in the two pollen types. However, oilseed rape was found to contain a greater proportion of the most essential amino acids required for growth of the honey bee, indicating that it is of greater nutritive value than field bean pollen. As the nutritional quality of pollen varies with plant species, pollen-seeking insects may be able to detect these differences, enabling selection of those that are most nutritious. The effect of pollen nutritional quality on the choice-behaviour of pollen-collecting honey bees is discussed.

9.2 INTRODUCTION

9.2.1 Pollen preferences of foraging honey bees

Honey bees (*Apis mellifera* L.) are oligolectic; they generally collect pollen from a single plant species per foraging trip (Betts, 1935; Percival, 1947; Free, 1963), but may collect pollen from several different species over their life time (Free, 1993). At the colony level, honey bees are polyphagous, and use pollen from many different plant species collected by the pollen foragers to feed the larvae. Without pollen, brood rearing ceases (Herbert, Shimanuki & Caron, 1977; Imdorf *et al.*, 1998), but it is also important for the development of young adults (De Groot, 1953). Analysis of the pollen brought back to the hive reveals that the foraging force concentrate their efforts on a smaller proportion

of plants than the total locally available (Percival, 1955; Free, 1963). Thus, bees appear to exhibit pollen preferences. Preference suggests the maximization of some currency that confers fitness benefits. Bumble bees forage for pollen efficiently, maximizing the ratio of gross foraging benefits to total costs (Rasheed & Harder, 1997a&b). It is likely that honey bees do likewise, preferring to forage on a particular type of pollen because it is easier to collect and/or it provides superior nutrition to others in the location.

Experiments conducted under laboratory conditions also indicate that honey bees prefer the pollens of some species over others (Levin & Bohart, 1955; Doull, 1966; Boch, 1982; Schmidt, 1982; Schmidt, 1984; Schmidt & Johnson, 1984; Schmidt *et al.*, 1995). As described in the previous chapter (8) bees appeared to prefer oilseed rape (*Brassica napus* L.) pollen over field bean (*Vicia faba* L.) pollen. The basis for these preferences is unclear, although since the pollens were removed from the flower before presentation, they were not based on differences in ease of collection from the flower. This indicates that honey bees may perceive actual differences, or cues to differences in the nutritional quality of pollen between species.

9.2.2 The nutritional value of pollen

The nutritional qualities of pollen have been the subject of many investigations (e.g Todd & Bretherick, 1942; Vivino & Palmer, 1944; Herbert & Shimanuki, 1978; Day *et al.*, 1990). These studies have shown that besides protein, pollen also contains lipids, carbohydrates, starch, sterols, vitamins and minerals. However, since protein is the major constituent of pollen, the nutritive value of pollen is most often judged on its protein content (Day *et al.*, 1990). Considerable variation in the total protein content of pollen from different species exists; reported values range between 8-40% (Herbert, 1992). Several bioassays performed to compare the nutritional qualities of different pollens found that pollens containing the most protein generally contributed more to colony growth or bee development than those containing less protein (Levin & Haydak, 1957; McCaughey, Gilliam & Standifer, 1980; Schmidt & Johnson, 1984; Regali & Rasmont, 1995). However, others found that total protein content was not always entirely correlated with performance (Schmidt, 1982; Schmidt, 1984; Schmidt, Thoenes & Levin, 1987). In some cases, the lack of correlation can be attributed to imbalances of the amino acid composition of the pollen proteins (Herbert, Bickley & Shimanuki, 1970; Loper & Cohen, 1987; Stace & White, 1994).

9.2.3 Amino acid requirements of the honey bee

The amino acid requirements of honey bees was investigated by De Groot (1953). Newly emerged adult bees were reared on an artificial diet from which one of the 20 amino acids generally occurring in proteins was omitted in turn, and the dry weight and total nitrogen content of bees after 14 days were compared. Methionine, arginine, tryptophan, lysine, isoleucine, phenylalanine, histidine, valine, leucine and threonine were found to be essential in the diet. Tyrosine, cysteine, serine, hydroxyproline, alanine, glycine and proline were deemed non-essential, although glycine, serine and proline were found to exert a 'stimulatory' effect on growth. De Groot (1953) also determined the quantitative requirements of each of the 10 essential amino acids. Leucine, isoleucine and valine were required in the greatest amounts (most essential amino acids) tryptophan, methionine, and histidine in the lowest amounts (least essential amino acids) whilst threonine, phenylalanine, arginine and lysine were intermediate.

9.2.4 Analysis of the amino acid content of proteins

The nutritional quality of pollen from a particular species that is high in protein is reduced if it lacks an essential amino acid required for insect growth. Thus, the nutritional value of pollen is more accurately defined by its amino acid content rather than its protein content (Crailsheim, 1990). Pollens from different plant species differ in the number and/or amounts of amino acids they contain (Bathurst, 1954; Bieberdoff, Gross & Weichlen, 1961; Stace, 1996). Pollens with the highest quantities of essential amino acids are assumed to be of higher nutritional value than pollens with lower quantities, although the proportions in which they occur is most important (Day *et al.*, 1990).

To determine the total protein amino acid composition of a sample, the proteins must be hydrolysed to their constituent amino acids. This is achieved by acid hydrolysis: samples are placed in 6 N hydrochloric acid containing 0.1% phenol and heated at 110 °C in a vacuum for up to 72 hours. During the acid hydrolysis, some amino acids are destroyed. Therefore aliquots of the hydrolysate are removed at different times for analysis (e.g. Robyt & White, 1987). This is a time consuming (and somewhat dangerous!) exercise. Stanley & Linskens (1974) reported that although the concentration of all amino acids is higher in the protein-bound form than in the free form, their distribution in both fractions tends to follow the same pattern. Therefore, an estimate can be obtained of the total amino acid content of pollen protein from analysing those in the free form.

There are several methods that may be used for the separation, detection and quantification of amino acids in pollen. Thin layer chromatography has been used to separate amino acids from pollen on paper or a silica gel/cellulose medium. Separated amino acids were detected with ninhydrin sprays (Bieberdoff, Gross & Weichlen, 1961; Shellard & Jolliffe, 1968; Singh & Singh, 1991). Ion-exchange chromatography has been used to separate and quantify amino acids present in acid hydrolysates of pollens. The amino acids are eluted using various buffers (pH 3.4-11.0) and temperatures ranging from 27-75 °C and the fractions reacted with ninhydrin (McLellan, 1977; McCaughey, Gilliam & Standifer, 1980; Rayner & Langridge, 1985; Loper & Cohen, 1987; Agarwal, 1989; Clark & Lintas, 1992). This method has been automated, and commercial amino acid analysers are available, making this the most common method of pollen amino acid analysis in the literature. However, reverse-phase high performance liquid chromatography (HPLC) is rapidly becoming the method of choice for the analysis of amino acids, superseding the older methods that are costly, time-consuming and require large amounts of the sample (Robyt & White, 1987). It involves the use of a nonpolar stationary phase, such as a column packed with carbon particles, and a polar mobile (liquid) phase. Amino acids are injected onto the stationary phase where they are adsorbed. Separation is achieved by variation in the composition of the solvents used in the mobile phase. To generate a solvent gradient over time, two pumps are used to deliver programmed amounts of solvents; one polar, one non-polar. The program is begun with 100% proportion of the polar solvent flowing through the column. As time progresses the proportion of the polar solvent decreases as that of the less-polar solvent increases, so that by the end of the program, 100% of the less-polar solvent is passing through the column. Separation of mixtures is achieved because polar molecules have a higher affinity for the mobile phase than the less polar ones, and these elute more quickly than the less polar ones, which elute last. This system has been used to analyse the amino acids present in pollen using post-column derivatization by ninhydrin (Grünfeld, Vincent & Bagnara, 1989) and *o*-phthalaldehyde (OPA) (Day *et al.*, 1990).

Jones, Pääbo & Stein (1981) described a technique of analysing amino acids using reverse-phase HPLC in which the amino acids were pre-column derivatized by OPA enabling a greater sensitivity of detection than methods of post-column derivitization. OPA in the presence of 2-mercaptoethanol reacts with primary amino acids to form intensely fluorescent thio-substituted isoindole derivatives. These are then separated by HPLC and detected by a fluorescent detector.

9.3 AIM

The aim of this study was to determine the identity and quantity of the free amino acids present in oilseed rape (*Brassica napus*) and field bean (*Vicia faba*) pollens as a measure of their total protein amino acid content (Stanley & Linskens, 1974). This measure of amino acid content was then used to compare their relative nutritive values for pollen-seeking insects, in particular, the honey bee.

9.4 MATERIALS & METHODS

9.4.1 Pollen

Oilseed rape (OSR) and field bean (FB) honey bee-collected pollen loads (2.3.2) stored frozen at –20 °C for the six months before analysis were used. Approximately 30-40 pollen loads of each type were thawed, then oven dried for 30 minutes at 40 °C to attain constant weight by removal of excess moisture (Serra Bonvehi & Escolà Jordà, 1997). Of these dried loads, ten of each species were selected at random and individually weighed on a microbalance (Analytical Plus, Ohaus Corporation, New York, U. S. A.). Pollen loads of known weight were placed in marked 0.5 ml Eppendorf tubes, and 200 µl HPLC-grade methanol (Sigma-Aldrich, Poole, Dorset, UK) was pipetted into each tube to extract the methanol-soluble free amino acids present in the pollen. Each sample was homogenized with an electrical vortex for one minute then centrifuged for five minutes at a relative centrifugal force of 13,500 g/min (14,000 revolutions/min in an Eppendorf Centrifuge series 5415 C). The supernatant was decanted into a 1.5 ml Eppendorf, and taken up into a 1 ml disposable syringe (Plastipak, Becton Dickinson UK Ltd., Cowley, Oxford, UK). The sample was then filtered through a syringe filter tip (4 mm, pore size 0.2 µm; Phenomenex, Torrance, California, USA) to remove particulates that could block the HPLC column. Samples were placed in labelled 1.5 ml Eppendorf tubes and frozen until used.

9.4.2 Amino acid standards and supplements

Sigma-Aldrich AA-S-18 amino acid calibration standards were used to help identify amino acids present in the samples. These comprised the following amino acids: alanine (Ala), arginine (Arg), aspartic acid (aspartate; Asp), cysteine (Cys), glutamic acid (glutamate; Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine

(Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr), and valine (Val). These were supplemented with asparagine (Asn), glutamine (Gln) tryptophan (Trp), and the non-protein amino acids, γ-aminobutyric acid (GABA) and homoarganine (Harg). Each amino acid was present at 2.5 mM. Standards and supplements were diluted separately to 1/100 in distilled water, filtered using 0.2 μm Acrodisc disposable filters (Gelman Sciences, USA) to give a concentration of 25 μM. Aliquots of 50 μl were pipetted into Eppendorf tubes and frozen until use.

9.4.3 Buffers

The two buffers used in the mobile phase of the HPLC analysis consisted of a mixture of 83 mM sodium acetate (NaAc) (Fisons HPLC grade, 11.3 g per litre of distilled water, pH adjusted to 5.9 with glacial acetic acid), and HPLC grade methanol:

- *Buffer A*: 200 ml methanol + 800 ml NaAc + 10 ml tetrahydrofuran (Sigma-Aldrich)
- *Buffer B*: 800 ml methanol + 200 ml NaAc

Buffer A acted as the aqueous solvent, to wash hydrophilic amino acids off the HPLC column, and Buffer B as the alcohol-based solvent to wash hydrophobic amino acids from the column. Buffers were filtered using 0.2 μm filters (Rainin Instrument Co., USA) and degassed under vacuum followed by ultrasonics for 10 minutes (Ultrasonic water bath, QH Kerry Ulrtrasonics Ltd. UK).

9.4.4 Reagents

Two reagents were used in the analysis: OPA to derivatize the amino acids before injection onto the column, and phosphate to halt the derivatization reaction. These were prepared as follows:

- Derivatizing solution: *o*-phthaldialdehyde (OPA) (5ml) and 2-mercaptoethanol (10 μl) (both Sigma-Aldrich) were mixed and frozen as 0.5 ml aliquots in Eppendorf tubes until use.
- Phosphate buffer (0.1 M) was prepared by diluting 1.36 g KH_2PO_4 in 100 ml distilled water. The solution was adjusted to pH 4 using hydrochloric acid (HCl), and was also frozen as 0.5 ml aliquots in Eppendorf tubes.

9.4.5 HPLC analysis

The methanol-soluble free amino acids present in the pollen samples were identified and quantified by reversed-phase high performance liquid chromatography (HPLC) after derivatisation with OPA following the procedure of Jones, Pääbo & Stein (1981). The system comprised of a 20 µl injector column (Rheodyne, California, USA) and a Gilson (Middleton, WI, 53562, USA) computerised solvent delivery system (consisting of two pumps (G306), a G805 manometric module, and a G811B dynamic mixer) equipped with a Kingsorb C18 guarded column (C18 150 x 4.6 mm; Phenomenex, Macclesfield, Cheshire, UK) and a CMA/280 fluorescence detector (Roslagsvägen, 101 S-104 05 Stokholm, Sweeden)). A Gilson computerised integrator program was used to calculate the area under each peak for quantification purposes and this was linked to a computer consul display. A schematic diagram of this system and the mode of operation of the HPLC system is shown in Figure 9.1.

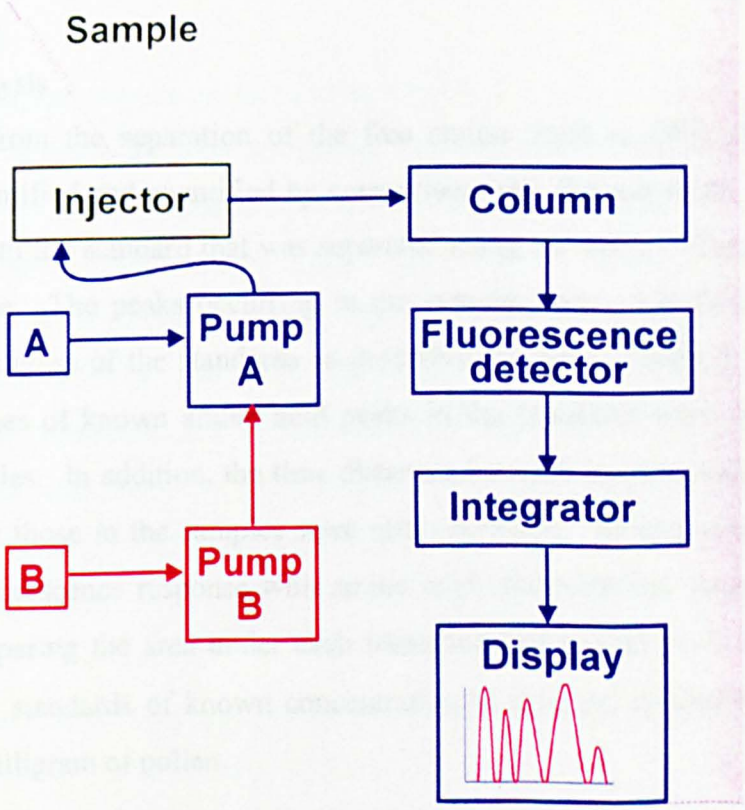


Figure 9.1 Schematic diagram of the mode of operation of HPLC method

All reagents, amino acid standards and supplements, as well as samples, were thawed and maintained over ice until used for HPLC. A standard was processed at the beginning of each day and every time the buffers were replaced. Standards (2.5 µM) were prepared by

adding 2 µl Sigma standards and the same amount of supplements into 16 µl HPLC grade water. Amino acids in 10 µl of this standard solution were derivatized by the addition of 10 µl OPA. The mixture was left for 60 seconds, and the reaction stopped by the addition of 20 µl phosphate. The standard was then injected onto the HPLC column using a 40 µl syringe. Plastic surgical gloves were worn during pipetting procedures to reduce contamination by proteins present in the oils from the surface of the skin. Separation occurred over a programmed time of 36 minutes. The gradient programme was as follows: 0% buffer B upon initiation on the program, with linear step to 14% B over 4 minutes, isocratic step at 14% B for 5 mins., linear step to 50% B over 3 mins., isocratic step at 50% B for 8 mins., linear step to 100% B over 10 mins., isocratic step at 100% B for 2 mins., linear step to 0% B over 4 mins.

Pollen samples were processed in the same way using 10 µl of a 1/500 dilution of sample in place of the standard amino acid mixture. Ten OSR and ten FB pollen loads were analysed in total.

9.4.6 Data analysis

Peaks resulting from the separation of the free amino acids in OSR and FB pollen samples were identified and quantified by comparison with the standards. Each sample was compared with the standard that was separated using the same buffers, on the same day as the sample. The peaks occurring in the samples were identified by using the known order of elution of the standards as described by Jones, Pääbo & Stein (1981). The retention times of known amino acid peaks in the standards were compared with those in the samples. In addition, the time distances between known peaks occurring in the standards and those in the samples were also compared. Since the technique used gives a linear fluorescence response with amino acid concentration, quantification was achieved by comparing the area under each identified amino acid peak in the samples with those of the standards of known concentration to give the concentration (µM) of amino acid per milligram of pollen.

The proportions of each free amino acid (µM/mg) present in OSR and FB pollens were calculated from the total free amino acid content of these pollens. To determine if there were differences between the amount of essential amino acids between the two pollens types, each amino acid was placed into one of four groups, according to De Groot's

(1953) ranking of required amounts for honey bees: non- least-, intermediate- and most-essential amino acids. These were as follows:

1. non-essential = tyrosine, cysteine, serine, alanine, glycine, proline (asparagine, aspartate, glutamate, glutamine and GABA were also included in this group)
2. least-essential = tryptophan, methionine and histidine
3. intermediate-essential = threonine, phenylalanine, arginine and lysine
4. most-essential = leucine, isoleucine and valine

Ratios of each of the three essential groups (least-, intermediate- and most- essential) to the non-essential group were calculated and these were transformed ($\log_{10}$) to normalize the ratios. A multivariate analysis of variance (MANOVA) was performed to test for differences within the three groups between the two pollen types. This test accounted for any dependence within the groups. An analysis of variance was then conducted to test for differences between the two pollen types within each of the three groups.

9.5 RESULTS

Figure 9.2a shows the elution profile and identities of amino acid standards and the supplements. All amino acids were adequately separated and were consistently found in each replicate. Cysteine and proline are absent from standards (and pollen samples) as this method is unable to detect them. This is due to the low fluorescence response of cysteine and the lack of reaction of secondary amino acids such as proline with OPA (Jones, Pääbo & Stein, 1981). Figure 9.2 b and c show the elution profiles and identities of free amino acids present in OSR and FB pollens, respectively. Other than proline and cysteine, all amino acids tested for were found in the pollen samples with the exception of homoarganine (Harg), which was absent from all pollen samples. Thus, eighteen amino acids and one non-protein amino acid were identified in both pollen types.

Six peaks were present in the pollen samples that could not be identified (or quantified) due to their absence in the standards. However, there was some inconsistency in their detection between replicates. Peak 1 with a mean retention time (RT) of 5.4 occurred in OSR pollen samples only. Peak 2, with a mean RT of 8.0 occurred in both OSR and FB pollens, although it did not occur in the OSR replicate shown in Fig. 9.2. Peak 3 had a mean RT of 12.8 and occurred in OSR only. Peak 4 occurred in both OSR and FB samples and had a mean RT of 16.4. Peak 5 occurred in both OSR and FB samples and

had a mean RT of 20.2. Peak 6, with a mean RT of 31.6 occurred in both OSR and FB pollen samples, but is not present in the OSR replicate shown in Fig. 9.2. These unidentified peaks are likely to be non-protein amino acids.

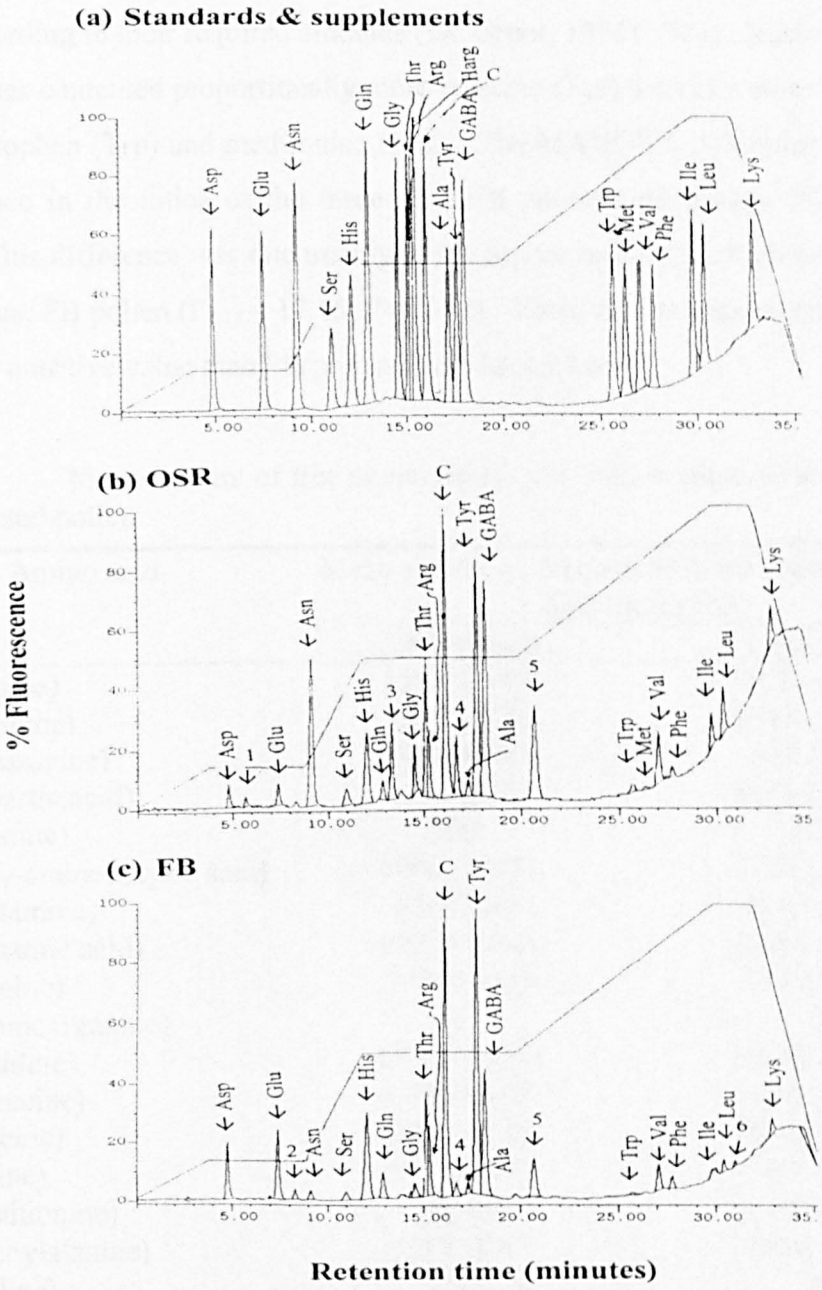


Figure 9.2 Elution profile of amino acids present in (a) standard & supplements; (b) oilseed rape (OSR) pollen; (c) field bean (FB) pollen, after pre-column derivatization with *o*-phthaldialdehyde (OPA) & 2-mercaptoethanol and separation by high performance liquid chromatography. Gradient bar shows % buffer B. Asp = aspartic acid/aspartate; Glu = glutamic acid /glutamate; Asn = asparagine; Ser = serine; His = histidine; Gln = glutamine; Gly = glycine; Thr = threonine; Arg = arginine; Harg = homoarganine; C = contaminant from OPA; Ala = alanine; Tyr = tyrosine; GABA = γ-aminobutyric acid; Trp = tryptophan; Met = methionine; Val = valine; Phe = phenylalanine; Ile = isoleucine; Leu = leucine; Lys = lysine; 1,2,3,4,5,6 = unidentified compounds

Table 9.1 shows the amount (μM /mg) of free amino acids present in OSR and FB pollens. The quantities of free amino acids found in the two pollen samples were relatively similar. Figure 9.3 shows the proportions of each amino acid of the total free content, ordered into 4 groups: non-essential, and the three groups of essential amino acids according to their required amounts (De Groot, 1953). This clearly shows that both pollen types contained proportionally more tyrosine (Tyr) than any other amino acid, and little tryptophan (Trp) and methionine (Met). The MANOVA determined that there was a difference in the ratios of the three essential amino acid groups ($F_{3,16}$ = 8.52; P = 0.001). This difference was due mainly to the higher ratio of most essential amino acids in OSR than FB pollen ($F_{1,18}$ = 17.15; P<0.001). These results suggest that OSR pollen is of greater nutritive value than FB pollen to the honey bee.

Table 9.1 Mean amount of free amino acids (μM /mg) in oilseed rape and field bean bee-collected pollen

Amino acid	Mean amount of free amino acids present in pollen (μM /mg) (±SE)	
	Oilseed rape	Field bean
Ala (alanine)	1263 (398)	1827 (402)
Arg (arginine)	1910 (793)	8518 (1712)
Asn (asparagine)	6330 (1000)	832 (225)
Asp (aspartic acid)	2986 (827)	8498 (2054)
Cys (Cystine)	ND	ND
GABA (γ-aminobutyric acid)	6048 (1028)	9743 (2373)
Gln (glutamine)	863 (200)	1802 (503)
Glu (glutamic acid)	8923 (1984)	11571 (2857)
Gly (glycine)	2189 (445)	1447 (467)
Harg (homoarganine)	0	0
His (histidine)	8722 (1801)	15648 (2930)
Ile (isoleucine)	1795 (405)	676 (335)
Leu (leucine)	3022 (539)	2728 (586)
Lys (lysine)	2956 (515)	3211 (740)
Met (methionine)	24 (24)	42 (42)
Phe (phenylalanine)	1052 (349)	1986 (495)
Pro (proline)	ND	ND
Ser (serine)	3006 (915)	2188 (821)
Thr (threonine)	8976 (1678)	12984 (2166)
Trp (tryptophan)	295 (147)	82 (56)
Tyr (tyrosine)	14949 (2428)	37615 (5514)
Val (valine)	3795 (664)	3916 (826)

ND = not determined in standards.

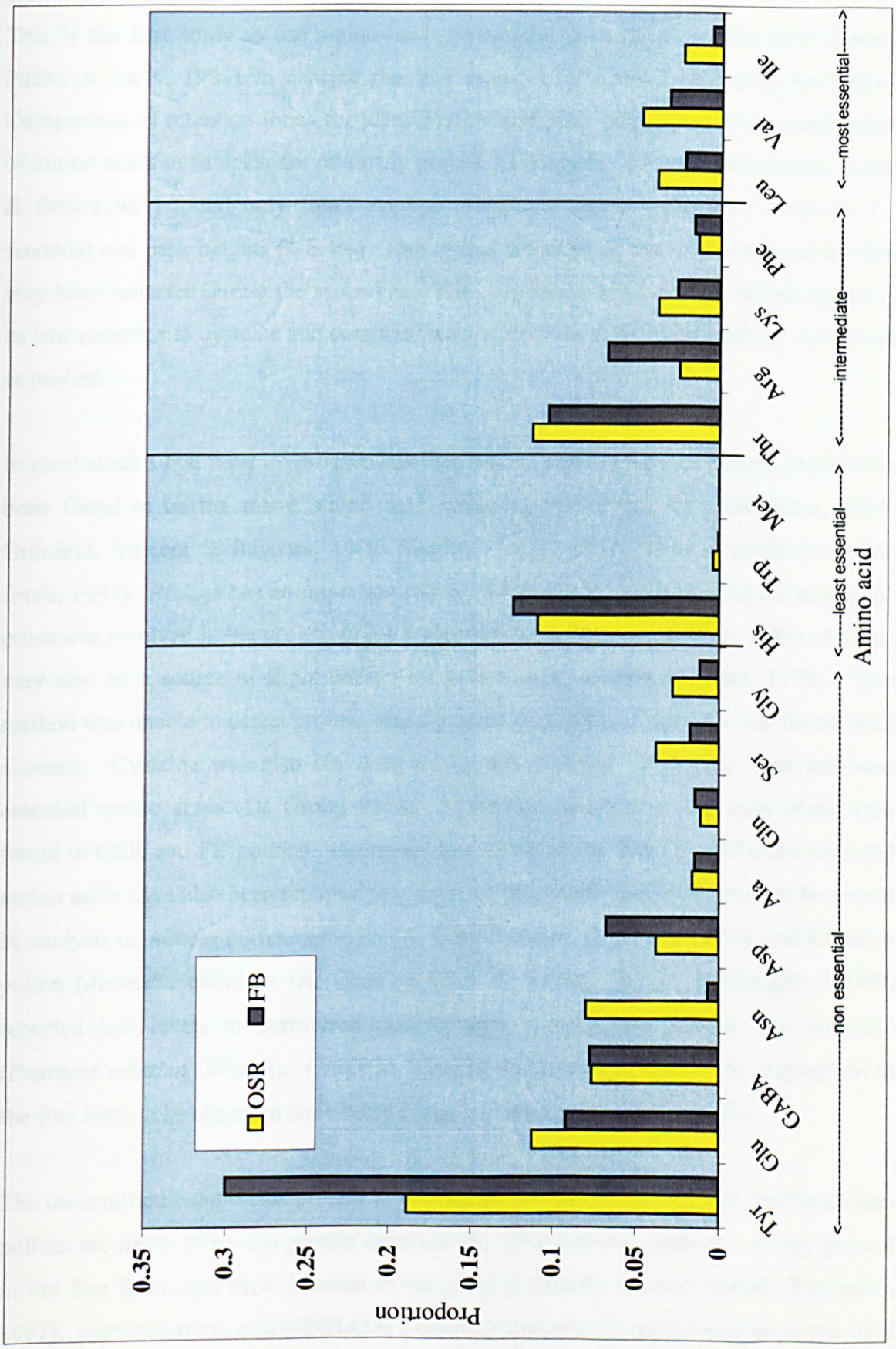


Figure 9.3 Proportions of amino acids of the total free content, with amino acids ordered into four groups: non- least- intermediate- and most- essential, according to their relative required amounts in the diet of the honey bee (De Groot, 1953)

9.6 DISCUSSION

This is the first study to use amino acid pre-column derivatization with OPA (Jones, Pääbo & Stein, 1981) to analyse the free amino acid content of pollen by HPLC. Comparison of retention times for identification and peak height area for quantification of amino acids in samples are relatively precise methods for data analysis; Jones, Pääbo & Sein (1981) found only small average deviations between retention times (± 1.1 seconds) and peak heights (± 6.4%). Part of this deviation is due to pipetting errors that may have occurred during the procedure. The only major disadvantage of this system is its low response to cysteine and complete lack of reaction to secondary amino acids such as proline.

In most studies that have investigated the free amino acid contents of pollen, proline has been found to be the major amino acid occurring in the free form (Bathurst, 1954; Grünfeld, Vincent & Bagnara, 1989; Singh & Singh, 1991; Serra Bonvehi & Escolà Jordà, 1997). Proline has an important role in pollen fertility; it is used in the metabolic processes involved in the growth of the pollen tube (see Agarwal & Nair, 1989). Proline may also be a source of flight energy for pollen-eating insects (Haydak, 1970). This method was unable to detect proline, and a greater proportion of tyrosine was found in its absence. Cysteine was also not detected by this method. However, both are non-essential amino acids (De Groot, 1953). Little free methionine and tryptophan were found in OSR and FB pollens. However, low levels in the free form of these essential amino acids have also been reported for other pollens; methionine was found to be absent in analysis of *Acacia auriculaeformis* (A. Cunn.) pollen (Agarwal, 1989), and kiwifruit pollen (*Actinidia deliciosa* (A. Chev.)) (Clark & Lintas, 1992). McCaughey (1980) reported low levels in burroweed (*Haplopappus tenuisectus* (Blake)) and mesquite (*Prosopis velutina* (Woot.)). Grünfeld, Vincent & Bagnara (1989) found tryptophan in the free form to be absent in strawberry (*Fragaria ananassa*, Dutch.) pollen.

The unidentified compounds present in the elution profiles of oilseed rape and field bean pollens are likely to be non-protein amino acids. These amino acids are usually present in the free form, and their function in the plant is usually defence-related (Rosenthal, 1991). γ-aminobutyric acid (GABA) is a common non-protein amino acid in plants. It is synthesised by decarboxylation from glutamate, and is known to be an inhibitory neurotransmitter in the nervous system of vertebrates. It was first reported in pollen in the free amino acid fraction of grass pollens by Shellard & Jolliffe (1968). It was also

found in the free fraction of kiwifruit pollen (Clark & Lintas, 1992). Peak 6 is possibly ornithine (Orn); a non-defence related non-protein amino acid. It is an intermediate in the reactions of the urea cycle and in arginine synthesis. Ornithine is reported to be detectable under this system (Jones, Pääbo & Stein, 1981) with a RT in this region, and has previously been reported in dandelion (*Taraxacum officinale* (Webber)) pollen (Loper & Cohen, 1987).

Both OSR and FB pollens contained, in the free form, the ten amino acids stipulated by De Groot (1953) as essential in the diet for growth of the honey bee. Therefore, qualitatively, both pollens represent good nutrition for the honey bee. However, OSR pollen was found to contain significantly more of the 'most-essential' amino acids in the free form than FB pollen. This suggests that OSR is of greater nutritional quality to the honey bee than FB pollen. The nutritional requirements of other insects have also been investigated (see reviews by Dadd, 1973; House, 1974). The ten amino acids required in the diet of the honey bee are also essential in the diet for the normal growth of most other insects investigated (Dadd, 1973). Thus conclusions about the nutritional values of pollens in this study may also apply to other insects, and hold so long as the free amino acid content is an accurate measure of the total protein amino acid content (Stanley & Linskens, 1974). The protein amino acid composition of pollens of the Brassicaceae (=Cruciferae), including oilseed rape, (McLellan, 1977; Rayner & Langridge, 1985) and of field bean (see Stace, 1996) have been previously reported, although these authors did not conduct an analysis of the free amino acid content to enable an evaluation of the similarity in their distribution. Therefore, to verify the measure, a comparison of the total protein amino acid content from an acid hydrolysis of pollen proteins with those present in the free-fraction could be conducted using the method described in this chapter.

Several bioassays have demonstrated the superior nutritional quality of OSR (and other members of the Brassicaceae) pollen compared with many other species, although none have yet involved a comparison with FB pollen. Schmidt *et al.*, (1995) showed that bees fed on a diet of OSR pollen had increased longevity compared with sunflower, (*Helianthus annus* L.) or sesame (*Sesamum indicum* L.) pollen. Queen-less bumble bee (*Bombus terrestris* L.) colonies fed on an OSR pollen diet had greater productivity and increased male longevity compared with colonies fed a sunflower pollen diet (Regali & Rasmont, 1995). *Apis cerana* (Fabr) colonies caged on mustard (*Brassica campestris* L.)

showed increased egg laying and increased brood area and larval fresh weight in comparison with colonies caged on non-mustard companion plants (*Ageratum*, *Cynodon* and *Antigonan* spp) (Singh & Singh, 1996). Feeding different pollen types to worker bees influenced the amino acid composition of the larvae they reared; those fed on mustard pollen had higher bound protein amino acid levels than larvae reared on non-mustard pollens (Singh & Singh, 1996). The effect of *Brassica* pollens in exerting high colony growth and bee development in these examples was probably at least partially due to their high content of essential amino acids. However, the presence of phagostimulants (Schmidt, 1985), phago-deterrents (e.g. Levin & Bohart, 1955; Moffett *et al.*, 1975), toxins (Schmidt, Buchmann & Glaiim, 1989) as well as digestibility factors (Barker & Lehner, 1972; Peng *et al.*, 1985), may also affect the nutritional qualities of pollen, and any of these factors may influence bees' preferences for certain pollen types.

Collection of beneficial pollen by foraging bees could only occur if bees can perceive measures of pollen quality and are able to recognize pollen characteristics that enable discrimination. Honey bees can discriminate between pollens on the basis of pollen odour (chapter 7 & 8) and colour (chapter 8). There is also evidence that differences in nutritional quality between pollens may be perceived by bees. The round dances (von Frisch, 1967) of bees in the hive was measured to quantify the pollen quality assessment of bees (Waddington, Nelson & Page, 1998). The dance rate (number of 180° turns per minute) was greater when bees had foraged for pure pollen compared with a lower quality mixture of pollen and non-nutritious alpha-cellulose, indicating that dance rate could be used to quantify a foragers subjective evaluation of pollen quality. A similar study could be conducted to compare the round dance rate of bees that had foraged on either OSR or FB pollen. Such a measure of bees' subjective evaluation or perceived value of OSR and FB pollens could help to explain their choice behaviour.

The criteria by which bees select one nectar or sugar solution over another has been well documented (von Frisch, 1967; Waller, 1972). However, the measures by which they perceive quality differences in pollen are still unclear. Pollen quality could be measured directly by tasting the free amino acids present in the surface layers of the pollen grains. Several insects have sensory receptors that detect amino acids (Shiraishi & Kuwabara, 1970; Mullin *et al.*, 1994) and behavioural responses have been recorded to some. Females of the peacock butterfly, *Inachis io* (L.) preferred an amino acid-containing sucrose solution to plain sucrose solution (Erhardt & Rusterholz, 1998). Furthermore,

they could not discriminate between the mixture and the single amino acids arginine and proline, suggesting that these butterflies can select for high amino acid concentrations in nectar, and perhaps use arginine and proline to do so (Erhardt & Rusterholz, 1998). The free amino acid proline was found to trigger the gustatory response in syrphids (Hansen *et al.*, 1998; Wacht, Lunau & Hansen, 2000). Investigations into the responses of free-flying honey bees to amino acids using an artificial flower feeder found that high concentrations of some amino acids are repulsive, and there appeared to be no correlation between the reactions of bees to essential and non-essential amino acids (Inouye & Waller, 1984). However, in another study, honey bees preferred to feed on sucrose solution containing relatively lower concentrations of glycine (Kim & Smith, 2000). Interestingly, it was also shown that the addition of glycine to a sucrose solution reward had a significant effect in improving the learning performance of bees to odours in the conditioned proboscis extension (CPE) learning bioassay (Kim & Smith, 2000). These authors concluded that honey bees can learn to modify their responses to an odour-conditioned stimulus based on the presence of an amino acid. Similarly, locusts could be conditioned to associate a protein diet with olfactory and visual cues (Simpson & White, 1990; Raubenheimer & Tucker, 1997). The ability of honey bees to learn to modify their behaviour based on the detection of an amino acid in a feeding stimulus could enhance the bees' ability to evaluate the quality of pollen, which could be associated with both pollen and /or floral odours and colours. Further studies, as described by Kim & Smith (2000), could be used to identify which amino acids are detected by bees, and allow insight into their role in honey bee choice behaviour.

10. Discussion

By offering pollen and/or nectar as a food resource, angiosperm plants utilize flower visitors for pollen transport. Pollen therefore acts as both a transport medium for male gametes and as a food reward for potential pollinators. This thesis has presented evidence that in addition, pollen from some species may act as an attractant, providing visual and olfactory signals which pollen-seeking insects may use as cues in resource-location. The use of these signals as cues by the pollen beetle (*Meligethes aeneus* (F.)), and the honey bee (*Apis mellifera* L.), are summarized in this chapter. The importance of pollen in functioning as an attractive signal in oilseed rape (*Brassica napus* L.) and field bean (*Vicia faba* L.) plants is also discussed.

10.1 The use of pollen cues in resource-location by the pollen beetle

This thesis has presented evidence that pollen cues, in particular pollen odour, may be used by the pollen beetle in locating both food and egg-laying sites. A scheme by which this might occur is proposed below.

Upon emergence from over-wintering diapause, pollen beetles, particularly females, must feed on pollen for their sexual maturation (Fritzsche, 1957). Pollen beetles are polyphagous, and this feeding can take place on the flowers of many plants, although they must eventually find cruciferous plants, such as oilseed rape (OSR), upon which they can reproduce. Pollen beetles may have an innate 'search image' of cruciferous plants that they follow, namely UV- yellow floral colour and floral volatiles. Some of these cues are present in many plants, and some are specialized; some being re-enforced after experience of them. However, they accept as food hosts non-cruciferous flowers upon which they may temporarily feed until a crucifer is eventually located. Thus, pollen beetles are stimulated to search for plants upon which they feed and mature by favourable temperatures, sufficient illumination and/or by hunger. Random or downwind movement (Evans & Allen-Williams, 1994) brings them into visual or olfactory range of plant colour and odour cues. Pollen beetles are attracted to yellow (Láska, Zelenková & Bicík, 1986; Buechi, 1990; Ekbom & Borg, 1996; Blight & Smart, 1999) (the petal and pollen colour of most of their cruciferous hosts), and many of the non-cruciferous

flowers upon which they feed are yellow. Thus, colour is an important cue used in this early-season search for flowers. However, in chapter 5 it was demonstrated that beetles are attracted to the odour of some non-cruciferous flowers as well as to OSR floral odour, so the use of general floral volatiles, that are present in many species, also plays a role in the location of hosts at this polyphagous phase in their life cycle. Upon odour perception, beetles orient upwind towards the plant, by odour-induced anemotaxis (Evans & Allen-Williams, 1994). If the first flowering plant located by the pollen beetle is not a crucifer, after temporary feeding the beetle will move on, being guided by its search image to new hosts. Upon finding a cruciferous host, beetles seem to become conditioned to it, and subsequently cease to respond to the odours of non-cruciferous food plants (chapter 5). This phenomenon may enable pollen beetles to remain on cruciferous hosts upon which they can reproduce, perhaps by restricting behavioural responses to odours that are specific to cruciferous flowers. These odours, and their use at this stage of the pollen beetle life cycle, require further investigation.

Pollen beetles are attracted to cruciferous plants upon which they can both feed and reproduce by their odour and the yellow colour of the flowers. Previous work investigating the attraction of pollen beetles to cruciferous-host odour has focused on their attraction to isothiocyanates, (the catabolites of glucosinolates that are specific to plants of the Brassicaceae (= Cruciferae) (Kjaer, 1976; Fenwick, Heaney & Mullin, 1983). These emanate mainly from the green parts of the plant, and although they are specific indicators of cruciferous host plants, they may not indicate that the plant is at the correct growth stage for the availability of pollen and flower-bud resources. Interactions between isothiocyanates and yellow colour on attraction of the pollen beetle and the seed weevil (*Ceutorhynchus assimilis* Payk. – another coleopteran pest of OSR) have been reported (Evans & Allen-Williams, 1989; Blight & Smart, 1999, respectively). The presence of isothiocyanates and yellow colours may synergise attraction; the isothiocyanates used to reliably indicate a cruciferous host and the yellow floral cues used to reliably indicate the presence of resources (pollen and flower buds). In chapter 3, the attraction of pollen beetles to the colour of OSR flowers was once again demonstrated. However, the attraction to floral odour in the absence of the yellow floral colour was also demonstrated, and odour was more important in this (short) distance attraction than colour. There was no evidence for an interaction between flower colour and odour cues. Similarly, Evans & Allen-Williams (1989) found no interaction between OSR floral odours and yellow colour in their study of seed weevil attraction. These

results indicate that pollen beetles may use OSR floral cues as direct indicators of both cruciferous hosts and the availability of pollen and flower bud resources. However, the crucifer-specific flower volatiles that may be involved in this attraction have not yet been identified.

Thus, pollen beetles can be attracted from a distance by both the colour and odour of OSR flowers. When they reach the plant, a combination of colour and short-range odour cues (such as pollen odour) stimulate them to land (chapter 3). Pollen colour-contrasts against petal colour could serve as an attractant at this range, and help insects to position themselves towards the centre of the flower for landing. Tactile and short-range odour cues induce examination of the flower; the presence of flower/pollen odour appears to motivate the beetles to remain in the locality of the source to search (chapters 3 & 4) whereas colour cues do not (chapter 3). Similar behaviour has been observed in other insects, such as moths (Brantjes, 1978) and bees (chapter 8). These short-range odour cues include pollen odours: it was shown that part of the attractive odour of flowers emanates from pollen (chapter 4). If OSR pollen odour is distinct from the other floral structures (Appendices 1.1 & 1.3), as demonstrated for some other species (Dobson, Bergström & Groth, 1990; Bergström, Dobson & Groth, 1995; Dobson, Groth & Bergström, 1996), then these odours could aid insect visitors, including the pollen beetle, in efficiently locating the pollen once they have alighted on the flower.

Once pollen beetles have located pollen, olfactory and/or tactile and gustatory phagostimulants (or the absence of phagodeterrents) induce feeding. The availability of dehisced pollen had the effect of retaining beetles on a plant more than in its absence (chapter 6). Since pollen beetles remain on the plant for some time to feed, this has the effect of causing aggregations of beetles on flowers. This effect could, however, also be due to aggregation pheromones, released in the presence of suitable hosts for reproduction (chapter 4), although this possibility requires further investigation. Aggregation of pollen beetles on host plants upon which they can reproduce facilitates mating, which, as for some other insects, may stimulate female's motivation to lay eggs.

The criteria used by female pollen beetles in host-quality assessment during oviposition have been little studied. They prefer to lay their eggs in OSR buds 2-3 cm long and this is measured by physical means (Borg & Ekbom, 1996). Tactile, visual and olfactory cues induce the female to bite a hole at the base of the bud. Gustatory and olfactory cues

are perceived whilst biting the hole, and these may include volatile isothiocyanates released from the damaged tissues. The female undergoes a complex pattern of behaviours including walking around the bud and placing her abdomen over the bite hole before egg-deposition occurs (Borg & Ekbom, 1996). The ovipositor bears sensillae (Kirk-Spriggs, 1996) and these may have various tactile and chemosensory functions, involved in the perception of cues that indicate host quality and/or oviposition stimuli from within the bud. These may include isothiocyanates (Borg & Borg-Karlson, 1996) or cues related to the presence of anthers within the bud: more male-sterile buds with reduced anthers lacking pollen were found devoid of eggs or larvae than male-fertile ones, indicating that male-sterile buds were often rejected for oviposition (chapter 6). The function of the ovipositor's sensilla and the cues perceived from within the damaged bud whilst pollen beetle females have their abdomens over the oviposition hole require further investigation. The number of eggs laid per bud may reflect oviposition preferences related to host quality assessments (Borg & Ekbom, 1996; Ekbom & Borg, 1996; Hopkins & Ekbom, 1999). However, the presence of pollen did not influence the number of eggs laid in accepted buds of male-sterile and male-fertile plants (chapter 6), indicating that pollen cues do not influence clutch size.

Pollen beetle larvae are not obligatory pollen-feeders: larvae developed on male-sterile OSR flowers without pollen, but did not develop on the flowers of field bean (FB), a non-cruciferous host, that did contain pollen (chapter 6). This suggests that larvae have a requirement for characteristics not present in FB flowers that are present in the flowers of OSR. Further work, comparing development on a wider range of cruciferous and non-cruciferous flowers could identify these requirements. A pollen-diet from OSR flowers was, however, facultative. Larvae reared on male-fertile OSR flowers had an increased survival rate to adulthood, increased weight at every developmental stage and a reduced developmental time compared to larvae reared on male-sterile OSR flowers without pollen (chapter 6). Thus, larvae that are able to receive an adequate diet of pollen will develop faster, which may reduce risks of predation and parasitism through reduced exposure time (Benrey & Denno, 1997); have a greater chance of surviving to adulthood; and will become heavier adults, which in turn may increase overwintering survival (Hokkanen, 1993) and reproductive fitness (Ekbom, 1997). The larval requirements for characteristics of cruciferous flowers, and the benefits that a pollen-diet confers on their development, may therefore have driven the reproductive-specialization of pollen beetles on cruciferous flower buds.

Reproductive specialization on cruciferous plants may have led to the (probably) innate responses of pollen beetles to visual and olfactory cues, including pollen cues, from these host plants: the response to yellow colours and the life-long preference for OSR floral odour over other odours tested (chapter 5). Honey bees, conversely, are generalists, and collect pollen from a wide variety of flowers upon which brood may be reared. Thus, the ability to learn and discriminate cues that indicate highly rewarding flowers as opposed to less-rewarding flowers is an important aspect of their behaviour during foraging. The next section summarizes the importance of the learning and the use of pollen cues in resource-location by the honey bee.

10.2 Learning, generalizing and discrimination of pollen cues by the honey bee

Honey bees are able to learn floral odour and colour cues from rewarding flowers that enable them to locate further rewarding flowers, and since floral colours and odours are species-specific, this promotes floral constancy and increases foraging efficiency (e.g. Menzel & Müller, 1996). In this thesis, it was demonstrated for the first time that honey bees have the ability to associatively learn pollen odours with a reward: OSR and FB pollen odours were learned quickly and efficiently by restrained bees in the conditioned proboscis extension (CPE) bioassay (chapter 7). This test also showed that bees generalize between the odours of OSR and FB pollens, indicating that these odours contain similar volatile compounds, but when differentially conditioned, bees discriminated between them, indicating that the pollens contained differences in their odours that bees could perceive (chapter 7). Both the generalization and the discrimination of odours are important adaptive functions that enable honey bees to forage efficiently given the variability of floral odours over time and space (Smith, 1993). The studies using the CPE bioassay, which measures the reflex responses to different odours of conditioned bees in a restrained preparation, propose the hypothesis that bees learn pollen odours and use them to discriminate between species in the foraging situation. Studies conducted in more natural foraging situations were used to examine this in more detail by examining the choice behaviours of free-flying bees, which involve 'higher neural processing' of information compared with reflex responses (Mauelshagen & Greggers, 1993).

Free-flying bees conditioned to either OSR or FB pollens which served as both the conditioning stimuli and the reward, discriminated between them when conditioned to OSR (i.e. chose OSR in the choice test), but when conditioned to FB, they showed no

more responses to FB than OSR (chapter 8). This result could have been due either to differences in the quality of rewards offered, or to preferences for OSR odour or colour over those of FB. Pollens of different species differ in their nutritional contents (e.g. Day *et al.*, 1990). OSR contains proportionally more of the essential amino acids required for the development of the honey bee than FB (chapter 9). Therefore, OSR pollen offered as reward could form stronger associations with its stimuli (odour and yellow colour) than the association formed between FB pollen as a reward and its odour and grey colour. Differences in the strength of associations formed with stimuli and different concentrations of nectar have been previously demonstrated (Loo & Bitterman, 1992). If this is the case, it indicates that bees are able to assess pollen quality and associate these measures of quality with pollen stimuli such as odour or colour. Pollen cues could therefore be used by bees as indicators of pollen quality. The mechanisms by which bees assess pollen quality and the possibility that pollen cues may be used as indicators of quality warrant further study. The possibility that OSR pollen cues are preferred over those of FB was studied in an experiment that used sucrose solution as a fixed reward, and separated pollen colour and odour cues. Conditioned responses of free-flying bees to these cues were examined. Conditioned bees were able to discriminate between OSR and FB pollens on the basis of their odours alone (chapter 8), supporting the hypothesis that bees can learn pollen odour and use it in the foraging situation to discriminate between different species. No preferences for either odour were exhibited. However, a preference for the yellow colour of OSR pollen was evident over that of the grey colour of FB grey (chapter 8). This preference may represent an innate response to the visual pollen cues, as displayed by other pollen-feeding insects such as syrphids and bumble bees (Lunau, 1990; Lunau & Wacht, 1994).

Pollen cues may therefore be important in resource-location by honey bees by functioning as both visual and olfactory cues that can be learned. If pollen volatiles are distinct from other floral parts, pollen-seeking bees could learn to associate pollen odours from rewarding flowers and use them to discriminate further rewarding flowers from unrewarding flowers with undehisced anthers or from flowers already depleted of pollen. Visual cues may similarly be learned. Pollen (UV-absorbing) can form colour contrasts against UV-reflecting petals (Lunau, 2000) and bees could learn to associate these colour patterns from rewarding flowers and use them to discriminate rewarding flowers from non-rewarding flowers as with pollen odour cues. The demonstration of the use of these cues during pollen-foraging requires further investigation.

10.3 The importance of pollen from different species as a signal to pollinators

The importance of pollen as a signal to pollinators may vary between plants of different species. In general, pollen of wind-pollinated pants does not serve an important attractive function; it has little pollenkitt, inconspicuously-coloured pigments and weak odours. Conversely, the pollen of insect-pollinated plants often serves as an important attractive signal, as demonstrated by their plentiful pollenkitt that usually contains brightly coloured pigments and from which strong odours emanate (von Aufsess, 1960; Dobson & Bergström, 2000; Lunau, 2000). Insect-pollinated plants may offer pollen or nectar as primary rewards to entice visitation by flower-visitors, and, in general, the pollen of plants that offer pollen as the primary reward smells more strongly, and smells different to other floral parts, compared with those that produce nectar as the main reward - particularly those pollinated by insects such as Lepidotera that do not feed on pollen (von Aufsess, 1960; Dobson, 1988). However, few investigations have been made on the importance of pollen signals in plants that offer nectar as a reward but are pollinated by both nectar and pollen-seeking insects.

OSR and FB are both important crop plants in the UK. They both benefit from insect pollination and both offer nectar as a reward. However, they differ in their floral structure and the way pollen is presented to pollinators. OSR has horizontally positioned open, yellow symmetrical flowers that present anthers and pollen openly; thus displaying features typical of beetle/fly pollinated flowers. The flowers of FB are vertically positioned, papilionoid in shape, have complex patterns, and completely enclose the pollen. These are typical bee-specialized flowers and have probably evolved to reduce exploitation by less specialized bees, beetles and flies that are able to collect or eat vast amounts of the limited pollen, by hiding it away. OSR has bright yellow pollen that has a strong odour, whilst FB pollen is an unattractive grey colour and has a very faint smell. Honey bees were able to learn the odour of OSR pollen better than that of FB (chapter 7) and bees preferred the yellow colour of OSR to the grey colour of FB in a choice test (chapter 8). Thus, OSR pollen is as an attractive signal to pollinators, and it is used by pollen-seeking insects as a cue in resource location (chapters 3, 4, 6, & 8). FB pollen, on the other hand, is less successful in attracting pollinators due to its unattractive colour and weak odour. However, since it is enclosed within the petals, it is unlikely to be important in this role, and the attractive functions of the flower are probably fulfilled by the properties of the petals. Thus, the advantage to plants in having attractive pollen properties varies with each plant's reproductive biology, and therefore the likelihood that

pollen signals are used as cues by their flower-visiting insects must be evaluated on a species by species basis.

References

Adegas, J.E.B. & Couto, R.H.N. (1992). Entomophilous pollination in rape (*Brassica napus* L var *oleifera*) in Brazil. *Apidologie,* **23**: 203-209.

Agarwal, A., Nair, P. K. K. (1989). Free and protein-bound amino acids of pollen of *Acacia auriculaeformis* (Mimosaceae). *Grana,* **28**: 155-157.

Agelopoulos, N.G. & Pickett, J.A. (1998). Headspace analysis in chemical ecology: effects of different sampling methods on ratios of volatile compounds present in headspace samples. *Journal of Chemical Ecology,* **24**: 1161-1172.

Agelopoulos, N.G. Hooper, A. M. Maniar, S. P. & Pickett, J.A. (1999). A novel approach for isolation of volatile chemicals released by individual leaves of a plant in situ. *Journal of Chemical Ecology,* **25**: 1144-1425.

Alford, D.V., Cooper, D.A. & Williams, I.H. (1991). *Insect pests of oilseed rape.* (H.G.C.A. Oilseeds Research Review OS1). Home-Grown Cereals Authority, London. 130 pp.

Alford, D.V. & Gould, H.J. (1975). Surveys of pest incidence on oil-seed rape in the UK. *Proceedings of the 8th British Insecticide and Fungicide Conference,* pp. 489-495. British Crop Protection Council, Farnham,.

Attah, P.K. & Lawton, J.H. (1984). The invasion of rape crops by insect pests, with special reference to pollen beetles, *Meligethes* spp. *Aspects of Applied Biology,* **6**: 265-274.

Axelsen, J. & Nielsen, P.S. (1990). Compensation in spring sown oilseed rape after attack by pollen beetles (*Meligethes aeneus* F.). *Tidsskrift for Planteavl,* **94**: 195-199.

Backhaus, W. (1992). Color vision in honeybees. *Neuroscience and Biobehavioral Reviews,* **16**: 1-12.

Backhaus, W. (1993). Color vision and color behavior of the honey bee. *Apidologie,* **24**: 309-331.

Baker, H. (1977). Non-sugar chemical constituents of nectar. *Apidologie,* **8**: 349-356.

Baker, H.G. & Baker, I. (1973). Amino acids in nectar and their evolutionary significance. *Nature,* **241**: 543-545.

Banschbach, V.S. (1994). Colour association influences honey bee choice between sucrose concentrations. *Journal of Comparative Physiology A,* **175**: 107-114.

Barbier, M. (1970). Chemistry and biochemistry of pollens. *Progress in Phytochemistry,* **2**: 1-34.

Barker, R.J. & Lehner, Y. (1972). The resistance of pollen grains and their degradation by bees. *Bee World,* **53**: 173-177.

Bartelt, R.J., Dowd, P.F., Plattner, R.D. & Weisleder, D. (1990). Aggregation pheromone of driedfruit beetle, *Carpophilus hemipterus*: wind-tunnel bioassay and identification of two novel tetraene hydrocarbons. *Journal of Chemical Ecology,* **16**: 1015-1039.

Bartlet, E. (1996). Chemical cues to host plant selection by insect pests of oilseed rape. *Agricultural Zoology Reviews,* **7**: 89-116.

Bartlet, E., Blight, M.M., Hick, A.J. & Williams, I.H. (1993). The responses of the cabbage seed weevil (*Ceutorhynchus assimilis*) to the odour of oilseed rape (*Brassica napus*) and to some volatile isothiocyanates. *Entomologia Experimentalis et Applicata,* **68**: 295-302.

Bathurst, N.O. (1954). The amino-acids of grass pollen. *Series of Experimental Botany,* **5**: 253-256.

Bedinger, P. (1992). The remarkable biology of pollen. *Plant Cell,* **4**: 879-887.

Benrey, B. & Denno, R.F. (1997). The slow-growth-high-mortality hypothesis: a test using the cabbage butterfly. *Ecology,* **78**: 987-999.

Berdegué, M., Reitz, S.R. & Trumble, J.T. (1998). Host plant selection and development in *Spodoptera exigua*: do mother and offspring know best? *Entomologia Experimentalis et Applicata,* **89**: 57-64.

Bergström, G. (1978). Role of volatile chemicals in *Ophrys*-pollinator interactions. In *Biochemical Aspects of Plant and Animal Co-evolution* (Ed. G. Harborne), pp.207-230. Academic Press, New York.

Bergström, G., Dobson, H.E.M. & Groth, I. (1995). Spatial fragrance patterns within the flowers of *Ranunculus acris* (Ranunculaceae). *Plant Systematics and Evolution,* **195**: 221-242.

Bernays, E. & Chapman, R. (1986). The evolution of deterrent responses in plant-feeding insects. In *Perspectives in Chemoreception and Behavior* (Ed. R.F. Chapman, E.A. Bernays & J.G. Stoffolano Jr.), pp.159-173. Springer-Verlag, London.

Bernays, E. & Graham, M. (1988). On the evolution of host specificity in phytophagous arthropods. *Ecology,* **69**: 886-891.

Betts, A.D. (1935). The constancy of the pollen-collecting bee. *Bee World,* **16**: 111-113.

Bhagavan, S. & Smith, B.H. (1997). Olfactory conditioning in the honey bee, *Apis mellifera*: effects of odor intensity. *Physiology and Behavior,* **61**: 107-117.

Bieberdoff, F.W., Gross, A.L. & Weichlen, R. (1961). Free amino acid content of pollen. *Annals of Allergy,* **19**: 867-876.

Bitterman, M.E., Menzel, R., Fietz, A. & Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *Journal of Comparative Psychology,* **97**: 107-119.

Blazejewska, A. (1960). Studia nad biologia slodyszka rzepakowego (Studies on biology of *Meligethes aeneus* Fabr.). *Studia Societatis Scientiarum Torunensis Torun - Polonia,* **Sectio E (Zoologia) 5**: 66- 70 (120-189).

Blight, M.M. (1990). Techniques for isolation and characterization of volatile semiochemicals of phytophagous insects. In *Chromatography and Isolation of Insect Hormones and Pheromones* (Ed. A.R. McCaffrey & I.D. Wilson), pp.281-288. Plenum Press, New York.

Blight, M.M., Le Métayer, M., Pham-Delègue, M.-H., Pickett, J.A., Marion-Poll, F. & Wadhams, L.J. (1997). Identification of floral volatiles involved in recognition of oilseed rape flowers, *Brassica napus* by honeybees, *Apis mellifera. Journal of Chemical Ecology,* **23**: 1715-1727.

Blight, M.M., Pickett, J.A., Ryan, J., Wadhams, L.J. & Woodcock, C.M. (1995). Recognition of oilseed rape volatiles by pollen beetles, *Meligethes spp.*: Electrophysiological and chemical studies. *9th International Rapeseed Congress: Rapeseed Today and Tomorrow* (Ed. D.C. Murphy). **3**: 1043-1045

Blight, M.M. & Smart, L.E. (1999). Influence of visual cues and isothiocyanate lures on capture of the pollen beetle, *Meligethes aeneus* in field traps. *Journal of Chemical Ecology,* **25**: 1501-1516.

Boch, R. (1982). Relative attractiveness of different pollens to honeybees when foraging in a flight room and when fed in the hive. *Journal of Apicultural Research,* **21**: 104-106.

Borg, A. & Borg-Karlson, A.-K. (1996). Differences in female behaviour in two *Meligethes* species on buds of *Brassica napus, B. juncea* and *Sinapsis alba.* In *Oviposition Behaviour of Two Pollen Beetles (Meligethes aeneus and M. viridescens) on Different Host Plants.* PhD Thesis: A. Borg. Chapter IV. Swedish University of Agricultural Sciences, Uppsala, Sweden.

Borg, A. & Ekbom, B. (1996). Characteristics of oviposition behavior of the pollen beetle, *Meligethes aeneus* on four different host plants. *Entomologia Experimentalis et Applicata,* **81**: 277-284.

Borg-Karlson, A.-K. (1990). Chemical and ethological studies of pollination in the genus *Ophys* (Orchidaceae). *Phytochemistry,* **29**: 1359-1387.

Börjesdotter, D. (2000). *Barbarea verna* and *Barbarea vulgaris* as host plants for the pollen beetle (*Meligethes aeneus*). *Journal of Agricultural Science, Cambridge,* **134**: 213-220.

Brantjes, N.B.M. (1978). Sensory responses to flowers in night-flying moths. In *The Pollination of Flowers by Insects* (Ed. A.J. Richards), pp.13-19. Academic Press, London.

Bromand, B. (1983). Possibility of continuous rearing of *Meligethes aeneus* Fabr. (Col.). *Journal of Applied Entomology,* **96**: 419-422.

Brunel, E., Mesquida, J., Renard, M. & Tanguy, X. (1994). Distribution of pollinating entomofauna on rapeseed (*Brassica napus* L) and turnip rape (*Brassica camperstris* L) flowers: the effect of the turnip rape's apetalous character. *Apidologie,* **25**: 12-20.

Buchanan, G.M. & Bitterman, M.E. (1988). Learning in honeybees as a function of amount of frequency of reward. *Animal Learning and Behaviour,* **16**: 247-255.

Buechi, R. (1990). Investigations on the use of turnip rape as trap plant to control oilseed rape pests. *Bulletin IOBC/WPRS,* **13**: 32-39.

Bunting, E.S. (1984). Oilseed rape in perspective: with particular reference to cop expansion and distribution in England 1973-1983. *Aspects of Applied Biology: Agronomy, physiology, plant breeding and crop protection of oilseed rape,* **6**: 11-23.

Butt, T.M., Carreck, N.L., Ibrahim, L. & Williams, I.H. (1998). Honey-bee-mediated infection of pollen beetle (*Meligethes aeneus* Fab.) by the insect-pathogenic fungus, *Metarhizium anisoplia. Biochemical Science and Technology,* **8**: 533-538.

Cane, J.H. (1993). Reproductive role of sterile pollen in cryptically dioecious species of flowering plants. *Current Science,* **65**: 223-225.

Caprio, M.A. & Grafius, E.J. (1990). Effects of light, temperature and feeding status on flight initiation in postdiaiapuse Colorado potato beetles (Coleoptera: Chrysomelidae). *Environmental Entomology,* **19**: 281-285.

Carisey, N. & Bauce, E. (1997). Impact of balsam fir flowering on pollen and foliage biochemistry in relation to spruce budworm growth, development and food utilization. *Entomologia Experimentalis et Applicata,* **85**: 17-31.

Carreck, N. & Williams, I.H. (1998). The economic value of bees in the UK. *Bee world,* **79**: 115-123.

Carruthers, S.P., Marsh, J.S., Turner, P.W., Ellis, F.B., Murphy, D.J., Slabas, T. & Chapman, B.A. (1995). *Industrial Markets for UK-Produced Oilseeds* (OS9), 84 pp Home-Grown Cereals Authority. .

Cassidy, M.D. (1978). Development of an induced food plant preference in the Indian stick insrct *Carausius morosus. Entomologia Experimentalis et Applicata,* **24**: 87-93.

Charpentier, R. (1985). Host plant selection by the pollen beetle *Melegethes aeneus. Entomologia Experimentalis et Applicata,* **38**: 277-285.

Charpentier, R. & Charpentier, B. (1986). Resistance to insects in cruciferous oil crops: Influence of chemical stimuli on the host plants selection by the pollen beetle (*Meligethes aeneus*). *Working Group Eucarpia/IOBC: Breeding for resistance to insects and mites*, pp. 83-84. SROP WPRS Bulletin.

Charpentier, R. & Weibull, J. (1994). Secondary sexual differences of pollen beetle, *Meligethes aeneus* Fabr. (Col., Nitidulidae) pupae. *Journal of Applied Entomology,* **118**: 100-102.

Chittka, L. (1996). Does bee colour vision predate the evolution of flower colour? *Naturwissenscaften,* **83**: 136-138.

Chittka, L. & Menzel, R. (1992). The evolutionary adaption of flower colours and the insect pollinators' colour vision. *Journal of Comparative Physiology A,* **171**: 171-181.

Clark, C.J. & Lintas, C. (1992). Chemical composition of pollen from kiwifruit vines. *New Zealand Journal of Crop and Horticultural Science,* **20**: 337-344.

Cole, R.A. (1980). Volatile compounds produced during ontogeny of some cultivated Crucifers. *Journal of the Science of Food and Agriculture,* **31**: 549-557.

Coleman, J.R. & Coleman, M.A. (1982). Reproductive biology of an andromonoecious *Solanum* (*S. palinacanthum* Dunal). *Biotropica,* **14**: 69-75.

Collet, D. (1991). *Modelling Binary Data.* 369pp. Chapman & Hall, London.

Cook, S., Murray, D.A. & Williams, I.H. (1999). Pollen beetle, *Meligethes aeneus* Fabricius incidence in the composite hybrid winter oilseed rape, Synergy. *Proceedings of the 10th International Rapeseed Congress: New Horizons for an Old Crop.* CD Rom & Abstract book p248 (383pp). Canberra, Australia, GCIRC.

Corbet, S.A., Williams, I.H. & Osborne, J.L. (1991). Bees and the pollination of crops and wild flowers in the European community. *Bee World,* **72**: 47-59.

Courtney, S.P. & Kibota, T.J. (1990). Mother doesn't know best: selection of hosts by ovipositing insects. In *Insect-Plant Interaction II* (Ed. E.A. Bernays), pp.161-188. CRC Press, Boca Raton.

Couvillon, P.A. & Bitterman, M.E. (1984). The overlearning-extinction effect and successive negative contrast in honeybees (*Apis mellifera*). *Journal of Comparative Psychology,* **98**: 100-109.

Couvillon, P.A. & Bitterman, M.E. (1991). How honeybees make choices. In *The behaviour and physiology of bees* (Ed. L.J. Goodman & R.C. Fischer), pp.116-130. CAB International, Wallingford.

CPB Twyford, L. (1996). *Break the barrier: Growers Guide to Synergy Hybrid Winter Oilseed rape.* 18pp. CPB Twyford Ltd., Church Street, Thriplow, Royston, Hertfordshire, UK.

Crailsheim, K. (1990). The protein balance of the honey bee worker. *Apidologie,* **21**: 417-429.

Crepet, W.L. (1983). The role of insect pollination in the evolution of angiosperms. In *Pollination Biology* (Ed. L. Real), pp.29-50. Academic Press, Orlando.

Crepet, W.L., Friis, E.M. & Nixon, K.C. (1991). Fossil evidence for the evolution of biotic pollination. *Philosophical Transactions: Royal Society of London Series B,* **333**: 187-195.

Crowson, R.A. (1981). *The biology of the Coleoptera.* 802 pp Academic Press, London.

Crowson, R.A. (1988). Meligethinae as possible pollinators (Coleoptera: Nitidulidae). *Entomologia Generalis,* **14**: 061-062.

Dadd, R.H. (1973). Insect nutrition: current developments and metabolic implications. *Annual Review of Entomology,* **18**: 381-420.

Dade (1977). *Anatomy and Dissection of the Honeybee.* 158 pp + plates. IBRA, Cardiff.

Dafni, A. (1992). *Pollination Ecology.* 250pp. IRL Press, Oxford.

Dafni, A., Giurfa, M. & Menzel, R. (1997). *Insect Vision and Flower recognition.* 278pp. Laser Pages Publishing, Israel.

D'Arcy, W.G., D'Arcy, N.S. & Keating, R.C. (1990). Scented anthers in the Solanaceae. *Rhodora,* **92**: 50-53.

Darwin, C. (1878). *The effects of Cross and self fertilization in the vegetable Kingdom.* 487 pp, see Chapters 10-11. John Murray, London.

Daumer, K. (1956). Reizmetrische untersuchung des farbensehens der bienen. *Zeitschrift für Vergleichende Physiologie,* **38**: 413-478.

Day, S., Beyer, R., Mercer, A. & Ogden, S. (1990). The nutrient composition of honeybee-collected pollen in Otago, New Zealand. *Journal of Apicultural Research,* **29**: 138-146.

De Groot, A. P. (1953). Protein and amino acid requirements of the honeybee (*Apis mellifera* L.). *Physiologia Comparata et Oecologia,* **3**: 197-285.

Delapane, K.S. & Mayer, D.F. (2000). *Crop Pollination by Bees.* 344pp. CABI, Oxon.

Delisle, J., McNeil, J.N., Underhill, E.W. & Barton, D. (1989). *Helianthus annuus* pollen, an oviposition stimulant for the sunflower moth, *Homoeosoma electellum. Entomologia Experimentalis et Applicata,* **50**: 53-60.

Dixon, A.F.G. & Wratten, S.D. (1971). Laboratory studies on aggregation, size and fecundity in the black bean aphid, *Aphis fabae* Scop. *Bulletin of Entomological Research,* **61**: 97-111.

Dobson, H.E.M. (1987). Role of flower and pollen aromas in plant-host recognition in solitary bees. *Oecologia,* **72**: 618-623.

Dobson, H.E.M. (1988). Survey of pollen and pollenkitt lipids- chemical cues to flower visitors? *American Journal of Botany,* **75**: 170-182.

Dobson, H.E.M. (1989). Pollenkitt in plant reproduction. In *The Evolutionary Ecology of Plants* (Ed. J.H. Bock & Y.B. Lindhart), pp.227-246. Westview, London.

Dobson, H.E.M. (1991a). Analysis of flower and pollen volatiles. In *Essential oils and waxes* (Ed. H.F. Linskens & J.F. Jackson), pp.231-251. Springer - Verlag, Berlin.

Dobson, H.E.M. (1991b). Do pollen odors signal food-reward availability to flower visitors? *1991 Annual Meeting of the Botanical Society of America with the American Institute of Biological Sciences, San Antonio, Texas, USA, August 4-8, 1991. American Journal of Botany,* **78**: 54.

Dobson, H.E.M. (1991c). Pollen and flower fragrances in pollination. *6th Pollination Symposium,* Acta Horticulturae **288**: 313-317.

Dobson, H.E.M. (1994). Floral volatiles in insect biology. In *Insect-Plant Interactions* (Ed. E. Bernays), pp.47-81. CRC Press, Boca Raton.

Dobson, H.E.M., Arroyo, J., Bergstrom, G. & Groth, I. (1997). Interspecific variation in floral fragrances within the genus *Narcissus* (Amaryllidaceae). *Biochemical Systematics and Ecology,* **25**: 685-706.

Dobson, H.E.M. & Bergström, G. (2000). The ecology and evolution of pollen odors. In *Pollen and Pollination* (Ed. A. Dafni, M. Hesse & E. Pachini), pp.63-87. Springer-Verlag, Wein.

Dobson, H.E.M., Bergström, G. & Groth, I. (1990). Differences in fragrance chemistry between flower parts of *Rosa rugosa* Thunb. (Rosaceae). *Israel Journal of Botany,* **39**: 143-156.

Dobson, H.E.M., Bergström, J., Bergström, G. & Groth, I. (1987). Pollen and flower volatiles in two *Rosa* species. *Phytochemistry,* **26**: 3171-3173.

Dobson, H.E.M., Danielson, E.M. & Van Wesep, I.D. (1999). Pollen odor chemicals as modulators of bumble bee foraging on *Rosa rugosa* Thunb. (Rosaceae). *Plant Species Biology,* **14**: 153-166.

Dobson, H.E.M., Groth, I. & Bergström, G. (1996). Pollen advertisment: Chemical contrasts between whole-flower and pollen odors. *American Journal of Botany,* **83**: 877-885.

Doull, K.M. (1966). The relative attractiveness to pollen-collecting honeybees of some different pollens. *Journal of Apicultural Research,* **5**: 9-14.

Dowd, P.F. & Bartelt, R.J. (1991). Host-derived volatiles as attractants and pheromone synergists for driedfruit beetle, *Carpophilus hemipterus*. *Journal of Chemical Ecology*, **17**: 285-308.

Du, Y.-J., Poppy, G.M. & Powell, W. (1996). Relative importance of semiochemicals from first and second trophic levels in host foraging behaviour of *Aphidius ervi*. *Journal of Chemical Ecology*, **22**: 1591-1605.

Dudai, N., Werker, E., Putievsky, E., Ravid, U., Palevitch, D. & Halevy, A.H. (1988). Glandular hairs and essential oils in the leaves and flowers of *Majorana syriaca*. *Israel Journal of Botany*, **37**: 11-18.

Dungey, S.G., Sang, J.P., Rothnie, N.E., Palmer, M.V., Burke, D.G., Knox, R.B., Williams, E.G., Hillard, E.P. & Salisbury, P.A. (1988). Glucosinolates in the pollen of rape seed and Indian Mustard. *Phytochemistry*, **27**: 815-817.

Eisikowitch, D. (1981). Some aspects of pollination of oil-seed rape (*Brassica napus* L.). *Journal of Agricultural Science, Cambridge*, **96**: 321-326.

Ekbom, B. (1997). Rapsbaggens val av värdväxter (Host plant choice by pollen beetles (*Meligethes* spp)). *Vaxtskyddsnotiser*, **61**: 87-91.

Ekbom, B. (1998). Clutch size and larval performance of pollen beetles on different host plants. *Oikos*, **83**: 56-64.

Ekbom, B. & Borg, A. (1996). Pollen beetle (*Meligethes aeneus*) oviposition and feeding preference on different host plant species. *Entomologia Experimentalis et Applicata*, **78**: 291-299.

Erhardt, A. & Rusterholz, H.-P. (1998). Do peacock butterflies (*Inachis io* L) detect and prefer nectar amino acids and other nitrogenous compounds? *Oecologia*, **117**: 536-542.

Evans, K.A. & Allen-Williams, L.J. (1989). The response of the cabbage seed weevil (*Ceutorhynchus assimilis* Payk.) and the brassica pod midge (*Dasineura brassicae* Winn.) to flower colour and volatiles of oilseed rape. *Aspects of Applied Biology*, **23**: 347-353.

Evans, K.A. & Allen-Williams, L.J. (1992). Electroantennogram responses of the cabbage seed weevil, *Ceutorhynchus assimilis*, to oilseed rape, *Brassica napus* spp. *olifera*, volatiles. *Journal of Chemical Ecology*, **18**: 1641-1659.

Evans, K.A. & Allen-Williams, L.J. (1994). Laboratory and field response of the pollen beetle, *Meligethes aeneus*, to the odour of oilseed rape. *Physiological Entomology*, **19**: 285-290.

Evans, K.A. & Scarisbrick, D.H. (1994). Integrated insect pest management in oilseed rape crops in Europe. *Crop Protection*, **13**: 403-412.

Faber, F., Fischer, G. & Kalt, B. (1920). Die biologische Bedeutung des Rapsglanzkafers fur Raps, Rubsen und Senf. *Landwirtschaftliche Jahrbucher,* **54**: 681-701.

Fadamiro, H.Y., Wyatt, T.D. & Birch, M.C. (1998). Flying beetles respond as moths predict: Optomotor anemotaxis to pheromone plumes at different heights. *Journal of Insect Behaviour,* **11**: 549-557.

Fægri, K. & van der Pijl, L. (1979). *The Principles of Pollination Ecology.* (3rd ed.), 244pp. Pergamon, Oxford.

Feeney, P. (1977). Defensive ecology of the Cruciferae. *Annals of the Missouri Botanical garden,* **64**: 221-234.

Fenwick, G.R., Heaney, R.K. & Mullin, W.J. (1983). Glucosinolates and their breakdown products in food and food plants. *CRC Critical Reviews in Food Science and Nutrition,* **18**: 123-201.

Finch, S., Collier, R.H. & Elliott, M.S. (1990). Seasonal variations in the timing of attacks of bronzed blossom beetles *Meligethes aeneus / Meligethes viridescens* on horticultural brassicas. *Brighton Crop Protection Conference: Pests and Diseases*, pp. 349-354. Brighton, UK, British Crop protection Council, Farnham.

Free, J.B. (1962). The behaviour of honey bees visiting field beans (*Vicia faba*). *Journal of Animal Ecology,* **31**: 497-502.

Free, J.B. (1963). Flower constancy of honey bees. *Journal of Animal Ecology,* **32**: 119-131.

Free, J.B. (1993). *Insect Pollination of Crops.* (2nd ed.), 684pp. Academic press, London.

Free, J.B. & Nuttall, P.M. (1968). The pollination of oilseed rape (*Brassica napus*) and the behaviour of bees on the crop. *Journal of Agricultural Sciences, Cambridge,* **71**: 91-94.

Free, J.B. & Williams, I.H. (1978a). The responses of the pollen beetle *Meligethes aeneus*, and the seed weevil *Ceuthorhynchus assimilis*, to oilseed rape, *Brassica napus* and other plants. *Journal of Applied Ecology,* **15**: 761-774.

Free, J.B. & Williams, I.H. (1978b). A survey of the damage caused to crops of oil-seed rape (*Brassica napus* L.) by insect pests in south-central England and their effect on seed yield. *Journal of Agricultural Sciences, Cambridge,* **90**: 417-424.

Free, J.B. & Williams, I.H. (1979). The distribution of insect pests on crops of oil-seed rape (*Brassica napus* L.) and the damage they cause. *Journal of Agricultural Science Cambridge,* **92**: 139-149.

Frings, H. (1944). The loci of olfactory end-organs in the honey bee *Apis mellifera*. *Journal of Experimental Zoology,* **97**: 123-134.

Fritzsche, R. (1955). Zur morphologie von *Meligethes aeneus* Fabr., *M. viridescens* Fabr., *M. coracinus* Sturm., und *M. picipes* Sturm. *Beitrage zur Entomologie,* **5**: 309-333.

Fritzsche, R. (1957). Zur biologie und ökologie der räpschadlinge aus der gattung *Meligethes*. *Zeitschrift fur Angrewandte Entomologie,* **40**: 220-280.

Gack, C. (1981). Zur bedeutung von staubgefäßattrappen als signale für die bestäuber experimente mit hummeln (*Bombus terrestris*) (The effect of imitation stamina in flowers on flower-visiting bumblebees). *Zoologische Jahrbucher Abteilung für Systematik Okologie und Geographic der Thiere,* **108**: 229-246.

GenStat, (Payne, R.W., Lane, P.W., Digby, P.G.N., Harding, S.A., Leech, P.K., Morgan, G.W., Todd, A.D., Thompson, R., Tunnicliffe-Wilson, G., Welham, S.J. & White, R.P.) (1993). *Genstat 5 Release 3 Reference Manual.* Oxford University Press, Oxford.

GenStat, (Payne, R.W. & Arnold, G.M.) (1998). *Genstat 5 Release 4.1 Procedure Library Manual PL11.* Numerical Algorithms Group Ltd., Oxford.

Gerber, B., Geberzahn, N., Hellstern, F., Klein, J., Kowalksy, O., Wüstenberg, D. & Menzel, R. (1996). Honey bees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. *Animal Behaviour,* **52**: 1079-1085.

Getz, W.M. & Smith, K.B. (1987). Olfactory sensitivity and discrimination of mixtures in the honeybee *Apis mellifera*. *Journal of Comparative Physiology A,* **160**: 239-245.

Giamoustaris, A. & Mithen, R. (1996). The effect of flower colour and glucosinolates on the interaction between oilseed rape and pollen beetles. *Entomologia Experimentalis et Applicata,* **80**: 206-208.

Gilbert, F.S. (1986). *Hoverflies*. 66pp. Camberidge University Press, Cambridge.

Giurfa, M. (1991). Colour generalization and choice behaviour of the honeybee *Apis mellifera ligustica*. *Journal of Insect Physiology,* **37**: 41-44.

Giurfa, M. & Núñez, J. (1989). Colour signals and choice behaviour of the honeybee (*Apis mellifera ligustica*). *Journal of Insect Physiology,* **35**: 907-910.

Giurfa, M. & Núñez, J.A. (1992). Honey bees mark with scent and reject recently visited flowers. *Oecologia,* **89**: 113-117.

Giurfa, M., Núñez, J.A., Chittka, L. & Menzel, R. (1995). Colour preferences of flower-naive honeybees. *Journal of Comparative Physiology A,* **177**: 247-259.

Golding, Y.C., Sullivan, M.S. & Sutherland, J.P. (1999). Visits to manipulated flowers by *Episyrphus balteatus* (Diptera: Syrphidae): Partitioning the signals of petals and anthers. *Journal of Insect Behavior,* **12**: 39-45.

Gori, D.F. (1983). Post-pollination phenomena and adaptive floral changes. In *Handbook of Experimental Pollination Biology* (Ed. C.E. Jones & R.J. Little), pp.31-45. Scientific and Academic Editions, Van Nostrand Reinhold, London.

Görnitz, K. (1956). Weitere untersuchungen uber insekten-attraktivstoffe aus Cruciferen. *Nachrichtenblatt für den Deutschen Pflanzenscutzdienst neue folge,* **10**: 137-147.

Gould, H.J. (1975). Surveys of pest incidence on oil-seed rape in in south central England. *Annals of Applied biology,* **79**: 19-26.

Goulson, D. (2000). Are insects flower constant because they use search images to find flowers? *Oikos,* **88**: 547-552.

Green, T.A., Prokopy, R.J. & Hosmer, D.W. (1994). Distance of response to host tree models by female apple maggot flies, *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae): Interaction of visual and olfactory stimuli. *Journal of Chemical Ecology,* **20**: 2393-2413.

Gregg, K.B. (1983). Variation in floral fragrances and morphology: incipient speciation in Cycnoches? *Botanical Gazette,* **144**: 566-576.

Greggers, U. & Menzel, R. (1993). Memory dynamics and foraging strategies of honey bees. *Behavioural Ecology and Sociobiology,* **32**: 17-29.

Grünfeld, E., Vincent, C. & Bagnara, D. (1989). High-performance liquid chromatography analysis of nectar and pollen of strawberry flowers. *Journal of Agriculture and Food Chemistry,* **37**: 290-294.

Gupta, P.D. & Thorsteinson, A.J. (1960). Food plant relationships of the diamond black moth (*Plutella maculipennis* (Curt.)). *Entomologia Experimentalis et Applicata,* **3**: 305-314.

Hansen, K., Wacht, S., Seebauer, H. & Schnuch, M. (1998). New aspects of chemoreception in flies. *Annals New York Academy of Sciences,* **855**: 143-147.

Hardie, J., Holyoak, M., Nicholas, J., Nottingham, S.F., Pickett, J.A., Wadhams, L.J. & Woodcock, C.M. (1990). Aphid sex pheromone components: age-dependent release by females and species-specific male response. *Chemoecology,* **1**: 63-68.

Hardie, J., Isaacs, R., Pickett, J., A, Wadhams, L., J. & M, W.C. (1994). Methyl salicylate and (-) - (*1R, 5S*)-myrtenal are plant-derived repellents for black bean aphid, *Aphis fabae* Scop. (Homoptera: Aphididae). *Journal of Chemical Ecology,* **20**: 2847-2855.

Hassell, M.P. & Southwood, T.R.E. (1978). Foraging strategies of insects. *Annual Review of Entomology,* **9**: 75-98.

Haydak, M.H. (1970). Honey bee nutrition. *Annual Review of Entomology,* **15**: 143-156.

Heeger, E. (1855). Beitrage zur naturgeschichte der insecten. *Sitzungsberichte der Akademie der Wissenschaften. Mathematisch - Naturwissenschaftliche Classe. Wein,* **14**: 273-281.

Heikertinger, F. (1920). Untersuchungen uber die Standpflanzen der Blutenkafergattungen *Meligethes*, *Brachypterus*, und *Brachypterolus* (*Heterostomus*). *Entomologische Blatter fur Biologie und Systematik der Kafer Berlin,* **16**: 126-143.

Heinrich, B. (1983). Insect foraging energetics. In *Handbook of Experimental Pollination Biology* (Ed. C.E. Jones & R.J. Little), pp.187-214. Van Nostrand Reinhold, London.

Henning, J.A., Peng, Y.-S., Montague, M.A. & Teuber, L.R. (1992). Honey bee (Hymenoptera: Apidae) behavioural responses to primary alfalfa (Rosales: Fabaceae) floral volatiles. *Journal of Economic Entomology,* **85**: 233-239.

Herbert, E.W., Shimanuki, H., Shasha, B. S. (1980). Brood rearing and food consumption by honeybee colonies fed pollen substitutes supplimented with starch-encapsulated pollen extracts. *Journal of Apicultural Research,* **19**: 115-118.

Herbert, E.W. (1992). Honey bee nutrition. In *The hive and the honey bee* (Ed. J.M. Graham), pp.197-233. Dadant & Sons, Hamilton, USA.

Herbert, E.W., Bickley, W.E. & Shimanuki, H. (1970). The brood-rearing capability of caged honey bees fed dandelion and mixed pollen diets. *Journal of Economic Entomology,* **63**: 215-218.

Herbert, E.W. & Shimanuki, H. (1978). Chemical composition and nutritive value of bee-collected and bee-stored pollen. *Apidologie,* **9**: 33-40.

Herbert, E.W., Shimanuki, H. & Caron, D. (1977). Optimum protein levels required by honey bees (*Hymenoptera, Apidae*) to initiate and maintain brood rearing. *Apidologie,* **8**: 141-146.

Hodges, D. (1952). *The pollen loads of the honeybee. A guide to their identification by colour and form.* 51pp. Bee Research Association, London.

Hodges, D. (1984). *The pollen loads of the honey bee. A guide to their identification by colour and form: 2nd facsimile reprint of original edition; with addenda.* 30 pp + plates. International Bee research Association, London.

Hokkanen, H., Granlund, H., Husberg, G.-B. & Markkula, M. (1986). Trap crops used successfully to control *Meligethes aeneus* (Coleoptera: Nitidulidae the rape blossom beetle). *Annales Entomologici Fennici,* **52**: 115-120.

Hokkanen, H.M.T. (1989). Biological and agrotechnical control of the rape blossom beetle *Meligethes aeneus* (Coleoptera, Nitidulidae). *Acta Entomologica Fennica,* **53**: 25-29.

Hokkanen, H.M.T. (1993). Overwintering survival and spring emergence in *Meligethes aeneus*: effects of body weight, crowding, and soil treatment with *Beauveria bassiana*. *Entomologia Experimentalis et Applicata,* **67**: 241-246.

Hokkanen, H.M.T. (2000). The making of a pest: recruitment of *Meligethes aeneus* onto oilseed Brassicas. *Entomologia Experimentalis et Applicata,* **95**: 141-149.

Hopkins, R.J. & Ekbom, B. (1996). Low oviposition stimuli reduce egg-production in the pollen beetle *Meligethes aeneus*. *Physiological Entomology,* **21**: 118-122.

Hopkins, R.J. & Ekbom, B. (1999). The pollen beetle, *Meligethes aeneus*, changes egg production rate to match host quality. *Oecologia,* **120**: 274-278.

Hori, M. (1998). Repellency of rosemary oil against *Myzus persicae* in a laboratory and in a screenhouse. *Journal of Chemical Ecology,* **24**: 1425-1432.

House, H.L. (1974). Nutrition. In *The physiology of insecta* (Ed. M. Rockstein), pp.1-62. Academic Press, London.

Hügel, M.-F. (1962). Étude de quelques constituants du pollen. *Annales Abeille,* **5**: 97-133.

Imdorf, A., Rickli, M., Kilchenmann, V., Bogdanov, S. & Wille, H. (1998). Nitrogren and mineral constituents of honey bee worker brood during pollen shortage. *Apidologie,* **29**: 315-325.

Inouye, D.W. & Waller, G.D. (1984). Responses of honey bees (*Apis mellifera*) to amino acid solutions mimicking floral nectars. *Ecology,* **65**: 618-625.

Irvin, N.A., Wratten, S.D., Frampton, C.M., Bowie, M.H., Evans, A.M. & Moar, N.T. (1999). The phenology and pollen feeding of three hover fly (Diptera: Syrphidae) species in Canterbury, New Zealand. *New Zealand Journal of Zoology,* **26**: 105-115.

Isaacs, R., Hardie, J., Hick, A.J., Pye, B.J., Smart, L.E., Wadhams, L.J. & Woodcock, C.M. (1993). Behavioural responses of *Aphis fabae* to isothiocyanates in the laboratory and field. *Pesticide Science,* **39**: 349-355.

Jayanth, K.P., Mohandas, S., Asokan, R. & Ganga Visalakshy, P.N. (1993). Parthenium pollen induced feeding by *Zygogramma bicolorata* (Coleoptera: Chrysomelidae) on sunflower (*Helianthus annus*) (Compositae). *Bulletin of Entomological Research,* **83**: 595-598.

Jermy, T., Hanson, F.E. & Dethier, V.G. (1968). Induction of specific food preference in Lepidopterous larvae. *Entomologia Experimentalis et Applicata,* **11**: 211-230.

Jones (1985). Chemical mediation of insect behaviour. In *Progress in Pesticide Biochemistry and Toxicology* (Ed. D.H. Hutson & T.R. Roberts), pp.311-373. Wiley New York, .

Jones, B.N., Pääbo, S. & Stein, S. (1981). Amino acid analysis and enzymatic sequence determination of peptides by an improved *o*-phthaldialdehyde precolumn labelling procedure. *Journal of Liquid Chromatography,* **4**: 565-586.

Jones, R.E. (1991). Host location and oviposition on plants. In *Reproductive Behavior of Insects: Individuals and Populations* (Ed. W.J. Bailey & J. Ridsdill-Smith), pp.108-138 Chapman & Hall, London.

Judd, G.J.R. & Borden, J.H. (1991). Sensory interaction during trap-finding by female onion flies: implications for ovipositional host-plant finding. *Entomologia Experimentalis et Applicata,* **58**: 239-249.

Juliano, S.A. (1985). The effects of body size on mating and reproduction in *Brachinus lateralis* (Coleoptera: Carabidae). *Ecological Entomology,* **10**: 271-280.

Kaiser, R. (1991). Trapping, investigation and reconstitution of flower scents. In *Perfumes: Art, Science and Technology* (Ed. P.M. Muller & D. Lamparsky), pp.213-250. Blackie Academic & Professional, London.

Kalish, H.I. (1969). Stimulus generalization. In *Learning: Processes* (Ed. M.H. Marx), pp.207-297. Macmillan, New York.

Kay, Q.O.N. (1976). Preferential pollination of yellow-flowered morphs of *Raphanus raphanistrum* by *Peris* and *Eristalis* spp. *Nautre,* **261**: 230-233.

Kennedy, J.S. (1977). Olfactory responses to distant plants and other odor sources. In *Chemical Control of Insect behavior: Theory and Application* (Ed. H.H. Storey & J.J.J. McKelvey), pp.67-91. Wiley, London.

Kevan, P.G. (1983). Floral colours through the insect eye: what they are and what they mean. In *Handbook of Experimental Pollination Biology* (Ed. C.E. Jones & R.J. Little), pp.3-30. Scientific & Academic Editions, Van Nostrand, London.

Kevan, P.G. & Baker, H.G. (1983). Insects as flower visitors and pollinators. *Annual Review of Entomology,* **28**: 407-53.

Kim, Y.S. & Smith, B.H. (2000). Effect of an amino acid on feeding preferences and learning behaviour in the honey bee, *Apis mellifera. Journal of Insect Physiology,* **46**: 793-801.

Kirk, W.D.J. (1985a). Effect of some floral scents on host finding by thrips (Insecta: Thysanoptera). *Journal of Chemical Ecology,* **11**: 35-43.

Kirk, W.D.J. (1985b). Pollen-feeding and the host specificity and fecundity of flower thrips (Thysanoptera). *Ecological Entomology,* **10**: 281-289.

Kirk, W.D.J. (1992). *Insects on Cabbages and Oilseed rape.* 66pp. Richmond Publishing Co. Ltd., Slough, UK.

Kirk, W.D.J. (1994). *A Colour Guide to Pollen Loads of the Honey bee.* 54pp. International Bee Research Association, Cardiff, UK.

Kirk, W.D.J., Ali, M. & Breadmore, K.N. (1995). The effects of pollen beetles on the foraging behaviour of honey bees. *Journal of Apicultural Research,* **34**: 15-22.

Kirk-Spriggs, A.H. (1991). Nitidulidae. In *A Coleopterist's handbook* (Ed. J. Cooter & G.e.P.W. Cribb), pp.123-128. The Amateur Entomologist's Society, Feltham, Middx.

Kirk-Spriggs, A.H. (1996). *Pollen beetles. Coleoptera:Kateretidae and Nitidulidae:Meligethinae.* 157pp. Royal Entomological Society of London, London.

Kjaer, A. (1976). Glucosinolates in the cruciferae. In *The biology and Chemisrty of the Cruciferae* (Ed. J.G. Vaughan, A.J. Macleod & B.M.G. Jones), pp.207-219. Academic Press, London.

Knoll, F. (1930). Über pollenkitt und bestaubungsart. *Zeitschrift fur Botanik.,* **23**: 609-675.

Knudsen, J.T. & Tollsten, L. (1991). Floral scent and intrafloral scent differentiation in *Moneses* and *Pyrola* (*Pyrolaceae*). *Plant Systematics and Evolution,* **177**: 81-91.

Knudsen, J.T., Tollsten, L. & Bergström, L.G. (1993). Floral scents - A checklist of volatile compounds isolated by head-space techniques. *Phytochemistry,* **33**: 253-280.

Knuth, p. (1908-1909). *Handbook of Flower Pollination: Observations in flower pollination made in Europe and the Arctic regions on species belonging to the natural orders.* Vol I (1908) 703pp; Vol II (1909) 644 pp. Translated by Ainsworth Davis, J. R. Oxford press, Oxford.

Kostal, V. (1992). Monitoring of activity and abundance of adult pollen beetle (*Meligethes aeneus* F) and cabbage stem weevil (*Ceutorhynchus pallidactylus* Marsh) in winter rape stand. *Rostlinna Vyroba,* **38**: 297-306.

Laloi, D., Roger, B., Blight, M.M., Wadhams, L.J. & Pham-Delègue, M.-H. (1999). Individual learning ability and complex odour recognition in the honey bee, *Apis mellifera* L. *Journal of Insect Behaviour,* **12**: 585-597.

Lance, D.R. (1983). Host-seeking behavior of the gypsy moth: the influence of polyphagy and highly apparent host plants. In *Herbivorous Insects: Host-seeking Behaviour and Mechanisms* (Ed. S. Ahmad), pp.201-224. Academic press, New York.

Laska, M., Galizia, C.G., Giurfa, M. & Menzel, R. (1999). Olfactory discrimination ability and odour structure-activity relationships in honeybees. *Chemical Senses,* **24**: 429-438.

Láska, P. & Kocourek, F. (1991). Monitoring of flight activity in some crucifer-feeding pests by means of yellow water-traps. *Acta Entomologica Bohemoslovaca,* **88**: 25-32.

Láska, P., Zelenková, I. & Bicík, V. (1986). Colour attraction in species of the genera: *Delia* (Diptera, Anthomyidae), *Ceutorhynchus, Meligethes* and *Phyllotreta* (Coleoptera: Curculionidae, Nitidulidae, Chrysomelidae). *Acta Entomologica Bohemoslovaca,* **83**: 418-424.

Le Métayer, M., Marion-Poll, F., Sandoz, J.C., Pham-Delègue, M.-H., Blight, M.M., Wadhams, L.J., Masson, C. & Woodcock, C.M. (1997). Effect of conditioning on discrimination of oilseed rape volatiles by the honeybee: use of a combined gas chromatography-proboscis extension behavioural assay. *Chemical Senses,* **22**: 391-398.

Le Métayer, M., Pham-Delègue, M.-H., Thiery, D. & Masson, C. (1993). Influence of host- and non-host plant pollen on the calling and oviposition behaviour of the European sunflower moth *Homoeosoma nebulellum* (Lepidoptera: Pyralidae). *Acta Oecologia,* **14**: 619-626.

Leather, S.R. (1994). Life history traits of insect herbivores in relation to host quality. In *Insect Plant Interactions* (Ed. E.A. Bernays), pp.175-207 CRC, Boca Raton.

Lepage, M. & Boch, R. (1968). Pollen lipids attractive to honeybees. *Lipids,* **3**: 530-534.

Lerin, J. (1984). Effect de deux isothiocyanates sue les niveaux de capture en cuvettes jaunes d'insectes ravageurs du colza. *Acta Œcologica / Œcologia Applocata,* **5**: 61-70.

Levin, M.D. & Bohart, G.E. (1955). Selection of pollens by honey bees. *American Bee Journal,* **95**: 392-393 & 402.

Levin, M.D. & Haydak, M.H. (1957). Comparitive value of different pollens in the nutrition of *Osmia lignaria. Bee World,* **38**: 221-226.

Lewis, J.A., Moore, C.J., Fletcher, M.T., Drew, R.A. & Kitching, W. (1988). Volatile compounds from the flowers of *Spathiphyllum cannaefolium. Phytochemistry,* **27**: 2755-2757.

Lin, H. & Phelan, P.L. (1991). Identification of food volatiles attractive to dusky sap beetle, *Carpophilus lugubris* (Coleoptera: Nitidulidae). *Journal of Chemical Ecology,* **17**: 1273-1286.

Lin, S. & Mullin, C. (1999). Lipid polyamide and flavanol phagostimulants for adult western corn rootworm from sunflower (*Helianthus annus* L.) pollen. *Journal of Agricultural Food Chemistry,* **47**: 1223-1229.

Linsley, E.G. (1961). The role of flower specificity in the evolution of solitary bees. *Proceedings of the 11th International Congress of Entomology. 1960, Vienna,* **1**: 593-596.

Loo, S.K. & Bitterman, M.E. (1992). Learning in honey bees (*Apis mellifera*) as a function of sucrose concentration. *Journal of Comparative Psychology A,* **108**: 29-36.

Loper, G.M. & Cohen, A.C. (1987). Amino acid content of dandelion pollen: a honey bee (Hymenoptera: Apidae) nutritional evaluation. *Journal of Economic Entomology,* **80**: 14-17.

Lunau, K. (1988). Angeborenes und erlerntes verhalten beim blütenbesuch von schwebfliegen - attrappenversuche mit *Eristalis pertinax* (Scopoli) (Diptera, Syrphidae) (Innate and learned behaviour of flower-visiting hoverflies - flower-dummy

experiments). *Zoologische Jahrbucher Abteilung für Allemeine Zoologie und Physiologie der Tiere,* **92**: 487-499.

Lunau, K. (1990). Colour saturation triggers innate reactions to flower signals: Flower dummy experiments with bumble bees. *Journal of Comparative Physiology A,* **166**: 827-834.

Lunau, K. (1991). Innate flower recognition in bumble bees (*Bombus terrestris, B. lucorum*; Apidae): Optical signals from stamens as landing reaction releasers. *Ethology,* **88**: 203-214.

Lunau, K. (1992). Innate recognition of flowers by bumble bees: orientation of antennae to visual stamen signals. *Canadian Journal of Zoology,* **70**: 2139-2144.

Lunau, K. (1996). Signalling functions of floral colour patterns for insect flower visitors. *Zoologischer Anzeiger,* **235**: 11-30.

Lunau, K. (2000). The ecology and evolution of visual pollen signals. In *Pollen and Pollination* (Ed. M. Hesse & E. Pacini), pp.89-112. Springer-Verlag, New York.

Lunau, K. & Wacht, S. (1994). Optical releasers of the innate proboscis extension in the hoverfly *Eristalis tenax* L. (Syrphidae, Diptera). *Journal of Comparative Physiology A,* **174**: 575-579.

Magin, N. & Classen, R. & Gack, C. (1989). The morphology of false anthers in *Craterostigma plantagineum* and *Torenia polygoponides* (Scrophulariaceae). *Canadian Journal of Botany,* **67**: 1931-1937.

Manning, R. (1995). *Honeybee Pollination* (Bulletin 4298), Western Australia Department of Agriculture.

Marfaing, P., Rouault, J. & Laffort, P. (1989). Effect of the concentration and nature of olfactory stimuli on the proboscis extension of conditioned honey bees *Apis mellifica ligustica. Journal of Insect Physiology,* **35**: 949-955.

Mauelshagen, J. & Greggers, U. (1993). Experimental access to associative learning in honeybees. *Apidologie,* **24**: 249-266.

McCaughey, W.F., Gilliam, M. & Standifer, L.N. (1980). Amino acids and protein adequacy for honey bees of pollens from desert plants and other floral sources. *Apidologie,* **11**: 75-86.

McLellan, A.R. (1977). Minerals, carbohydrates and amino acids of pollens from some woody and herbaceous plants. *Annals of Botany,* **41**: 1225-1232.

McNeil, J.N. & Delisle, J. (1989). Host plant pollen influences calling behaviour and ovarian development of the sunflower moth, *Homoeosoma electellum. Oecologia,* **80**: 201-205.

Menzel, R. (1967). Untersuchungen zum erlernen von spektralfarben durch die honigbiene (*Apis mellifera*). *Zeitschrift für Vergleichende Physiologie,* **56**: 22-62.

Menzel, R. (1985). Learning in honeybees in an ecological and behavioural context. In *Experimental behavioural Ecology* (Ed. B. Hölldobler & M. Lindauer), pp.55-74. Fischer Verlag, Stuttgart.

Menzel, R. (1990). Learning, memory and 'cognition' in honey bees. In *Neurobiology of Comparative Cognition* (Ed. R.P. Kesner & D.S. Olten), pp.237-292. Lawrence Erlbaum Associates, Hillsdale, New Jersey, USA.

Menzel, R. & Backhaus, W. (1991). Colour vision in insects. In *Vision and Visual Dysfunction* (Ed. P. Gouras), pp.262-288. Macmillan Press, London.

Menzel, R., Erber, T. & Mashur, J. (1974). Learning and memory in the honeybee. In *Experimental Analysis of Insect Behavior* (Ed. L.B. Browne), pp.195-217. Springer-Verlag, Berlin.

Menzel, R., Greggers, U. & Hammer, M. (1993). Functional organization of appetitive learning and memory in a generalist pollinator, the honey bee. In *Insect Learning: Ecological and Evolutionary Perspectives* (Ed. D. Papaj & A.C. Lewis), pp.79-125. Chapman and Hall, London.

Menzel, R. & Müller, U. (1996). Learning and memory in honeybees: from behavior to neural substrates. *Annual Review of Neuroscience,* **19**: 379-404.

Menzel, R. & Shmida, A. (1993). The ecology of flower colours and the natural colour vision of insect pollinators: the Israeli flora as a study case. *Biological Review,* **68**: 81-120.

Mesquida, J., Marilleau, R., Pham-Delègue, M.-H. & Renard, M. (1988). A study of rapseed (*Brassica napus* L. var. *Oleifera* metzger) flower nectar secretions. *Apidologie,* **19**: 307-318.

Metcalf, R.L. (1987). Plant volatiles as insect attractants. *CRC Critical Reviews in Plant Sciences,* **5**: 251-301.

Miller, J.R. & Strickler, K.L. (1984). Finding and accepting host plants. In *The Chemical Ecology of Insects* (Ed. W.J. Bell & R.T. Cardé), pp.127-155. Chapman & Hall, London.

Milne, J.R., Walter, G.H., Kaonga, D. & Sabio, G.C. (1996). The importance of non-pollen plant parts as food sources for the common blossom thrips, *Frankliniella schultzei. Entomologia Experimentalis et Applicata,* **78**: 271-281.

Minnich, D.E. (1921). An experimental study of the tarsal chemoreceptors of two nymphalid butterflies. *Journal of Experimental Zoology,* **33**: 173-203.

Minnich, D.E. (1932). The contact chemoreceptors of the honey bee *Apis mellifera* Linn. *Journal of Experimental Zoology,* **61**: 375-393.

Moericke, V. (1953). Wie finden geflugelte blattlause ihre wirtspflanze? *Mitteilungen Biologischen Zentralanstalt fur Land und Forstwirtschaft,* **75**: 90-97.

Moffett, J.O., Stith, L.S., Burkhart, C.C. & Shipman, C.W. (1975). Honey bee visits to cotton flowers. *Environmental Entomology,* **4**: 203-206.

Mullin, C.A., Chyb, S., Eichenseer, H., Hollister, B. & Frazier, J.L. (1994). Neurorecepter mechanisms in insect gustation: a pharmacological approach. *Journal of Insect Physiology,* **40**: 913-931.

Nair, K.S.S. & McEwen, F.L. (1976). Host selection by the adult cabbage maggot *Hylemya brassicae* (Diptera : Anthomyiidae): effect of glucosinolates and common nutrients on oviposition. *Canadian Entomologist,* **121**: 1030.

Nielsen, P.S. & Axelsen, J. (1988a). Developmental time and mortality of the immature stages of the pollen beetle *(Melegethes aeneus* F.) under natural conditions. *Journal of Applied Entomology,* **105**: 198-204.

Nielsen, P.S. & Axelsen, J. (1988b). Spatial distribution of the pollen beetle (*Meligethes aeneus* F.) in spring sown rape (*Brassica napus* L.) in Denmark, and its importance for sampling. *Journal of Applied Entomology,* **105**: 35-40.

Nilsson, C. (1987). Yield losses in summer rape caused by pollen beetles (*Meligethes* spp.). *Swedish Journal of Agricultural Research,* **17**: 105-111.

Nilsson, C. (1988a). The pollen beetle (*Meligethes aeneus*) in winter and spring rape at Alnarp 1976-1978. I. Migration and sex ratio. *Växtskyddsnotiser,* **52**: 134-138

Nilsson, C. (1988b). The pollen beetle (Meligethes aeneus) in winter and spring rape at Alnarp 1976-1978. II. Oviposition. *Växtskyddsnotiser,* **52**: 139-144.

Nilsson, C. (1988c). The pollen beetle (*Meligethes aeneus* F.) in winter and spring rape at Alnarp 1976-1978. III. Mortality. *Växtskyddsnotiser,* **52**: 145-150.

Nilsson, C. (1988d). Pollen beetles (*Meligethes aeneus* F.) and flowering in rape. *Swedish Journal Of Agricultural Research,* **18**: 113-118.

Nilsson, C. & Andreasson, B. (1987). Parasitiods and predators attacking pollen beetles (*Meligethes aeneus* F). in spring and winter rape in southern Sweden. *IOBC/WPRS bulletin,* **10**: 64-73.

Nolte, H.-W. (1955). Die verwendungsmöglichkeit von gelbschalen nach moericke für sammler und angewandte entomologen. Bericht über die 7. *Wanderversammlung Deutscher Entomologen,* (Ed. P.D.H. Sachtleben). pp.201-212 Berlin.

Nolte, H.-W. (1959). Untersuchungen zum farbensehen des rapsglanzkäfers (*Meligethes aeneus* F.). *Biologisches Zentralblatt,* **78**: 63-107.

Nottingham, S.F. & Coaker, T.H. (1985). The olfactory response of cabbage root fly *Delia radicum* to the host plant volatile allylisothiocyanate. *Entomoligia Experimentalis et Applicata,* **39**: 307-316.

Nottingham, S.F., Hardie, J., Dawson, G.W., Hick, A.J., Pickett, J.A., Wadhams, L.J. & Woodcock, C.M. (1991). Behavioural and electrophysiological responses of aphids to host and nonhost plant volatiles. *Journal of Chemical Ecology,* **17**: 1231-1242.

Osborne, P. (1964). Morphology of the immature stages of *Meligethes aeneus* (F.) and *M. viridescens* (F.) (Coleoptera, Nitidulidae). *Bulletin of Entomological Research,* **55**: 747-759.

Osche, G. (1979). Zur evolution optischer signale bei blutenpflazen. *Biologie in unserer Zeit,* **9**: 161-170.

Osche, G. (1983). Optische signale in der coevolution von pflanze. Berichte der Deutschen Botanischen Gesellscaft, **96**: 1-27.

O'Toole, C. & Raw, A. (1991). *Bees of the World.* 192pp. Blanford, London.

Pacini, E. (2000). From anther and pollen ripening to pollen presentation. In *Pollen and Pollination* (Ed. A. Dafni, M. Hesse & E. Pachini), pp.19-43. Springer-Verlag, Wein.

Pacini, E. & Franchi, G.G. (1993). Role of the tapetum in pollen and spore dispersal. *Plant Systematics and Evolution,* **7**: 1-11.

Patt, J.M., Wainright, S.C., Dietrick, J., Hamilton, G.C., Bosley, K., Whittinghill, D. & Lashomb, J.H. (in prep.). Assimilation of floral nutrients by a predaceous insect larva. *Submitted to Ecological Entomology.*

Pavlov, I.P. (1927). *Conditioned Reflexes.* Translated by Anrep, G. V. 430pp. Oxford University Press, Oxford.

Payne, T.L., Birch, M.C. & Kennedy, C.E.J. (1986). *Mechanisms in Insect Olfaction.* 364pp. Clarendon Press, Oxford.

Pellmyr, O. & Thein, L.B. (1986). Insect reproduction and floral fragrances: keys to the evolution of angiosperms? *Taxon,* **35**: 76-85.

Pelz, C., Gerber, B. & Menzel, R. (1997). Odorant intensity as a determinant for olfactory conditioning in honeybees: roles in discrimination, overshadowing and memory consolidation. *The Journal of Experimental Biology,* **200**: 837-847.

Peng, Y.-H., Nasr, M.E., Marstan, J.M. & Fang, Y. (1985). The digestion of dandelion pollen by adult worker honeybees. *Physiological Entomology,* **10**: 75-82.

Percival, M. (1947). Pollen collection by *Apis mellifera. The New Phytologist,* **46**: 142-167.

Percival, M.S. (1955). The presentation of pollen in certain angiosperms and its collection by *Apis mellifera. New Phytologist,* **54**: 353-368.

Pesho, G.R. & van Houten, R.J. (1982). Pollen and sexual maturation of the pea weevil (Coleoptera: Bruchidae). *Annals of the Entomological Society of America,* **75**: 439-443.

Peto, R. & Peto, J. (1972). Asymptotically efficient rank invariant procedure. *Journal of the Royal Statistical Society, Series A,* **135**: 185-207.

Petroski, R.J., Bartelt, R.J. & Vetter, R.S. (1994). Male-produced aggregation pheromone of *Carpophilus obsoletus* (Coleoptera: Nitidulidae). *Journal of Chemical Ecology,* **20**: 1483-1493.

Pham-Delègue, M.-H., Bailez, O., Blight, M.M., Masson, C., Picard-Nizou, A.L. & Wadhams, L.J. (1993). Behavioural discrimination of oilseed rape volatiles by the honeybee *Apis Mellifera* L. *Chemical Senses,* **18**: 483-494.

Pham-Delègue, M.-H., Blight, M.M., Kerguelen, V., Le Métayer, M., Marion-Poll, F., Sandoz, J.C. & Wadhams, L.J. (1997). Discrimination of oilseed rape volatiles by the honeybee: combined chemical and biological approaches. *Entomologia Experimentails et Applicata,* **83**: 87-92.

Pham-Delègue, M.-H., De Jong, R. & Masson, C. (1990). Effet de l'âge sur la réponse conditionnée d'extension du proboscis chez l'abeille domestique (Age-dependency of the conditioned proboscis extension response in honeybees). *Comptes Rendus de l'Académie des Sciences Paris, série III,* **310**: 527-532.

Pham-Delègue, M.-H., Etievant, P., Guichard, E. & Masson, C. (1989). Sunflower volatiles involved in honeybee discrimination among genotypes and flowering stages. *Journal of Chemical Ecology,* **15**: 329-343.

Pham-Delègue, M.-H., Masson, C., Etievant, P. & Azar, M. (1986). Selective olfactory choices of the honeybee among sunflower aromas: a study by combined olfactory conditioning and chemical analysis. *Journal of Chemical Ecology,* **12**: 781-793.

Phillips, W.M. (1977). Modification of feeding 'preference' in the flea beetle *Haltica lythri* (Coleoptera: Chrysomelidae). *Entomologia Experimentalis et Applicata,* **21**: 71-80.

Pichersky, E., Raguso, R.A., Lewinsohn, E. & Croteau, R. (1994). Floral scent production in *Clarkia* (Onagraceae). *Plant Physiology,* **106**: 1533-1540.

Pierre, J., Mesquida, J., Marilleau, R., Pham-Delègue, M.-H. & Renard, M. (1999). Nectar secretion in winter oilseed rape, *Brassica napus*: quantitative and qualitative variability among 71 genotypes. *Plant Breeding,* **118**: 471-476.

Pinochet, X. & Bertrand, R. (2000). Oilseed rape grain yield productivity increases with hybrid varietal types: a first balance sheet with post registration tests in France and Europe. *OCL-Oleagineux Corps Gras Lipides,* **7**: 11-16.

Poppy, G.M. & Williams, I.H. (1999). A bee flight room designed for studies of bee foraging behaviour. *Journal of Apicultural Research,* **38**: 177-189.

Procter, M., Yeo, P. & Lack, A. (1996). *The Natural History of Pollination.* (2nd ed.), 479pp. Harper Collins, London.

Prokopy, R.J. (1986). Visual and olfactory stimulus interaction in resource finding by insects. In *Mechanisms in Insect Olfaction* (Ed. T.L. Payne, M.C. Birch & C.E.J. Kennedy), pp.81-89. Clarendon Press, Oxford.

Prokopy, R.J. & Owens, E.D. (1983). Visual detection of plants by herbovorous insects. *Annual Review of Entomology,* **28**: 337-364.

Prüffer, K. (1958). Zerowanie slodyszków (*Meligethes* spp.) w kwiatach róznych gatunków roslin (Feeding of *Meligethes* sp. in the flowers of differnt species of plants). *Studia Societatis Scientiarum Torunensis Torun - Polonia,* **Sectio E (Zoologia) 4**: 1-48.

Rasheed, S.A. & Harder, L.D. (1997a). Economic motivation for plant species preferences of pollen-collecting bumble bees. *Ecological Entomology,* **22**: 209-219.

Rasheed, S.A. & Harder, L.D. (1997b). Foraging currencies for non-energetic resources: pollen collection by bumblebees. *Animal Behaviour,* **54**: 911-926.

Rathcke, B.J. (1992). Nectar distributions, pollinator behavior, and plant reproductive success. In *Effects of Resource Distribution on Animal-Plant Interactions* (Ed. M.D. Hunter, T. Ohgushi & P.W. Price), pp.113-138. Academic Press, California.

Raubenheimer, D. & Tucker, D. (1997). Associative learning by locusts: pairing of visual cues with consumption of protein and carbohydrate. *Animal Behaviour,* **54**: 1449-1459.

Ray, S. & Ferneyhough, B. (1997a). Seasonal variation of proboscis extension reflex conditioning in the honey bee (*Apis mellifera*). *Journal of Apicultural Research,* **36**: 108-110.

Ray, S. & Ferneyhough, B. (1997b). The effects of age on olfactory learning and memory in the honey bee *Apis mellifera. NeuroReport,* **8**: 789-793.

Rayner, C.J. & Langridge, D.F. (1985). Amino acids in bee-collected pollens from Australian indigenous and exotic plants. *Australian Journal of Experimental Agriculture,* **25**: 722-726.

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

Regali, A. & Rasmont, P. (1995). Nouvelles méthodes de test pour l'évaluation du régime alimentaire chez des colonies orphelines de *Bombus terrestris* (L) (Hymenoptera, Apidae) (New bioassays to evaluate diet in *Bombus terrestris*). *Apidologie,* **26**: 273-281.

Rehder, V. (1987). Quantification of the honeybee's proboscis reflex by electromyographic recordings. *Journal of Insect Physiology,* **33**: 501-507.

Rescorla, R.A. (1988). Behavioural studies of Pavlovian conditioning. *Annual Review of Neuroscience,* **11**: 329-352.

Ribbands, C.R. (1949). The foraging method of individual honey-bees. *Journal of Animal Ecology,* **18**: 47-66.

Ribbands, C.R. (1954). The scent perception of the honeybee. *Proceedings of the Royal Society, Series B,* **143**: 367-379.

Riedel, I.B.M. & Wort, D.A. (1960). The pollination requirement of the field bean (*Vicia faba*). *Annals of applied Biology,* **48**: 121-124.

Robaker, D.C., Flottum, P.K., Sammataro, D. & Erickson, F.H. (1982). Effects of climatic factors on soybean flowers. *Field Crops Research,* **122**: 267-278.

Robertson, G.W., Griffiths, D.W., MacFarlane Smith, W. & Butcher, R.D. (1993). The application of thermal-dissorption-gas chromatography-mass spectrometry to the analysis of five varieties of oilseed rape (*Brassica napus* spp. *oleifera). Phytochemical Analysis,* **4**: 152-157.

Robyt, J.F. & White, B.J. (1987). *Boichemical Techniques: Theory and Practice.* 407 pp. Brooks & Cole, Monterey, California.

Rogers, C.E. (1978). Sunflower moth: feeding behaviour of the larva. *Environmental Entomology,* **7**: 763-765.

Rosenthal, G. A. (1991). In *Herbivores: Their Interactions with Secondary Plant Metabolites Volume 1, The Chemical Participants.* (Ed. G. A. Rosenthal, M. R. Berenbaum) 2nd Ed., pp.1-30. Academic Press, London.

Roulston, T.H. & Cane, J.H. (2000). Pollen nutritional content and digestibility for animals. In *Pollen and Pollination* (Ed. A. Dafni, M. Hesse & E. Pacini), pp.188-209. Springer-Verlag, Wein.

Ruther, J. & Thiemann, K. (1997). Response of the pollen beetle *Meligethes aeneus* to volatiles emitted by intact plants and conspecifics. *Entomologia Experimentalis et Applicata,* **84**: 183-188.

Sakuma, M. & Fukami, H. (1985). The linear track olfactometer: An assay device for taxes of the German cockroach, *Blattella germanica* (L.) (Dictyoptera : Blattellidae) toward their aggregation pheromone. *Applied Entomology and Zoology,* **20**: 387-402.

Salom, S.M. & McLean, J.A. (1991). Flight behaviour of scolytid beetle in response to semiochemicals at different wind speeds. *Journal of Chemical Ecology,* **17**: 647-661.

Sandoz, J.C., Roger, B. & Pham-Delègue, M.-H. (1995). Olfactory learning and memory in the honeybee: comparison of different classical conditioning procedures of the proboscis extension reponse. *Comptes Rendus de l'Académie des Sciences, Série III - Sciences de la Vie,* **318**: 749-755.

Sawyer, R. (1981). *Pollen identification for beekeepers.* 111pp. University College Cardiff Press, Cardiff, UK.

Scherney, F.V. (1953). Zur biology der an Raps vorkommenden Meligethesarten. *Zeitschrift fur Pflanzenbau und Pflanzenschutz,* **4**: 154-176.

Schmidt, J.O. (1982). Pollen foraging preferences of honey bees. *The Southwestern Entomologist,* 7: 255-259.

Schmidt, J.O. (1984). Feeding preferences of *Apis mellifera* (Hymenoptera:Apidae): Individual versus mixed pollen species. *Journal of the Kansas Entomological Society,* **57**: 323-327.

Schmidt, J.O. (1985). Phagostimulants in pollen. *Journal of Apicultural Research,* **24**: 107-114.

Schmidt, J.O., Buchmann, S.L. & Glaiim, M. (1989). The nutritional value of *Typha latifolia* for bees. *Journal of Apicultural Research,* **28**: 155-165.

Schmidt, J.O. & Johnson, B.E. (1984). Pollen feeding preference of *Apis mellifera,* a polylectic bee. *The Southwestern Entomologist,* **9**: 41-47.

Schmidt, J.O., Thoenes, S.C. & Levin, M.D. (1987). Survival of honey bees, *Apis mellifera* (Hymenoptera: Apidae), fed various pollen sources. *Annals of the Entomological Society of America,* **80**: 176-183.

Schmidt, L.S., Schmidt, J.O., Rao, H., Wang, W.Y. & Xu, L.G. (1995). Feeding preference and survival of young worker honey bees (Hymenoptera: Apidae) fed rape, sesame, and sunflower pollen. *Journal of Economic Entomology,* **88**: 1591-1595.

Schoonhoven, L.M., Jermy, T. & van Loon, J.J.A. (1998). *Insect-Plant Biology: from physiology to evolution.* 409pp. Chapman & Hall, London.

Scriven, W.A., Cooper, B.A. & Allen, H. (1961). Pollination of field beans. *Outlook on Agriculture,* **3**: 69-75.

Sedivy, J. & Kocourek, F. (1994). Flight activity of winter rape pests. *Journal of Applied Entomology,* **117**: 400-407.

Seeley, T.D. (1985). *Honeybee Ecology: A Study of Adaption in Social Life.* 201pp Princeton University Press, Princeton.

Seeley, T.D. (1994). Honey bee foragers as sensory units of their colonies. *Behavioural Ecology and Sociobiology,* **34**: 51-62.

Serra Bonvehi, J. & Escolà Jordà, R. (1997). Nutrient composition and microbiological quality of honeybee-collected pollen in Spain. *Journal of Agricultural and Food Chemistry,* **45**: 725-732.

Shapiro, M.S. (2000). Quantitative analysis of risk sensitivity in honeybees (*Apis mellifera*) with variability in concentration and amount of reward. *Journal of Experimental Psychology,* **36**: 196-205.

Shellard, E.J. & Jolliffe, G.H. (1968). The identification of the free amino acids present in some grass pollens by thin layer chromatography. *Journal of Chromatography,* **38**: 257-266.

Shiraishi, A. & Kuwabara, M. (1970). The effects of amino acids on the labellar hair chemosensory cells of the fly. *The Journal of General Physiology,* **56**: 768-782.

Simpson, S.J. & White, P.R. (1990). Associative learning and locust feeding: evidence for a 'learned hunger' for protein. *Animal Behaviour,* **40**: 506-513.

Singh, R.P. & Singh, P.N. (1991). A study on the biochemical composition of fresh and stored mustard pollen and honey. *Apicata,* **26**: 38-44.

Singh, R.P. & Singh, P.N. (1996). Amino acid and lipid spectra of larvae of honey bee (*Apis cerana* Fabr) feeding on mustard pollen. *Apidologie,* **27**: 21-28.

Smart, L.E. & Blight, M.M. (2000). Response of the pollen beetle, *Meligethes aeneus* to traps baited with volatiles from oilseed rape, *Brassica napus*. *Journal of Chemical Ecology,* **26**: 1051-1064.

Smart, L.E., Blight, M.M. & Hick, A.J. (1993). Development of a monitoring system for the cabbage seed weevil and the pollen beetle. *Bulletin IOBC/WPRS,* **16**: 351-354.

Smart, L.E., Blight, M.M. & Lane, A. (1996). Development of a monitoring trap for spring and summer pests of oilseed rape. *Brighton Crop Protection Conference- Pests & Diseases*, pp. 167-172. Brighton, UK,.

Smart, L.E., Blight, M.M. & Ryan, J. (1995). Response of pollen beetles, *Meligethes* spp., to volatiles from *Brassica napus*. *Nineth International Rapeseed Congress*, pp. 1040-1042. (Ed. D.C. Murphy). Organizing Committee of the 9th International Rapeseed Congress, Cambridge,.

Smith, B.H. (1993). Merging mechanism and adaption: an ethological approach to learning and generalization. In *Insect Learning: Ecological and Evolutionary Perspectives* (Ed. D. Papaj & A.C. Lewis), pp.126-157. Chapman and Hall, London.

Smith, B.H., Abramson, C.I. & Tobin, T.R. (1991). Conditional withholding of proboscis extension in honeybees (*Apis mellifera*) during discriminative punishment. *Journal of Comparative Pyschology,* **105**: 345-356.

Smith, B.H. & Cobey, S. (1994). The olfactory memory of the honeybee, *Apis mellifera*: II. Blocking between odorants in binary mixtures. *The Journal of Experimental Biology,* **195**: 91-108.

Smith, B.H. & Getz, W.M. (1994). Nonpheromonal olfactory processing in insects. *Annual Review of Entomology,* **39**: 351-375.

Smith, B.H. & Menzel, R. (1989). The use of electromyogram recordings to quantify odourant discrimination in the honey bee, *Apis mellifera*. *Journal of Insect Physiology,* **35**: 369-375.

Sprengel, C.K. (1793). Das entdeckte Geheimnifs der Natur im Bau in der Befruchtung der Blumen: Discovery of the secret nature in the structure and fertilization of flowers. Translated by Peter Haase. In *Floral Biology: Studies on Floral Evolution in Animal-Pollinated Plants* (Ed. D.G. Lloyd, S.C.H. Barrett) (1996), pp.3-43. Chapman & Hall, New York.

Stace, P. (1996). *Protein content and amino acid profiles of honeybee-collected pollens.* Bees 'N Trees Consultants, Lismore, NSW, Australia. Available on-line at: http://www.honeybee.com.au/library/pollen/nutrition.html.

Stace, P. & White, E. (1994). The use of iso-leucine as a supplement feed for honey bees (*Apis mellifers*) in Australia. *The Australian Beekeeper,* **96**: 159-166.

Stanley, R.G. & Linskens, H.F. (1974). *Pollen: Biology Biochemistry Management.* 307pp. Springer - Verlag, New York.

Stoddard, F.L. (1986). Pollination and fertilisation in commercial crops of field beans (*Vicia faba* L.). *Journal of Agricultural Science, Cambridge,* **106**: 89-97.

Stoddard, F.L. & Bond, D.A. (1987). The pollination requirements of the Faba bean. *Bee World,* **68**: 144-152.

Stone, J.C., Abramson, C.I. & Price, J.M. (1997). Task-dependent effects of dicofol (kelthane) on learning in the honey bee (*Apis mellifera*). *Bulletin of Environmental Contamination and Toxicology,* **58**: 177-183.

Sutton, C.J., Keegans, S.J., Kirk, W.D.J. & Morgan, E.D. (1992). Floral volatiles of *Vicia faba. Phytochemistry,* **31**: 3427-3428.

Sylven, E. & Svensson, G. (1976). Effect on yield of damage caused by *Meligethes aeneus* F. (col.) to winter rape, as indicated by cage experiments. *Annales Agriculturae Fenniae,* **15**: 24-33.

Sylvester-Bradley, R. & Makepeace, R.J. (1984). A code for stages of development in oilseed rape (*Brassica napus* L.). *Aspects of Applied Biology,* **6**: 399-419.

Szalai, Z. (2000). Differences of pollen and pollenkitt attractiveness of some cultivated and ornamental apples for honeybees. *International Journal of Horticultural Science,* **6**: 145-151.

Taber, S. (1963). Why bees collect pollen. *Proceedings of the International Beekeeping Congress, Prague,* **19**: 114.

Takeda, K. (1961). Classical conditioned response in the honey bee. *Journal of Insect Physiology,* **6**: 168-179.

Tamir, L., Sedivý, J., Bergmannová, E. & Hanker, I. (1967). Further experience obtained in studies on dispersal flights of *Meligethes aeneus* F. marked with P32 (Coleoptera). *Acta Entomologica Bohemoslovaca,* **64**: 325-332.

Teulon, D.A.J. & Penman, D.R. (1991). Effects of temperature and diet on oviposition rate and development time of the New Zealand flower thrips, *Thrips obscuratus*. *Entomologia Experimentalis et Applicata,* **60**: 143-155.

Thiery, D., Bluet, J.M., Pham-Delegue, M.H., Etievant, P. & Masson, C. (1990). Sunflower aroma detection by the honeybee. *Journal of Chemical Ecology,* **16**: 701-711.

Thompson, J.N. (1988). Evolutionary ecology of the relationship between oviposition preference and performance of offspring in phytophagous insects. *Entomologia Experimentalis et Applicata,* **47**: 3-14.

Todd, F.E. & Bretherick, O. (1942). The composition of pollens. *Journal of Economic Entomology,* **35**: 312-317.

Tollsten, L. & Bergström, G. (1988). Headspace volatiles of whole plants and macerated plant parts of *Brassica* and *Sinapis*. *Phytochemistry,* **27**: 4013-4018.

Trichilo, P.J. & Leigh, T.F. (1988). Influence of resource quality on reproductive fitness of flower thrips, Thysanoptera: Thripidae. *Annals of the Entomological Society of America,* **81**: 64-70.

Tulisalo, U. & Wuori, T. (1986). Blossom Beetle *Meligethes aeneus* Fab. as a yield factor in turnip rape *Brassica campestris* L. *Journal of Agricultural Sciences in Finland,* **58**: 221-238.

van der Pijl, L. (1960). Ecological aspects of flower evolution. *Evolution,* **14**: 403-416

van Praagh, J.P. (1972). Towards a controlled environment room suitable for normal colony life of honey bees; 1. Descriptions and general observations. *Journal of Apicultural Research,* **11**: 77-87.

Vareschi, E. (1971). Duftunterscheidung bei der honigbiene - einzelzell-ableitungen und verhaltensreaktionen. *Zeitscrift fur Vergleichende Physiologie,* **75**: 143-173.

Visser, J.H. (1986). Host odour perception in phytophagous insects. *Annual Review of Entomology,* **31**: 121-144.

Vivino, A.E. & Palmer, L.S. (1944). The chemical composition and nutritional value of pollens collected by bees. *Archives of Biochemistry,* **4**: 129-136.

Vogel, S. (1963). *The role of scent glands in pollination: On the structure and function of osmophores*. 271pp Amerind Publishing Co., New Delhi.

von Aufsess, A. (1960). Geruchliche nahorientierung der biene bei entomophilen und ornithophilen blüten. *Zeitscrift fur Vergeichende Physiologie,* **43**: 469-498.

von Frisch, K. (1914). Der Farbensinn und Formensinn der Biene. *Zoologische Jahresbücher der Physiologie,* **35**: 1-188.

von Frisch, K. (1919). Uber den Geruchsinn der biene und seine blutenbiologisce bedeutung. *Zoologische Jahresbücher der Physiologie* **37**: 1-238.

von Frisch, K. (1923). Uber die 'sprache' der bienen. *Zoologische Jahrbucher Abteilung fur allgemaine Zoologie und Physiologie der Tiere,* **40**: 1-186.

von Frisch, K. (1967). *The Dance Language and Orientation of Bees.* 566pp Harvard University Press, Cambridge, M.A. USA.

Wacht, S., Lunau, K. & Hansen, K. (1996). Optical and chemical stimuli control pollen feeding in the hoverfly *Eristalis tenax. Entomologia Experimentalis et Applicata,* **80**: 50-53.

Wacht, S., Lunau, K. & Hansen, K. (2000). Chemosensory control of pollen ingestion in the hoverfly *Eristalis tenax* by labellar taste hairs. *Journal of Comparative Physiology A,* **186**: 193-203.

Wäckers, F.L. (1994). The effect of food deprivation on the innate visual and olfactory preferences in the parasitoid *Cotesia rubecula. Journal of Insect Physiology,* **40**: 641-649.

Waddington, K.D., Nelson, C.M. & Page, R.E. (1998). Effects of pollen quality and genotype on the dance of foraging honey bees. *Animal Behaviour,* **56**: 35-39.

Wadhams, L.J., Blight, M.M., Kerguelen, V., Le Métayer, M., Marion-Poll, F., Masson, C., Pham-Delègue, M.H. & Woodcock, C.M. (1994). Discrimination of oilseed rape volatiles by honey bees: Novel combined gas chromatographic-electrophysiological behavioural assay. *Journal of Chemical Ecology,* **20**: 3221-3231.

Waller, G.D. (1972). Evaluating responses of honey bees to sugar solutions using an artificial flower feeder. *Annals of the Entomological Society of America,* **65**: 857-862.

Waller, G.D., Loper, G.M. & Berdel, R.L. (1974). Olfactory discrimination by honeybees of terpenes identified from volatiles of alfalfa flowers. *Journal of Apicultural Research,* **13**: 191-197.

Waser, N.M. (1986). Flower constancy; definition, cause and measurement. *American Naturalist,* **127**: 593-603.

Wasman, E. (1926). Versuche über den fabensinn des rapskäfers (*Meligethes aeneus* F.). *Zeitschrift fur Wissenschaftliche Insektenbiologie,* **21**: 147.

Whalon, M. & Croft, B. (1985). Managing arthropod immigration and colonization: a community ecology approach. In *The Movement and Dispersal of Agriculturally Important Biotic Agents* (Ed. D.R. Macanzie, *et al*), pp.511-526. Claitor's Publishing Division, Baton Rouger, Louisiana.

Wheeler, D. (1996). The role of nourishment in oogenesis. *Annual Review of Entomology,* **41**: 407-431.

Wiklund, C. (1973). Host plant suitability and the mechanism of host selection in larvae of *Papilio machaon*. *Entomologia Experimentalis et Applicata,* **16**: 232-242.

Williams, C.S. & Poppy, G.M. (1997). Responses of individual honey bees to artificial feeders visited by themselves and to feeders visited by hivemates. *Journal of Apicultural Research,* **36**: 105-108.

Williams, I.H. (1978). The pollination requirements of swede rape (*Brassica napus* L.) and of turnip rape (*Brassica campestris* L.). *Journal of Agricultural Sciences, Cambridge,* **91**: 343-348.

Williams, I.H., Corbet, S.A. & Osborne, J.L. (1991). Beekeeping, wild bees and pollination in the European community. *Bee World,* **72**: 170-180.

Williams, I.H. & Free, J.B. (1978). The feeding and mating behaviour of pollen beetles (*Meligethes aeneus* Fab.) and seed weevils (*Ceutorhynchus assimilis* Payk.) on oilseed rape (*Brassica napus* L.). *Journal of Agricultural Science, Cambridge,* **91**: 453-459.

Williams, I.H., Martin, A.P. & White, R.P. (1987). The pollination requirements of oil-seed rape (*Brassica napus* L.). *Journal of Agricultural Science, Cambridge,* **115**: 67-73.

Williams, N.H. (1983). Floral fragrances as cues in animal behaviour. In *Handbook of Experimental Pollination Biology* (Ed. C.E. Jones, Little, R. J.), pp.50-72. Van Nostrand Reinhold, New York.

Winfield, A.L. (1961). Studies on the relationship between three species of Coleoptera and certain species of annual mustard and rape. *Entomologia Experimentalis et Applicata,* **4**: 123-132.

Winfield, A.L. (1992). Management of oilseed rape pests in Europe. In *Agricultural Zoology Reviews* (Ed. K. Evans), pp.51-94. Intercept, Andores.

Winston, M.L. (1987). *The Biology Of The Honey bee.* 281pp. Harvard University Press, Cambridge, M. A. USA.

Wood, D.L. (1982). The role of pheromones, kairomones, and allomones in the host selection and colonization behaviour of bark beetles. *Annual Review of Entomology,* **27**: 411-446.

Woodford, J.A.T., Birch, A.N.E., Robertson, G.W. & Griffiths, D.W. (1992). The perception of flower odours by the raspberry beetle. *Scottish Crop Research Institute Annual Report,* **1991**: 97-99.

Zhang, Z.-Q. & McEvoy, P.B. (1996). Factors affecting the response of *Longitarsus jacobaeae* (Coleoptera: Chrysomelidae) to upwind plant odours. *Bulletin of Entomological Research,* **86**: 307-313.

Zimmerman, M. (1982). Optimal foraging: random movement by pollen collecting bumblebees. *Oecologia,* **53**: 394-398.

silver foil boat within the air-entrainment vessel, and an air entrainment was conducted over a one-hour period within the laboratory where the pollen sample was collected.

Results & Discussion

There were distinct differences between the floral volatiles of the male-fertile and male-sterile Synergy plants. Figure A.1.1 shows a comparison of volatiles eluted from the male-fertile plants (top line) and the male-sterile plants (bottom line) showing the compounds that were identified by comparisons with compounds identified in the solvent sample. Most of the components that could be identified were found in both male-fertile and male-sterile flowers. However, benzaldehyde (RT 13.3) was found in male-fertile flowers but not male-sterile ones, linalool (RT 13.35) was found in male-sterile flowers but not male-fertile ones (this was surprising), much more phenylacetaldehyde (RT 14.85) was found in male-fertile flowers than male-sterile flowers, as was (*E, E*)-α-farnesene (15.5). Some of the unidentified components were only present in male-sterile flowers and may represent examples of varietal differences (these had RT's of: 9.66, 14.07 and 16.6). Some components were found in male-fertile flowers but not male-sterile ones. These could represent further varietal differences or could be pollen-derived differences (compounds with retention times of 5.8, 8.2, 9.01, 10.05, 12.1, 13.3 and 16.3).

Unfortunately, the pollen sample contained many contaminants from the silver-foil used as a boat to hold the pollen in the air entrainment. However, those differences between male-fertile and male-sterile flowers that were due to pollen could still be identified by comparing the RT's of the compounds absent in male-sterile flowers that are present in both male-fertile flowers and the pollen sample. These were compounds with RT's of: 8.2, 9.01, 12.1, 13.3 and 16.3. These compounds require further chemical study if they are to be identified. This could be achieved by making further air entrainments of these samples and analyzing the collected volatiles by combined GC-MS using the optic GC-system.

Conclusion

Male-fertile and male-sterile Synergy flowers have volatile differences in their odours that are attributable to both varietal differences and the presence of pollen in the male-fertile flowers and its absence in male-sterile ones. Further experiments are required to resolve these differences.

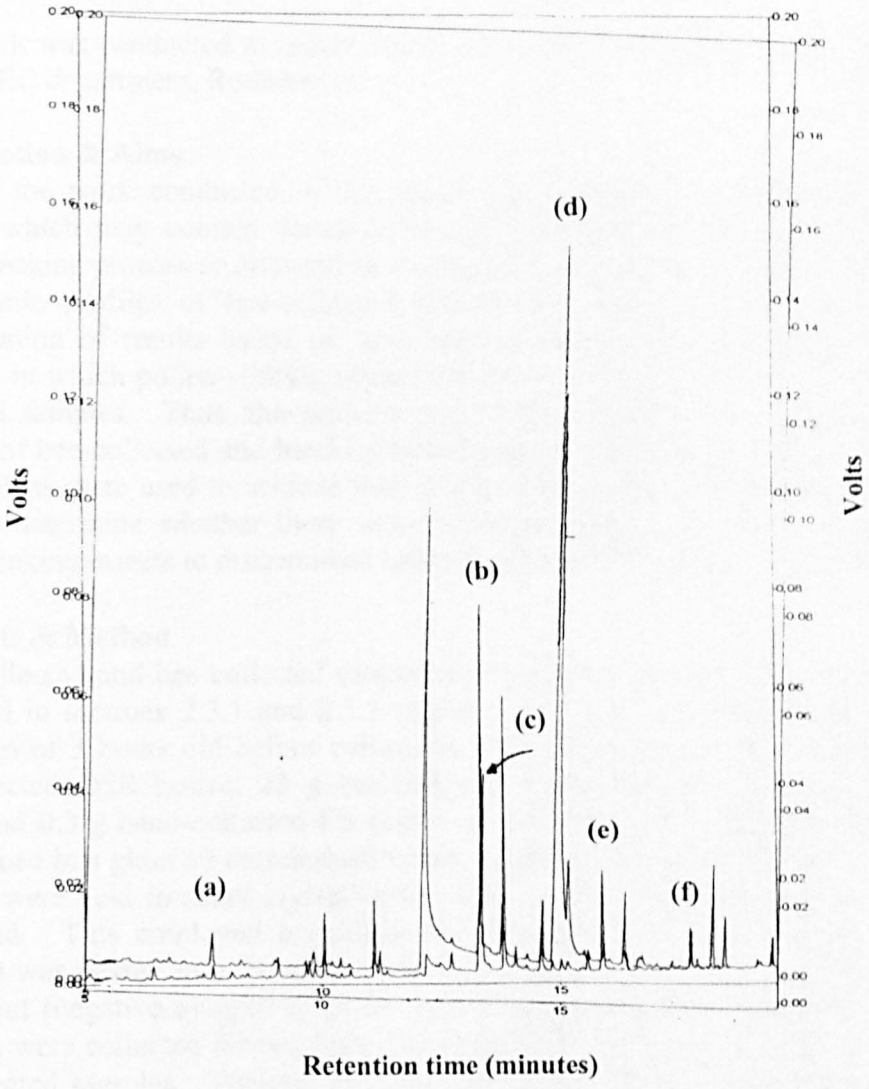


Figure A.1.1 Volatile compounds produced by male-fertile (top line) and male-sterile (bottom line) Synergy oilseed rape plants, showing the compounds that were identified: a=myrcene; b=benzaldehyde; c=linalool; d=phenyacetaldehyde; e=(E, E)-α-farnesene; f=benzyl alcohol

The identified compounds from the samples are listed in Table A.1.1, and shows in which of the four pollen treatments the compounds were found. Only around 20% of the identified components of hand-collected pollen are found in the respective bee-collected sample. This indicates that there are common volatiles, but overall, hand-collected and bee-collected pollens are quite different. Further study is required to determine whether the insects use these similar components in pollen recognition or the different ones (either those volatiles present in hand-collected pollen in the natural situation that are not present in the bee-collected pollens or compounds in bee-collected pollens used in experiments that are not present in the natural situation). In the first case, it is valid to substitute hand-collected pollen with bee-collected pollen; in the second, generalizing the results gained using bee-collected pollens to natural situations would require caution.

Table A1.1 also highlights the differences between the species; there were distinct differences between volatiles occurring in OSR samples and those occurring in FB samples. Few compounds were specific to the mode of collection, with toluene and ethylbenzene being the only compounds common to both hand-collected pollens and octane being the only compound common to both bee-collected pollens, although 6-methyl-5-hepten-2-one, nonanal and decanal were common to all four samples. This indicates that there are both similar compounds that could be used in generalization and differences that could be used for discrimination between these two species, irrespective of their mode of collection.

Conclusions

Hand-collected and bee-collected pollens do contain common volatile components, although overall they are quite different. Oilseed rape and field bean pollens contain some similar compounds and some species-specific ones, irrespective of the mode of collection.

Table A.1.1. Volatile compounds identified by optic GC-MS analysis of headspace air entrainment of hand-collected and bee-collected oilseed rape and field bean pollens

RT	Compound	Samples in which the compound is found			
		Oilseed rape Pollen		Field bean pollen	
		Hand-collected	Bee-collected	Hand-collected	Bee-collected
159-60	acetic acid	+	+		+
206	1-penten-3-ol				+
218	3-hydroxy-2-butanone		+		
285	Toluene	+		+	
295	2-methyl-propanoic acid		+		
328-29	Octane		+		+
328	butyl acetate			+	
354	(*E*)-2-hexanal				+
376-77	Ethylbenzene	+		+	
381	2-methyl-butanoic acid		+		
385	1,2- dimethyl benzene			+	
406	1-chloro-octane	+			+
410	Benzene			+	
425	Nonane			+	
452	Benzaldehyde				+
454	1-methyl-4-(1-methyl)-		+		

	1,4-cyclohexadiene				
481-84	6-methyl-5-hepten-2-one	+	+	+	+
487, 498	(*E, E*)-2,4-heptadienal				+
499-500	Octanal		+	+	
507-09	1,3-dichloro-benzene	+		+	+
526	benzyl alcohol				+
533	Limonene	+			
534	β-phellandrene		+		
545	3-carene				+
546	Ocimene		+		
550	(*E, E*)-3,5-octadien-2-one				+
573	Methyl ester, benzoic acid	+			
582-84	Nonanal	+	+	+	+
660-63	Decanal	+	+	+	+
683	Aminocaproic acid	+			
724	Indole	+			
732	1-methyl-napthalene	+			
741	Tridecane	+			
829	Trans-geranylacetone	+			+
849	(bicyclo-hexan-2-one)?			+	
849	2,6,10,14-tetramethyl-pentadecane				+
857	Acenaphthene	+			
871	Pentadecane	+			+
871	1-iodo-tetradecane			+	
922	Isocarvomenthol			+	
926	Dodecyl ester, acetic acid			+	
929	Dodecane	+			
962	1,1'-oxybis-octane	+			
986-87	Heptadecane			+	+
1040	Octadecane			+	
1057	Hexahydrofarnesyl acetone			+	+
1090	Nonadecane	+			+
1144	hexadecanoic acid	+			

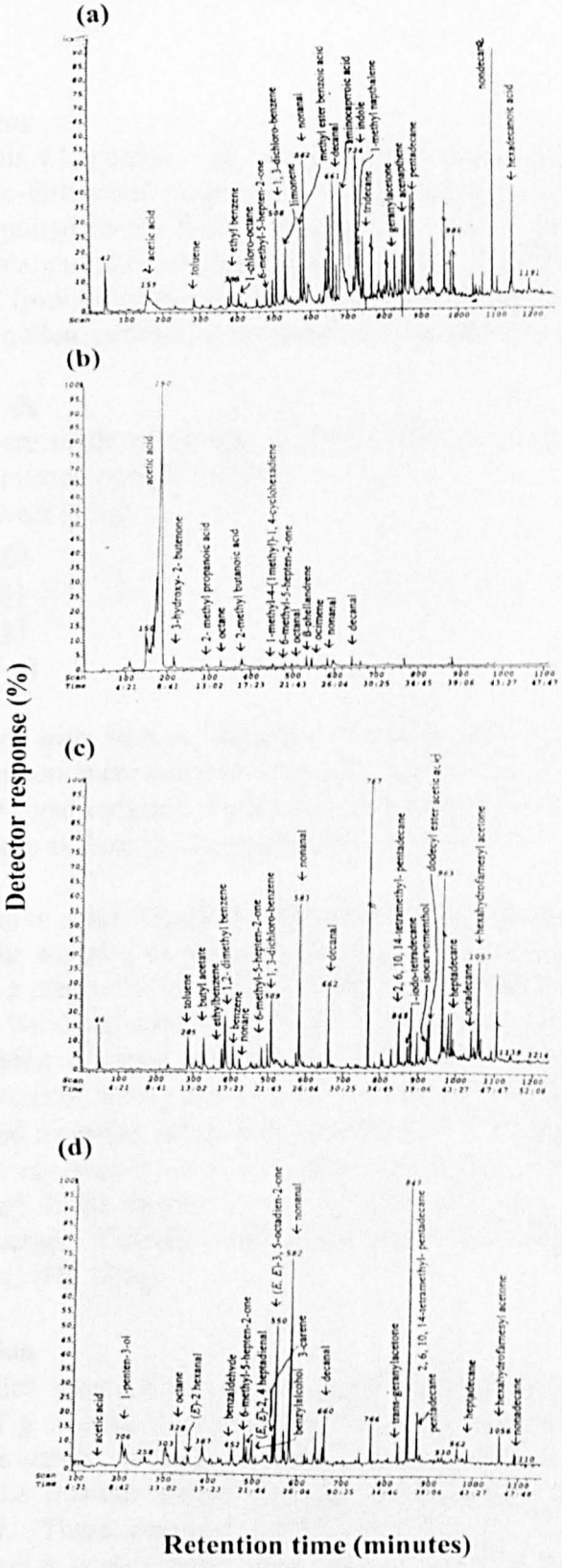


Figure A.1.2. Volatile components of (a) hand-collected oilseed rape pollen (b) bee-collected oilseed rape pollen (c) hand-collected field bean pollen (d) bee-collected field bean pollen

1.3 POLLEN VOLATILES: SOMETHING SPECIAL, OR MORE OF THE SAME?

Introduction & Aims

Previous experiments (Appendix 1.1) suggested that some differences in the volatile emissions from male-fertile and male-sterile oilseed rape (OSR) flowers were attributed to the presence of pollen in the former and not the latter. This experiment aimed to determine if volatiles emitted from pollen are specific to pollen, or whether they are released in addition from other floral parts, and, to determine the relative importance of volatiles from OSR pollen, petals and sepals to the overall odour of OSR flowers.

Materials & Methods

Air entrainments were made of freshly prepared OSR floral samples from male-fertile flowers of Synergy oilseed rape (2.2.1.2):

- Whole OSR flowers (15 g)
- OSR sepals (15 g)
- OSR petals (15 g)
- OSR anthers (2 g)
- OSR pollen (0.5 g)

Petals were removed with forceps, the ends of which were wrapped in Teflon tape to reduce damage. Anthers were cut with surgical scissors to leave the filament (Flower – petals and anthers = sepal sample). Pollen was removed from the anthers using the blunt edge of a scalpel blade as described in appendix section A.1.1.

Samples were placed in glass 'Quickfit' chambers (500 ml for samples of flowers, sepals and petals; 50 ml for samples of anthers and pollen), and sealed with Teflon tape. Air was drawn through a molecular sieve and through the samples at a rate of 0.7 l/min and headspace volatiles were collected by polymer trap (50 mg poropak). Two samples and one control (consisting of empty glassware) were conducted simultaneously (see Fig. A.1.3). Air entrainments were conducted in a room maintained at 25 °C for 8 hours. Volatiles were eluted from the polymer by passing 500 µl of solvent (re-distilled diethyl ether) into a glass chromacol microvial, that was sealed with a Teflon-coated cap. Samples were stored in the freezer at –20 °C until analysed. Five replicates of each sample were conducted. Volatiles were separated by gas chromatography (GC) on a Hewlett Plackard GC (HP 5890).

Results & Discussion

As expected, volatiles were most numerous and of greater amounts in the samples from the whole flower (Fig. A.1.4). Unfortunately, technical difficulties prevented separation of samples from the sepals. In their absence, anthers contributed most to the quantity of volatiles seen in the flowers. Petals produced surprisingly few volatiles, and pollen released fewer still. There was some variation within the replicates of each sample, however, general trends were evident from the GC traces: volatiles present in the petals were not generally present in the pollen sample and vice-versa and the retention times of compounds consistently found in all replicates of each sample are shown in Table A.1.2.. Volatiles present in pollen were also present in the anthers. These results suggest that pollen may release volatiles that are detectable within the volatile profile of the whole flower that are not released by petals. However, further work is required to confirm this, to determine the contribution of the sepals and the relative proportions that

each floral part contributes to the overall floral odour and to distinguish between qualitative and quantitative differences.

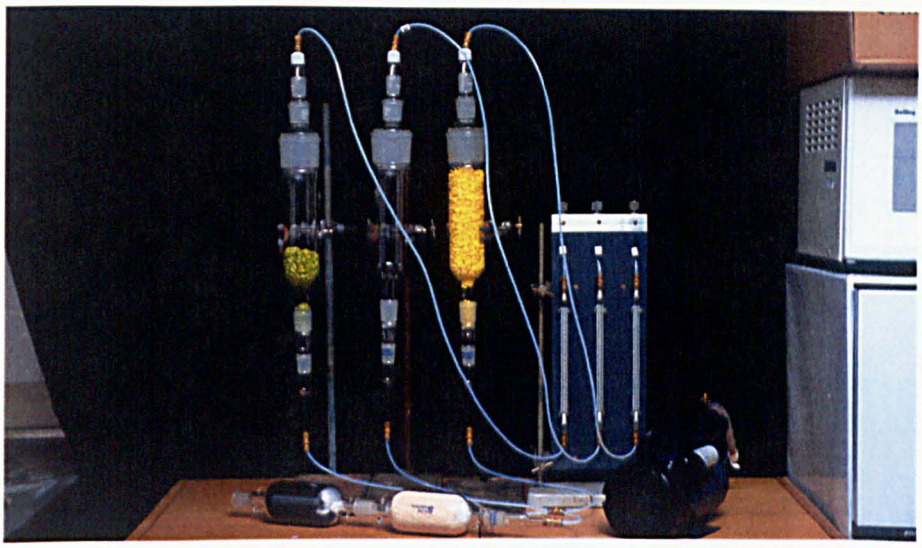


Figure A.1.3 Air entrainment system, showing pump drawing air through molecular sieve, through 3 simultaneously-conducted samples (in this case, OSR sepals, control and OSR petals) located in glass vessels, through polymer traps located at the top of each of these vessels; airflow regulated by flowmeters.

Table A.1.2. Retention times of compounds present in the volatiles of oilseed rape whole flowers, petals, anthers and pollen

Retention time of compound, and sample in which it was found			
Whole flowers	Petals	Anthers	Pollen
	5.31		
	6.05		
		6.5	
7.54			
7.797		7.7	
		7.9	
8.2		8.2	
		8.5	
	8.8	8.8	8.8
9.1			
	9.9		
	10.02		
11.05		11.05	
11.5			
12.3			
	12.8		
		13.4	13.4
		13.6	
14.1	14.1	14.1	
15.6	15.6		
			16.7
17.7	17.8		
18.1			
	18.7	18.65	
			19.9
20.3			20.3
21.4		21.5	
22.8			
			24.1
	28.98		

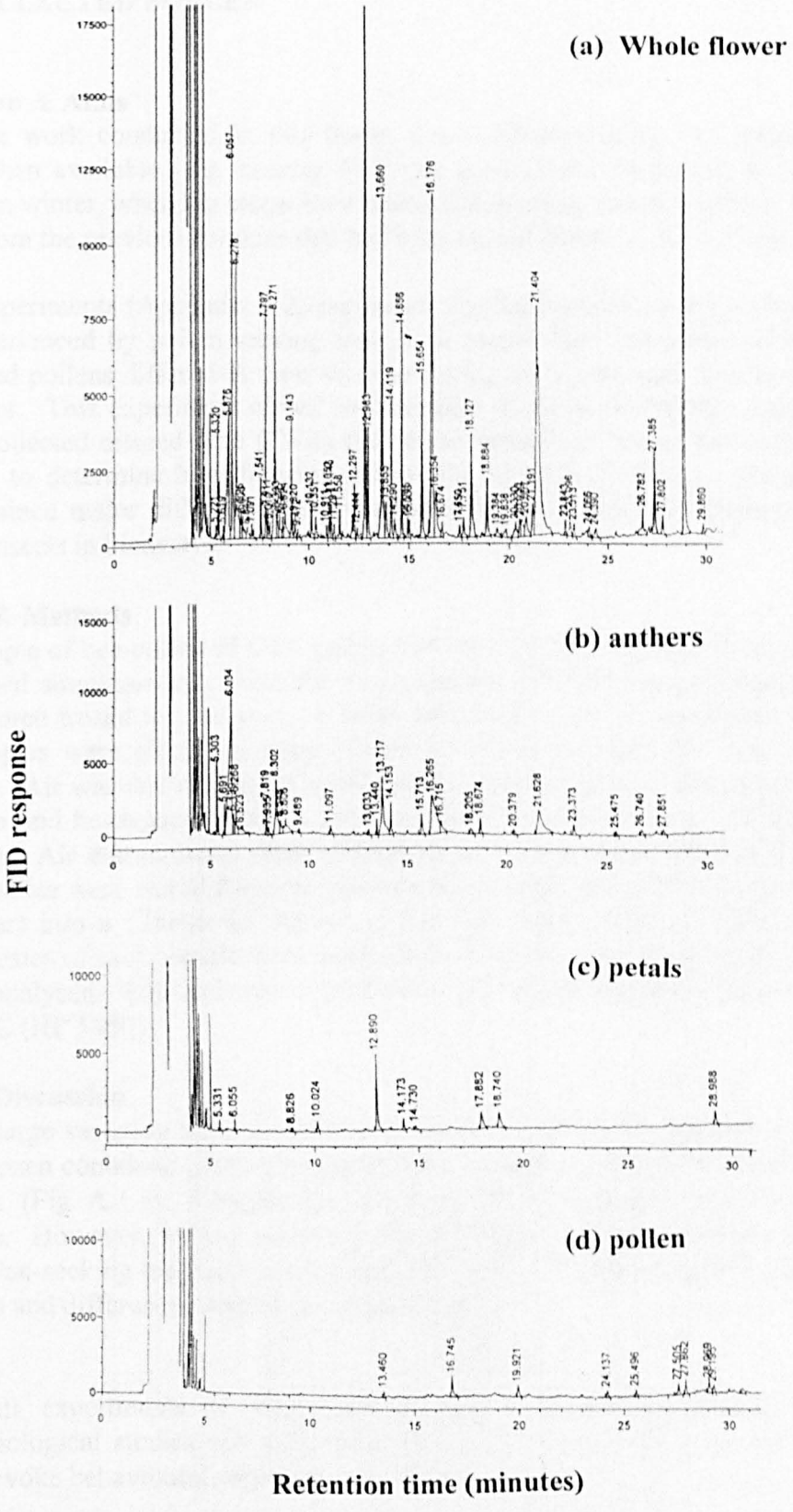


Figure A.1.4. Volatile profile of headspace volatiles released from oilseed rape (a) whole flowers (b) petals (c) anthers (d) pollen

1.4 VOLATILE DIFFERENCES BETWEEN FRESH AND FROZEN BEE-COLLECTED POLLEN

Introduction & Aims

Most of the work conducted in this thesis was conducted using bee-collected pollen (2.3.2). When available (e.g. chapter 4) it was used fresh. However, in experiments conducted in winter, when the crops have finished flowering, and bees forage less, pollen collected from the previous summer that had been stored frozen (–20 °C) was used.

Previous experiments (Appendix 1.2) suggested that hand-collected pollen (more similar to that experienced by pollen-seeking insects in nature than bee-collected pollen) and bee-collected pollens differed in their volatile components, although similar compounds were evident. This experiment aimed to determine if the main volatile components of fresh bee-collected oilseed rape (OSR) pollen are present in frozen bee-collected OSR pollen, and to determine how freezing affects the volatile profile in general. This is important, since major differences could influence the behavioural responses to these pollens of insects in bioassays.

Materials & Methods

A fresh sample of bee-collected OSR pollen (between 10-20 g depending on availability) was entrained simultaneously with the same amount of OSR bee-collected pollen that had been stored frozen for one year. A blank air control was also conducted at the same time. Samples were placed in glass 'Quickfit' chambers (500 ml), and sealed with Teflon tape. Air was drawn through a molecular sieve and through the samples at a rate of 1.1 l/min and headspace volatiles were collected by polymer trap (50 mg poropak) (Fig. A.1.3). Air entrainments were conducted in a room maintained at 25 °C for 24 hours. Volatiles were eluted from the polymer by passing 500 µl of solvent (re-distilled diethyl ether) into a Chromacol microvial that was sealed with a Teflon-coated cap. Seven replicates of each sample were conducted. Samples were stored in the freezer at –20 °C until analysed. Volatiles were separated by gas chromatography (GC) on a Hewlett Plackard GC (HP 5880).

Results & Discussion

There was large variation between replicates within a sample, but generally, pollen that had been frozen contained fewer compounds and the quantities present were less than in fresh pollen (Fig. A.1.5). Freezing may destroy some components, into several smaller components. However, without knowing which components are perceived and used as cues by pollen-seeking insects, it is difficult to determine how freezing affects them. The major peaks and differences remain to be identified.

Conclusion

As with all experiments in this section, only extensive behavioural work and electrophysiological studies can determine which compounds are perceived by insects and which evoke behavioural responses.

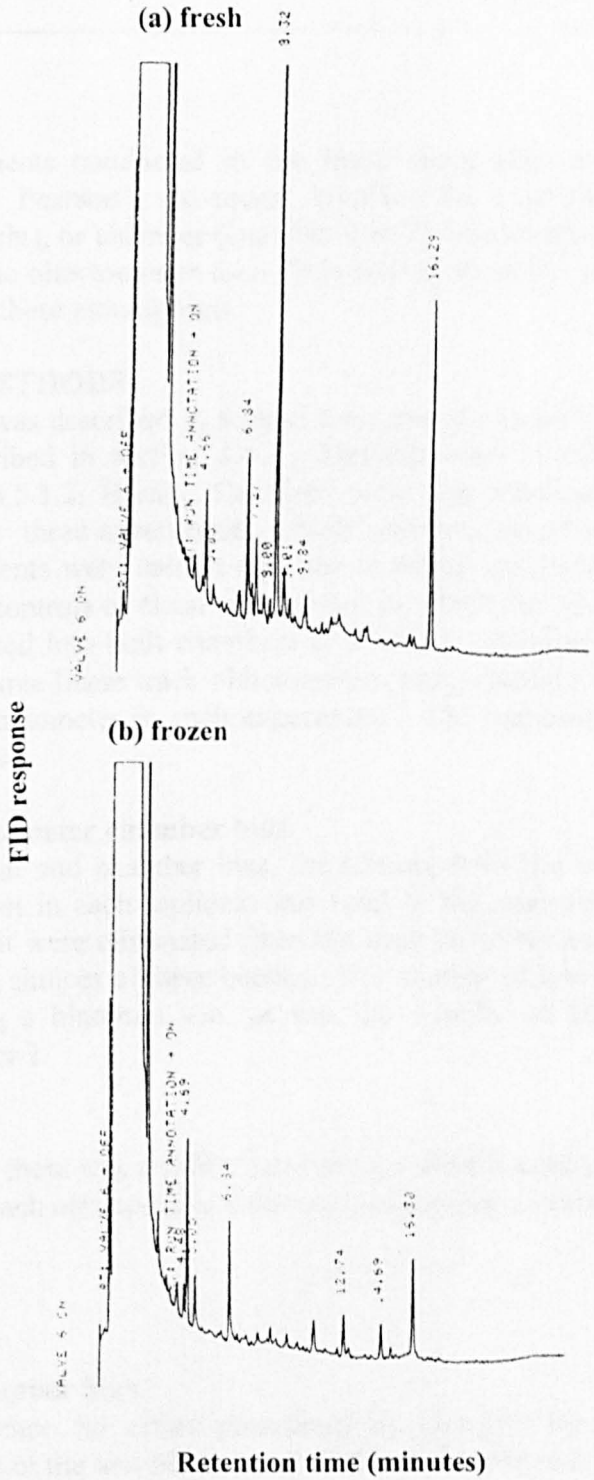


Fig. A.1.5. Volatile profile of oilseed rape bee-collected pollens: (a) fresh (b) stored frozen for 1 year

Appendix 2. Linear track olfactometer bias tests

INTRODUCTION

Analysis of experiments conducted in the linear track olfactometer (LTO; used in chapters 4 & 5) by Pearson's chi-square involves the assumption that there is no directional (left or right), or chamber (chamber 1 or 2) bias within olfactometers, and no difference between the olfactometers used. This section describes experiments conducted to test the validity of these assumptions.

MATERIALS & METHODS

The LTO apparatus was described in section 4.4.1 and the experimental set-up in which it is used was described in section 4.4.2. Methods used to conduct bias tests were described in section 4.5.1.2. Briefly, The same odour was introduced into both chambers of the olfactometer in these experiments, which were conducted as described in section 4.4.5. Two experiments were carried out, one in which no odour was introduced into either chamber (2 x controls of clean air) and one in which the odour of 5 g oilseed rape flowers was introduced into both chambers (2 x OSR). Pre-diapause beetles collected from the field, and three linear track olfactometers were tested in both experiments with 20 replicates per olfactometer in each experiment. The responses of beetles at the T-junction were recorded.

Directional & olfactometer chamber bias

To test for directional and chamber bias, the turning response of only the first beetle coming out of the pot in each replicate was used in the analysis. Subsequent beetles coming out of the pot were eliminated from the analysis to remove possible bias due to any non-independent choices of these beetles. The number of beetles turning left or right was compared using a binomial test, as was the number of beetles turning towards chamber 1 or chamber 2.

Olfactometers

To examine whether there was a difference between olfactometers, the turning responses at the T-junction in each olfactometer were compared using a Pearson's chi-square test.

RESULTS

Directional and chamber bias

There was no evidence for either directional or chamber bias of the first beetle's responses, regardless of the test odours used (Table A.2.1 and A.2.2, respectively).

Difference between olfactometers

There was no difference in results obtained between the three olfactometers (Table A.2.3). When two controls were introduced into both chambers of the LTOs, there was no significant difference between olfactometers in terms of the number of beetles turning left or right ($\chi^2_2 = 2.8$; $P = 0.247$) or turning towards chamber 1 or 2 ($\chi^2_2 = 2.8$; $P=0.812$). Similarly, when the odour of OSR flowers was introduced into both sides of the olfactometer, there was no difference between olfactometers in the number of beetles going left or right ($\chi^2_2 = 0.42$; $P=0.812$) or to chamber 1 or 2 ($\chi^2_2 = 1.76$; $P=0.414$).

Table A.2.1 Bias test: Direction. The number of pollen beetles that turned left and right in the linear track olfactometer (LTO)

Test	L T O no.	Direction of beetles' choice		Binomial Test		
		Left	Right	K	N	P
2 x Controls	1	9	11	9	20	0.824
	2	8	12	8	20	0.504
	3	13	7	7	20	0.264
2 x OSR	1	9	11	9	20	0.824
	2	8	12	8	20	0.504
	3	7	13	7	20	0.264

Table A.2.2 Bias test: Olfactometer chambers. The number of pollen beetles turning towards linear track olfactometer (LTO) chamber 1 or 2

Test	L T O no.	Chamber of beetles' choice		Binomial Test		
		1	2	K	N	P
2 x Controls	1	11	9	9	20	0.824
	2	12	8	8	20	0.504
	3	13	7	7	20	0.264
2 x OSR	1	9	11	9	20	0.824
	2	12	8	8	20	0.504
	3	13	7	7	20	0.264

Table A.2.3 Olfactometer bias: The number of pollen beetles orientating left or right and towards chamber 1 or 2 in each of the three linear track olfactometers (LTO)

Test	LTO no.	No. beetles turning towards			
		Direction		Chamber	
		Left	Right	C1	C2
2 x Controls	1	9	11	11	9
	2	8	12	12	8
	3	13	7	13	7
2 x OSR	1	9	11	9	11
	2	8	12	12	8
	3	7	13	13	7

DISCUSSION & CONCLUSION

There was no evidence for either directional, chamber or olfactometer bias in these experiments. These results validate the assumptions made in the Pearson's chi-square test that there are no such biases.

Appendix 3. Survival and development of pollen beetle larvae on male-fertile and male-sterile Synergy oilseed rape flowers and on field bean flowers

This experiment was described in Chapter 6, section 6.4.2

This appendix reports the daily weights and developmental stage of larvae reared on male-fertile Synergy oilseed rape flowers (Table A3.1); male-sterile Synergy oilseed rape flowers (Table A.3.2) or field bean flowers (Table A.3.3)

Key to tables:

Black font	=	**eggs**
Red font	=	**1st instar larva**
Blue font	=	**2nd instar larva**
Green font	=	**prepupa**
Pink font	=	**pupa**
Brown font	=	**adult**
*	=	**missing data**
P	=	**pupal stage (not weighed again until eclosion)**

Table A.3.1 Pollen beetle larvae developing on male-fertile oilseed rape flowers

POLLEN BEETLE LARVAE DEVELOPING ON FERTILE FLOWERS - replicate 1																										
		DAYS AFTER HATCHING																								
Egg number	egg weight	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	0.035	dead																								
2	0.033	dead																								
3	0.038	0.03	dead																							
4	0.035	dead																								
5	0.04	0.03	0.108	*	0.15	0.613	1.266	1.273	1.308	1.035	1.007	0.995	1.079	0.987	dead											
6	0.033	0.033	0.13	0.396	**	0.599	1.267	1.938	1.546	1.56	1.308	1.322	1.307	1.429	dead											
7	0.038	dead egg																								
8	0.035	0.037	dead																							
9	0.037	0.039	dead																							
10	0.037	0.033	0.167	*	0.235	0.885	1.105	1.279	1.167	0.872	0.81	0.785	dead													
11	0.037	0.03	0.16	0.264	**	0.73	1.406	1.645	1.803	dead																
12	0.031	0.03	dead																							
13	0.038	dead egg																								
14	0.034	0.028	dead																							
15	0.043	0.033	dead																							
16	0.043	0.034	0.194	0.192	*	0.423	1.442	1.118	1.129	1.13	1.05	1.02	0.977	1.005	1.032	1.05	1.034	(M?) p	p	dead pupa						
17	0.044	0.035	0.073	0.239	*	0.28	0.792	1.249	1.19	1.193	1.029	0.991	1.241	1.3	1.29	1.265	(M?) p	p	p	p	p	p	1.467	adult (male)		
18	0.041	dead																								
19	0.04	0.037	0.1	0.219	*	0.287	dead																			
20	0.036	0.037	dead																							
21	0.042	0.035	0.187	*	0.195	0.814	0.999	0.905	1.153	0.962	0.933	0.935	1.074	1.3	1.29	1.258	(M?) p	p	p	p	p	1.333	adult (male)			
22	0.038	dead																								
23	0.033	0.035	dead																							
24	0.043	0.034	0.149	*	0.193	0.209	1.63	1.694	1.618	1.272	1.345	1.225	1.268	dead												
25	0.041	0.042	0.07	0.436	**	0.812	0.999	1.523	1.443	1.657	1.622	1.54	1.53	(F?) p	p	p	p	p	p	1.47	adult (female)					
26	0.037	0.035	0.16	0.523	*	0.19	0.886	1.227	1.35	1.35	1.124	1.065	1.228	1.281	dead											
27	0.038	0.03	0.17	0.39	**	1.125	1.193	1.529	1.332	1.495	1.281	1.326	1.374	1.401	1.378	(F?) p	p	p	p	p	p	p	dead pupa			
28	0.04	0.033	0.213	0.413	*	0.424	2.05	2.3	1.471	1.56	1.322	1.429	1.445	1.476	1.504	1.46	(F?) dead pupa									
29	0.04	0.028	0.149	0.207	*	0.293	0.953	1.301	1.508	1.452	1.245	1.133	1.104	1.13	1.288	1.28	1.31	1.196	(M?) p	p	p	p	p	p	p	1.2	adult (male)
30	0.036	0.036	0.142	0.325	**	0.722	1.528	2.06	1.466	1.548	1.72	1.638	1.649	1.64	(F?) p	p	p	p	p	1.605	adult (female)					
31	0.036	0.03	0.129	0.3	**	1.072	1.774	1.822	1.204	1.471	1.385	1.38	1.291	(M?) p	p	p	p	p	p	1.315	adult (male)					
32	0.038	0.031	0.125	*	0.373	1.269	1.773	1.428	1.441	1.102	1.18	1.182	1.265	1.342	1.324	dead										
33	0.04	0.04	0.279	*	0.326	0.937	1.266	dead																		
34	0.038	0.033	0.192	*	0.147	0.316	0.345	dead																		
35	0.038	0.036	0.198	**	0.456	1.541	1.243	1.121	dead																	
36	0.035	0.033	0.232	*	0.241	0.705	1.167	1.199	1.199	1.038	1.02	0.974	dead													
37	0.038	0.031	0.125	*	0.132	0.517	0.925	1.661	1.467	1.13	1.08	1.028	1.081	1.084	1.08	1.1	1.092	1.05	dead							
38	0.042	0.039	0.183	0.275	dead																					
39	0.038	0.033	0.193	*	** dead																					
40	0.037	0.033	0.058	0.094	dead																					
41	0.036	0.033	0.112	0.372	**	0.497	1.373	1.954	1.422	1.444	1.284	1.246	1.565	1.586	1.568	(F?) p	p	p	p	p	p	1.398	adult (female)			
42	0.036	dead egg																								
43	0.035	0.037	0.106	0.333	dead																					
44	0.034	0.035	0.18	0.285	*	0.274	0.904	1.475	1.336	1.336	1.109	1.108	1.012	dead												
45	0.034	0.038	0.153	0.4	*	0.18	0.784	1.493	1.532	1.455	1.095	1.066	1.109	1.412	1.539	1.536	1.64	1.543	(F?) p	p	p	dead pupa				
46	0.031	dead																								
47	0.032	0.036	0.197	dead																						
48	0.035	0.036	0.121	0.361	*	0.27	1.007	1.554	1.194	1.396	1.109	1.408	1.377	1.378	(M?) p	p	p	p	p	p	1.303	adult (male)				
49	0.035	0.04	dead																							
50	0.033	dead egg																								

POLLEN BEETLE LARVAE DEVELOPING ON FERTILE FLOWERS - replicate 2

Egg number	egg weight	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
51	0.033	0.033	*	0.082	0.143	0.22	0.483	1.148	1.137	0.802	0.732	0.96	1.046	1.1	1.175	1.152	(F?) p	p	p	p	p	1.1	adult (female)			
52	0.033	0.036	dead																							
53	0.033	0.045	*	0.263	0.534	1.292	1.87	1.342	1.504	1.47	1.556	1.526	(M?) p	p	p	p	p	p	1.485	adult (male)						
54	0.032	dead egg																								
55	0.035	dead egg																								
56	0.034	dead egg																								
57	0.032	0.037	*	0.344	0.743	1.068	1.355	1.133	1.46	1.58	1.556	1.525	(F?) p	p	p	p	p	1.5	adult (female)							
58	0.03	0.032	*	0.278	0.759	1.499	1.417	1.19	0.989	1.288	1.3	1.3	(F?) p	p	p	p	p	p	1.221	adult (female)						
59	0.033	0.028	*	0.285	0.522	1.222	1.675	1.25	1.228	1.356	1.2	1.405	1.596	1.431	pupa (M?)	p	p	p	p	1.431	adult (male)						
60	0.033	0.031	*	0.404	0.562	1.491	1.533	1.416	1.196	1.5	1.621	1.617	1.225	(F?) p	p	p	p	p	1.558	adult (female)						
61	0.033	0.032	dead																							
62	0.041	dead egg																								
63	0.03	0.03	dead																							
64	0.033	dead egg																								
65	0.039	dead egg																								
66	0.039	0.04	*	0.48	0.801	1.404	1.358	1.222	1.15	1.19	1.299	1.275	1.5	1.332	(M?) p	p	p	p	p	1.271	adult (male)						
67	0.04	0.038	*	0.398	0.782	1.479	1.381	1.446	1.44	1.4	1.737	1.728	1.622	(M?) p	p	p	p	p	p	1.532	adult (male)						
68	0.032	dead egg																								
69	0.043	0.034	0.284	0.309	0.979	1.528	1.28	1.16	1.32	1.151	1.112	1.114	1.114	(M?) p	p	p	p	p	1.175	adult (male)						
70	0.048	0.038	*	0.302	0.73	1.059	1.773	1.061	1	dead																
71	0.04	0.048	*	0.426	0.724	1.309	1.174	dead																		
72	0.042	0.042	*	0.404	0.856	1.5	1.54	1.45	1.245	1.541	1.7	1.16	1.669	(M?) p	p	p	p	p	p	1.522	adult (female)						
73	0.04	0.04	0.265	0.327	0.549	1	0.653	dead																		
74	0.041	0.039	0.172	0.271	0.599	1.037	1.64	1.7	1.581	1.259	1.42	1.785	1.785	1.873	1.845	(M?) p	p	p	p	p	1.655	adult (male)					
75	0.037	dead egg																								
76	0.037	0.035	0.272	0.371	0.728	1.375	1.994	1.659	1.457	1.496	1.416	1.667	1.659	1.659	(F?) p	p	p	p	p	1.745	adult (female)						
77	0.04	0.034	dead																							
78	0.039	dead egg																								
79	0.037	0.035	dead																							
80	0.036	0.041	0.286	0.298	0.983	1.61	1.59	1.424	1.798	1.838	1.857	1.785	(F?) p	p	p	p	p	1.635	adult (female)							
81	0.036	0.04	0.126	0.16	0.169	0.25	dead																			
82	0.039	0.036	dead																							
83	0.038	0.038	dead																							
84	0.037	0.045	*	0.402	0.559	0.836	1.373	1.172	1.117	1.2	0.962	1.181	1.315	1.355	(M?) p	p	p	p	p	p	p	p	p	dead pupa		
85	0.032	dead egg																								
86	0.03	dead egg																								
87	0.038	0.04	*	0.314	0.703	1.3	1.505	1.417	1.174	1.48	1.753	1.725	1.69	(F?) p	p	p	p	p	1.855	adult (female)						
88	0.035	0.035	*	0.339	0.484	1.391	1.457	1.15	0.895	1.24	1.404	1.38	1.424	1.374	(F?) p	p	p	p	p	1.315	adult (female)						
89	0.038	0.031	dead																							
90	0.038	0.04	*	0.272	0.611	1.22	1.387	1.063	1.038	0.898	0.775	0.822	1.007	1.285	1.4	1.45	dead									
91	0.035	0.036	*	0.299	missing																					
92	0.035	dead egg																								
93	0.032	0.034	*	0.303	0.654	1.407	1.364	1.095	0.999	1.124	1.106	1.14	1.127	1.122	(M?) p	p	p	p	p	p	1.117	adult (male)					
94	0.034	0.03	*	0.444	0.577	1.076	1.287	1.1	1.075	1.17	1.046	1.119	dead													
95	0.036	dead egg																								
96	0.037	0.048	*	0.404	dead																					
97	0.035	0.041	*	0.332	0.712	1.345	1.455	1.177	0.886	1.56	1.622	1.671	1.633	(F?) p	p	p	p	p	p	p	p	dead				
98	0.035	0.039	dead																							
99	0.04	0.037	dead																							
100	0.04	0.045	0.163	0.319	0.819	1.626	1.508	1.21	1.734	1.6	1.487	1.5	1.461	(F?) p	p	p	p	p	1.475	adult (female)						
n	100	78	39	47	36	52	50	46	45	43	43	43	36	25	14	10	5	6	7	10	4	4	2			2
mean	0.03672	0.035727	0.166395	0.322913	0.535143	0.912314	1.308755	1.394067	1.311409	1.323833	1.279024	1.290024	1.338543	1.345083	1.362538	1.283444	1.269	1.3848	1.4615	1.467333	1.358333	1.277	1.467			1.2
SD	0.003516	0.004512	0.058969	0.091063	0.256751	0.471666	0.363256	0.298038	0.228175	0.233612	0.283559	0.274261	0.236584	0.213006	0.217868	0.169234	0.274394	0.249316	0.246905	0.15391	0.273235	0.156694	0			0
SE	0.000352	0.000514	0.009567	0.013427	0.043399	0.06605	0.051894	0.04443	0.0344	0.036046	0.043752	0.042318	0.039991	0.043479	0.060351	0.056411	0.137197	0.111501	0.100819	0.051303	0.157757	0.09047	0			0

Table A.3.2 Pollen beetle larvae developing on male-sterile oilseed rape flowers

LARVAE DEVELOPING ON STERILE FLOWERS replicate 1																										
		DAYS AFTER HATCHING																								
Egg number	egg weight	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	0.042	0.03	0.132	0.295	*	0.077	0.276	0.412	0.19	dead																
2	0.044	0.037	dead																							
3	0.045	0.036	dead																							
4	0.041	0.033	dead																							
5	0.04	0.042	0.18	0.125	*	0.102	0.559	dead																		
6	0.041	0.034	0.133	0.165	*	0.298	1.03	1.235	1	dead																
7	0.042	0.035	dead																							
8	0.041	0.033	dead																							
9	0.037	0.039	dead																							
10	0.037	0.036	0.147	0.187	dead																					
11	0.035	egg dead																								
12	0.035	* dead																								
13	0.023	0.025	dead																							
14	0.042	0.034	0.07	0.089	dead																					
15	0.038	0.03	0.034	0.084	*	0.199	0.476	0.931	1.448	2.072	1.023	1.002	dead													
16	0.037	0.031	dead																							
17	0.037	0.041	dead																							
18	0.037	0.031	0.174	0.229	**	0.315	0.541	1.563	1.224	1.049	0.64	dead														
19	0.036	* dead																								
20	0.037	0.036	0.086	0.167	*	0.215	0.301	1.248	1.255	1.188	0.869	0.97	1.263	1.262	1.263	1.25	(F?) p	p	p	p	p	p	1.14	adult (female)		
21	0.035	0.031	dead																							
22	0.035	0.032	0.118	dead																						
23	0.045	dead egg																								
24	0.037	0.036	0.169	0.217	*	0.236	0.741	0.901	2.462	1.14	dead															
25	0.044	0.033	0.086	0.156	*	0.179	dead																			
26	0.042	0.033	0.05	dead																						
27	0.037	0.036	0.039	0.289	*	0.196	0.206	0.691	missing																	
28	0.038	0.036	0.08	0.152	*	0.156	0.155	dead																		
29	0.041	0.034	dead																							
30	0.036	0.03	0.163	0.214	dead																					
31	0.037	* dead																								
32	0.038	* dead																								
33	0.039	0.04	0.083	0.165	*	0.186	0.63	0.787	1.255	0.894	dead															
34	0.04	0.034	0.052	0.209	*	0.199	0.375	dead																		
35	0.039	0.028	dead																							
36	0.032	0.03	0.036	dead																						
37	0.035	0.032	dead																							
38	0.035	0.03	dead																							
39	0.033	0.034	dead																							
40	0.036	* dead																								
41	0.036	dead egg																								
42	0.035	* dead																								
43	0.035	0.037	0.07	0.15	dead																					
44	0.035	0.033	0.071	0.213	*	0.211	dead																			
45	0.035	0.032	0.081	0.254	*	0.288	0.675	0.682	0.687	1.009	1.128	1.126	1.371	1.095	0.829	0.76	0.867	0.941	0.932	0.976	(F?) p	dead pupa				
46	0.035	* dead																								
47	0.031	dead egg																								
48	0.033	0.03	0.13	0.267	*	0.254	0.506	0.595	0.342	dead																
49	0.032	0.034	dead																							
50	0.037	dead egg																								

LARVAE DEVELOPING ON STERILE FLOWERS replicate 2

		DAYS AFTER HATCHING																								
Egg number	egg weight	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
51	0.039	0.036	dead																							
52	0.033	0.037	0.053	0.048	missing																					
53	0.037	dead egg																								
54	0.037	dead egg																								
55	0.032	dead egg																								
56	0.032	0.031	0.049	0.063	0.09	0.1	0.295	0.483	0.992	1.577	1.43	1.236	1.309	1.335	1.315	(F?) p	p	p	p	p	p	1.362	adult (female)			
57	0.035	0.038	0.062	0.058	0.073	0.076	0.128	0.206	0.247	0.91	1.368	1.27	1.031	1.2	1.015	1.275	1.275	1.22	(F?) p	dead pupa						
58	0.033	0.037	0.147	0.096	0.058	0.058	dead																			
59	0.037	dead egg																								
60	0.03	0.03	0.075	0.05	0.184	0.141	0.17	0.102	0.1	0.108	0.15	0.286	0.375	0.426	0.362	0.2	dead									
61	0.035	0.03	0.047	0.133	0.168	0.143	0.448	0.255	0.533	0.841	0.84	0.725	0.82	0.906	1.005	dead										
62	0.035	0.034	missing																							
63	0.04	0.036	0.113	0.102	0.184	0.147	0.189	0.199	0.4	0.459	0.599	1.14	0.925	0.942	0.7	0.775	0.777	0.759	(M?) p	p	p	p	p	p	1.006	adult (female)
64	0.04	dead egg																								
65	0.034	0.034	0.034	dead																						
66	0.04	0.04	0.162	0.144	0.149	0.111	0.204	0.07	0.271	dead																
67	0.04	0.04	0.236	0.169	0.183	0.273	0.383	0.355	dead																	
68	0.04	0.044	0.164	dead																						
69	0.04	0.037	0.164	0.131	0.146	0.267	0.902	1.09	1.268	dead																
70	0.04	0.03	0.05	dead																						
71	0.036	dead egg																								
72	0.036	0.033	dead																							
73	0.039	0.039	0.106	dead																						
74	0.039	0.037	0.151	0.139	0.153	0.289	0.26	0.845	1.584	1.241	0.9	0.82	1.132	1.118	0.188	1.355	(M?) p	p	dead pupa							
75	0.035	0.033	0.159	0.254	0.303	0.312	0.443	0.583	1.311	1	0.745	0.742	0.931	0.93	0.962	0.952	0.943	(M?) p	p	p	p	p	0.885	adult (male)		
76	0.035	0.032	0.229	0.232	0.161	0.18	0.088	0.244	0.189	0.303	0.762	0.833	1.123	1.056	0.928	0.8	1.016	1.006	1.002	(F?) p	p	p	p	p	0.894	adult (male)
77	0.033	0.033	0.042	0.045	dead																					
78	0.034	0.037	0.154	0.184	0.105	0.104	0.071	dead																		
79	0.04	0.041	0.202	0.159	0.143	0.108	0.435	1.084	0.561	1.216	1.075	0.983	1.179	1.2	1.2	(F?) p	dead pupa									
80	0.04	0.043	0.211	dead																						
81	0.042	0.031	0.148	0.102	0.097	0.095	0.162	0.126	0.179	0.197	0.211	0.375	0.69	0.815	0.502	0.675	0.785	0.762	dead							
82	0.034	0.04	dead																							
83	0.033	0.038	0.159	0.384	0.132	0.478	0.494	1.02	1.401	1.127	1.304	1.483	1.637	1.6	1.516	(F?) p	p	p	p	p	1.436	adult (female)				
84	0.03	dead egg																								
85	0.031	0.037	dead																							
86	0.033	0.035	0.118	0.123	0.136	0.243	0.355	0.853	1.249	1.3	1.175	1.13	1	0.943	0.95	0.93	(M?) p	dead pupa								
87	0.036	0.044	0.05	dead																						
88	0.037	0.033	dead																							
89	0.044	0.035	0.057	dead																						
90	0.043	0.037	0.213	0.25	0.233	0.275	0.56	0.658	0.804	dead																
91	0.04	dead egg																								
92	0.04	0.037	0.119	0.192	0.215	0.106	0.27	0.65	1.1	1.535	1.27	1.166	dead													
93	0.036	dead egg																								
94	0.035	0.03	dead																							
95	0.03	0.033	0.415	dead																						
96	0.031	dead egg																								
97	0.029	dead egg																								
98	0.03	dead egg																								
99	0.034	dead egg																								
100	0.032	0.043	0.294	0.153	dead																					
n	100	76	53	42	20	35	32	28	26	20	18	17	15	15	15	11	7	6	3	2	2	2	3	1	3	
mean	0.03661	0.03484	0.122442	0.16678	0.153316	0.194618	0.397677	0.661778	0.88208	1.008737	0.911118	0.955438	1.056143	1.059143	0.909643	0.8972	0.9396	0.9396	0.967	0.976	1.436	1.362	1.0125		0.95	
SD	0.003941	0.003976	0.074451	0.075601	0.058491	0.091693	0.233872	0.39725	0.582726	0.488692	0.372018	0.317714	0.311201	0.274847	0.372451	0.372451	0.189527	0.189527	0.049497	0	0	0	0.180312		0.079196	
SE	0.000394	0.000459	0.010325	0.011813	0.013419	0.015725	0.042003	0.076453	0.116545	0.112111	0.09023	0.079429	0.083164	0.073449	0.107273	0.11779	0.077358	0.084761	0.035	0		0	0.127519		0.056008	

Table A.3.3 Pollen beetle larvae developing on field bean flowers

LARVAE DEVELOPING ON FIELD BEAN FLOWERS													
		DAYS AFTER HATCHING											
Egg number	egg weight	1	2	3	4	5	6	7	8	9	10	11	12
Replicate 1													
1	0.045	0.066	missing										
2	0.045	dead egg											
3	0.041	0.03	dead										
4	0.042	dead egg											
5	0.04	0.035	0.047	0.336	dead								
6	0.04	0.041	0.054	0.1	dead								
7	0.034	dead egg											
8	0.036	0.031	dead										
9	0.032	0.027	0.137	dead									
10	0.031	0.025	dead										
11	0.036	0.026	0.131	dead									
12	0.035	0.04	0.063	dead									
13	0.025	0.025	dead										
14	0.025	0.02	dead										
15	0.034	0.031	dead										
16	0.036	0.028	0.035	dead									
17	0.036	0.024	0.063	dead									
18	0.035	0.025	dead										
19	0.036	0.028	0.075	dead									
20	0.035	0.024	0.054	0.054	0.043	dead							
21	0.039	0.045	dead										
22	0.04	0.031	0.061	0.08	0.641	0.915	0.36	dead					
23	0.034	0.029	0.142	0.045	0.515	0.515	0.515	0.087	dead				
24	0.04	0.03	0.117	0.098	0.13	dead							
25	0.035	0.026	dead										
26	0.037	0.044	0.045	dead									
27	0.039	dead egg											
28	0.031	0.037	dead										
29	0.032	dead egg											
30	0.043	0.049	0.037	dead									
31	0.03	dead egg											
32	0.039	0.035	0.133	dead									
33	0.033	0.025	missing										
34	0.032	0.035	dead										
35	0.026	0.037	0.128	0.153	missing								
36	0.031	0.028	dead										
37	0.03	0.049	0.038	0.051	0.045	dead							
38	0.031	dead egg											
39	0.03	0.03	dead										
40	0.034	0.032	0.04	missing									
41	0.04	0.047	0.047	0.056	0.199	dead							
42	0.04	0.051	0.06	0.447	0.304	0.07	dead						
43	0.036	0.033	0.036	dead									
44	0.039	0.044	0.039	0.653	0.482	0.803	0.299	0.303	0.505	0.483	0.346	0.329	dead
45	0.036	dead egg											
46	0.04	0.04	dead										
47	0.037	0.03	dead										
48	0.032	0.035	dead										
49	0.038	0.032	0.135	dead									
50	0.038	0.041	0.043	dead									
Replicate 2													
51	0.04	0.032	dead										
52	0.04	0.048	0.064	0.069	0.053	dead							
53	0.038	0.044	0.044	0.046	0.043	dead							
54	0.038	0.038	0.038	0.035	dead								
55	0.04	0.042	0.04	0.043	dead								
56	0.04	0.041	0.059	dead									
57	0.04	0.042	0.038	0.066	0.052	0.063	0.113	0.067	0.073	dead			
58	0.04	0.04	0.047	dead									
59	0.034	0.034	dead										
60	0.035	0.036	dead										
61	0.04	0.043	0.053	0.068	missing								
62	0.04	0.03	dead										
63	0.04	0.039	0.048	dead									
64	0.04	0.036	0.054	missing									
65	0.04	0.039	0.039	0.04	0.061	0.063	0.201	dead					
66	0.04	0.036	0.062	0.062	dead								
67	0.03	0.033	dead										
68	0.031	0.039	dead										
69	0.035	0.032	dead										
70	0.033	0.042	dead										
71	0.036	0.034	0.056	0.085	0.12	missing							
72	0.035	0.044	0.063	0.102	dead								
73	0.033	0.041	dead										
74	0.033	0.03	missing										
75	0.03	0.047	0.037	0.049	dead								
76	0.035	0.03	missing										
77	0.033	0.032	dead										
78	0.034	0.033	0.1	dead									
79	0.033	0.043	dead										
80	0.035	0.032	0.039	0.072	dead								
81	0.036	0.04	0.076	0.119	0.147	0.261	dead						
82	0.036	0.037	0.056	dead									
83	0.035	0.038	0.038	dead									
84	0.034	0.036	0.04	0.047	dead								
85	0.033	0.033	missing										
86	0.034	0.035	0.049	0.052	dead								
87	0.034	0.032	missing										
88	0.034	0.033	0.065	0.069	0.092	0.081	dead						
89	0.033	0.035	dead										
90	0.034	0.036	0.036	dead									
91	0.038	0.031	dead										
92	0.038	0.03	0.044	missing									
93	0.03	0.03	dead										
94	0.031	0.035	0.041	dead									
95	0.034	0.032	0.047	0.053	0.067	0.068	0.075	0.086	dead				
96	0.034	0.032	missing										
97	0.036	0.03	dead										
98	0.037	0.045	0.055	0.089	0.089	dead							
99	0.036	0.035	0.062	0.073	0.085	0.076	dead						
100	0.036	0.033	dead										
n	100	92	53	30	18	10	6	4	2	1	1	1	0
mean	0.03565	0.035446	0.061321	0.1104	0.176	0.2915	0.2605	0.13575	0.289	0.483	0.346	0.329	
SD	0.003935	0.007316	0.030516	0.134298	0.184525	0.332575	0.164839	0.111879	0.30547	0	0	0	
SE	0.000393	0.000763	0.004192	0.02452	0.043489	0.105179	0.067309	0.05594	0.216033	0	0	0	

Appendix 4. Conditioning efficiency of honey bee-collected and hand-collected oilseed rape and field bean pollen odours in the conditioned proboscis extension test conducted on restrained honey bees

EXPERIMENTAL DETAILS

This appendix refers to experiments conducted in chapter 7.

Table A.4.1. Conditioning efficiency (after Pham-Delègue, *et al.* 1993) of honey bees conditioned to the odour of bee-collected pollen.

Conditioning odour	No. bees at start	'learners' (a)	'nonlearners' (b)	'non-selective learners' (c)	No. PER (d)	No. bees used in test	
						T1 OSR	T1 FB
Oilseed rape	84	82	0	1	1	41	41
Field bean	84	72	8	4	0	37	35
Control	35	3	32	N/A	0	N/A	

(a) No. bees exhibiting conditioned proboscis extension (CPE) response at least once during conditioning
(b) No. bees not exhibiting CPE response once during conditioning
(c) No. bees exhibiting CPE response to the blank air control
(d) No. bees without proboscis extension response (PER) at end of testing

Table A.4.2. Conditioning efficiency (after Pham-Delègue, *et al.* 1993) of honey bees conditioned to the odour of hand-collected pollen.

Conditioning odour	No. bees at start	'learners' (a)	'nonlearners' (b)	'non-selective learners' (c)	No. PER (d)	No. bees used in test	
						T1 OSR	T1 FB
Oilseed rape	74	54	13	7	0	25	29
Field bean	73	32	25	16	0	16	16
Control	37	6	30	N/A	1	N/A	

(a) No. bees exhibiting conditioned proboscis extension (CPE) response at least once during conditioning
(b) No. bees not exhibiting CPE response once during conditioning
(e) No. bees exhibiting CPE response to the blank air control
(f) No. bees without proboscis extension response (PER) at end of testing

CONCLUSION

In each experiment, the conditioning efficiency of oilseed rape and field bean pollen odours were higher than a blank air control. Also, bee-collected pollen has a higher conditioning efficiency than hand-collected pollen.

Appendix 5. Floweroid bias

INTRODUCTION & AIM

This experiment was conducted to determine any preferences for direction (left/right) or feeder (1 or 2) of bees tested in choice tests conducted using the 'floweroid' artificial flower that may bias results gained from such experiments.

MATERIALS & METHOD

The 'floweroid' artificial flower (8.4.5) operating as an odour-distribution unit (8.5.2.2) was tested for bias in the bee flight room (8.4.1) using a colony of honey bees (8.4.2) maintained there. The method was essentially the same as described in the thesis section 8.5.3. Free-flying bees were conditioned to the odour of 10 g oilseed rape (OSR) pollen loads emitted by the central conditioning feeder of the floweroid by offering 60% sucrose solution as an associative reward. Once a bee had learned the position of the feeder and returned of its own accord, it was marked, and allowed five visits to collect the reward, after which it was considered to be conditioned to the odour. It's 6^{th} visit was tested; the bee was presented in an unrewarded choice test, the OSR conditioning odour in both outer test feeders. The first choice of the test bee (whether it landed on feeder 1 or 2 when on the left or right) were recorded and the number of visits to each feeder and their duration was recorded over a three-minute period. Ten bees were tested, individually, and the direction (left or right) of the feeders were alternate between tests.

RESULTS & DISCUSSION

Number of first landings

More bees landed first on feeder 2 than feeder 1 (Table A.5.1), although the result was borderline in terms of significance at the 5% level.

TABLE A.5.1 Number of first landings by honey bees on feeder 1 and feeder 2 when both these emit the odour of the conditioning pollen odour

No first landings				FEXACT Probability
Feeder1 on Left	Feeder 2 on right	Feeder 2 on Left	Feeder 1 on Right	
0	5	4	1	P=0.048

Total number of visits

There was no difference in the total number of visits made on feeder 1 or 2 in the choice tests (Table A.5.2)

TABLE A.5.2 Mean number of visits to oilseed rape pollen (OSR) yellow or field bean pollen (FB) grey – coloured feeders made by honey bees in a choice test after conditioning to either OSR yellow or FB grey

Mean no. visits (±SE)		n	Wilcoxon Test statistic	Normal approximation	P
Feeder 1	Feeder 2				
5.9 (1.059)	8.5 (1.47)	10	14.5	1.325	P>0.05

Mean duration of each visit

There was no difference in time spent searching feeder 1 or 2 in the choice tests (Table A.5.3).

TABLE A.5.3 Mean time per visit (in seconds) spent searching yellow or grey coloured feeders in a choice test after conditioning to either oilseed rape (OSR) yellow or field bean (FB) grey pollen colours

Mean time (s) (±SE) searching		Mean difference (±SE)	N	T	df	P
Feeder 1	Feeder 2					
1.48 (0.346)	1.33 (0.136)	0.146 (0.939	10	0.48	9	>0.05

CONCLUSION

There is evidence to suggest that there is no bias between feeders evident when measuring the mean number of visits to each feeder or their duration. However, there is some evidence to suggest there may be bias towards feeder 2 when the first landings are analysed, but this could be due to the small sample size involved in this bias test (n=10), and samples are larger in the experiments (n=20).

Appendix 6. Preliminary experiment to test the conditioned responses of free-flying honey bees to oilseed rape and field bean pollen

INTRODUCTION & AIMS

Foraging bees learn as a consequence of their foraging decisions. The aim of this experiment was to determine whether free-flying bees could discriminate between two crops species based on their pollens alone.

MATERIALS & METHODS

The principles of associative learning are described in the thesis section 8.4.6. Honey bees (8.4.2) from a colony maintained in the bee flight (8.4.1) were conditioned (8.5.1.1) to pollens using the test arena with artificial flowers (8.4.4). Briefly, bees were conditioned over a period of two hours to 5 g oilseed rape (OSR) bee-collected pollen placed in the central artificial flower. After this period, foraging bees were removed from the arena, the conditioning artificial flower was removed, and after cleaning, two test artificial flowers were put into place at equal distances apart from where the conditioning artificial flower had been. One of the test artificial flowers was filled with either 0.5 g OSR (conditioned pollen) and the other one with the same amount of field bean (FB) bee-collected pollen (the novel pollen).

The bees foraging at each dish were counted once every minute over a five-minute period. Then, the test feeders were removed and a fresh artificial flower with 5 g OSR pollen was repositioned in the central space. Bees were re-conditioned to the OSR pollen over a 30-minute period. After this reconditioning session, the arena was again cleaned and two fresh test dishes were repositioned; this time, the position of the conditioning and novel pollens were reversed. This sequence was repeated until 10 replicates had been completed. The mean numbers of bees foraging on each pollen type at each half-hour interval was calculated and plotted.

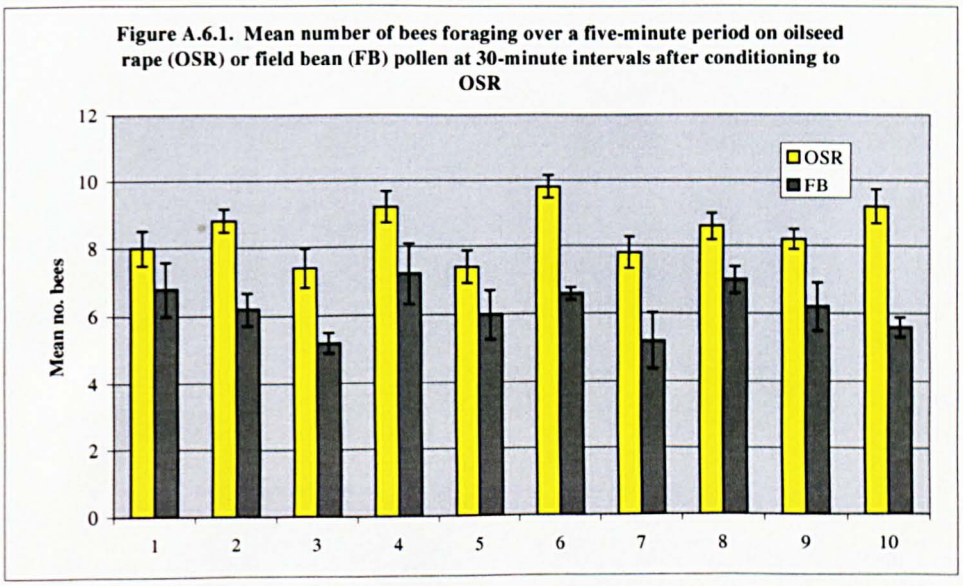
In a second experiment, conducted one day later, bees were conditioned to FB pollen, and OSR was the unconditioned type. Again, the mean numbers of bees foraging on each pollen type at each half-hour interval was plotted. For each experiment, the mean number of bees foraging on each dish throughout all 10 replicates were compared using a T-test.

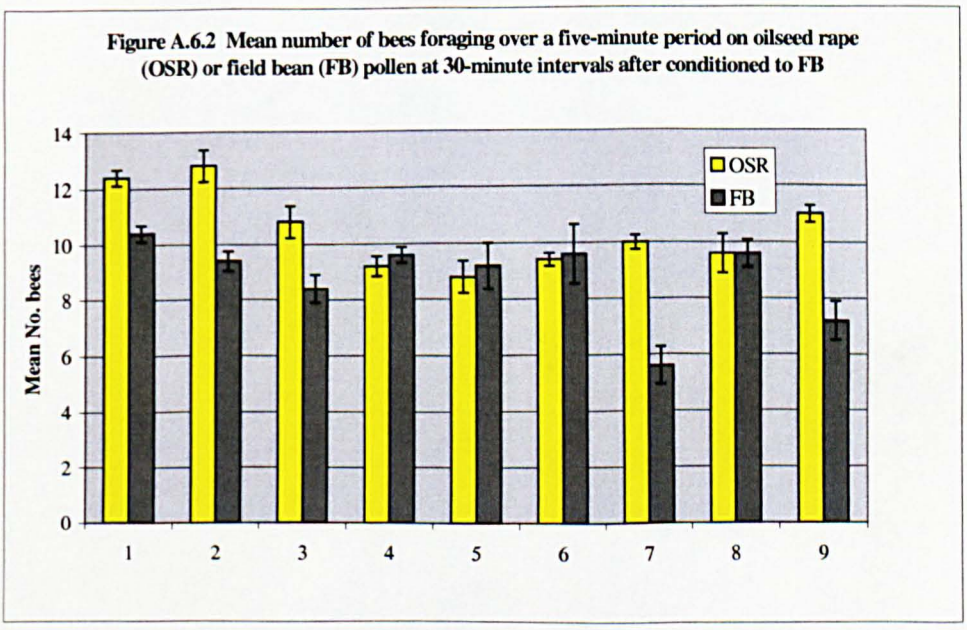
RESULTS & DISCUSSION

When conditioned to OSR pollen, the mean number of bees foraging at the OSR dish was greater than the number foraging at the FB dish throughout a day's replicates (Fig. A.6.1). The overall mean number of bees foraging at the OSR a dish was greater than that on the FB dish ($P<0.001$) (Table A.6.1). When bees were conditioned to FB pollen the next day, only nine of the ten replicates were completed. More bees were found foraging on the OSR than the FB for the first three replicates, but after this, the numbers foraging on each dish became more equal (Fig. A.6.2). Despite this, significantly more bees were recorded foraging on OSR than FB overall ($P<0.05$) (Table A.6.1). Footprint markings and the presence of groups of foraging bees may have influenced these results. Tests on individual bees in the absence of others should therefore be conducted to remove these influences in future tests.

Table A.6.1 Mean number of bees foraging on oilseed rape (OSR) and field bean (FB) pollens after conditioning to either OSR or FB.

Conditioning pollen type	Mean no. of foragers		Mean difference	T	df	P
	OSR	FB				
OSR	8.440	6.200	2.240	9.19	9	<0.001
FB	10.440	8.770	1.667	2.56	8	<0.05





CONCLUSION

These results suggest that bees can discriminate between OSR and FB pollens but that bees have a preference for OSR over FB.

Hallelujah! ~ This work is done.