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The importance of nutrition for bumblebees and solitary bees; implications for pesticides and stewardship.

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Abstract

Critical to supporting populations of wild and managed species in agricultural systems are an improved understanding of mechanisms by which bees enhance crop quality and yield; the factors influencing pollen selection by foraging bees (including pollen diversity and amino acid concentrations); the influence of pollen profiles in determining individual and colony performance; and the response of individuals to insecticides in nectar. Laboratory and field studies addressed these questions using *Osmia bicornis* and *Bombus terrestris audax*.

In a mixed lowland farm system, *O. bicornis* reproduction and plant utilisation was investigated in florally-enriched vegetation. A high selectivity for pollen was observed and in the laboratory larval survival was related to the pollen profile of the diet. The role of pollen diversity and amino acid concentrations were investigated in both foraging behaviour and survival, but requires clarification, although poor survival in the laboratory was associated with low amino acid concentrations in a single-species diet of *Pinus* pollen. In *B. terrestris audax*, laboratory colony performance was greatest in pollen diets with high concentrations of essential amino acids, but again no consistent relationship with the diversity of pollen species was detected. Results suggest that composition of off-crop vegetation, including those offered in agri-environment schemes, could be modified to better support wild and managed bee populations.

Managed *O. bicornis*, in a cherry orchard experiment, were shown to improve fruit quality, demonstrating a potential commercial application of improved understanding of pollinator biology. Management interventions in farm systems, however, need to consider the potential impacts of insecticides. In laboratory experiments, foraging *B. terrestris* used colour cues when selecting flowers, but chemical cues to detect nectar spiked with neonicotinoids. Observed sub-lethal toxicity was reversible when clean nectar was offered but further work is required to confirm the mechanism (behavioural or physiological) leading to these findings.

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1.1 Introduction

The UK is currently reflecting global trends in pollinator decline (Potts *et al.,* 2010; González-Varo *et al.,* 2013). Pollinator decline in the UK has been observed across a range of natural and agricultural landscapes (Potts *et al.,* 2010; Goulson *et al.,* 2011); the underlying causes of this decline are subject to wide debate and controversy (Potts *et al.,* 2010; Walters, 2013), but habitat loss, nutrition, disease, climate change and pesticide use are frequently proposed (Potts *et al.,* 2010; Walters, 2013). Many studies focus on one or a limited number of these factors whereas it is likely that each contribute.

Floral resources are recognised as a significant factor in pollinator dynamics (Sagili & Pankiw, 2007; Potts *et al.,* 2010; Eckhardt *et al.,* 2014) and are of particular significance for bee species (Hymenoptera: Apidae), in which both adults and larvae are dependent on flowers for nutrition (Michener, 2007; Goulson, 2010). One response to rising world population has been agicultural intensification, which in turn has resulted in reduction of floral diversity/non-crop habitats in the agricultural landscape (Williams & Osborne, 2009; Breeze *et al*., 2011). Floral resource limitations significantly affect bee species diversity, but can be mitigated or minimised through the provision of off-crop habitats offering increased floral diversity (Jauker *et al.,* 2012; Eckhardt *et al.,* 2014). Optimisation of the floral composition of these habitats however, is currently limited by a lack of knowledge of the effects of specific nutritional components on pollinators at both the individual and colony level.

Pollen is required for development of ovaries in females and provisioning of larval offspring (Biliñski & Teper 2004; Goulson, 2010). Nectar is utilised as a carbohydrate fuel source in order to sustain flight during foraging trips (Tasei & Aupinel, 2008). Pollen is key to colony development and offspring mass (Ribeiro, 1994; Sagili & Pankiw, 2007; Jauker *et al.,* 2012; Eckhardt *et al.,* 2014), and pollen quality has been linked to antimicrobial immune response (Moret & Schmid-Hempel, 2004; Mapalad *et al.,* 2008), and the detoxification of pesticides (Alaux *et al.*, 2010; Di Pasquale *et al*., 2013; Corby-Harris *et al*., 2014). Most work leading to improved understanding of pollen quality, however, has been directed at designing artificial diets for supplementary feeding of honeybees and there has been limited

biochemical analysis of pollen beyond crude protein/lipid analysis and fewer investigations have considered bumblebees or solitary bees (Cook *et al.,* 2003; Sagili & Pankiw, 2007; James & Pitts-Singer, 2008; Abou-Shaara, 2014). The specific nutritional requirements of bees include 10 essential amino acids that are needed for growth of honey bees (DeGroot, 1953; Ruedenauer *et al*., 2016). Deficiencies in any of these amino acids in the pollen collected by foraging bees can result in serious consequences; e.g. bees fed a diet of dandelion pollen, which is low in arginine, were shown to be unable to rear brood until their diet was supplemented (Vanderplanck *et al*., 2014).

If a lack of pollen diversity is contributing to declining bee health due to nutritional imbalances and stress, as suggested by several authors (Cnaani *et al*., 2006; Campbell, 2013; Baloglu & Gurel, 2015), the introduction of a range of sources into an agricultural landscape (e.g. pollinator strips) could help to ameliorate any nutritional deficiencies. In designing these both the nutritional quality and temporal constancy of the floral resource needs to be taken into account. Any recommendations on plant pollen sources should also consider the effects of larval or adult nutrition on the susceptibility of these stages to pesticides. As indicated above, pollen ingestion has been shown to reduce honeybee susceptibility to pesticides and pathogens.

The majority of pollinator based studies focus on honeybees, but recent studies have concluded that wild pollinators (non-*Apis* bee species) are more effective than honeybees in many crops worldwide (Winfree *et al.,* 2011; Garibaldi *et al.,* 2013). Studies of nutrition in other invertebrate pollinators are urgently required.

1.2 Pollinators and their importance

Pollination is a key ecosystem service; foods such as fruits and vegetables are vital for balanced diets, make up 77% of world crop varieties and require insects for full or partial pollination (Klein *et al.,* 2007). As the human population continues to expand the demand for stable food sources is greater than ever, with a predicted crop production increase of 70% required by 2050 (Royal Society, 2009). Efficient pollination services are essential for obtaining this target, in order to maximise yield and quality (Klein *et al*., 2007; Ollerton *et al*., 2012).

The Apiformes (bees) are widely considered the most effective pollinating insects for food crops (Tepedino, 1979). Co-evolution of Apiformes with Angiosperms (flowering plants) has resulted in physiological, morphological and behavioural characteristics aiding in pollen collection and transfer (Vaknin *et al.,* 2000). Furthermore, nearly all Apiformes require pollen for larval provision driving high rates of flower visitation close to the nesting site (Falk & Lewington, 2015).

Many Apiformes, especially *Apis, Bombus* and specialist foraging solitary bees are known to maintain high levels of floral consistency and this high selectivity for particular pollens at a spatial and temporal scale in turn can result in effective crop pollination in both agricultural and horticultural environments (Grant, 1950*;* Rasheed & Harder, 1997).

Wild bee species play an important role in pollination being responsible for 35% of crop pollination globally (Garibaldi *et al*., 2013). Wild bees further represent an important group of UK insect pollinators (Carreck & Williams, 1998). Within the UK pollination is valued at £430 million per annum (Vanbergen *et al.*, 2014). Klein *et al.* (2007) further concluded at the European level that yields of 84% of crop varieties are enhanced to some degree via animal pollination services. Although honeybees and other managed pollinators have received much attention in the scientific literature and public debate on pollinator decline, it has been suggested that wild pollinators are responsible for the majority of pollination in most cropping systems (Garibaldi *et al.,* 2013). In total the European based pollination service has been valued at an estimated £17 billion (Potts *et al*., 2010). Thus making it a key service and one of great importance.

1.3 Pollinator decline

Widespread decline in wild pollinators has been reported over the last 60 years, linked to factors driven by agricultural intensification (Potts *et al*., 2010). This decline has in turn resulted in pollination deficits in some commercial cropping systems (Ollerton *et al*., 2014) and commercial managed pollinators are often utilised to address this deficit, most commonly honeybees (*Apis mellifera)* (Aizen & Harder, 2009). The use of *Apis mellifera* is, however, meeting problems associated with a variety of issues, including loss of suitable forage and off crop habitats, diseases and use of pesticides (Aizen & Harder, 2009; Neumann & Carreck, 2010; Potts *et*

al., 2010). In addition the widespread use of *A. mellifera* has begun to be questioned as the use of large numbers of bees through introduction of managed hives can interact negatively with wild bee populations raising concerns relating to their conservation (Mallinger *et al.*, 2017). A clear consensus on this issue has yet to be reached, however. For example, Garibaldi *et al.* (2013) reports that fruit set in crop systems worldwide is enhanced by a diverse range of wild bees regardless of *A. mellifera* abundance, which may suggest that supplementation is a viable option. Kleijn *et al.* (2015) suggests that a few common generalist species of wild bees dominate crop pollination services.

Recognition of the importance of solitary bees and bumblebees as pollinators has increased in recent years. Widespread decline is also reported in these groups, but detailed population data to track this decline is lacking because of the absence of coordinated recording schemes such as those used for managed *A. mellifera* . This limits the ability to define the extent of decline accurately and to identify causes (Potts *et al*., 2010). Although it has been suggested that decline trends are driven by only a few specific species (Ghazoul, 2005), more recent assessments indicate that widespread decline has occurred though a diverse range of pollinator assemblages, including solitary bees, lepidopteran and dipteran pollinators (Potts *et al*., 2010).

A range of mechanisms underpin this decline but habitat change, resulting from agricultural intensification is thought to be a key driver (Heard *et al*., 2007). With 70% of land use in Britain is currently dedicated to agriculture (Ollerton *et al.*, 2014), this in turn has not only resulted in loss of floristic diversity but also the degradation of nesting sites. Other factors such as climate change, disease and parasites and pressures from pesticide use that have been associated with declines of other pollinator species have also been implicated for solitary bees and bumblebees (Potts *et al*., 2010; Brown *et al.*, 2016).

With regard to pesticides, there has been a particular focus on neonicotinoid insecticides in recent years, resulting in a preliminary two year moratorium on these insecticides in Europe (EC, 2013) and a total ban as of 2018 (EC, 2018a, b, c). It is important to stress however that these factors often work in combination with each other, often making it difficult to identify key stressors and mitigate for them (Vanbergen, 2013; Goulson *et al*., 2015). There has been limited research on one potential factor, the impact of nutrition and quality of forage of bee populations, with the few studies available focussed mainly on honey bees and some bumblebee studies. Furthermore the reduction in floral resource availability resulting from

habitat change is a major issue for bee populations with pollen the primary nutrition source for developing bee larvae. Landscape effects on bee diversity are therefore likely to be mainly driven by limited availability of pollen resources of the necessary quality (Holzschuh *et al.*, 2007; Kennedy *et al.,* 2013; Baude *et al*., 2016). Targeted, cost effective conservation measures are required for pollinators to address drivers and mitigate their impact (Carvell *et al*., 2011).

Currently under the Common Agricultural Policy (CAP) there are a range of options as we move from the previous entry level (ELS) and higher level (HLS) agri-environment schemes in the UK. The ELS/HLS will run to 2024 on 10 year agreements made in 2014, but since 2015 agreements have been implemented under the new Countryside Stewardship Scheme. Stewardship options within agri-environment schemes are available to producers to mitigate some of the direct threats faced by pollinators (Brown *et al*., 2016). Such schemes commonly attempt to address the issue of reduced floral resource availability with the introduction of floristically enriched off crop habitats (Wratten *et al*., 2012). These measures however, have largely been considered incomplete; failing to address key factors such as nest site availability or take into account their position both spatially and temporally. Furthermore, many of the plant species chosen for mixes do not provide early season forage, and focus more on *Bombus* species and their need for late season nectar forage rather than the requirements of other wild bees (Mallinger *et al.,* 2017). Greater care is required to improving bee nutrition, increasing abundance of key stones species at suitable temporal and spatial scales, thus tailoring schemes to their respective environments and cropping systems (Carreck & Williams, 2002).

1.4 *Osmia bicornis* – importance as a pollinator

The red mason bee; *Osmia bicornis* (Linnaeus, 1758), previously *Osmia rufa*; is a small solitary bee of the family Megachilidae (James & Pitts-Singer, 2008). The genus *Osmia* comprises over 300 species residing mainly in Holarctic and temperate areas (Raw, 1972; O'Toole & Raw, 1999; Michener, 2007). The majority of *Osmia* species nest in either pre-established or self-excavated cavities, in which females build a series of cells each provisioned with pollen and separated with leaf or mud walls (Raw, 1972; Else, 2012). *Osmia bicornis* belongs to the less common group, that use mud to build cell walls (Raw, 1972; Else, 2012), one of only two such *Osmia* species to do so in the UK (Else, 2012).

Osmia species are polylectic foragers which emerge and forage earlier in the year than many other pollinators (Wood *et al.,* 2016). Consequently there is considerable interest in the use of *Osmia* species as pollinators of spring crops (Biliński & Teper, 2004; Gruber *et al.,* 2011). In Japan *Osmia cornifrons* was developed for orchard pollination in the 1960's and it is currently used in 70% of the country's apple orchards (Maeta & Kitamura, 1974; Maeta *et al.,* 1990). The success of *O. cornifrons* as an orchard pollinator has resulted in it being adopted more widely and it now utilised in mainland Asia (Xu *et al.,* 1995), and the USA (Batra, 1979, 1998). Issues with introducing non-native pollinators to new areas, however, have also become more widely recognised in recent years (Atsumura *et al.,* 2004), resulting in a native North American species *O. lignaria* now being more commonly used in orchard pollination in the USA (Torchio, 1976, 1985, 2003; Bosch & Kemp, 2001, 2002; Bosch *et al.,* 2006).

Similar developments have been observed in Europe where *O. bicornis* has been used since the 1970s as a pollinator for fruit trees and a range of other crops (Hansted *et al*., 2014; Sedivy & Dorn, 2014). *O. cornuta* was also developed for use in European orchards and has since been adopted for use in almond crops in California (Asensio, 1984; Torchio & Asensio, 1985; Bosch, 1994; Monzón *et al.,* 2004). *Osmia* species have also demonstrated potential scope for use in protected crops (Dogterom, 1999; Abel *et al.,* 2003).

Limitations in assessment methods have made it difficult to accurately assess *Osmia* flight distances. With original estimates for forage range constricted to 600m of the nesting site (Gathman, 1998), ranges as high as 900m have been recorded by Gathmann & Tscharntke (2002), although this has been debated (Bosch & Vicens, 2002; Greenleaf *et al*., 2007). This range, however, does greatly exceed that of many other solitary bee species. A range of factors such as proximity of floral resources or physical barriers in the landscape may influence such foraging behaviour (Garthman & Tscharntke, 2002; Gruber *et al*., 2011; Saunders & Luck, 2014).

1.5 *Osmia bicornis* - life history

Osmia bicornis is an univoltine species; completing a single brood development cycle per year (Raw, 1972; Else, 2012). As with all univoltine *Osmia* species, the life

history *O. bicornis* may be divided into six distinct periods: 1. Overwintering, 2. Emergence and mating, 3. Pre-nesting, 4. Nesting, 5. Development and 6. Pre-wintering (Leather *et al*., 1995; James & Pitts-Singer, 2008).

Osmia bicornis overwinters as fully formed adults (Raw, 1972; O'Toole & Raw, 1999; James & Pitts-Singer, 2008). This is a derived trait within the family of Megachilidae, with many other members such as the leaf cutter bees (genus: *Megachile*) overwintering as pre-pupae (Bosch *et al.*, 2001; James & Pitts-Singer, 2008). Adult overwintering facilitates a variable emergence date, allowing for early spring emergence when conditions are favourable (Biliński & Teper, 2004; James & Pitts-Singer, 2008; Radmacher & Strohm, 2010) and *O. bicornis* to utilise floral resources not accessible to other species (Gruber *et al.,* 2011; Jauker *et al.,* 2012).

1.5.1 Emergence and mating

When early spring temperatures exceed the developmental threshold, overwintering adults emerge from their cocoons and leave the natal nest (Raw, 1972; O'Toole & Raw, 1999; James & Pitts-Singer, 2008; Else, 2012). Males typically emerge 2-4 days prior to females (Raw, 1972; James & Pitts-Singer, 2008). Following emergence, males forage for and consume floral nectar and congregate near nest site entrances anticipating female emergence (Raw, 1972; James & Pitts-Singer, 2008). This behaviour is triggered by a low persistence sex pheromone released by female *O. bicornis* (Rosner, 1994). Timing of emergence is highly variable for both sexes; with emergence dependant on both overwintering temperatures and spring incubation temperatures, prior to eclosion (Radmacher & Strohm, 2010; Jauker *et al.,* 2012). Studies of other *O. lignaria* and *O. cornifrons* have shown a reduction in the time before emergence with higher ambient temperatures during the incubation period (Bosch & Kemp, 2001, 2004; Maeta *et al.,* 2006). One can assume similar trends apply to *O. bicornis* although further confirmatory studies are required.

1.5.2 Pre-nesting behaviour

At the point of emergence female oocytes are not fully developed (James & Pitts-Singer, 2008), thus a brief pre-nesting period after mating is required to allow females to reach ovarian maturity (Monzón, 1998; Sgolastra, 2007). Females often

disperse from the natal nest site following mating, seeking sheltered areas such as loose bark (James & Pitts-Singer, 2008). The duration of pre-nesting stage is approximately 2-5 days in *O. bicornis*; however in other *Osmia* species this period can be increased if adverse weather conditions occur (Maeta, 1978; Bosch & Kemp, 2001; Bosch & Vicens, 2006).

Following oocyte development females search for suitable nest sites; such as degraded buildings, large areas of stacked wood or broken *Umbellifera* stems (Else, 2012). Nests are built in blind cavities with a single entrance, and consist of a linear series of individual cells, each of which will contain a single insect. Optimal nest sites occur in shady raised south facing areas, protected from strong winds, and with a nearby source of damp soil for the construction of nest cell walls and sufficient floral resources for foraging (Raw, 1972). *Osmia bicornis* is capable of excavating a suitable nest site in mud or loose mortar in buildings leading to the common name of the red mason bee. Eventually females will reduce their search to a particular area before commencing nest construction (Biliński & Teper, 2004; Gruber *et al.,* 2011).

1.5.3 Nesting

Following nest site selection *O. bicornis* females start collecting mud to form the cell walls (O'Toole & Raw, 1999; Biliński & Teper, 2004; James & Pitts-Singer, 2008; Else, 2012). It is not uncommon for large numbers of individuals to congregate on suitable loose soil banks at this time (Else, 2012). Mud is carried by the female back to the nest site in the mandibles, it is then packed into position using a pair of horn like extensions from the clypeus plate (Raw, 1972; Else, 2012). In the UK only *O. bicornis* and *O. lignaria* utilise mud for nest construction; other *Osmia* species rely on leaf pulp (Else, 2012).

Nest construction begins with a basal partition at the innermost end of the nest cavity. After this partition is completed, the female will begin to provision the cell with pollen. Despite a maximum forage range between 600-900m recorded (Gathman, 1998; Gathmann & Tscharntke, 2002), females usually forage for suitable pollen resources within a few hundred meters of the nest site although longer foraging flights have been reported (Raw, 1972; O'Toole & Raw, 1999; Else, 2012). Approximately 15-20 foraging flights are required to collect sufficient pollen for a

single cell (Raw, 1972). Pollen collected in this period is often of varying composition and there is some evidence that poor quality pollens are supplemented by mixing with higher quality pollen provisions (Bosch & Kemp, 2001; Jauker *et al.,* 2012; Eckhardt *et al.*, 2014). Pollen is mixed with a small amount of nectar to bind it together; the pollen clump is then formed into a rough ball referred to as a pollen loaf (James & Pitts-Singer, 2008). A single egg is laid onto the surface of the pollen loaf, before females seal the cell with another mud partition (Raw, 1972; O'Toole & Raw, 1999; James & Pitts-Singer, 2008; Else, 2012). This process is repeated an average of 7 times for a single cavity nest site (Raw, 1972), after which the nest is finally sealed with a thick mud plug (Raw, 1972; O'Toole & Raw, 1999; James & Pitts-Singer, 2008; Else, 2012). Sides of the nest tunnels used by *O. bicornis* are not lined with mud (Raw, 1972). Rather individual cells are cyclindrical in shape with mud partitions rough and convex on the inner side and smooth and concave on the outer (Raw, 1972).

Females will continue to found nest sites thoughout the flight period. Later founded nests often contain a higher proportion of males, with final nest sites often containing exclusively male progeny (Raw, 1972; Maeta, 1978; Tepedino & Torchio, 1982; Sugiura & Maeta, 1989; Biliński & Teper, 2004; Bosch & Vicens, 2006; Else, 2012). Male progeny are considered to require less nest cell resource than females, partially due to the haploid diploid nature of hymenopera (Radmacher & Strohm, 2010; Seidelmann, 2014). The flight period lasts approximately two months, during which females may construct 4-7 nests, although number of cells within each nest will decrease as nest number from individual females increases (Raw, 1972). However, this may be a reflection of the size of cavities utilised in the study, or the female's reproductive capacity. Following the end of the flight period females will succomb to senecense (Raw, 1972).

1.5.4 Egg and larval development

Eggs of *O. bicornis* hatch approximately one week after cell completion, depending on environmental variables (Biliński & Teper, 2004; James & Pitts-Singer, 2008; Seidelmann, 2014). Larvae feed on the pollen loaf provisions and similar to other hymenopterans, *Osmia* are haplo-diploid allowing for the female to select the sex of offspring (Wasielewski *et al.,* 2013; Seidelmann, 2014).

Osmia bicornis develop though five larval instars (Raw, 1972; Torchio, 1989; Else, 2012). Once the pollen loaf is consumed and the fifth instar larvae has completed defecation, and thus have cleared their gut, larva will begin the initial stages of pupation at 31 ± 1 days after egg hatching (Raw, 1972; James & Pitts-Singer, 2008). Over a period of a few days the larvae begin to spin cocoons (Bosch & Kemp, 2000; Maeta *et al*., 2006). Cocoons are constructed from multiple layers of silken secretions originating from the salivary glands (Torchio, 1989).

1.6 Bombus terrestris - importance as a pollinator

The buff-tail bumble bee (*Bombus terrestris audax* (L.)) is one of the most common and recognisable bee species within the UK (Sladen, 1912; Goulson, 2010). As with all bumblebee (*Bombus)* species, *B. terrestris* is a member of the family Apidae (Edwards & Jenner, 2009; Goulson, 2010). The family contains the most advanced social bees outside of *Apis* bee species and the majority of its members are eusocial insects (Benton, 2006; Holland, 2013) forming colonies containing a non-reproductive worker class. The majority of bumblebees, differ from the *Apis* species (honey bees) and *Meliponinae* (tropical stingless bee) species by having a non-continuous colony (Benton, 2006; Edwards & Jenner, 2009; Goulson, 2010). Bumblebee colonies undergo an annual life cycle, with mated Queens founding new nest or colony sites at the start of each season (Sladen, 1912; Edwards & Jenner, 2009; Goulson, 2010). As with all Hymenopterans, *Bombus terrestris* have haplodiploid reproductive strategies (Free & Butler, 1959), in which males are haploid (produced from unfertilised eggs containing a single set of chromosomes) and females are diploid and develop from a fertilised egg. Queens are able to determine the sex of their offspring (Free & Butler, 1959; Benton, 2006; Goulson, 2010).

Bombus terrestris forms an ideal model species for research. In addition to ease of recognition, it is widely used as a pollinator throughout much of Europe, where commercially produced colonies are introduced to promote fruit set in crops such as tomatoes and soft fruit crops. It has also established in North America and New Zealand (Estoup *et al.,* 1996; Atsumura *et al.,* 2004; Ings *et al.,* 2005; Coppée, 2010). Although the species is an important and effective crop pollinator (Velthuis, 2002; Goulson, 2010), under some circumstances it has been considered economically damaging due to advancing invasion fronts of introduced non-native

sub species. Consequently *B. terrestris* is a highly studied organism including molecular studies and studies of physiology, biology and ecology (Bourke, 1999; Baer & Schmid-Hempel, 2003a, b; Macdonald & Nisbet, 2006). Furthermore much molecular work has also been conducted (Estoup *et al.,* 1996; Pereboom *et al.,* 2005; Coppée, 2010), with the genome of *B. terrestris* currently undergoing sequencing (Bumble Bee Genome Project, 2014). This rich basis of knowledge makes it an ideal model species for research.

1.6.1 *Bombus terrestris* - life history

The life cycle of *B. terrestris audax* reflects the standard annual life cycle observed in many temperate *Bombus* species (Sladen, 1912; Goulson, 2010). Pre-mated queen bees overwinter in burrows and abandoned nest sites surviving on fat reserves (Prys-Jones & Corbet, 1987; Goulson, 2010); hibernation sites are commonly found in close proximity to trees (Sladen, 1912), which may be a factor necessary for consideration when conservation methods are proposed. During the initial weeks of overwintering queens remain reasonably active and are able to leave the hibernation chamber in search of a more suitable site if disturbed (Sladen, 1912), but subsequently lower temperatures result in a period of inactivity. Queens emerge from hibernation in spring, most commonly in early in February to March (Sladen, 1912; Prys-Jones & Corbet, 1987; Macdonald & Nisbet, 2006). This pattern is however changing, possibly as a result of climate change (Goulson *et al.,* 2011), with shifts towards earlier emergence reported with increased temperature leading to a potential phenological divergence between *B. terrestris* and its early forage species.

Following emergence the queen's search for a suitable nest site. *B. terrestris* commonly nests in underground burrows; often utilising pre-existing holes such as abandoned rodents' nests (Prys-Jones & Corbet, 1987). Such abandoned burrows often contain adequate supplies of loose plant material accompanied by hair or feathers, which are used in the construction of the single entrance and central nest chamber (Sladen, 1912; Prys-Jones & Corbet, 1987; Macdonald & Nisbet, 2006; Goulson, 2010). In the UK *B. terrestris* shows a strong preference for north facing abandoned burrows located in loose soil (Alford, 1975).

Initial foraging flights are undertaken to collect pollen, which is formed into a ball onto which the queen lays the first batch of eight eggs (Ribeiro, 1994; Goulson, 2010). The brood and pollen resource are then covered with a layer of wax mixed with small amounts of pollen; wax is secreted between abdominal segments on the queen's ventral surface (Sladen, 1912; Goulson, 2010). A small wax cell or pot is created near to the entrance to the brood chamber, and is filled with regurgitated nectar collected by the queen which is used as an energy source during initial nest construction, thus reducing risk by undertaking fewer foraging flights during this critical period (Sladen, 1912; Benton, 2006).

The queen incubates the brood by touching the cell surface with the ventral surface of her abdomen, thus maintaining an egg temperature of 30-32°C (Heinrich, 1975, 1976; Heinrich & Heinrich, 1983; Goulson, 2010). Eggs hatch in approximately 4 days (Sladen, 1912; Free & Butler, 1959; Prys-Jones & Corbet, 1987; Mapalad *et al.*, 2008), after which the larvae will feed on the pollen provisions.

During the early larval stage individuals form a brood clump (Benton, 2006; Macdonald & Nisbet, 2006) and the queen divides her time between incubation and foraging to maintain pollen and nectar stores (Prys-Jones & Corbet, 1987; Macdonald & Nisbet, 2006). Consequently the early stage of colony development is reliant on flowering plant abundance in close proximity to the nest (Macdonald & Nisbet, 2006; Goulson *et al.,* 2011; Dicks *et al.,* 2013; González-Varo *et al.,* 2013).

Bombus species may be sub divided into two distinct groups relating to the behaviour associated with brood provisioning (Macdonald & Nisbet, 2006; Goulson, 2010). Pocket-makers include the majority of long- tongued species (Free & Butler, 1959; Prys-Jones & Corbet, 1987; Goulson, 2010). The brood clump of pocket-makers is maintained in a single cell and are fed on a bed of pollen forced at intervals though holes on the side of the brood clump; later stages of development are fed by regurgitation from the queen (Goulson, 2010). *Bombus terrestris* belongs to the secondary sub-division referred to as the pollen-storers and are typical of short- tongued *Bombus* species (Macdonald & Nisbet, 2006; Goulson, 2010). In these short-tongued bees the brood clump breaks up, with individual larvae constructing separate wax cells in which they will continue to develop (Couvillon & Dornhaus, 2009). Notably, short-tongued species respond better than long-tongued species to commercial production processes, consequently the majority of studies of ecology and life histories of bumblebees is based on commercially available short-tongued species, such as *B. terrestris* (Velthuis, 2002; Goulson, 2010).

Although poikilothermic, bumblebees are able to exert greater control over body temperature compared to many other insect species (Heinrich, 1975; Heinrich & Heinrich, 1983). Development time of larvae is however, highly dependent on temperature, in addition to the availability of food resources (Ribeiro, 1994; Couvillon & Dornhaus, 2009). Temperature variation is significantly mitigated in established larger colonies in which workers are able to thermo-regulate the nest temperature by either fanning their wings to cool the nest, or by disconnecting wing couplings and contracting flight muscles to increase body temperature (Mapalad *et al.,* 2008; Goulson, 2010). This sophisticated thermoregulation system limits temperature variation to less than 2.5°C in developed nests (Mapalad *et al.,* 2008; Goulson, 2010).

Bombus terrestris larvae develop through four instars, until at the final moult (approx. 10-14 days), a silken cocoon is spun in which they pupate (Edwards & Jenner, 2009; Holland, 2013). The queen typically continues to lay more eggs and forages for pollen reserves (Sladen, 1912; Prys-Jones & Corbet, 1987; Goulson, 2010). Adult workers emerge approximately 14 days after pupation (Ribeiro, 1994; Couvillon & Dornhaus, 2009). Former cocoons of hatched workers may be used to house further pollen and nectar supplies (Sladen, 1912; Macdonald & Nisbet, 2006). The life span of workers is variable but they are known to survive for up to two months before senescence (Smeets & Duchateau, 2003).

After the initial founding stages, nest development increases exponentially (Goulson, 2010). In large colonies, production will switch from workers to new queens and drones (males) (Pereboom *et al.,* 2003, 2005; Moret & Schmid-Hempel, 2004; Goulson, 2010). In *B. terrestris* successful nests can comprise of up to 350 workers (Benton, 2006; Holland, 2013). The transition to the reproductive stage of the colony is typically observed between April and August (Sladen, 1912; Edwards & Jenner, 2009; Shykoff & Muller, 2009), and is triggered by worker density, following which the queen ceases to suppress worker ovarian development via the use of a pheromone (Pereboom *et al.,* 2003, 2005; Goulson, 2010; Evans & Raine, 2014). This switch to reproductive production seems to be synchronised in many other *Bombus* species and colonies within similar geographical areas (Shykoff & Muller, 2009; Goulson, 2010). Thus late founded nests will have shorter optimal forage duration before the switch to the reproductive stage. This in turn may result in only drones being produced due to resource limitation (Velthuis, 2002; Goulson, 2010). During this period workers may also lay eggs; all such worker laid broods are

haploid males but overall significantly fewer drones originate from workers than queens (Bourke, 1999; Velthuis, 2002).

Young queens will spend much time in the nest consuming large amounts of pollen and nectar provisions in order to build fat reserves for the winter diapause (Ribeiro, 1994; Pereboom *et al.*, 2003; Benton, 2006; Goulson, 2010), after which they leave the nest and locate a mate. Following the departure of reproductives, workers cease to maintain the nest resulting in colony decline through a combination of senescence, parasitism and disease (Alford, 1975; Benton, 2006; Edwards & Jenner, 2009). Males leave the colony within a few days of adult emergence (Shykoff & Muller, 2009; Goulson, 2010), and patrol the local area feeding on flower nectar in order to sustain flight (Free & Butler, 1959; Goulson, 2010). Few studies have demonstrated, however, the levels of attraction of females to male patrolled sites and pheromones under natural conditions. Thus further in-depth studies are required to investigate *Bombus* mating behaviour in the wild. Following mating, males die and mated females leave the colony permanently to search for a suitable nest site in which to overwinter.

1.7 Use of managed non-*Apis* bees in crop pollination

1.7.1 Managed *Bombus* in crop pollination

Increased understanding of nutritional requirements and mimicking of hibernation conditions allowed for the production of *Bombus* under laboratory conditions for pollen storing species (i.e. *Bombus terrestris, Bombus lucorum, Bombus hypnorum*) (Velthuis, 2002; Velthuis & Van Doorn, 2006). This has in turn allowed for the expansion of commercial production with mass produced *B. terrestris* and *B. terrestris audax* colonies available for purchase for pollination services across Europe and the UK respectively (Velthuis & Van Doorn, 2006). Much of the initial success in this area related to *Bombus* use for tomato pollination within glass house systems, resulting in increased quality (Velthuis & Cobb, 1990; Velthuis, 2002). This was followed by use in a variety of other glasshouse crops such as sweet peppers (Velthuis & Cobb, 1990).

Issues have arisen however, with commercially produced colonies producing males in larger numbers than young queens (Duchateau & Velthuis, 1988; Bourke, 1997).

This reduced queen production leads to potential problems with in-breeding (Duchateau *et al.,* 1994). Furthermore the large scale of movement of commercial colonies may have resulted in the introduction and spread of parasites and diseases affecting native bee populations (Goulson, 2010; Goulson *et al.,* 2011; Goulson *et al.,* 2015). Consequently alternative pollinator sources or increasing wild populations of pollinators should be seen as preferable were possible and cost effective.

1.7.2 Use of managed solitary bees in crop pollination.

Multiple successful commercial production systems and pollinator supplementation systems have been developed that utilise members of the genus *Osmia*. Such systems have focussed on fruits crops. In Japan successful use of *Osmia cornifrons* began in the 1960s, with 80% of apple acreage relying on *Osmia cornifrons for* pollination since 1996 (Sekita & Yamada, 1993; Sekita, 2000; Bosch & Kemp, 2002). Further success of *O. cornifrons* in commercial orchard settings was recorded following its introduction to China and the USA (Batra, 1979). The USA however, also utilises the native *Osmia lignaria* for orchard pollination (Morales-Ramos *et al.,* 2013) and in California the European *Osmia cornuta* was introduced for almond pollination (Torchio & Asensio, 1985).

As previously mentioned, interest in *O. bicornis* has increased in recent years, with use expanding across Europe and its continued development by commercial enterprises within the UK (Teper & Bilinski, 2009; Gruber *et al.,* 2011). Although *O. bicornis* is utilised as a managed commercial pollinator for apple and other top fruit and soft fruit production within Europe and parts of the UK (Wilkaniec & Radajewska, 1996; Bilinski & Teper, 2004; Teper & Bilinski, 2009; Gruber *et al.,* 2011), *ex-situ* commercial production of bees in the UK is largely on a small scale. Development of larger scale commercial production processes would benefit from further investigation of the physiology, biology and behaviour of *O. bicornis*, identification of key pollen species and more precise information of nutritional requirements (Bosch & Kemp, 2002).

Aspects of the ecology, behaviour and biology of *Osmia bicornis* contribute its potential as a successful pollinator. The early spring emergence of *O. bicornis* often occurs during periods of scarce floral resources and sub-optimum weather

conditions for managed *Api*s bees (Kirk & Howes, 2012), offering potential for the pollination of early season crops. Timing of emergence is, however, strongly dependant on latitude (Bosch *et al.,* 2008), with later emergence in more northern latitudes. Consequently this may lead to potential phenological mismatch with some top fruit crops in northern regions, reducing pollination efficacy. *O. bicornis* does however, have lower temperature thresholds for flight compared to other managed pollinator species such as *A. mellifera* (Guler & Dikmen, 2013). This in turn increases the number of hours foraging by *Osmia* in the early season. The forage range of *O. bicornis*, however, is greatly reduced compared to that of *A. mellifera* and *Bombus* spp. (Gathmann & Tscharntke, 2002; Zurbuchen *et al*., 2010). The maximum forage distance of 900m recorded by Gathmann & Tscharntke (2002) shortens when there is increased floral resource available closer to nesting sites. This behaviour results in high flower visitation frequency close to nests. Fewer individuals per unit land area are therefore required to pollinate flowers when compared to *A. mellifera*; allowing for targeted localised bee release in the chosen crop (Gruber *et al.,* 2011).

The management of *O. bicornis* overwintering is one of the most important challenges in the commercial rearing process, with careful attention required to ensure the viability of populations in the following spring (Biliński & Teper, 2004). Diapause may be artificially manipulated to induce either earlier emergence (by increasing temperatures) or to elongate diapause for later season crops, thus maximising pollination services. There is however, limited information supporting the husbandry approach required for this process. Studies by Dmochowska *et al.* (2012) question whether increasing diapause length results in reduced fitness and earlier senescence due to oxidative stress and consumption of fat reserves. Conversely decreasing the diapause period may raise metabolic rate and thus nutrient use potentially increasing mortality and rate of failed emergence, or decreasing body weight of adults emerging from overwintering (Fliszkewicz *et al.,* 2012).

1.7.3 Pollination of early season crops; sweet cherry, *Prunus avium* (L.)

Sweet cherry *Prunus avium* (L.), is an early flowering top fruit crop, in which the efficacy of *O. bicornis* as an early season pollinator is currently being debated. In the UK the flowering period of commercial *P. avium* begins in late March or early April, and as the majority of commercial cultivars are self-sterile there is a high level of

reliance on entomophilous pollination services. In addition, as rapid ovule degeneration occurs during flowering the majority of self-fertile varieties that are grown in commercial orchards are thought to benefit from supplementary pollination by insects (Lane, 1979; Delaplane *et al.,* 2000). Early pollination has been shown to influence fruit set, and it has been suggested that flowers pollinated later in the blossom period may show a reduction in eventual fruit quality (Mayer *et al*., 1987; Ughini & Roversi, 1993).

With flowering occurring early in the UK, there is limited diversity of pollinating insects offering this ecosystem service. Diptera provide the majority of *P. avium* flower visitations, but early emerging *Bombus* spp and solitary bees such as *Andrena spp.* and *Osmia spp.* are also important (Kirk & Howes, 2012; Guler & Dikmen, 2013). Managed *Apis mellifera* are frequently used in orchard systems, but are less effective during periods of adverse weather conditions, with fewer foraging flights undertaken during rainfall and requiring a minimum flight temperatures of 12°C (Guler & Dikmen, 2013). Furthermore a study by Holzschuh *et al.,* (2012) in Germany show wild bee presence to increase fruit set and yield despite high *A. mellifera* abundance.

Although *O. bicornis* has been used successfully in other systems and is considered an efficient pollinator, potential issues are raised by Bilinski & Teper (2004). They found that only one third of pollen collected in an apple and cherry orchard system came from orchard plants, with preferences for *Salix spp.* and *Acer spp.* observed. Based on such evidence it has been suggested that necessary release rates of *O. bicornis* in commercial systems would be too high to be commercially viable (Hansted *et al.,* 2014). Increased visitation rate, and improved pollination efficiency and fruit quality (the latter associated with increased pollen deposition) may result however in commercial advantage being gained from *O. bicornis* in situations where alternative pollinators are scarce (Coudrain *et al.,* 2015).

In *B. terrestris* despite high levels of selectivity, increased diversity of floral resources is linked to increased colony fitness and size (Baloglu & Gurel, 2015). Low floral diversity during the flowering period cherry may, amongst other factors, reduce its potential impact on yield or quality.

1.8 Nutrition

Floral resources are recognised as a significant factor governing pollinator dynamics (Sagili & Pankiw, 2007; Potts *et al.,* 2010; Eckhardt *et al.,* 2014), and are especially important for bee species (Hymenoptera: Apidae), in which both adults and larvae are solely dependent on flowers for nutrition (Michener, 2007; Goulson, 2010). Pollen is required for development of ovaries in females and provisioning of larval offspring (Ribeiro, 1994; Biliński & Teper, 2004; Sagili & Pankiw, 2007; Goulson, 2010; Jauker *et al.,* 2012; Eckhardt *et al.,* 2014). In addition, pollen quality has been linked to antimicrobial immune responses of bees (Moret & Schmid-Hempel, 2004; Mapalad *et al.,* 2008), and the ability to detoxify pesticides (Alaux *et al.*, 2010). This may be partly because pollen consumption can increase the expression of particular genes, with p-coumaric acid a key component of pollen (Mao *et al*., 2013). Nectar is utilised as a carbohydrate fuel source in order to sustain flight and foraging trips (Mapalad *et al.,* 2008; Evans & Raine, 2014).

Despite the significance of pollen in behaviour and development for all taxonomic groups, the majority of research has been aimed at the improvement of diets in *Apis* bees with a focus on supplementary feeding (Cook *et al.,* 2003; Sagili & Pankiw, 2007; James & Pitts-Singer, 2008; Abou-Shaara, 2014). The work often includes limited chemical analysis of pollen, with experiments focusing on the role of crude protein and lipid content (Sagili & Pankiw, 2007; Eckhardt *et al.,* 2014). Very limited data is available for both bumblebees and solitary bees, with more in-depth analysis of pollen content and its potential effect on pesticide resistance, larval development and behaviour required.

Nectar provides insect pollinators with carbohydrates, and pollen provides protein, amino acids, fatty acids, lipids, vitamins and minerals. If a lack of pollen diversity is contributing to declining bee health due to nutritional imbalances and stress, as suggested by several authors, the introduction of a range of off-crop sources into an agricultural landscape (e.g. pollinator strips) could help to ameliorate any nutritional deficiencies (Williams & Kremen, 2007; Carvell *et al*., 2011). The nutritional quality and temporal constancy of flower species need to be taken into account in the design of these off-crop resources. Any recommendations on plant pollen sources should also consider the effects of larval or adult nutrition on their susceptibility to pesticide.

The focus of research on four closely related species of honeybee in the genus *Apis*, may limit its application to other invertebrate pollinators. Recent studies have concluded that wild pollinators (non-*Apis* bee species) are more effective than honey bees for crop production (Winfree *et al.,* 2011), resulting in many companies moving to supply bumblebee species such as *B. terrestris* and solitary bee species such as the red mason bee *O. bicornis* for pollination (Gruber *et al.,* 2011).

Nutrition is a key component in population dynamics of both *Osmia* and *Bombus*; linking directly to floral resource availability (Strohm *et al*., 2002). *Osmia* fitness, as measured by nest cell production, varies with floral resource availability in combination with other abiotic factors (Fliszkiewicz *et al*., 2015). Thus work in this field is beneficial for maximising management options and informing off-crop habitat choices.

Both total protein content and at least ten individual essential amino acids have been correlated with individual bee or colony performance, with *Bombus* shown to triple forage attempts when crude protein content was artificially doubled (Kitaoka & Nieh, 2009). This trend is not as apparent in honeybees, which select highly abundant pollen and nectar resources, displaying less selective foraging behaviour (Leonhardt & Blüthgen, 2012; Brunet *et al*., 2015). The mechanisms underpinning *Bombus* selective behaviour are however, currently not understood.

1.8.1 *Osmia* nutrition

Many bee species are expected to preferentially select pollen sources native to the biome they inhabit in order to maximise survival and population growth (Williams & Kremen, 2007). In addition pollen selection throughout the foraging season is also correlated with both distance between the nest site and location of preferred plant species and their density (Williams & Tepedino, 2003). The characteristics used by adult *O. bicornis* when selecting plants to visit are only partially understood. Drivers may include the nutritional value of the pollen or levels of harmful secondary metabolites but appear to vary between species and groups. For example, a preference for *Ranunculus spp.* pollen in *Osmia* has been observed and associated with reduced larval mortality (Sedivy *et al.,* 2011), whereas larvae of other bee genera experience mortality arising from the toxic secondary metabolite biochemical ranunculin (Sedivy *et al*., 2012). Thus, pollen selectivity may contribute to niche

separation and avoidance of competition from other pollinators. Even within the *Osmia* genus, however, an elevated proportion of *Ranunculus spp.* pollens in larval pollen provisions results in mortality and pollen mixing is a viable strategy to mitigate such negative effects (Ekhart *et al*., 2014).

The reduction of pollinator populations at a landscape scale is thought to be strongly driven by floral resource reduction (Heard *et al*., 2007), and both pollen and nectar availability has been correlated with this decline (Baude *et al*., 2016). Increased floral diversity at the landscape level is often proposed as an important factor when addressing the decline, and although it is undoubtedly beneficial, selectivity in pollen foraging by bees should also be taken into account when designing man made habitats, such as pollinator headlands, that contribute to biodiversity uplift (M*ü*ller *et al.,* 2006; Walters, 2016). Such improved understanding of the requirements of wild bees, offers the opportunity to provide more targeted action in support of their populations. For example, diversity of wild pollinators has been shown to benefit when selected keystone floral species are added to non-targeted floral diversity (Saunders *et al.,* 2015). The approach is further enhanced when the temporal and spatial abundance of each species within the range of keystone floral resources is considered when designing recommended plant assemblages for use in agri-environment schemes intended to support more complex pollinator assemblages. Current schemes often fail to address these more complex issues (Wood *et al.,* 2015).

1.8.2 *Bombus* nutrition

Although bumblebees (*Bombus* spp.) utilise a wide diversity of flora for both nectar and pollen provision (Reynolds & Fenster, 2008), floral selection is again highly targeted (Harmon-Threatt *et al*., 2017). Selectivity and foraging behaviour has been shown to be influenced by a variety of pollen quality characteristics. The presence or absence of specific amino acid or lipids, and their relative proportions (ratio) in which they appear in pollen diet have been shown to be an important determinant of both fitness of individual larvae fed on the diet and thus the performance of the colony, together with vitamins and minerals (Moller, 1995; Robertson *et al.*, 1999; Willmer, 2011).

Recent studies have moved away from crude protein levels to focus more on specific amino acids, with pollen diversity collected by *Bombus* colonies considered to be a mitigation strategy facilitating the balancing of nutritional requirements by mixing those pollens high in specific amino acids with others containing lower levels (Moerman *et al.*, 2017). There is a need to conduct a range of nutritional studies using a variety of pollen mixes and analysis of their biochemical composition, in order to further develop our understanding in this area. This would allow for accurate linkage between *Bombus* nutritional components of the diets with specific physiological mechanisms leading to the observed colony level outcomes.

1.9 Neonicotinoid insecticides

1.9.1 Classification and usage

Neonicotinoids are neurotoxic compounds falling under the IRAC Group 4 classification (Tomlin, 2003). This IRAC group consists of nicotinic acetylcholine receptor (nAChRs) agonists which disrupt synaptic nerve impulses in invertebrates (Matsuda *et al*., 2001). Use of neonicotinoids for pest management in commercial agriculture and horticulture increased greatly over the last twenty years, partly due to their systemic activity which allowed pre-emptive seed treatment reducing the need in some crops for multiple repeat spray treatments during plant establishment (Noleppa & Hahn, 2013; Rundolf *et al.,* 2015). This trend however is seeing reversal, with a now total ban of neonicotinoid products in the EU following an extended moratorium (EC, 2013, 2018a, b, c).

Due to their systemic activity and limited potential for translocation in the soil, risk of exposure of non-target organism to this group of insecticides was originally viewed as minimal (EFSA, 2013a, b, c). In addition, they were effective when used against significant pest species for which resistance to other pesticides had been recorded (Elbert *et al*., 2008). Accordingly they were adopted as components of integrated pest management strategies developed for major crops globally that targeted a wide range of pest guilds and species (Tomizawa & Casida, 2003; Walters, 2013; Walters, 2016).

Initial neonicotinoid products contained the active ingredient imidacloprid, first registered in 1985 (Thany, 2010) as part of the chloronicotinoid neonicotinoids

(imidacloprid). This family of neonicotinoids expanded with the introduction of thiamethoxam and clothianidin in the early 2000's (Tomlin, 2003). Since their introduction, and up to the recent moratorium in 2013, thiamethoxam and clothianidin have increased in popularity overtaking the usage of imidacloprid (Walters, 2013).

Co-incident with the introduction of the neonicotinoid seed treatments beekeepers in Europe began to report colony decline in hives placed within the foraging distance of neonicotinoid seed-treated fields (Rortais *et al*., 2005). These incidents were thought to result from dust generation during drilling of seed, with a major incident related to maize seed treated with clothianidin (Forster, 2009). Studies also reported low levels of neonicotinoids within both pollen and nectar of seed-treated crops (Krupke *et al*., 2012). Furthermore the UK production of oilseed rape (OSR) increased significantly from 2003 to 2013 to over 700,000 ha (DEFRA, 2014) raising concerns over the potential exposure of pollinators. Consequently the EU Commission proposed to issue a moratorium on the use of three commonly used neonicotinoids; namely imidacloprid, thiamethoxam and clothianidin, as seed treatments in flowering crops under the EU Regulation No 485/2013. The moratorium ran for two years from December 2013. Although the decision drew from a variety of studies and EFSA complied data, the ban was widely criticized for being based on limited evidence, and drawing heavily on laboratory based studies (Walters, 2013; Carreck & Ratnieks, 2014; Cutler *et al*., 2014; McGrath, 2014). This moratorium has since been replaced by total ban on use of neonicotinoids in Europe from 2018 (EC, 2018a, b, c). Different conclusions, however, have been arrived at elsewhere, with no ban in many countries (including Australia, Canada, USA, India and many Asian countries). There are continuing discussions in major South American countries, but no action at present. Therefore, there remains a need for studies using field realistic exposure levels, especially on non-*Apis* species.

1.9.2 Contamination of off-crop habitats

Presence of neonicotinoid pesticides in off-crop habitats was considered to be limited in a review by EFSA (EFSA, 2013a, b, c). Neonicotinoid pesticides show limited ability for lateral migration through the soil profile, but there is currently little published information on the ability for vertical movement through soil (EFSA, 2013a, b, c). Based on the evidence available it has however, been assumed that

field margins and off-crop habitats will contain little or no neonicotinoid contamination (EFSA, 2013a, b, c). A recent study (Botías *et al*., 2015) has reported neonicotinoid presence within pollen and nectar of plants in field margins, forming a potential exposure route. However, figures quoted in this work only include data from the minority of sites where neonicotinoids were detected. Furthermore samples were collected a maximum of 2m into the field margin whereas many buffer strips are required to be 3-6m deep depending on the stewardship option in place.

1.9.3 Sub-lethal effects

Concentrations of neonicotinoids identified in pollen and nectar from seed-treated crops in the field tend to fall in the range 1-10µg/L (EFSA, 2013a, b, c). Concentrations within this range do not generally induce mortality in bumblebees (Blacquiere *et al.,* 2012); but work has reported significant reductions in feeding and memory, foraging efficiency, pollen collection and ultimately brood production and colony survival (Yang *et al*., 2008; Mommaerts *et al*., 2010; Gill *et al*., 2012; Raine & Gill, 2014; Feltham *et al*., 2014). Impacts on honeybee colonies are reported at levels that are an order of magnitude higher than the “field realistic” range defined above (Faucon *et al.,* 2005; Dively *et al.*, 2015).

The majority of evidence linked to brood decline is based on the neurotoxic properties of the neonicotinoids at sub-lethal levels resulting in the impairment of cognitive functions and thus a decreased ability to navigate effectively (Decourtye & Devillers, 2010; Dively *et al*., 2015), but this research has mainly been conducted using honey bees as a model species. Studies have reported no significant effects of imidacloprid and clothianidin treated crops on the ability of *B. terrestris* to return to the hive following foraging (Schmuck *et al*., 2001; Cutler *et al.*, 2014). However, other studies have linked exposure to neonicotinoids to reductions in reproductive success in *Bombus* (Raine & Gill, 2014; Rundlöf *et al*., 2015).

Although studies have linked concentrations of imidacloprid in nectar and pollen as low as 1µg/L to falls in brood production in *Bombus* micro-colonies (Laycock *et al*., 2012) it is worth noting that other studies with thiamethoxam, such as Elston *et al*. (2014) have not replicated the effect. Great care needs to be taken in choice of both dose level and extrapolating results across neonicotinoids in experimental studies to understand the effects of field realistic exposure (Walters, 2013).

A recent study by Budge *et al.* (2015) correlated imidacloprid usage with honey bee hive mortality at a UK regional scale. They fail however, to investigate confounding factors such as floral resource loss. Furthermore, issues arise with the reporting of continued decline in honeybee hives. Published trends by organisations such as the British Beekeepers Association (BBKA) showing that honeybee numbers and hive numbers have continued to increase or remain stable throughout the period of neonicotinoid usage in the UK and in Europe (FAO, 2015). Thus, the reported increase in honeybee colony loss needs to be interpreted in the context of an overall increase in hive numbers.

Studies have linked exposure to neonicotinoids to delayed development in honeybee larvae (Decourtye *et al*., 2004; Wu *et al.,* 2011; van de Sluijs *et al*., 2013) with potential negative effects to honeybee colonies resulting from the knock on delay as older workers die off, without younger individuals being available to take over hive hygiene activities and brood rearing (Jandt & Dornhaus, 2009; Blacquiere *et al*., 2012). It is worth noting that many studies reporting negative effects on reproductive output have been conducted with bumble bees given access to only treated feed (Whitehorn *et al*., 2012; Zarevúcka, 2013; Raine & Gill, 2014; Scholer & Krischik, 2014), but one field-scale experiment has detected negative effects on reproductive output when *Bombus* colonies were located and allowed to forage in treated areas; neonicotinoid levels in nectar approached the maximum field reported exposure rate (Rundlöf *et al*., 2015).

It is well established that neonicotinoid exposure affects the nervous system of insects resulting in uncontrolled firing on synapses associated with coordination (Decourtye & Devillers, 2010). However, these symptoms and other associated effects, have not been linked with specific dosages and thus may form an unreliable method of detecting the action of neonicotinoids (Aliouane *et al*., 2009). Despite this many studies rely on locomotive responses as response variables for laboratory investigations. It is also worth noting, despite claims of permanent memory damage, a study by Aliouane *et al*. (2009) reported decreases in learning potential for 24 hours following exposure to thiamethoxam, yet the effect was reversed after 48 hours suggesting recovery is rapid.

1.9.4 Anti-feeding and avoidance responses

Presence of neonicotinoid residues in nectar may reduce feeding leading to sub-optimal foraging. Dose-dependent reductions in feeding by both bumblebees and *Apis* bees offered sugar solution spiked with imidacloprid, clothianidin or thiamethoxam have been reported (Alaux *et al.*, 2010; Cresswell *et al*., 2014; Thompson *et al*., 2014). The effect of imidacloprid residues on bumblebee feeding is known to be reversible and delays before complete recovery reflect the clearance rate of ingested imidacloprid in bumblebees (Cresswell *et al*., 2014). Thompson *et al.* (2014) reported a reduction in feeding, particularly for the higher 10µg/L concentration, which may have indicated an anti-feeding response due to either sub lethal toxicity effects or detection and avoidance of contaminated nectar, but the study did not allow definitive conclusions regarding the underlying mechanism. Similar results were also reported by Blacquiere *et al*. (2012) and Elston *et al*. (2013). Reduced feeding could prevent the individual from consuming a lethal dose. Furthermore work by Thompson *et al.* (2014) demonstrates that feeding reduction induced over a four day period may be reversed following a switch to an uncontaminated nectar source, with recovery time ranging from 1-2 days. These results suggest a possible behavioural response which would inhibit exposure to contaminated nectar sources. Such studies lack a choice test or any associated cue that could be used to distinguish between feeders with different dosages, however, factors that may be relevant to forager selectivity of plants. Findings from a choice test (Kessler *et al.,* 2015), report a feeding reduction increasing as the dose of neonicotinoid increases; thus supporting previous findings from Thompson *et al.* (2014). Kessler *et al.* (2015) also reported preferential feeding on neonicotinoids; although this trend was not observed in newly emerged *Bombus terrestris* individuals. However, once again, no cues were provided by which the bees could distinguish feeders containing neonicotinoid contaminated or uncontaminated nectar sources.

Investigations utilising artificial flowers and foraging behaviour were expanded on by Arce *et al.* (2018). This study ran for 10 days utilising artificial flowers and associated sucrose feeders for *B. terrestris audax.* Flowers utilised were all of the same colour (blue) with a control and two dosages of thiamethoxam. The proportion of visits to pesticide spiked feeders was lower than to feeders offering untreated nectar at the start of the experimental run. The proportion of visits to thiamethoxam spiked feeders however, increased with time only at the lower of the two

concentrations utilised, thus seemingly demonstrating an attraction by worker bumblebees to thiamethoxam and associated detection. There is currently no explanation regarding this change in behaviour from avoidance to attraction, or for the attraction being only to the lowest concentration tested.

Recommendations regarding future commercial use of neonicotinoid insecticides that are based on such studies have, in many cases, not considered the alternative active ingredients that are currently available. Budge *et al.* (2015) demonstrated that systemic seed treatments significantly reduce the need for insecticide use up to the flowering period of major crops such as oilseed rape. With the removal of neonicotinoids under the moratorium many farmers must rely on older chemical treatments, such as pyrethoids, which may have other human health and environmental impacts. Moreover, given the known pyrethroid resistance of significant pests (aphids, cabbage stem flea beetles) of the establishment period in OSR, and the current absence of effective (commercially viable) cultural control or biocontrol methods for such pests, the increased reliance on older means of chemical control may increase the overall volume of products applied, exacerbate resistance and potentially reduce yield.

1.10 Aims and hypotheses

This study aimed to improve our understanding of a) the ecological mechanisms by which managed non- *Apis* bees may enhance crop production, b) the drivers of selectivity of foraging for pollen exhibited by bees in agroecological landscapes, c) the importance of the nutritional components of pollen to larval development, and as a contributor to foraging behaviour, and d) the response of bees to nectar and pollen contaminated with systemic pesticides.

The research programme addressed five primary hypotheses:

1. Supplementing pollination of *O. bicornis* will increase the quality and yield of fruit in commercial cherry orchards.
2. *Osmia bicornis* can identify favourable characteristics (amino acids) of pollens from different plant species, and optimise larval provisions in nest

cells by selective utilisation of a few plant species from the diverse range available.

3. The performance (development and survival) of individual *O. bicornis* larvae is determined by pollen species profile and associated amino acid content of wild collected and experimental pollen diets.
4. Colony performance of *B. terrestris* is determined by pollen species profile and associated amino acid content of experimental pollen diets.
5. *Bombus terrestris* displays behavioural mechanisms associated with visual and chemical cues that lead to avoidance of neonicotinoid contaminated food.

Chapter 2: Impact of enhanced *Osmia bicornis* (Hymenoptera; Megachilidae) populations on pollination and fruit quality in commercial sweet cherry (*Prunus avium* (L)) orchards

2.1 Abstract

The impact on pollination of supplementing wild pollinators with commercially reared *Osmia bicornis* in commercial orchards growing the self-fertile sweet cherry variety "Stella" was investigated in each of two years. The quality characteristics used by retailers to determine market value of fruit were compared when insect pollination was by wild pollinators only, or wild pollinators supplemented with *O. bicornis* released at recommended commercial rates. No effect of treatment on the number of fruit set or subsequent rate of growth was recorded. However, supplemented pollination resulted in earlier fruit set when compared to pollination by wild pollinators alone and offered the potential benefit of a larger proportion of the crop reaching optimum quality within a narrower time range, resulting in more consistent produce. Retailers use five key quality criteria in assessment of market value of cherries (the weight of individual fruit, width at the widest point, fruit colour, sugar content and firmness). Price paid to growers depends both on meeting the criteria and consistency between fruit in these characteristics. In both years, the commercial criteria were met in full in both treatments, but harvested fruit following supplemented pollination were consistently larger and heavier compared to those from the wild pollinator treatment. In the year where supplemented pollination had the greatest impact on the timing of fruit set, fruit size and sugar content were also less variable than when pollination was by wild species only. The implications for the commercial use of *O. bicornis* in cherry orchards are considered.

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2.2 Introduction

Insect pollination is a key ecosystem service, with estimates valuing it at £430 million per annum within UK agricultural systems alone (Vanbergen *et al.*, 2014). Insect pollinators (including wild bees) account for 35% of global crop pollination (Garibaldi *et al*., 2013) but a decline in bee populations has been observed over the last 60 years (Potts *et al.*, 2010) and linked to a range of drivers (Neumann & Carreck, 2010; Potts *et al*., 2010). The decline is more severe amongst specialist feeding species with many generalist feeders less affected due to their association with a wider range of plant species (Neumann & Carreck, 2010; Potts *et al*., 2010). It has been suggested that wild pollinator decline has had a greater impact on pollination of high value fruit crops such as top fruit orchards than on other crops, and to address shortfalls in pollination services in orchards wild pollinators are commonly supplemented with commercially managed species such as *Apis mellifera* (Allsopp *et al.*, 2008; Breeze *et al*., 2011; Garibaldi *et al*., 2013). Against a background of increasing costs associated with such managed bees and the decline in wild pollinators (Allsopp *et al.,* 2008; Potts *et al*., 2010; Breeze *et al*., 2011) interest in the potential commercial use of alternative organisms to supplement pollination is rising.

Solitary bees of the genus *Osmia* have been shown to be an effective alternative to existing commercial pollinators in several fruit crops. *Osmia cornifrons* is used for commercial apple and cherry pollination in China (Batra, 1978) and Japan (Sekita & Yamada, 1993; Sekita, 2000; Bosch & Kemp, 2002) and *Osmia lignaria* in orchards in the USA (Morales-Ramos *et al*., 2013). In Europe the use of *Osmia cornuta* was developed successfully for orchard pollination, and this was followed by its

introduction to California in the 1980s to pollinate almond crops (Torchio & Asensio, 1985). *Osmia bicornis* (Linnaeus, 1758) (previously *Osmia rufa*) has been used in European orchards since being developed from the 1970s as a pollinator for fruit trees (including sour cherries) and other crops including strawberries and oilseed rape (Hansted *et al*., 2014; Sedivy & Dorn, 2014). Although positive effects on both yield and quality (compared to background pollination by wild pollinators alone) have been reported when this species is released in later flowering fruit crops, more work is needed to determine its efficacy in earlier flowering crops such as cherries.

Osmia bicornis is a widely distributed univoltine, polylectic species, ranging from Scandinavia to the Mediterranean (O'Toole, 2000; L'homme, 2014). It is active in Europe from April onwards in most years (Raw, 1972; O'Toole, 2000), and commercial rearing techniques ensure the availability of adults for release sufficiently early in the year to pollinate earlier flowering orchard crops, such as cherries, at a time when fewer alternative wild pollinators are available (Gruber *et al*., 2011).

In the UK both self-fertile and self-sterile varieties of sweet cherry (*Prunus avium* (L.)) flower in late March or early April. Rapid ovule degeneration occurs during flowering and the majority of self-fertile varieties that are grown in commercial orchards (e.g. Stella), are thought to benefit from supplementary pollination by insects (Lane, 1979; Delaplane & Mayer, 2000). Early pollination has been shown to influence fruit set, and it has been suggested that flowers pollinated later in the blossom period may show a reduction in eventual fruit quality (Mayer *et al*., 1987; Ughini & Roversi, 1993). Thus pollinators that are actively foraging during the short flowering period may play an important role in maintaining yield or quality of produce (Lane, 1979; Delaplane & Mayer, 2000). The early flowering period of cherry trees coincides with activity of a restricted range of wild pollinators, amongst which some *Bombus spp*, *Andrena spp* and *Osmia spp* are thought to be of key importance (Kirk & Howes, 2012; Guler & Dikmen, 2013). These early flying wild species can increase fruit set and yield, even when honey bee colonies are placed in the orchards (Holzschuh *et al*., 2012).

Successful use of *O. bicornis* in commercial orchards is due in part to their morphology and, resulting from their nesting behaviour, the ease with which bees can be released and retained in a local area (Sekita, 2000; Hansted *et al.,* 2014). Mason bees, such as *O. bicornis,* collect pollen on the scopa, which is located on the ventral surface of the abdomen. The location of the scopa increases the

potential for contact with plant reproductive structures (Kuhn & Ambrose, 1984) and pollen is easily dislodged resulting in effective transfer between flowers (Raw, 1972). Pollination can thus be achieved by fewer floral visits than is required by social bee species (Klein *et al*., 2012). The utility of *O. bicornis* is also enhanced because it requires a high number of foraging trips for provisioning of larval nest cells, and these trips are commonly completed within a short foraging range (Gathmann & Tscharntke, 2002). These factors, coupled with the ability to fly in adverse weather (Stone & Willmer, 1989; Güler & Dikmen, 2013), increases the potential of the species to act as an effective early season commercial pollinator.

The crop production industry is currently investigating the potential of *O. bicornis* as a pollinator in oilseed rape, cherry orchards and soft fruit crops such as strawberry (Wilkaniec & Radajewska, 1996; Biliński & Teper, 2004; Teper & Biliński, 2009; Gruber *et al*., 2011). Little information is available on its impact in UK cherry (*Prunus avium*) production systems, and there is active debate regarding its potential efficacy. Research is therefore required to support optimisation of pollination services in this crop. This study investigates the hypothesis that supplementing pollination by *O. bicornis* release will increase the quality and yield of fruit in commercial cherry orchards.

2.3 Materials and methods

Experiments were conducted in a mature commercial sweet cherry orchard (*Prunus avium*) in North Herefordshire, UK (SO 583502), established in 2000 using the self-fertile cultivar “Stella” (RHS, 2016). The orchard is on south facing slopes with well-draining red Herefordshire soil, slightly acid loamy and clayey (Soilscapes, 2016), at 200m above sea level, and with a density of 1900 trees per hectare yielding a mean of 20 tonnes fruit per hectare. Trees were covered with open ended 100m polythene tunnels (poly-tunnels), each containing 2 rows separated by a narrow (2m) grass strip with occasional herbaceous flora including *Taraxacum* and *Ranunculus spp*. Normal commercial husbandry practice included opening tunnels during spring and summer, allowing access for pollinating insects during the flowering period.

2.3.1 Experimental design and treatments

A randomised design of four blocks each containing two treatment plots was established during early spring of 2015. The experiment was replicated in 2016. Each treatment plot occupied the central portion of a poly-tunnel, and contained 50 trees (two rows of 25 trees). Fourteen days prior to bud burst (average growth stage 2: 23rd March 2015, 21st March 2016; Chapman & Catlin, 1976) the polythene sides of each plot were replaced with an insect-proof mesh covering. Mesh walls were also constructed to seal both open ends, thus facilitating release and containment of known numbers of *O. bicornis*.

Within each block, in one treatment plot (control) insect pollination relied on the wild pollinators trapped when mesh walls were constructed. In the second, wild pollinating insects were supplemented with commercially reared *O. bicornis* released at the standard rate (2 adult bees/tree) and timing recommended for cherry orchards by the supplier (Mason Bees Ltd., Shropshire, UK). Two standard weatherproof release boxes (18 x16 x 8cm) were set at 1.5m above the soil surface according to normal commercial practice. Boxes were positioned at distances equivalent to approximately one-third and two-thirds along the length of each of the plots, with an exit slit facing towards the south to allow escape of adult bees. Fourteen days before commencement of bud burst, fifty *O. bicornis* pupae were placed in each release box and allowed to eclose (60:40 female to male ratio). To confirm the total number of adult *O. bicornis* that were active during flowering in each plot, empty cocoons were counted at 7 day intervals until all had emerged. Pupae that failed to eclose within the expected time period were removed and replaced.

With the exception of the treatment-specific procedures, all crop husbandry activities were identical in all plots and followed normal commercial practice for the orchard.

2.3.2 Assessments

2.3.2.1 Temperature: Temperatures within each treatment plot were recorded with a handheld digital thermometer at each assessment visit (TPI Digital Pocket

Thermometer, Crawley UK). Three measurements were recorded in each treatment/replicate at each visit, at 10:00, 13:00 and 15:00.

Abundance of wild pollinators - The abundance of wild pollinating fauna was established by taking sweep net samples in all plots on each of five days during the flowering period of the orchard. Sampling was conducted while walking at a standard speed (circa 2 ms^{-1}) along the full length of the central strip between the 2 rows of cherry trees, before the catch was transferred to a sealed plastic bag and returned to the laboratory where it was stored in a freezer at -20ºC until processing. To take account of diurnal activity cycles of different pollinators, sampling was undertaken during three periods (08:00-10:00, 11:30-13:30, 15:00-17:00), and was replicated on each of five days during the blossom period. Counts were only taken on days when temperatures were favourable for pollinator activity (>12°C), based on the temperature assessments described above. As available resources precluded identification of all individuals to species, the insects caught were recorded under six categories, wild (non-*O.bicornis*) solitary bees, bumblebees (*Bombus* spp.), honey bees (*Apis mellifera*), hoverflies (Syrphidae), "other" diptera, and "other" insects (Hymenoptera (mainly parasitoids, Coleoptera and Neuroptera). The assessment therefore recorded the groups found in each sample that potentially contributed to wild pollination, but as counts included both pollinating and some non-pollinating species it is likely that they overestimated the cumulative contribution of wild pollinators to cherry pollination in the treatment tunnels.

2.3.2.2 Fruit set and fruit drop: Prior to the start of bud burst (growth stage 2, Chapman & Catlin, 1976), ten trees were selected at random in each treatment/replicate (five from each row), and a branch from mid-canopy level was selected for assessments and labelled. The number of buds on the distal 50cm portion of each labelled branch was counted. After the end of all flowering (growth stage 7, Chapman & Catlin, 1976) the number of developing fruit was counted, with further counts of fruit being taken on 5, 10, 16, 23 June and 1 July in 2015, and 7, 12, 19, 24 June and 3 July 2016. The last count of fruit was taken at the commencement of ripening.

2.3.2.3 Fruit growth: The terminal fruit cluster from labelled branches was identified and the width at the widest point of each individual fruit was measured with digital callipers (Sealey, Suffolk UK). Measurements were repeated at weekly intervals (2015: 5, 10, 16, 23 June, 1 July; 2016: 7, 12, 19, 24 June and 3 July).

2.3.2.4 Fruit quality: Fruit quality assessments were taken within two days of the harvest date for the crop (10th July 2015; 9th July 2016) with a minimum of 40 fruit sampled from each plot. Fruit were harvested by commercial pickers, placed carefully in labelled punnets and returned immediately to the on-site cold storage facility. Five quality measurements were made for each individual fruit (weight), width at the widest point, fruit colour, sugar content and consistency (firmness), using the standard equipment and approaches used in commercial quality assessment procedures for determining market value (Sainsbury's Supermarkets Ltd, 2015). Weight was assessed using a 50g spring scale (Pesola Light-Line, Schindellegi, Switzerland), width using the callipers described above, fruit colour on the industry standard scale of 1 (light fruit) to 7 (dark fruit) using the standard commercial colour guide (Centre Technique Interprofessionel des Fruits et Legumes, Paris France), and sugar content (percentage brix) by piercing the skin of the fruit and squeezing the juice onto the receptor of an Atago digital refractometer (Atago, Tokyo, Japan). Fruit consistency was assessed using a digital firmness penetrometer (Agro Technologie, Serqueux, France) by averaging two measurements of fruit consistency taken at the widest point of the cherry (separated by 180 Degrees). In each measurement consistency was recorded as the pressure required to penetrate the flesh of the cherry and expressed (according to normal commercial practice) as percentage of the maximum pressure that could be exerted by the penetrometer, which corresponded to a pressure of 806g (Agrosta, 2015). Penetrometer assessments were only made in 2015 due to an equipment failure in 2016.

2.3.3 Statistical analysis

Statistical analysis was conducted using R Studio 0.99.903 (RStudio Team, 2015). All data were tested for normality and Log or sqrt transformations applied where necessary. Factor reduction was conducted allowing for the removal of non-significant terms and interactions in order to reach the minimum adequate model for all statistical tests as described by Crawley (2013). During factor reduction, ANOVA between models was conducted to verify that the validity of the statistical model was not affected.

Temperature data consisted of a continuous response variable with categorical explanatory variables, thus a two way analysis of variance (ANOVA) was utilised.

The number of buds and number of fruit set per unit length of branch was count data and thus was analysed using GLM with Poisson error structure. Where residual deviance was found to be greater than the degrees of freedom a Quasi-Poisson error structure was applied. The proportion of fruit set was analysed with a GLM with a Binomial error structure.

Impact of treatment on cherry development (fruit size over time) consisted of both a continuous and categorical response variable, due to this an ANCOVA was used for analysis.

For fruit quality post-harvest assessments, data for width, weight, firmness and brix were all subjected to ANOVA and Tukey post-hoc test to assess the impact of treatment.

Fruit colour data were collected on an ordinal scale and differences between treatments were investigated using a Kruskal-Wallis one-way analysis of variance and post-hoc Dunn test.

For all post-harvest quality assessments a Fisher's *F*-test was conducted to investigate whether the variability of fruit differed between treatments.

2.4 Results

For all assessments, block and plot were found to be non-significant in both years and therefore removed in both factor reduction and creation of the minimum adequate model.

2.4.1 Temperature

During the creation of the minimum adequate model, treatment was found to be non-significant and thus removed from analysis. Thus, there was no difference in temperature between treatment blocks. Temperature varied significantly between dates in both 2015 ($F = 2.87$, d.f. = 4, 91, $p = 0.027$) and 2016 ($F= 700.9$, d.f. = 1, 69, $p <0.001$) reflecting the transition from spring to summer. Higher temperatures were recorded in 2016 than 2015 ($F = 279.05$, d.f. = 2,160, $p <0.001$).

Wild pollinators - Very few wild pollinators from any of the six groups (wild (non-*O. bicornis*) solitary bees, bumblebees (*Bombus* spp.), honey bees (*Apis mellifera*), hoverflies (Syrphidae), "other" diptera, and "other" insects), were recorded in sweep net samples taken in either year (Table 1). The results of paired t-tests show no significant differences between treatments in the numbers of insects caught in either year (2015: t = -1.67, d.f. = 3, p =0.193; 2016: t = -1.71, d.f. = 3, p = 0.185). Although very low in both years, insect counts were significantly higher in 2016 compared to 2015 (t = 5.41, d.f. = 7, p <0.001).

2.4.2 Fruit set

2.4.2.1 Bud counts: There were more buds per branch in 2015 than in 2016 (t= 11.97, d.f. = 233, p <0.001), but there were no significant differences between treatments in the number of buds in either year (2015: t= 0.24, d.f. =152, p = 0.810; 2016: t= 1.056, d.f. = 79, p = 0.294, Figure 2.1a, b).

2.4.2.2 Proportion fruit set: In 2015 the proportion of buds from which fruit was set was lower than in 2016 (z = -29.61, d.f. = 233, p <0.001). Differences between treatments were not consistent between years. In 2015, the proportion of buds from which fruit was set was not significantly different between wild pollinator and *Osmia* supplemented treatments (z= 0.19, d.f. =152, p = 0.849) (Figure 2.1c), but in 2016 the proportion of fruit set was lower in *Osmia* supplemented treatments (z= -8.76, d.f. =79, p <0.001) (Figure 2.1d).

2.4.2.3 Fruit counts: The results for fruit counts mirrored those for fruit set. In 2015 total fruit count was lower than in 2016 (t = -6.59, d.f. = 233, p <0.001) and total fruit counts were not significantly different between wild pollinator and *Osmia* supplemented treatments (t= 0.19, d.f =152, p = 0.849) (Figure 2.1e). In 2016 total fruit count was found to be lower in *Osmia* supplemented treatments compared to the treatment with wild pollinators only (t = -2.599, d.f. = 79, p = 0.011) (Figure 2.1f).

2.4.3 Fruit growth

Following log transformation to normalise the residuals of the data, results of ANCOVA analysis show that fruit size increased as a function of time (t = 46.0, d.f. = 392, p < 0.001), but no significant differences were found between treatments in the slopes of the lines describing the growth in width of cherries with time (Figure 2.2a). This interaction was therefore removed from the minimum adequate model for both 2015 and 2016.

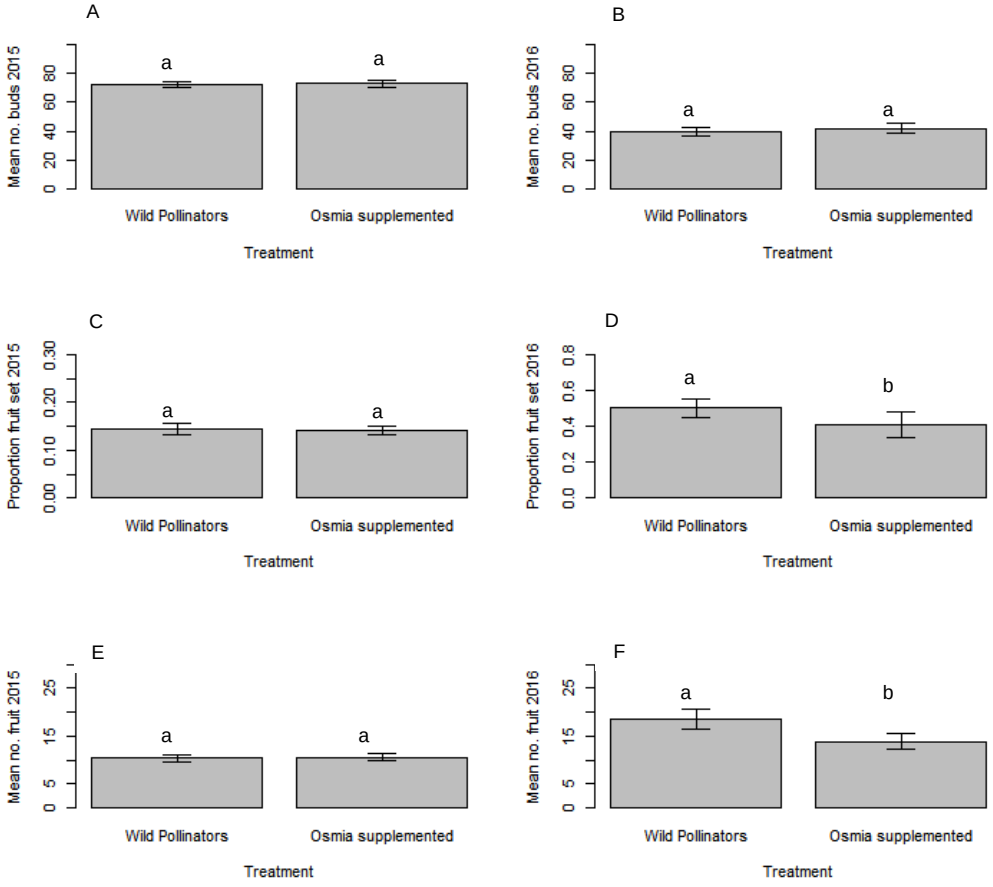


Figure 2.1. Mean (± S.E.) number of buds/branch in 2015 (A) and 2016 (B), proportion from which fruit set in 2015 (C) and 2016 (D), and mean total number fruit set/branch in 2015 (E) and 2016 (F) on 50cm lengths of branch in *Osmia* supplemented and wild pollinator treatments. Treatments sharing the same letter did not vary significantly from each other (p >0.05).

There was, however, another significant effect of treatment on cherry development in 2015 (F = 8.94, d.f. = 1, 392, p = 0.003), with the intercepts of the regression line for *Osmia* supplemented treatments occurring earlier than that of the wild pollinator treatments (t = 225.8, d.f. = 392, p <0.001), thus allowing to extrapolate this data to estimate the time of pollination, indicating that the mean time of commencement of

fruit growth (following fruit set) was earlier in the *Osmia* supplemented treatments (Figure 2.2a). As pollination could only commence when flowers opened, which occurred at the same time in each treatment, the earlier mean time for commencement of fruit growth in the *Osmia* supplemented treatment suggests that pollination/fertilisation was completed within a shorter time period when the bees were present. This extrapolation results in approximately 2 day earlier set in 2015 and 7 day earlier set in 2016.

A similar outcome was recorded in 2016 (Figure 2.2b). A significant effect of treatment on cherry development was recorded ($F = 100.56$, d.f. = 1,637, $p < 0.001$), with the intercept for the *Osmia* supplemented treatment significantly earlier than the wild pollinator treatment ($t = -165.71$, d.f. = 637, $p < 0.001$). All fruit widths increased as a function of time ($t = 37.56$, d.f. = 637, $p < 0.001$) (Figure 2.2).

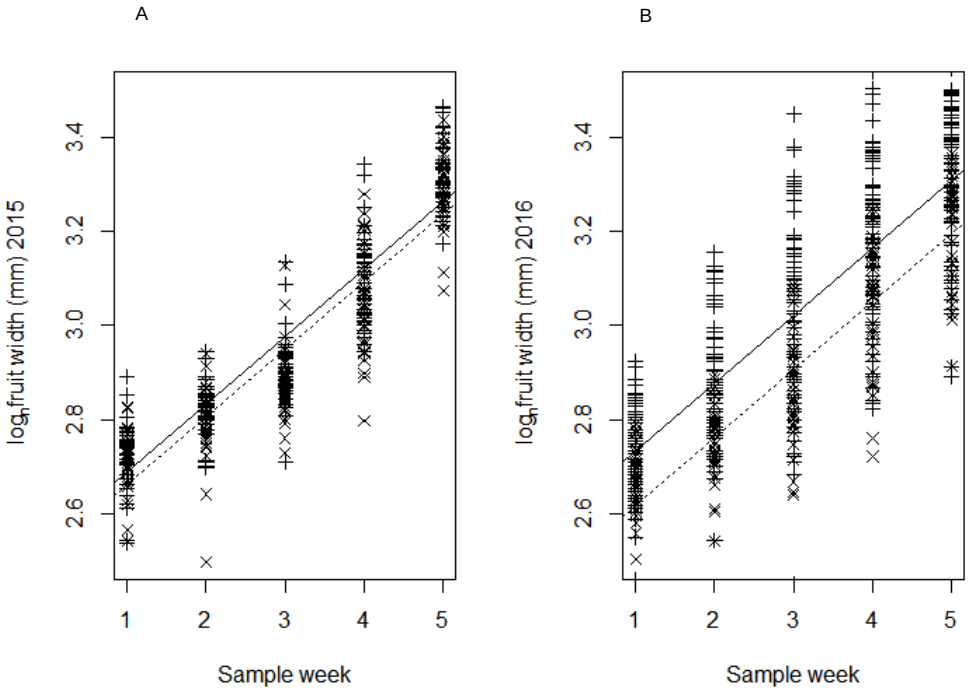


Figure 2.2. Increase in fruit width (mm) with time (sample week) in 2015 (A) and 2016 (B). + / —— = *Osmia* supplemented treatment; x / --- = wild pollinator treatment.

2.4.4 Post-harvest assessments

For all postharvest assessments block and plot were found to be non-significant in both years and were removed in factor reduction. Due to only two treatments being available, two tailed t-tests were utilised for analysis.

2.4.4.1 Weight and width: Fruit weight and width were both found to be higher in the *Osmia* supplemented treatment in both 2015 and 2016 ($p < 0.001$ in each case) (Figure 2.3). In 2015, however, there was no difference between treatments in the variability of individual cherry weight ($F = 0.96352$, d.f. = 501, 434, $p = 0.656$) or width ($F = 1.1637$, d.f. = 501, 433, $p = 0.052$). In 2016, the variability of fruit weight did not differ between treatments ($F = 0.91968$, d.f. = 306, 385, $p = 0.778$), however width was found to be significantly more variable for fruit from the wild pollinators treatment compared to the fruit from the *Osmia* supplemented treatment ($F = 1.353$, d.f. = 306, 385, $p = 0.002$).

2.4.4.2 Sugar content and consistency: The cherries from all treatments met commercial requirements for sugar content (Sainsbury's Supermarkets Ltd, 2015), but Brix did not vary as a function of treatment in 2015 ($p > 0.05$) or 2016 ($p > 0.05$) (Figure 2.4). Likewise firmness did not vary as a function of treatment in 2015 ($p > 0.05$). Cherries from the wild pollinators and *Osmia* supplemented treatments were found to be equally variable for both sugar content and consistency in 2015 (Sugar content: $F = 0.99197$, d.f. = 502, 432, $p = 0.535$; consistency: $F = 0.88373$, d.f. = 502, 435, $p = 0.909$). In 2016 however, sugar content was found to be more variable for fruit in the treatment with wild pollinators alone ($F = 16.917$, d.f. = 306, 385, $p < 0.001$).

2.4.4.3 Colour: In 2015 and 2016 the colour of cherries varied between treatments (2015: $H = 14.85$, d.f. = 1, $p < 0.001$), (2016: $H = 13.22$, d.f = 1, $p < 0.001$). Fruit from *Osmia* supplemented treatments were darker in colour than those harvested from the wild pollinator treatments in 2015, with this reversed in 2016 (Figure 2.5). However, fruit colour scored lower (overall lighter) in 2015. The variability in colour of cherries did not differ between treatments in either 2015 or 2016 (2015: $F = 0.90128$, d.f = 502,435, $p = 0.869$; 2016: $F = 1.001$, d.f = 306, 385, $p = 0.494$) and all required quality standards were met.

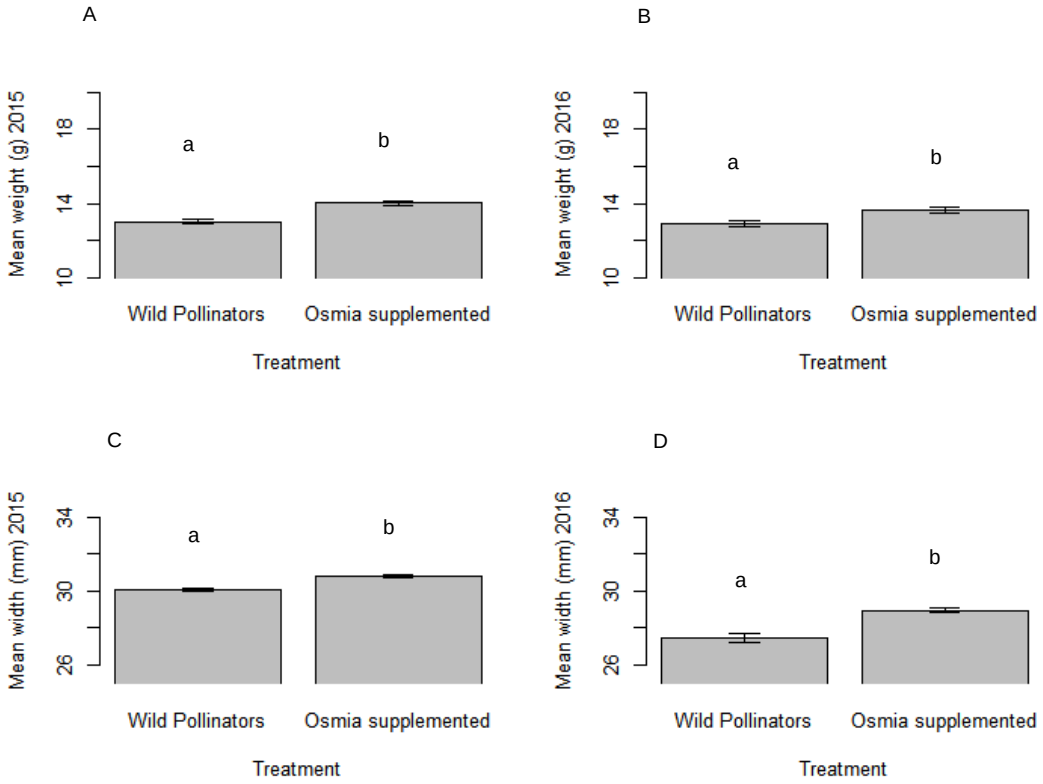


Figure 2.3. Mean (± S.E.) fruit weight (g) in 2015 (A) and 2016 (B), and mean fruit width (mm) in 2015 (C) and 2016 (D), on 50cm lengths of branch in *Osmia* supplemented and wild pollinator treatments. Treatments sharing the same letter did not vary significantly from each other ($p > 0.05$).

2.5 Discussion

The value of a sweet cherry crop is determined by yield, and quality characteristics of the produce (including weight, size, colour, sugar content, and firmness of the fruit), but simply meeting the set quality criteria is not sufficient to command the highest prices. The consistency between fruit in key quality factors is also an important consideration in commercial quality grading procedures determining the price paid to growers (Sainsbury's Supermarkets Ltd, 2015). In this study, all the quality characteristics of cherries from trees pollinated by wild pollinators only, and those exposed to both *O. bicornis* and wild pollinators, were within the ranges required by retailers. Significant differences between treatments in some key characteristics were, however, found.

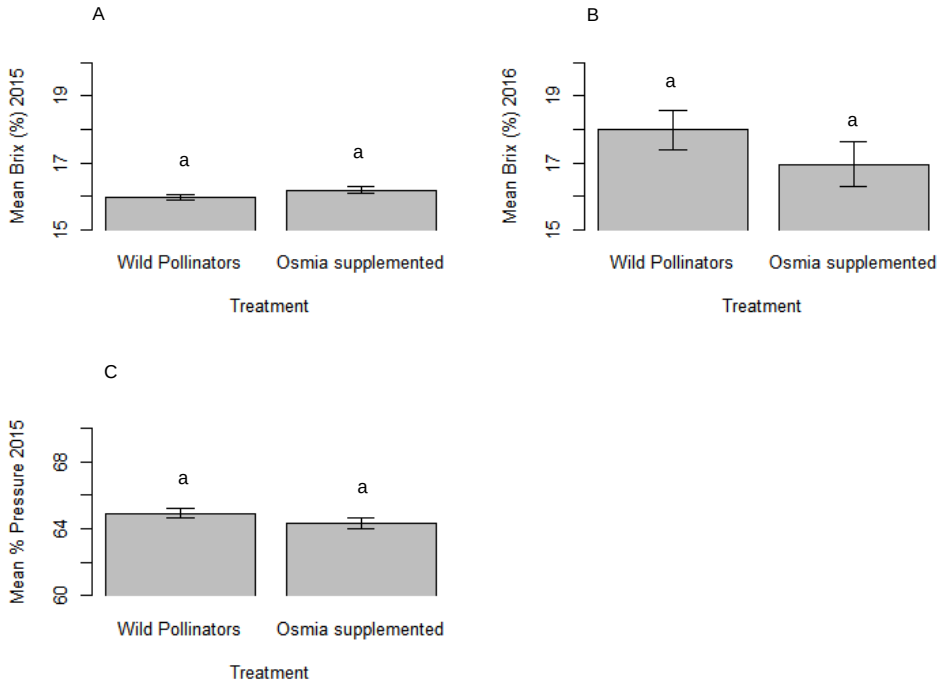


Figure 2.4. Mean (± S.E.) post-harvest sugar content (% Brix) in 2015 (A) and 2016 (B), and consistency (pressure required to penetrate the fruit expressed as percentage of maximum pressure exerted by the penetrometer) in 2015 (C), on 50cm lengths of branch in *Osmia* supplemented and wild pollinator treatments. Treatments sharing the same letter did not vary significantly from each other (p >0.05).

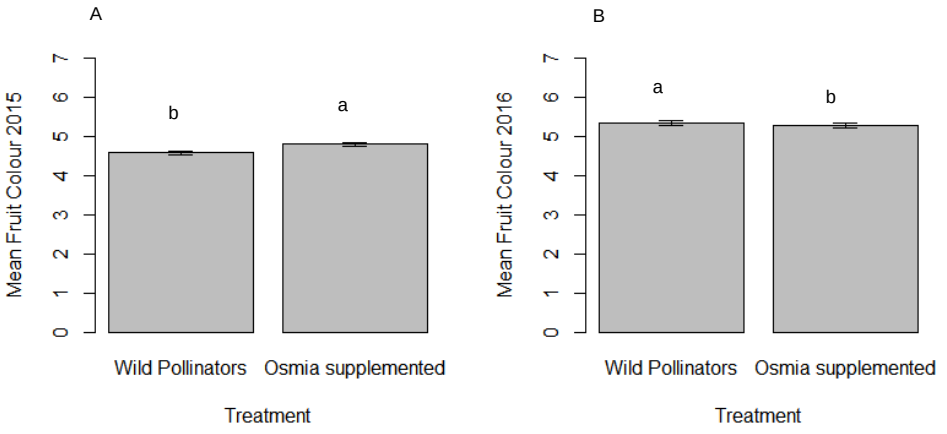


Figure 2.5. Mean (± S.E.) post-harvest colour measurements (Industry standard scale) in 2015 (A) and 2016 (B), on 50cm lengths of branch in *Osmia* supplemented and wild pollinator treatments. Treatments sharing the same letter did not vary significantly from each other (p >0.05).

No consistent relationship was established between number of flowers per unit length of branch, number of fruit set and treatment in either year, thus no evidence of increased fruit set following supplementation of wild pollinators by release of *Osmia* was recorded. In both years, however, fruit from the *Osmia* supplemented treatment were larger and heavier at harvest than those produced in the treatment with wild pollinators alone, which in combination with no differences being recorded in fruit count in 2015, results in a higher overall weight of cherries per unit branch length occurring in supplemented pollinator treatments in 2015. This difference did not occur in 2016.

The rate at which the fruit grew following fruit set did not differ between treatments in either year. In both years, however, consistent differences between treatments in the mean timing of fruit set were recorded. Trees in *Osmia* supplemented plots completed fruit set earlier than those with wild pollinators only. Flowering commenced at the same time in both treatments, but pollination occurred more rapidly after bud burst in supplemented pollinator plots, and fruit set from all flowers on a tree was completed during a shorter time window, particularly in 2016 (Figure 2.2).

An increase in fruit quality has been reported from a variety of crops when *O. bicornis* contributes to pollination, partly due to the mechanical action by which pollination is achieved in this species increasing pollen transfer (Kuhn & Ambrose, 1984; Wilkaniec & Radajewska, 1996; Klatt *et al*., 2014). Higher levels of pollen deposition have been shown to increase fruit set and quality in some *Prunus* species, and the high pollination efficiency established by other studies may have contributed to the shortening of the pollinations window when *O. bicornis* was released (Kuhn & Ambrose, 1984; Wilkaniec & Radajewska, 1996; Zhang *et al*., 2010). The importance of pollen deposition may be amplified in “Stella” cherries, as other self-fertilising crop species, such as blueberries, have been shown to require higher levels of pollen grain deposition on the pistil when compared to cross pollinating varieties (Parrie & Lang, 1992).

In the current study, shortening of the pollination window will result in greater synchronisation of cherry development within the crop, and it has been suggested that in some crops this contributes to the production of more uniform fruit size and quality at harvest (Stephenson, 1980; Hasegawa *et al*., 2003; Freihat *et al*., 2008).

This study provides supporting evidence as improved developmental synchrony of sweet cherries from pollinator supplemented plots can be linked to fruit uniformity through significantly lower variability in fruit size and sugar content. However, significant effects were only recorded in the year in which the largest differences between treatments in the length of the pollination window occurred (2016), and further work is required to establish both the factors influencing reliability of this outcome and its economic importance. In addition to improved market value, growers have commented that benefits are also accrued if synchronisation results in a larger proportion of the crop being ready for harvest within a narrow time range, reducing the number of passes pickers need to make and associated labour costs.

Although significant differences between treatments in fruit colour were recorded, they were not consistent between years, suggesting other factors may have influenced the findings. In 2015 fruit colour (an indicator of ripening) was darker in *Osmia* supplemented plots compared to those with only wild pollinators. Treatments were harvested simultaneously, suggesting that the earlier completion of fruit set in *Osmia* supplemented treatments resulted in optimal harvest time being slightly earlier. Results from 2016 however, suggested over-ripening of cherries in the wild pollinator treatment compared to those in the *Osmia* supplemented treatment. Fruit firmness (as measured using a penetrometer) is also, in part, related to degree of ripening if harvest is late, but did not vary as a function of treatment in 2015. Further work is required to improve understanding of factors influencing these important quality characteristics to support decisions on time of harvesting.

In conclusion the release of *O. bicornis* in cherry orchard plots significantly increased the quality of fruit produced by shortening the pollination window, resulting in greater size and uniformity, important fruit quality characteristics, compared to pollination by wild insects alone. Although the impact of *O. bicornis* pollination in other UK crops such as strawberry (Klatt *et al*., 2014) and apples (Garratt & Truslove, 2013) is more clearly established, the commercial potential of *Osmia* as a pollinator of cherries continues to be debated (Hansted *et al*., 2014). Further research is required to investigate yield and quality responses in both self–fertile and non-self-fertile *P. avium* varieties to support cost benefit analyses for its commercial use, and to enable comparisons with alternative managed pollinators. The effect of pollination treatment on shelf life of the crop, a key characteristic for both growers and retailers, also warrants investigation.

2.6 Acknowledgments

This work was supported by a Biotechnology and Biological Sciences Research Council (BBSRC) CASE Award under Grant BB/M503447/1. We thank Chris Whittles of the MasonBee Company Ltd for his generous support in providing the solitary bees used in this study, Andrew Hunt of Lower Hope Farm ltd., for provision of the experimental site and use of quality assessment equipment, and Keith Ward of Syngenta Ltd. for advice on statistical analysis.

Chapter 3: Investigating the influence of floral resource availability on the nesting behaviour of the solitary bee (*Osmia bicornis)* in a mixed lowland farm.

3.1 Abstract

The UK National Pollinator Strategy aims to boost the number of pollinating insects on commercial farms by creating specific habitats tailored to local conditions and native insects. There is currently, however, limited understanding of the suitability of floral resources for solitary bees, an important group including many early season pollinating species. This study investigates selective floral utilisation by *Osmia bicornis,* when foraging in either floristically enriched or unenriched habitats established as part of a government funded stewardship scheme. Within each habitat *O. bicornis* were released and allowed to forage freely. Weekly vegetation surveys were conducted and timing of larval cell construction by foraging bees assessed. Pollens present in larval cell provisions were identified to species, quantified and compared with relative availability of different flower species during the period of cell construction. Amino acid analysis of pollen was undertaken, establishing the quality of pollen mixes in larval provisions.

A greater number of cells were constructed in nest boxes positioned within floristically enriched vegetation. The plant species composition of nest cell pollen provisions was not however, directly correlated with the most common flowers and showed a bias towards specific herbaceous species, and several early flowering shrubs and tree. The “keystone” plant species highlighted in this study are not specifically addressed in current conventional agri-environment schemes. Thus, in order to support more diverse pollinator assemblages, future schemes could be modified to include these plants in pollen and nectar seed mixes, along with enhanced hedgerows and woodlands.

Results of amino acid analysis of nest cell provisions show some differences between times of the season and nesting sites in total protein content parameters, however, there were no consistent differences in concentrations related to proximity to floristically enriched vegetation. It is possible that foraged pollen met the minimum

required nutritional requirements of larvae at each nest site, but that there were trade-offs between foraging time and cell number. Specifically, reduced cell construction in nests situated within floristically unenriched areas may reflect greater expenditure of time and energy on foraging.

Key Words: Nutrition, *Osmia bicornis,* nesting behaviour, floral abundance, floral diversity, foraging behaviour, solitary bees, pollen

3.2 Introduction

Assemblages of pollinators are key in supporting a variety of ecosystem services within agricultural landscapes (Klein *et al.,* 2007). A reduction of pollination services may have particularly serious implications, with 35% of global food production directly relying on these insects (Klein *et al*., 2007). Pollination is thus directly tied to global agricultural economics (Meffe, 1998; Kevan & Phillips, 2001; Gallai *et al*., 2009), but many pollinators are declining (IPBES, 2017). Previous studies have focussed on a few pollinator groups which have shown significant decline over the last 60 years; namely honeybees (*Apis* spp), bumblebees (*Bombus* spp) and lepidopteran pollinators (Potts *et al*., 2010). There is growing evidence however, that other key pollinator groups have also experienced a reduction in diversity since the 1920s (Ollerton *et al*., 2014).

Many factors have been implicated in pollinator decline. Habitat loss, habitat degradation, climate change, parasites and pathogens, alien species and agrochemicals have all been identified as contributory factors (Potts *et al*., 2010). The contribution of the availability of floral resources, and specifically their nutritional value, however, requires further study at both individual and population levels (Williams & Osborne, 2009; Breeze *et al.,* 2011). This is especially true for the solitary bees, an important group of early season pollinating species (Batra, 1979; Bosch & Kemp, 2002; Teper & Biliński, 2009). Despite this, the UK National Pollinator Strategy aims to boost the number of pollinating insects on commercial farms by creating specific habitats tailored to local conditions and native insects (DEFRA, 2014). Indeed, current efforts to mitigate pollinator and bee declines in Europe focus on the provisioning of floristically rich off crops habitats adjacent to fields (Wratten *et al*., 2012), through implementation of agri-environment schemes under the Common Agricultural Policy (CAP) (EU, 2015). Many of these schemes

are, however, expensive to implement and their effectiveness is uncertain. It is paramount that these schemes are designed and implemented as effectively as possible (Batáry *et al*., 2015), whilst taking into account that different bee species respond differently to management options (Carvell *et al*., 2007). In order to maximise the effectiveness of pollinator support networks, further investigation into the relationship between floristic resources and bee behavioural population dynamics is therefore required. This in turn may allow for identification of key mechanisms driving foraging behaviour and population growth. This is of particular importance with recent studies advocating the significance of keystone plant species over floral diversity as the main factor driving population growth in pollinators (Mills *et al.,* 1993; Radmacher, & Strohm, 2010). This is especially true of *Bombus* bee species and solitary bees, such as those of the *Osmia* genus.

This study, therefore, aims to investigate patterns of temporal floral resource utilisation and foraging behaviour in the solitary bee *Osmia bicornis* at the field scale and in relation to available floral resources. If *O. bicornis* is able to identify favourable characteristics of pollens from different plant species it would be expected that it will optimise larval provisions in nest cells by preferentially foraging on a few plant species over the more diverse range available in the landscape.

3.3 Materials and methods

3.3.1 Study site

The study was conducted at Great Wollaston farm, Shropshire, UK (SJ329123). Great Wollaston farm consists of 97 ha, with a mixed arable and livestock production system. The main crops consist of winter wheat and grassland for grazing dairy cattle. It is a LEAF farm (Linking Enviorment and Farming) taking part in an array of outreach and educational activities. Furthermore during the study participated in a variety of HLS and ELS options as part of an agri-environment scheme run by natural England under the Department of Environment, Food and Rural affairs (Natural England, 2010; Natural England, 2013). Options included pollen-rich areas and 6m buffer strips of enhanced grass buffer strips, wild bird seed plant mixes or nectar-rich plant mixes Floristically-enhanced off crop habitats were unevenly distributed across the farm landscape allowing the recognition of relatively floristically rich areas for comparison with floristically unenriched areas.

The experiment run for two consecutive years in 2015 and 2016.

3.3.2 Habitat identification

Aerial photography, and historical cropping and planting records provided by the farmer, were combined to facilitate visual representation of the farm landscape. Broad landscape and habitat types were identified and this initial habitat classification was backed up by ground-truthing. Habitat types were distinguished as either agricultural (identified to crop variety), semi natural habitat (including semi-improved grassland and woodland), hedgerows and finally Higher Level stewardship options such as nectar rich plant areas, wild bird seed plant mixes and enhanced grass buffer strips.

Mapping of the habitats allowed for strategic placement of *O. bicornis* nest boxes to evaluate the effectiveness of the various agri-environment options in providing foraging habitat (Figure 3.1).

3.3.3 *Osmia bicornis* nest box design and placement

Nest boxes consisted of ten sheets of grooved wood (350mm wide) stacked on top of one other within a wooden housing unit. Each sheet had 15 grooves (with each groove being 10mm wide, with 10mm between grooves). Grooves were covered with a thin clear Perspex sheet. The nature of the design is such that the nest may be dismantled to observe nest construction. This allowed the date of nest cell completion to be recorded with minimal disturbance to the *Osmia* larvae. Furthermore, the Perspex sheet could be cut with a scalpel to allow extraction of larvae or pollen from cells as required.

A total of 6 nest boxes were used (Figure 3.1). Nest boxes were placed a minimum of 50m apart from one another, in a variety of areas to better invesgtigate *Osmia* foraging behaviour. Two nest boxes were located in floristically-enriched areas: one in an area of wild bird seed plant mix (WBS) and one in an area of nectar rich plant mix (NS). Two nest boxes were placed in a floristically-unenriched grass buffer strip alongside a cereal field and adjacent woodland edge (Wood1 and Wood2), but within 150m of one of the floristically enhanced areas containing boxes WBS and

NS. Finally, two nest boxes were placed in more intensively farmed grassland areas (Grass1 and Grass2) (Figure 3.1). Broad habitat types within the 150m flight radius of *Osmia* are summarised in Table 3.1.

Each nest box utilised the first row as a release box, under which the nesting trays were positioned. The placement of the nest box under the release box is typical of commercial practices and maximises the chance of nesting behaviour because *O. bicornis* typically will nest close to the parent nest (O'Toole, 2000). Wild individuals of other *Osmia* species tend not to be attracted towards them (Gruber *et al.,* 2011).

For each nest site release boxes were provisioned with 200 *Osmia* cocoons. Boxes were provisioned on the 6th April close to the anticipated timing of adult emergence. Release boxes were checked for emergence every 10 days.

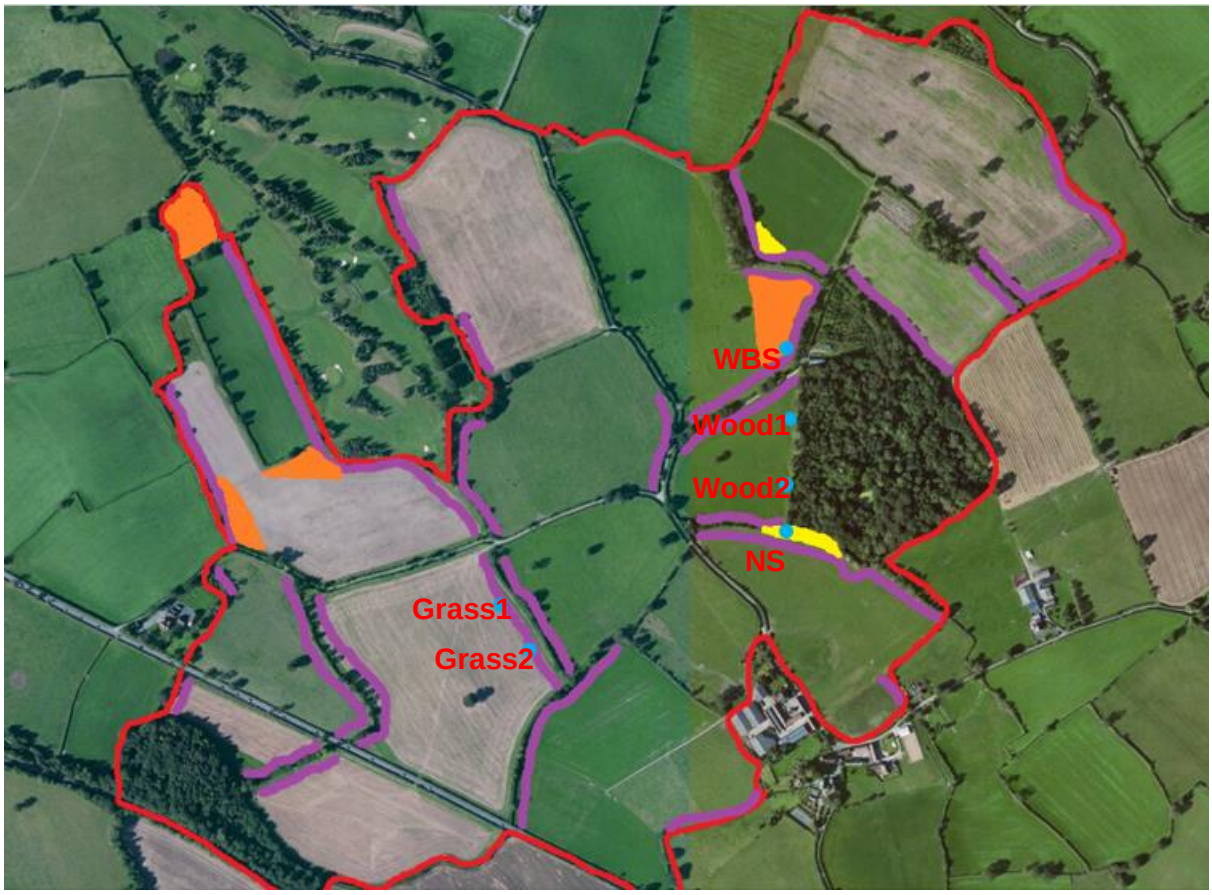


Figure 3.1. The location of *O. bicornis* release/nest boxes at the field study site. Key habitats are highlighted as follows: Orange = Wild bird seed mix (with location of associated nest box "WBS" marked with blue circle); Yellow = nectar-rich mix (with nest box "NS"); Purple = 6m enhanced buffer strips (with nest boxes "wood 1" and "wood2"); unenriched areas (with nest boxes "grass 1" and "grass2").

3.3.4 Mapping of floral resources

Surveying of floral resources was conducted on every 10th day from early April until completion of nest provisioning in August. Quadrats were used to quantify the availability of flowers. Only plant species in flower were recorded. Samples of every flower plant encountered were taken and pressed, accompanied with a colour photograph to create a botanical voucher reference collection. In addition, pollen grains of each species in flower were collected and preserved as a herbarium for use as a reference collection. Prior to slide preparations all pollen samples were stored at -20°C. Pollen samples were prepared as mounted slides and stained as described by Moore et al., (1991). Each pollen slide was photographed under high magnification for inclusion with the plant specimen in the voucher collection.

Table 3.1. Habitat types within a 150m flight radius of each nest box.

Habitat	Description	Grass1	Grass2	Wood1	Wood2	WBS	NS
Floristically enriched field margin	Environmental Stewardship option (6m wide) established through seed mix of 80% grasses and 20% wildflowers.	X	X	X	X	X	X
Grassland	Grassland for forage and silage	X	X				X
Winter wheat	Winter wheat crop			X	X		
Winter barley	Winter barley crop					X	
Nectar-rich plant mix	Environmental Stewardship option established through seeding with a prescribed seed mix on a two-year rotation.				X		X

Wild bird seed plant mix	Environmental Stewardship option established through seeding with a prescribed seed mix on a two-year rotation.			X		X	
Woodland	Wet wooded area, tree composition predominantly *Sambucus, Fraxinus, Quercus, Salix and Acer.*			X	X	X	X
Hedgerow	HLS hedgerows cut on three year rotation. Predominantly consisting of *Prunus spinosa* and *Crataegus monogyna*.	X	X	X	X	X	X

On each survey date, ten 6m by 2m fixed quadrats were placed in each habitat that fell within the 150 m of a nest site. This size of quadrats allowed for accounting of species in buffer strips close to the hedgerow and field margin without biasing results, thus giving a more inclusive view of floral availability.

3.3.5 Nest construction and identification of pollen used to provision cells

During the flight period nest production and progression was recorded every 10th day. Completed cells and cells under construction were recorded by marking key dates on to the overlying Perspex sheet with a waterproof pen. Cells were considered complete when capped with a mud partition and containing a pollen provision and egg. Parasitoid presence, larval development and mortality were also recorded on each date. When all the nest tubes (grooves) within a section were utilised, this section of the nest was removed and replaced.

On each sample date pollen samples were taken from 10 brood cells in each nest box in order to conduct a palynology study, these cells were selected to minamise samples from a single nest tube where possible, thus allowing a more representivie pollen profile. Prior to slide preparations all pollen samples were stored at -20°C. On dates when fewer than 10 cells were available as many samples as possible were recovered (Williams & Tepedino, 2003). Pollen was removed via a small incision made with a scalpel though the Perspex covering. A micro spatula was used to

remove a small amount of pollen from the middle of the provision. Clear sticky tape was then used to re-secure the Perspex flap to allow the larvae to continue development. Pollen samples were homogenised prior to slide preparation in glycerine gel on slides (Beil *et al*., 2008). Prepared slides were then placed under a compound light microscope for identification.

A sample of 50 grains from each cell was identified to species (or genus if that was not possible) (Sawyer & Pickard, 1981; Williams & Tepedino, 2003; Williams & Kremen, 2007). For analysis the data were presented as the percentage of each pollen species in each nest cell.

3.3.6 Amino acid analysis

Samples of pollen taken and used for palynological analysis were reserved for identification of amino acid content. Samples from Grass1 (floristically unenriched), WBS (wild bird seed) and NS (nectar-rich strip) boxes were selected for this analysis. In order to give maximum contrast samples were selected from early, mid and late season cells and repeated for both years, five samples from each date were selected for analysis. Amino acid content was determined according to European Pharmacopoeia methodology (https://www.edqm.eu/en/european-pharmacopoeia-ph-eur-9th-edition) by Alta Bioscience Ltd (Redditch, UK). The ISO 17025:2005-accredited methodology (limit of quantitation 5 nmol; creatine/creatinine content cannot be determined) utilises acid hydrolysis prior to separation using a sodium citrate buffer, such that both the total of both free and previously protein/peptide-bound amino acids are quantified and are referred to as total protein..

Methionine, tryptophan, arginine, lysine, histidine, phenylalanine, isoleucine, threonine, leucine, valine have been identified as the ten essential amino acids for honeybees (DeGroot, 1953). Therefore, in addition to the levels of individual amino acids in each treatment, total protein levels, total essential amino acids (EAA’s) and non-essential amino acids (NAAs) were determined.

3.3.7 Statistical analysis

Statistical analysis was conducted using R Studio 0.99.903 (RStudio Team, 2015), with use of multcomp package (Hothorn *et al.,* 2008). All data were checked for normality and Log or Sqrt transformations applied where necessary. Factor reduction was conducted allowing for the removal of non-significant terms and interactions in order to reach the minimum adequate model for all statistical tests conducted as described by Crawley (2013). During factor reduction, ANOVA between models was conducted to verify that the validity of the statistical model was not affected.

3.4 Results

3.4.1 Emergence of *Osmia* from the release boxes

GLM analysis of the number of *Osmia* successfully emerging from nest boxes found the residual deviance to be greater than the degrees of freedom and thus a quasi-Poisson error structure was adopted. During the factor reduction process and creation of the minimum adequate model the number of *Osmia* emerging from release boxes was shown to vary independently of both box and years. The overall mean number successfully emerging per release box was 150.8, representing an emergence rate of 75.4%. Retention of Osmia to nests, however, could not be guaranteed within this experimental design.

3.4.2 Nest cell construction

GLM analysis of the number of nest cells constructed per box showed the residual deviance to be greater than the degrees of freedom and thus a quasi-Poisson error structure was adopted. During the construction of the minimum adequate model Year was removed. The number of nest cells did not differ between all boxes in the floristically unenriched areas. These four boxes were therefore combined into a single factor for analysis. Nectar-rich site (NS) had a higher number of completed nest cells than unenriched areas ($t = 5.256$, $d.f = 99$, $p < 0.001$), but fewer than wild bird seed mix sites (WBS) ($t=3.309$, $d.f=99$, $p = 0.002$) (Figure 3.2).

3.4.3 Proportional representation of pollen in nest cells

The data on the sources of pollen in samples taken from nest cells was over-dispersed so was subjected to a GLM with a quasi-binomial error structure. An interaction between Year and Pollen Species was found (F = 10.445, d.f. = 20, 7130, p <0.001); indicating that although the abundance of pollen differed between species, these differences were not consistent between years (Figure 3.3a, b, c). For example, *Trifolium repens* pollen was very abundant in samples collected in 2015, but relatively scarce in samples collected from nests in 2016.

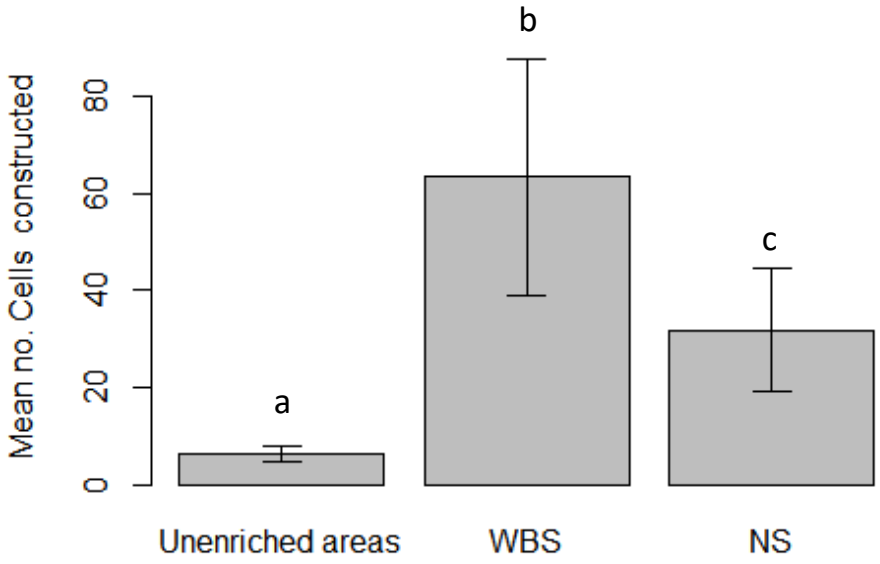
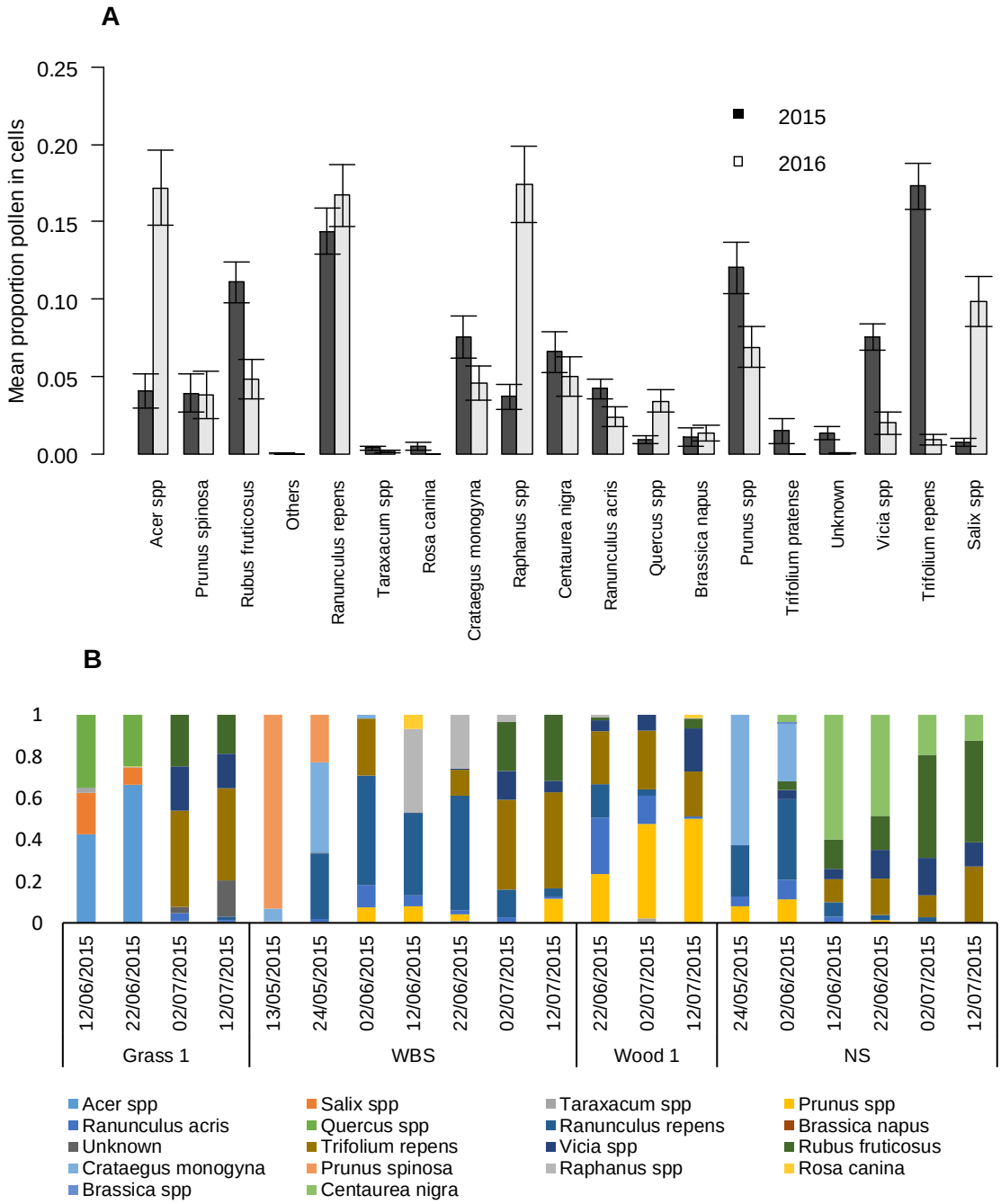


Figure 3.2. Mean number of nest cells (±SE) constructed per nest box placed in three different habitat types. Wild bird seed area (WBS); Nectar-rich area (NS). Unenriched grassland areas, Wild bird seed plant mix areas (WBS) and nectar-rich plant areas (NS) in 2015 and 2016 combined. Treatments with different letters were significantly different (p < 0.05).

A post-hoc General linear hypotheses test (glht) was utilised to further understand the interaction. Pollen sources were distributed in nine internally homogenous (p >0.05) groups with significant differences (p <0.001) between groups (Table 3.2). It is evident that while some species, such as *Prunus spinosa*, *Crataegus monogyna* and *Centaurea nigra*, showed no difference in representation in cell pollen provisions between years, the majority differed significantly between years. The extent of consistency between years appears to be independent of growth form

because trees and woody shrubs provide examples of both differences between years (e.g. *Salix* spp. and *Raphnus* spp.) and equal representation between years (e.g. *P. spinosa* and *C. monogyna*) (Figure 3.3a, b,c).



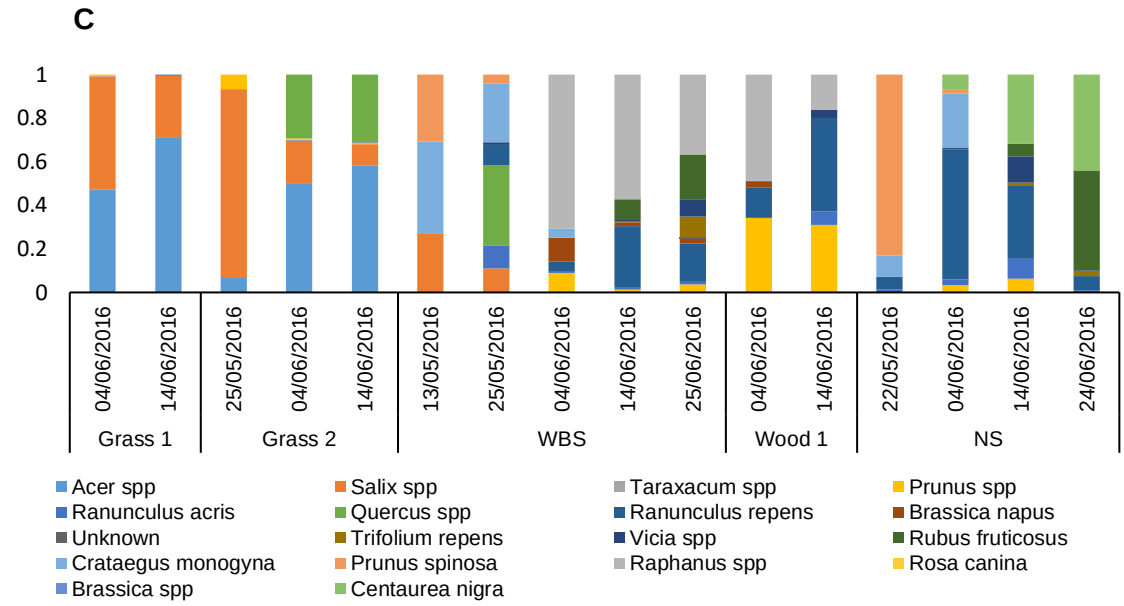


Figure 3.3. Mean proportion of pollen species (±SE) found within samples taken from nest cells over both years of the experiment (A). Pollen proportions at individual sample dates and nest box locations for both 2015 (B) and 2016 (C). Statistical comparisons between pollen species are shown in Table 3.2.

3.4.4 Proportional floral selectivity

The variation in the representation of plant species within pollen sampled from nest cells cannot be understood without taking into consideration the availability of flowers within the areas. The availability of flowers varied between plant species and years (Table 3.2). An index of selectivity was therefore developed based on the ratio of pollen species in nest cells to the availability of flower species in the landscape. In this way this analysis takes into account both the spatial and temporal availability of floral species and provides a basis for assessing selectivity. An index of 1.0 indicates that a pollen species was found in nest cells in the same proportion as the plant species' proportional availability in the landscape. Indices <1.0 indicate avoidance, while >1.0 indices indicate that the pollen species was actively selected by the foraging bees. For those plant species that were found in both the floral survey and as pollen in nest cells, a one-sample t-test was used to compare their observed index of selectivity to that expected if the plant species was represented equally in nest cells (as pollen) and the landscape (as flowers) (Table 3.3).

Table 3.2. Grouping of pollen species according to their proportional representation in samples taken from nest cells. There were no significant differences between pollen species within a group ($p > 0.05$); but significant differences between pollen species in different groups ($p < 0.001$).

Nominal group	**Plant species included in group**
Group 1	*R. repens, Acer* spp*., Raphanus* spp. in year 2; *T. repens* in year 1.
Group 2	*R. repens* in year 2; *Prunus* spp. in year 1, *R. fruticosus*
Group 3	*Salix* spp. in year 2
Group 4	*C. nigra, C. monogyna, Vicia* spp*.* in year 1; *Prunus* spp. in year 2.
Group 5	*C. monogyna, R. fruticosus, C. monogyna* in year 2
Group 6	*Raphanus* spp.*, Acer* spp.*, R. acris* year 1; *P. spinosa* year 1 & 2; *Quercus* spp. Year 2.
Group 7	*Vicia* spp*., R. acris* in year 2.
Group 8	*Quercus spp., T. pratense,* unknown*, Salix* spp. in year 1; *B. napus* year 1 & 2; *T. repens* in year 2.
Group 9	*Taraxacum officialis agg.,* unknown, *R. carnia* year 1 & 2

Prior to this analysis the index of proportional selectivity was normalised with a log transformation; hence an index equivalent to a lack of selectivity or avoidance was zero (Figure 3.4).

The index reveals that *Taraxacum* spp. was avoided by foraging bees in both years, while other plant species occurred at levels greater than expected from their representation in the landscape (Figure 3.4, Table 3.3). It is also important to recognise that some species were recorded in the floral survey, but were never found in nest cells as pollen. These are not shown in Figure 3.4, but are listed with their proportional abundance in the landscape in Table 3.2. These species were more strongly avoided than all species, including *Taraxacum* spp., because despite being available their pollen was not present in any samples of pollen from nest cells. Several of these species, including *Galium aparine* and *Urtica diocia*, were more abundant as flowers in the landscape than many of the species that were frequent as pollen in the nest cell provisions (Table 3.2). Only a single pollen, identified to genus *Brassica* spp., was found in the nest cell provisions yet was never encountered in the floral survey. This species was either extremely scarce and was overlooked in the floristic survey or was located beyond the anticipated flight radius of the bees.

3.4.5 Nest provisioning amino acid analysis

3.4.5.1. Total amino acid (AA) content g/100g: Results show there were significant differences in total amino acid content in g/100g between years ($F = 7.208$, d.f. = 1, 59, $p = 0.009$), between boxes ($F = 5.595$, d.f. = 2, 59, $p= 0.006$) and between sample periods ($F = 6.319$, d.f. = 2,59, $p = 0.003$).

Tukey post-hoc analysis confirmed that samples of nest provisions taken in year one had higher levels of protein than year two ($p <0.01$). Samples of nest provisions taken from boxes sited in unenriched grassland (Grass1) and wild bird seed plant areas (WBS) did not differ, however those from nectar-rich plant areas (NS) had higher levels of protein than boxes from the other 2 sites ($p <0.05$). Finally, samples of the nest provisions taken in early season and late season did not differ to each other, but samples taken from nest provisions in mid-season had higher levels of protein than both early and late season samples ($p <0.01$).

Table 3.3. Mean proportion plant species in the field (species not recorded in a given year are represented by NA).

Plant species	**Year**	
	2015	**2016**
Plant species utilised by *Osmia*		
Taraxacum spp	0.0190	0.0098
Rubus fruticosus	0.0095	0.0051
Ranunculus repens	0.1451	0.1233
Raphanus spp	0.0692	0.3921
Centaurea nigra	0.0017	0.0010
Ranunculus acris	0.0056	0.0140
Brassica napus	0.0841	0.1465
Trifolium pratense	0.0005	NA
Cirsium spp	0.0018	0.0013
Vicia spp	0.0081	0.0153
Trifolium repens	0.0760	0.0227
Non-utilised species		
Galium aparine	0.1930	0.0082
Urtica dioica	0.1496	0.1076
Hyacinthoides non-scripta	0.0239	0.0304
Fumaria officinalis	0.0219	NA
Geranium robertianum	0.0209	0.0032
Stellaria holostea	0.0185	0.0290
Glechoma hederacea	0.0184	0.0113
Brassica oleracea	0.0171	0.0062
Silene dioica	0.0169	0.0109
Rumex acetosa	0.0123	0.0038
Mercurialis perennis	0.0115	NA
Oenanthe crocata	0.0087	0.0031
Sinapis arvensis	0.0082	0.0042
Leucanthemum vulgare	0.0078	0.0061
Cerastium fontanum	0.0071	0.0013
Chaerophyllum temulum	0.0068	0.0065
Rumex obtusifolius	0.0055	0.0047
Sinapis alba	NA	0.0054
Ajuga spp	0.0048	0.0017
Veronica serpyllifolia	0.0046	0.0039
Heracleum sphondylium	0.0037	NA
Bellis perennis	0.0034	0.0036
Geum urbanum	0.0026	NA
Cardamine hirsuta	0.0021	NA
Cerastium arvense	0.0018	NA
Prunella vulgaris	0.0013	NA
Tripleurospermum inodorum	0.0011	NA
Ficaria verna	NA	0.0011
Stellaria neglecta	0.0010	0.0024
others	0.0049 (n = 15)	0.0036 (n= 6)

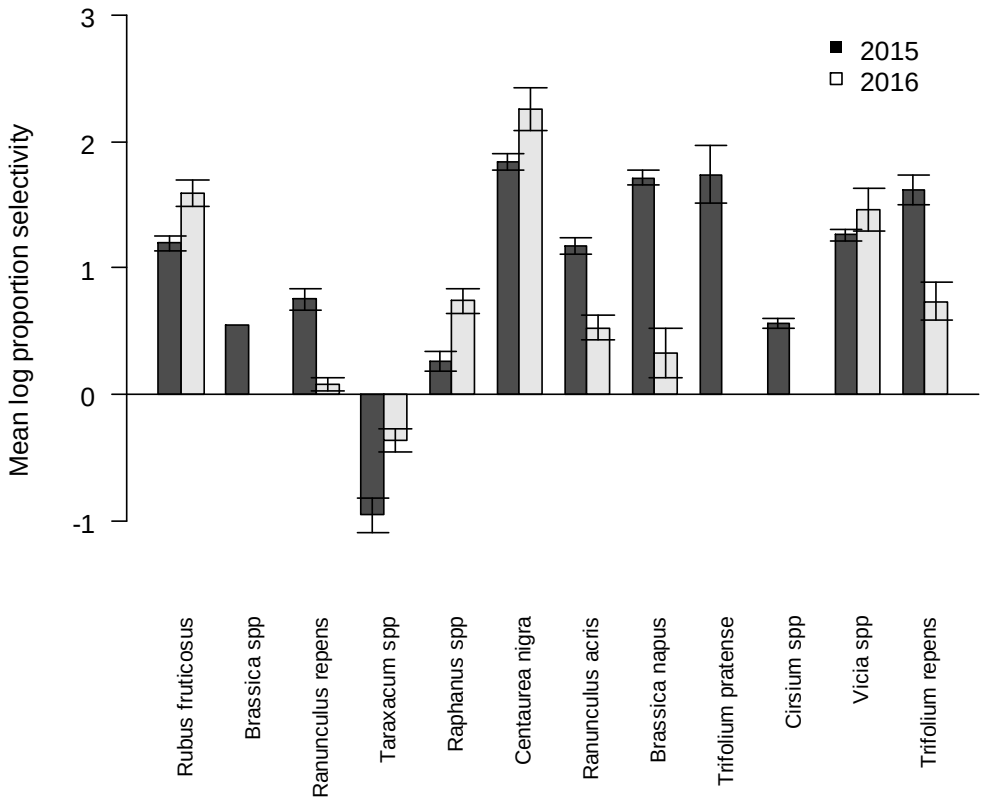


Figure 3.4. Mean (± SE) of the proportional selectivity (log) for plant sources of pollen based on the proportion of pollen source present in a sample taken from nest cells compared with its availability in the local area.

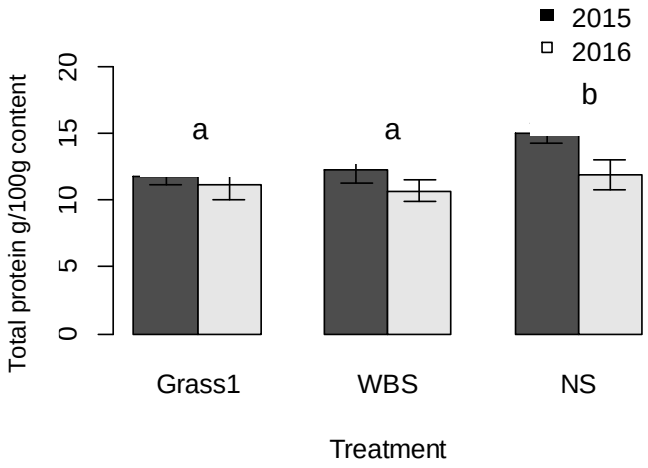


Figure 3.5. Mean total protein ±SE (g/100g) in nest provision samples taken from nest boxes placed in unenriched grassland (Grass1), wild bird seed plant mix (WBS) and nectar-rich plant mix (NS) areas. Treatments with different letters were significantly different ($p < 0.05$).

Table 3.4. Results of one tail t-test comparisons of the pollen species selectivity indices to zero (i.e. where representation in nest cells was equal to availability in the wider landscape).

Plant species	***Year 1***		***Year 2***	
	t	***p***	***T***	***p***
Rubus fruticosus	20.074	<0.001	14.731	<0.001
Ranunculus repens	8.7203	<0.001	1.44	>0.05
Taraxacum spp	-6.9348	<0.001	-4.0989	<0.05
Raphanus spp	3.4755	<0.01	7.5803	<0.001
Centaurea nigra	28.079	<0.001	13.059	<0.001
Ranunculus acris	17.88	<0.001	5.4719	<0.001
Brassica napus	29.85	<0.001	1.6286	>0.05
Trifolium pratense	7.6155	<0.01	NA	NA
Cirsium spp	15.816	<0.05	NA	NA
Vicia spp	27.132	<0.001	8.8449	<0.001
Trifolium repens	14.149	<0.001	4.9378	<0.01

3.4.5.2 Total non-essential amino acid content: There were significant differences in non-essential amino acid (NAA) content (g/100g) of the samples collected from nest provisions between years ($F = 15.782$, d.f. = 1, 59, $p < 0.001$), between boxes; grassland (Grass1), wild bird seed plant mix (WBS) and nectar-rich plant mix (NS) ($F = 5.362$, d.f. = 2, 59, $p = 0.007$) and between sample periods ($F = 10.057$, d.f. = 2,59, $p < 0.001$).

Tukey post-hoc analysis confirmed that the samples collected in year one had higher levels of NAA than year two ($p < 0.001$). Samples collected from boxes in areas of unenriched grassland (Grass1) and wild bird seed plant mixes (WBS) did not differ, however samples collected from boxes in nectar –rich plant areas (NS) had higher levels of NAA than those collected from boxes in the other two areas ($p < 0.05$) (Figure 3.6a). Finally, samples of nest provisions collected during early season and mid-season did not differ to each other, but samples collected in late season had higher levels of NAA than both early and mid-season samples ($p < 0.01$).

3.4.5.3 Total essential amino acid content: Total essential amino acid (EAA) content data was found to be over dispersed and was normalised following a sqrt transformation. There was no significant difference in EAA content (g/100g) between years. However there was a significant effect on EAA content of nest box ($F = 4.648$, d.f. = 2, 60, $p = 0.013$) and sample period ($F = 4.726$, d.f. = 2, 60, $p = 0.012$).

Tukey post-hoc analysis confirmed that the EAA content of samples of nest provision taken from boxes in the unenriched grassland (Grass1) and wild bird seed plant mix areas (WBS) did not differ, however samples from boxes in the nectar-rich plant mix area (NS) box had higher levels of EAA than WBS and Grass1 ($p < 0.05$) (Figure 3.6b). Finally samples of nest provisions taken early and mid-season did not differ in EAA content, neither did samples taken mid and late season. However, samples taken late season, contained higher levels of EAA than early season samples ($p < 0.05$). This result indicates more consistency in the EAA content of nest provisions than recorded in total protein content or NAA.

3.4.5.4 Individual essential amino acid content: Following sqrt transformation of the individual essential amino acid content data normality checks were carried out and the assumptions were met. During creation of the minimum adequate model, year was found to be non-significant and thus removed from the model.

There were significant differences in the levels (g/100g) of the individual amino acids EAAs of the samples of nest provisions ($F = 112.78$, d.f. = 8, 495, $p < 0.001$). As no interaction was found between the levels of the individual EAAs and the nest box and/or sample date, variation in levels of the individual EAAs were summarized independently of these factors.

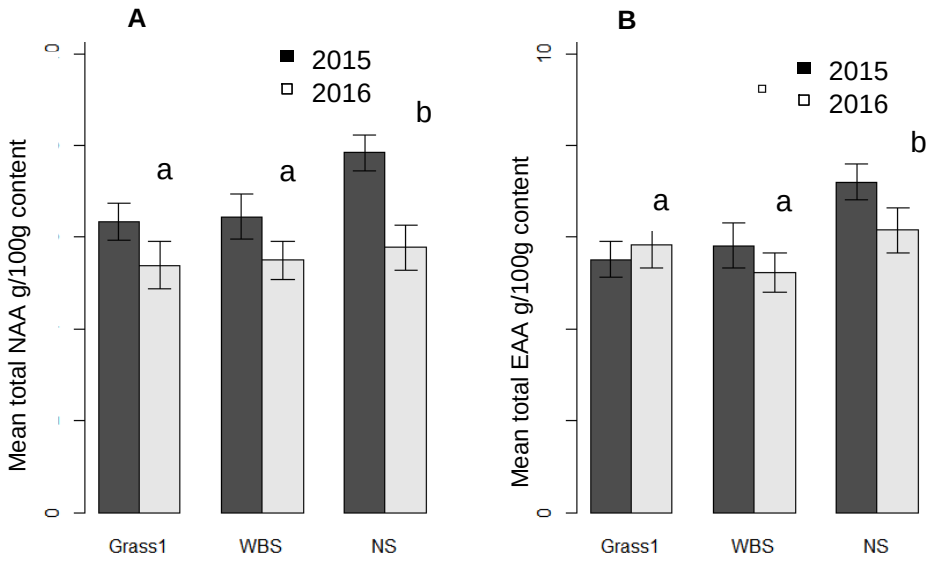


Figure 3.6. Mean (±SE) (A) total non-essential amino acid (NAA) and (B) total essential amino acid (EAA) content of nest provision samples taken from nest boxes placed in unenriched grassland (Grass1), wild bird seed plant mix (WBS) and nectar-rich plant mix (NS) areas. Treatments with different letters were significantly different ($p < 0.05$).

Tukey post-hoc analysis confirmed that the levels of leucine and lysine in the samples of nest provisions did not differ to each other and appeared in the higher amounts than all other individual EAAs ($p < 0.001$, Figure 3.7). The levels of valine, arginine, threonine, phenylalanine and isoleucine in nest provision samples did not differ from each other but all were at lower levels than those of leucine and lysine ($p < 0.001$) and higher than that of histidine ($p < 0.001$) which was higher than methionine ($p < 0.001$).

3.5 Discussion

This study investigated the utilisation of flowers in an agricultural landscape by *O. bicornis* when provisioning nest cells. The results provide indications of the potential effectiveness of current agri-environment schemes. This in turn allows for extrapolation of both the effectiveness of such schemes for solitary bees species,

which they were not developed specifically to target, but also to understand how the distribution and availability of specific floral resources may drive population dynamics. Overall effects of the different stewardship options were observed.

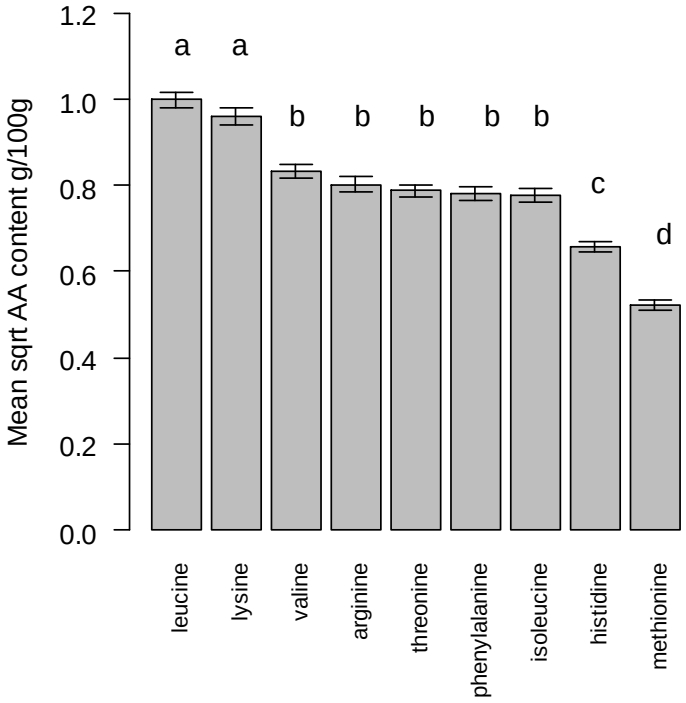


Figure 3.7. Mean (±SE) individual essential amino acid (EAA) content of nest cell provision samples taken from nest boxes placed in unenriched grassland (Grass1), wild bird seed plant mix (WBS) and nectar-rich plant mix (NS) areas. Different letters indicate significant differences between amino acid content of nest provision ($p < 0.001$). No significant differences occurred between the levels of each AA in nest cells constructed in the three habitat areas so data was combined. Treatments with different letters were significantly different ($p < 0.05$).

Nest boxes in the floristically enhanced areas, within the nectar-rich (NS) and wild bird seed plant mixes (WBS), yielded the highest numbers of nest cells (Figure 3.2). The boxes placed on the woodland edge (Wood1 and Wood2) contained low numbers of nest cells despite being within 100m of these floristically enhanced areas (Table 3.1, Figure 3.1). Although *O. bicornis* is reported to acquire pollen within a 150m flight radius of their nest (O'Toole, 2000) a number of factors may have been responsible for poor reproductive success in the woodland edge boxes. It is possible that hedgerows acted as barriers to foraging females and the shady

nature of the woodland edge may also have reduced the boxes' attractiveness for nesting. It is known that *O. bicornis* will leave their site of emergence to locate more favourable sites for nesting if conditions are sub-optimal (Teper, 2007). These factors may also have limited the reproductive success of *Osmia* in the unenriched grassland sites (Grass1 and Grass2). Reduction in nest cell construction in the floristically unenriched areas may however, simply reflect the increased energetic exposure of *O. bicornis* in such areas, as they have to travel further and expend more energy to collect sufficient suitable pollen in order to construct nests. This may also have knock-on indirect effects through increasing potential for parasitism of the cells, with the increased duration of absence during pollen collection of the female allowing more opportunities for parasitoids such as the sapygiid wasp (*Sapyga quinquepunctata*). One further factor towards cell construction may be that of migration, with individuals congregating towards areas nest boxes located closer to higher floral abundance or increased floral nutrition, whilst simultaneously leaving less suitable areas.

The results highlight the importance of both the floral composition of environmental stewardship options and their configuration in space and time. Indeed, the results challenge the accuracy of our current understanding of these aspects of *Osmia*'s biology. Placement of off-crop habitats to the correct scale in order to boost populations and maintain their presence within a crop area is of paramount importance. Current recommendations for the species composition of nectar-rich plants mixes, in which flower species are supposedly tailored towards boosting pollinators, might benefit from modification to suit the foraging season of specific species of bee. In the present study, the box sited on the WBS strip showed higher production of cells than the box sited on the bespoke nectar-rich strip designed to support pollinators (Figure 3.2). This may have been in part due to plants in the nectar strip flowering in the later summer outside of the effective foraging season of *O. bicornis*, whilst providing nectar sources for later flying *Bombus* and *Apis* bees (Carvell *et al.,* 2007).

The overall range of plants which *O. bicornis* utilised during this study was low, with high levels of consistency observed across year, although there was greater variation between areas. With more than 50 flower species available and observed throughout this study only a total of 19 species were utilised for nest provision of pollen (11 floral species, 8 tree species). Some of these were recorded at such low levels it is likely they resulted from adventitious collection, as demonstrated with the

low levels of *Taraxacum* spp. in nest cells (Figure 3.4). Extreme selectivity was however, demonstrated by *O. bicornis* females for other species, despite their low availability in the field, suggesting that pollen selection is not purely quantity driven but related to perceived value by the nesting females. Pollen sampled from nest provisions at each sample date typically consisted of a maximum of only 2-4 species. This seems to represent selection of specific species available over time and may reflect pollen mixing as required to match larval nutritional requirements (Eckhardt *et al.,* 2014).

In contrast to papers championing floral diversity for pollinators (Holzschuh *et al.,* 2007; di Pasquale *et al.,* 2013), results indicate high levels of selectivity by the females and may allow for the identification of “keystone” species. Species of particular importance based on the observed high levels of selectivity include members of *Prunus* and *Rosacea* families along with *Ranunculu*s, *Acer, Quercus* and *Salix*. These species are not specifically addressed in current conventional agri-environment schemes. To support more diverse pollinator assemblages, future schemes could therefore be expanded to promote favoured herbaceous plants, along with enhanced hedgerows and woodlands. Flower species which are selectively chosen either may produce pollen having greater nutritional value to support developing larvae or may represent lower foraging costs to adult females, when compared to alternative sources available. Greater analysis of nutritional characteristics of individual pollens is required in order to fully understand the basis of pollen selection. In some cases pollen mixing may be used as a strategy to alleviate the effects of otherwise harmful pollen constituents. Examples include the toxin ranuculin produced by members of the *Ranunculus* family which has been reported to increase mortality in bees in higher doses (Sedivy *et al*., 2012). Alkaloids present in some pollens have also been shown to affect survival of larval and adult honeybees and inhibit the development in the larvae of the solitary bee *O. bicornis* (Hitchcock, 1959; Detzel & Wink, 1993; Kevan & Ebert, 2005; Kempf *et al.,* 2010; de Mesquita *et al.,* 2010; Sedivy *et al.,* 2011; 2012; Gosselin *et al.,* 2013).

The present study only measures the response of one species, *O. bicornis*, and resource requirements for other solitary bee species may differ. Establishing the extent of interspecific variation would require extensive experimentation. In order to further explain the selectivity of such species exhibited by *O. bicornis*, examination of the basis underlying the differential values of pollens to them are required. The evidence from this study suggests that high quality pollen resources for pollinators,

especially solitary bees such as *O. bicornis,* may not be distinguishable solely based on amino acid content

The majority of research into nutrition in bees is based on work focusing on honeybees (DeGroot, 1953; Day *et al*., 1990; Cook *et al*., 2003). Within the *Apis* species, pollen is an essential source of protein, carbohydrates, lipids, minerals and vitamins, the lack of which may impede larval development (Day *et al*., 1990; Cook *et al*., 2003). DeGroot (1953) identified 10 essential amino acids for honey bees. Individual amino acid concentrations within pollens have been suggested to be more important in determining pollen quality for honeybees than total protein content alone (Nicolson, 2011) and isoleucine, leucine and valine are considered the most important EAA's in honey bees (DeGroot, 1953; Rogala & Szymaś, 2004). The results from analysis of the nest provisions of *O bicornis* in this study also suggest leucine and lysine are required in higher quantities for larval nutrition followed by isoleucine, phenylalanine, threonine and valine. This study was however, unable to follow *O. bicornis* development through to emergence to allow pollen and EAA profiles to be linked to emergence and potential for population growth and stability. Due to resource limitations for amino acid analysis this study could not undertake the analysis of the either the individual pollen sources found within the nest cells or of the abundant species not detected in nest provisions. Future studies are required to investigate the composition of non-targeted pollens and/or pollen mixes with different EAA profiles on development of *O. bicornis.*

Off-crop habitat enrichment under current agri-environment schemes currently do not meet requirements of a full range of insect pollinators (Wood *et al*., 2015). In order to better target the most prolific deliverers of pollination services, such as solitary bees (Breeze *et al.,* 2011), schemes could benefit from a higher degree of specificity. In order to further increase the value of agri-environment schemes to pollinators it may be necessary to introduce additional options which target aspects which are not currently addressed. For example, agricultural weeds have an important role within agroecosystems in supporting biodiversity (Marshall *et al*., 2003), and are shown here to be likely key resources providers for populations of solitary bees. Options which allow for the tolerance of a greater number of key flowering weed species, whilst minimising impact on crop yield, may be beneficial. A further issue highlighted by this study is the importance of the spatial arrangement of florally enriched vegetation. *Osmia bicornis* shows limited ability to nest successfully when floral resources are more than 50m distant. In order to improve the impact of

agri-environment schemes it is necessary to clearly both define the objectives and measure the outcome of interventions to allow a more targeted approach at a landscape scale.

3.6 Acknowledgements

I would like to thank Robert Kynaston for access to and accommodation at Great Wollaston farm throughout this field study.

Chapter 4: Amino acid composition of pollen and the larval development and survival of the generalist solitary bee (*Osmia bicornis)*

4.1 Abstract

The influence of amino acid content of larval pollen diet of the solitary bee, *Osmia bicornis*, was investigated. Six diets were offered to larvae in artificial nest cells, including pure *Camellia, Phacelia, Brassica napus*, or *Pinus spinose* pollen, a pollen mix consisting of 66% *Castanea sativa* pollen and 3 other species, and a pollen mix collected by naturally foraging *O. bicornis* adult females (60% *Ranunculus repens*, 25% *Crataegus monogyna* and 15% comprising three other species). The total protein, essential amino acid (EAA) and non-essential amino acid content, and levels of nine individual EAA in each diet were quantified. Lowest levels of all nutritional factors were found in the pure *Pinus spinosa* pollen, and the highest in the pure *Camellia* or *Phacelia* diets. Significantly lower survival of larvae fed the *Pinus spinosa* diet was recorded with the highest survival associated with the pollen mix collected by foraging *O. bicornis*. No significant effect of diet on larval development rate, pupal weight, pupal survival, or sex of emerging adult was recorded. The results are discussed in relation to the need for improved understanding of multiple nutritional factors that may contribute to both larval performance, and the definition of optimal plant species profiles that support large and stable solitary bee populations in the natural environment.

Key-words: Solitary bees, *Osmia bicornis,* pollen, amino acids, protein, larval diet, nutrition, larval development, mortality

4.2 Introduction

Pollination is a key ecosystem service within both wild and agricultural landscapes (Klein *et al*., 2007). The majority of flowering plants have evolved adaptations to attract animals in order to distribute pollen grains and enhance the potential for fertilisation. Bees (Apiformes) are an efficient group of pollinators, and widely considered the primary pollen vectors in most ecosystems (Michener, 2007). They

differ from other pollinating groups in that pollen and nectar are utilised both by the adults and for larval provisioning (DeGroot, 1953; Dobson & Peng, 1997), a relationship that has resulted in the evolution of specialised morphological and behavioural adaptations (Thorp, 2000).

The collection of pollen is energetically expensive, with individual floral visits yielding only small quantities (Müller *et al*., 2006), which presents a particular challenge to groups such as solitary bees in which there is no colony to contribute to brood provisioning. Furthermore the nutritional content of pollen varies significantly between plant taxa (Roulston & Cane, 2000), and within an individual plant species in relation to multiple abiotic factors (Suarez-Cervera *et al*., 1994; Dobson & Peng, 1997). In addition, some plant species have evolved to reduce pollen loss by attracting only a limited range of bees (e.g. deep corolla only accessible to long-tongued species) or producing pollen or nectar which contains toxins (Adler, 2000).

Osmia bicornis is a polylectic species that utilises flowers belonging to multiple plant families, providing larvae with nutritional resources containing mixtures of different pollen species (Cane & Sipes, 2006; Műller & Kuhlmann, 2008). This behaviour may have evolved to facilitate more efficient exploitation of spatially and temporally variable plant communities, and offers a variety of potential benefits. In addition to maximising floral resource utilisation to increase quantity of larval provisions (Williams & Tepedino, 2003), mixing of such resources can also mitigate the impact of pollens low in particular amino acids (or other nutritional components), and buffer pollens with harmful secondary metabolites (Budde & Lunau, 2007; Eckhardt *et al.,* 2014). This strategy may involve selective foraging based on real-time evaluation of pollen quality, and have resulted from co-evolution of the bee with multiple plant species. Currently there is limited evidence of the ability of solitary bees to assess pollen quality, but some *Bombus* species have been shown to preferentially select high protein pollens (Leonhardt & Bleuthgen, 2012). Improved understanding of the optimal chemical characteristics of pollens utilised by bees relies on a wider knowledge of the chemical composition of bee collected pollens, the physiology of developing bee larvae, the mechanisms of pollen digestion by pollen consumers, and resultant impact on individual fitness (Roulston & Cane, 2000; Eckhardt *et al*., 2014).

When offered different pollen diets, *O. bicornis* fed *Ranunculus acris* pollen has been shown to develop successfully but not when provided with *Echium vulgare* pollen, whereas the reverse has been reported for *Osmia cornuta* (Praz *et al*., 2008;

Sedivy *et al.,* 2011). Both species perform well on *Sinapsis arvensis* pollen but neither developed on *Tanacetum vulgare* pollen. Pollen of *E. vulgare* has been shown to contain pyrrolizidine alkaloids which are lethal to honey bees (Reinhard, 2011), and larval mortality of both solitary bee species differed significantly between diets. It has been concluded that the bees require physiological adaptations to address unfavourable chemical properties of particular pollen species (Sedivy *et al.,* 2011). Comparing diets with varying proportions of pollen from favourable and unfavourable species, Eckhardt *et al*. (2014) reported that feeding *O. cornuta* larvae with pure *R. acris* pollen resulted in high levels of mortality, whilst a 50% admixture with *S. arvensis* resulted in good larval survival, development times, and body mass. Thus, if it is mixed with a favourable pollen, larvae may benefit from the nutrient content of unfavourable pollen without being negatively affected. Pollen mixing may therefore be a strategy to optimise larval food quality and exploit pollen species with unfavourable chemical characteristics (Eckhardt *et al*., 2014), and has been reported for many bee species including *O. cornuta* and *O. bicornis* (Tasei, 1973).

Although the protein, carbohydrates, lipids, vitamins, minerals and starch contained in pollen are all required by honey bees, most studies have focused on amino acid composition an important indicator of nutritional value (Day *et al*., 1990; Cook *et al*., 2003). It has been suggested that ten amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) are essential for honeybees with isoleucine, leucine and valine needed in the largest quantities, and histidine, methionine and tryptophan in lowest quantities (DeGroot, 1953). Non-essential amino acids included alanine, cysteine, glycine, hydroxyproline, proline, serine and tyrosine (DeGroot, 1953). The amino acids are obtained from pollen but levels vary significantly between pollen species and can be related to larval performance (Cook *et al*., 2003; Somme *et al.,* 2015).

Thus the nutritional value of pollen is important for defining pollinator-plant interactions, but insufficient information on the chemical composition of bee-collected pollen is available to make generalizations on which plant taxa possess favourable chemical properties (Roulston & Cane, 2000; Eckhardt *et al*., 2014). This study investigated the influence of single and mixed pollen diets of known amino acid composition on the development of larvae of the generalist solitary bee *O. bicornis*.

4.3 Materials and methods

4.3.1 Insects

Osmia bicornis eggs were obtained from nest cells constructed within artificial nest boxes (Mason Bees Ltd., Shropshire, UK) by bees foraging on four lowland sites (Shrewsbury, Shropshire; Shawbury, Shropshire; Wakefield, Yorkshire; Hull, Humberside). Eggs were taken from nest cells using a micro spatula. Each egg was transferred to a covered Petri dish containing a damp filter paper and incubated in a constant environment (CE) cabinet (LEEC, SL2, Nottingham, England) at 5°C and 60% RH, in continuous darkness for 7 days prior to the start of the experiment. Collection of eggs from multiple sites reduced the potential for responses being affected by habituation of populations to local habitats, and to account for their differing origins, eggs from different sites were allocated between experimental treatments at random. Pollen from the nest cells constructed by bees at one of these sites (Shrewsbury, Shropshire) was also retained for use in one of the treatments.

4.3.2 Pollen

Treatments consisted of one of six pollen mixes. Four of these mixes were obtained from commercial sources, one from a commercial field, while the sixth was collected from nest cells constructed by free-foraging bees (henceforth referred to as “wild collected pollen”).

4.3.2.1 Commercially sourced pollen: Four pollens were purchased from commercial sources, including honey bee foraged sweet chestnut (“Chestnut mix”; *Castanea sativa*; TOCA®, Spain), *Camellia* (“Camellia“; Simianshan®, China), oilseed rape (“OSR”; *Brassica napus*; Simianshan®, China), and pine pollen (“Pine”; *Pinus spinosa*; Simianshan®, China). All China sourced pollens were collected from centrifuged flowers and thus contained no binding nectar or other apis secretions).

4.3.2.2 Field collected pollen: Phacelia pollen (“Phacelia”) collected by honey bees from an untreated commercial field-plot was obtained from Biochem Agrar GmbH, Germany.

4.3.2.3 Wild collected pollen: Pollen (“Wild pollen”) was collected from the same cells from which eggs were sourced at Great Wollaston farm, Shropshire, UK

(SJ329123). These nest cells were created within artificial nest boxes by free-foraging bees. Pollen was removed using a micro-spatula after the eggs had been removed for use in the laboratory experiment. The farm participates in the UK Higher Level Environmental Stewardship Scheme (Defra, 2018) and maintains areas sown with seed mixes designed to provide plant communities rich in pollen and nectar for pollinators.

All pollens sourced were no more than 3months old at the time of the experiment. Each of these pollens were homogenised using a wet and dry grinder (Andrew James Ltd., UK), and stored at -20°C prior to use in the experiment. This resulted in a fine powder consistency, both homogenising and breaking down individual pollen loads from honey bee collected pollens. All pollens were examined under a microscope to ensure no damage to the pollen grains had occurred, which may have affected the experiment.

4.3.3 Palynological analysis

Three samples of each of the homogenised pollen treatments were subjected to palynological analysis to confirm the plant species composition. In each case a sample of the pollen was added to 0.1ml of 50% isopropanol on a microscope slide. The sample was spread evenly across the glass surface (using a micro-pestle) before being heat fixed to the slide. Remnants of the lipid coating of the grains was removed by washing with 100% isopropanol, and the excess was evaporated by heating to 50ºC on a hotplate. A 2 x 2mm piece of standard safranin glycerol-gelatin (Brunel Microscopes Ltd., UK) was then placed over the residual pollen and heating continued until the gelatin had melted and dried, at which point a glass cover slip was applied to the stained preparation and the edges sealed using clear varnish. Fifty grains were identified from each slide using a compound light microscope (TEC Microscopes Ltd., UK) following the approach of Moore *et al*. (1991), and the percentage of each species found calculated. Where identification to species was not possible, genus was recorded.

4.3.4 Amino acid analysis

Three sub-samples of the homogenised pollen mixes used in each treatment where retained and analysed to determine amino acid content. Amino acid content of each of the homogenised pollen mixes was determined according to European Pharmacopoeia methodology (https://www.edqm.eu/en/european-pharmacopoeia-ph-eur-9th-edition) by Alta Bioscience Ltd (Redditch, UK). The ISO 17025:2005-accredited methodology (limit of quantitation 5 nmol; creatine/creatinine content cannot be determined) utilises acid hydrolysis prior to separation using a sodium citrate buffer, such that both the total of both free and previously protein/peptide-bound amino acids are quantified. Quantification of tryptophan and cysteine/cystine required an additional alkaline hydrolysis step and conversion of cysteine to cysteic acid prior to the acid hydrolysis. For further details see https://altabioscience.com/wp-content/uploads/2016/05/Amino-acid-analysis-tech-doc.pdf.

Methionine, tryptophan, arginine, lysine, histidine, phenylalanine, isoleucine, threonine, leucine, valine have been identified as the ten essential amino acids for honeybees (DeGroot, 1953). Therefore, in addition to the levels of individual amino acids in each treatment, total protein levels (which are equivalent to total amino acid levels), total essential amino acids (EAAs) and non-essential amino acids (NAAs) were determined.

4.3.5 Experimental procedure

Individual brood chambers (IBC) consisting of a beech wood block (40 x 40 x 22mm) with a 8 x 8 x 22mm channel milled into the top to form an artificial nest cell were constructed (Sedivy *et al.,* 2011; Eckhardt *et al.,* 2014). In each of the six treatments *O. bicornis* larvae were offered one of the pollen mixes described above. Each cell was provisioned with excess (500mg) of pollen and a single *O. bicornis* egg was laid on top of the provisions. The open side of the cell was covered with a glass cover slip (Fisher Scientific Ltd, Leicestershire, England) and secured with transparent tape (Sellotape, Cheshire, England) to allow observation of brood development. The IBCs were then incubated in a constant environment cabinet (LEEC, SL2, Nottingham, England) at 20°C and 60% RH in continuous darkness.

Larval development in each IBC was assessed at three-day intervals following daily checking for the first hatch date (Sedivy *et al*., 2011; Eckhardt *et al*., 2014). The

dates at which the following events were first observed were noted: egg hatch, feeding without defecating (larval stage 1), feeding and defecating (larval stage 2), and completion of the cocoon (defined as when the cocoon became opaque preventing observation of the larva within (Torchio, 1989). The cocoons were then carefully extracted and weighed, before being returned to the IBC for further observation.

Following the completion of the cocoons in late autumn (15 November) the incubation temperature was progressively reduced to 5°C over a period of 4 weeks (to avoid cold shock), and maintained until the following spring (22 February). The temperature in the CE cabinet was then progressively increased again to 20°C over a period of four weeks. Cocoons were monitored weekly to confirm successful emergence of adults. The sex of all insects (irrespective of survival) was identified using the method of Falk (2015). Insects that did not emerged were dissected from the cocoon prior to identification.

4.3.6 Statistical analysis

Statistical analysis were conducted using R Studio 0.99.903 (RStudio Team, 2015), and the following packages; survival (Therneau, & Grambsch, 2000), multcomp (Hothorn *et al.,* 2008), ggplot2 (Wickham, 2009) and lsmeans (Lenth, 2016). All data was tested for normality and Log or sqrt transformations applied where necessary. Factor reduction was conducted allowing for the removal of non-significant terms and interactions in order to reach the minimum adequate model for all statistical tests as described by Crawley (2013). During factor reduction ANOVA between models was used to verify that the validity of the statistical model was not affected, as per normal practice. For each treatment any eggs that failed to hatch were excluded from analysis.

4.3.7 Pollen amino acid composition

Total protein (g/100g) content, total non-essential and total essential amino acid (g/100g) content were all subjected to ANOVA. Individual essential amino acid content of each treatment was also subjected to ANOVA to investigate any potential relationship between relative EAA levels.

4.3.8 *Osmia* performance

Larvae that had completed their cocoon were defined as survivors irrespective of whether they later successfully completed metamorphosis. Kaplan–Meier survival statistics were used to compare larval survival on the different pollen diets (R Core Team, 2013). The number of days between egg hatch and completion of the cocoon was considered as 'censored data': individuals that died before the completion of the cocoon represented the exact observations for which the event (death) occurred, while those that completed the cocoon were the censored observations. GLM with binomial error structure was used to test for differences between survival distributions.

Dates of egg hatching, start of feeding, and start of defecation, were determined as the average of the two observation dates between which the respective event occurred. Due to the nature of the ranked larval development data, in order to investigate time to completion of cocoon, data was subjected to a Scheirer–Ray–Hare test nonparametric test. Post-hoc Dunn tests for each significant factor or interaction were conducted.

Finally, adult weight was subjected to an ANOVA.

4.4 Results

4.4.1 Palynological analysis of pollen

Palynological analysis of samples of the pollen resources offered to developing *O. bicornis* larvae showed that the commercially sourced pure *Camellia*, Pine and Oilseed rape pollen, and the *Phacelia* pollen obtained from commercial field plot, all contained only the expected pollen species (Table 4.1). The commercially sourced Chestnut pollen contained 66% *C. sativa*, with the remaining 34% consisting of pollen grains from three other plant species. Analysis of samples of wild collected pollen showed that 85% of the larval resource consisted of *Ranunculus repens* or *Crataegus monogyna*, with three different plant species represented amongst the remaining pollen grains.

4.4.2 Total protein content of larval pollen resources

Total protein content (g/100g) was found to be normally distributed and there was a significant effect of treatment ($F = 131.2$, d.f. = 5, 12, $p < 0.001$; Figure 4.1a). Tukey post-hoc tests confirmed that *Camellia* pollen diet had a higher total protein content than all other treatments except *Phacelia* ($p < 0.001$). Total protein was higher in *Phacelia* than in all treatments other than *Camellia* ($p < 0.01$), while that of Chestnut was higher than recorded in OSR, wild collected and pine pollen ($p < 0.001$). Wild collected and OSR pollen did not differ in total protein content, but both had higher levels than pine pollen ($p < 0.001$).

Table 4.1. The mean proportion of pollen grains from different plant species present in larval resources used in *O. bicornis* performance experiments.

Treatment Name	**Genus/Species**	**Mean proportion**
Wild collected pollen	*Ranunculus repens*	0.60
	Crataegus monogyna	0.25
	Centaurea nigra	0.10
	Lotus corniculatus	0.01
	Rubus fruticosus	0.04
Camellia Pollen	*Camellia spp*	1.00
Chestnut Pollen	*Castanea sativa*	0.66
	Prunus spp.	0.17
	Lotus corniculatus	0.10
	Brassica napus	0.07
Oilseed rape	*Brassica napus*	1.00
Phacelia	*Phacelia spp*	1.00
Pine	*Pinus spinosa*	1.00

4.4.3 Total non-essential amino acid content of larval pollen resources

Total non-essential amino acid content (NAA; g/100g) was normalised by log transformation prior to ANOVA. There was a significant effect of treatment on total non-essential amino acid content ($F = 230.1$, d.f. = 5,12, $p < 0.001$; Figure 4.1b).

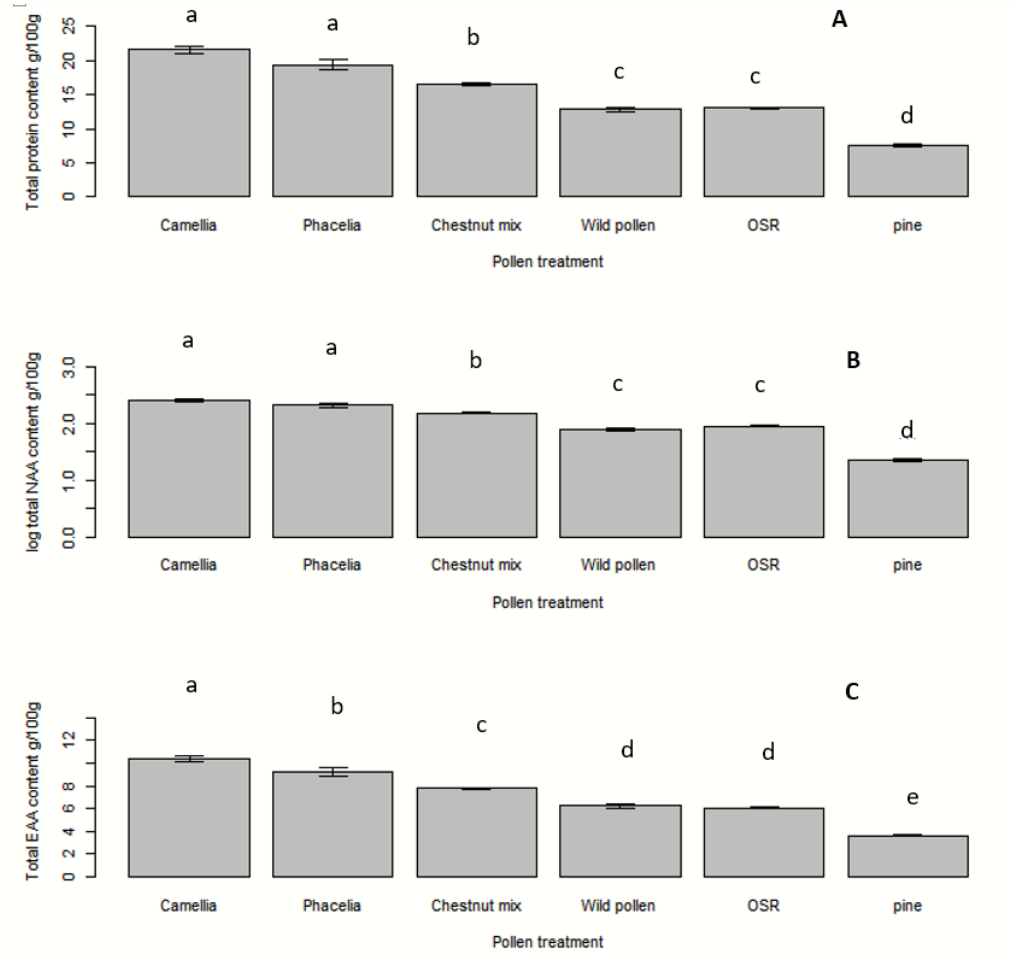


Figure 4.1. Protein and amino acid content (g/100g) of pollen resources offered to *O. bicornis* larvae. Mean (± S.E.) of assessments of three samples from each treatment for (A) Total protein content; (B) Total non-essential amino acid (NAA) content; (C) Total essential amino acid (EAA) content Bars with the same letter are not significantly different (p >0.05).

Levels of non-essential amino acids did not differ between *Camellia* and *Phacelia* pollen, and both were higher than in all other treatments (p <0.05). Higher levels were recorded in Chestnut pollen than in treatments (except *Camellia* and *Phacelia*; p <0.001). Non-essential amino acid content of wild collected and OSR pollens did not vary, but both had higher levels than pine pollen (p <0.001).

4.4.4 Total essential amino acids

Total essential amino acid content (EAA), (g/100g) was normally distributed, and a significant effect of treatment was identified ($F = 119.9.1$, d.f. = 5,12, $p < 0.001$; Figure 4.1c). Tukey post-hoc tests confirmed that *Camellia* pollen had a higher level of essential amino acid than *Phacelia* ($p < 0.05$), which in turn contained higher levels than all other treatments ($p < 0.01$). A significantly higher essential amino acid content was found in Chestnut pollen than in OSR, wild collected and pine pollen ($p < 0.01$). Wild collected and OSR pollen did not differ in essential amino acid content, but both had higher levels than was recorded in pine pollen ($p < 0.001$).

4.4.5 Individual essential amino acids

Following square root transformation normality checks were carried out and all assumptions were met. There was a statistically significant interaction between the effects of individual essential amino acids (EAA's) and treatment (g/100g; $F = 9.295$, d.f. = 40,108, $p < 0.001$). Since the interaction effect was significant, the 'Pollen treatment' effect cannot be generalised for all EAA's combined.

4.4.5.1 Leucine and isoleucine: *Camellia* pollen had higher levels of Leucine and Isoleucine compared with all other treatments ($p < 0.01$; Figure 4.2). *Phacelia* contained higher levels than all other treatments except *Camellia* ($p < 0.01$). More Leucine and Isoleucine was recorded in Chestnut pollen than in OSR, foraged pollen and pine pollen ($p < 0.001$). The amount of the two AAs recorded did not differ between wild collected and OSR pollen, but both contained higher levels than were found in pine pollen ($p < 0.001$).

4.4.5.2 Lysine and valine: *Camellia* and *Phacelia* pollen had similar Lysine and Valine content, but higher levels of both AAs compared to all other treatments ($p < 0.001$; Figure 4.2). Pine had lower levels than Chestnut, foraged pollen and OSR ($p < 0.001$). Lysine and valine content of wild collected pollen did not differ from that of Chestnut or OSR pollen, but Chestnut had higher levels than OSR alone ($p < 0.001$).

4.4.5.3 Arginine: *Camellia* and *Phacelia* pollen contained similar levels of Arginine, which were higher than in all other treatments ($p < 0.05$; Figure 4.2). Arginine content of wild collected and chestnut did no differ, both having higher levels than was

recorded for OSR pollen ($p < 0.05$). Pine pollen had a lower argenine content than Chestnut, wild collected ($p < 0.001$) and OSR ($p < 0.05$).

4.4.5.4 Phenylalanine: *Camellia* and *Phacelia* pollen did not differ from each other and *Camellia* had significantly higher Phenylalanine levels than all other treatments ($p < 0.05$; Figure 4.2). Levels in *Phacelia* and Chestnut were similar, but Chestnut pollen contained more phenylalanine than OSR, wild collected and pine pollen ($p < 0.001$). Wild collected and OSR pollen did not vary in phenylalanine content compared to each other, but both had higher levels than was recorded in pine pollen ($p < 0.001$).

4.4.5.5 Threonine and methionine: Threonine and methionine levels in *Camellia* and *Phacelia* pollen did not differ, and higher levels were present in both when compared all other treatments ($p < 0.05$; Figure 4.2). Chestnut pollen had higher levels than were recorded in the wild collected and Pine pollens ($p < 0.001$), but was similar to OSR. Wild collected and OSR pollens did not differ in threonine and methionine content, but had higher levels than pine pollen ($p < 0.001$).

4.4.5.6 Histidine: Similar histidine contents were recorded in *Camellia*, *Phacelia* and Chestnut pollens, and higher histidine levels were found in *Camellia* and *Phacelia* compared to the remaining treatments ($p < 0.01$; Figure 4.2). Chestnut pollen contained higher levels than was recorded in OSR ($p < 0.05$), wild collected and pine pollens ($p < 0.001$). Wild collected and OSR pollens did not differ from each other, but had higher levels of histidine than pine pollen ($p < 0.001$).

4.4.6 *Osmia bicornis* survival and development

During factor reduction no significant differences occurred between the survival of larvae offered *Camellia*, *Phacelia*, OSR or Chestnut pollen and as per common practice they were combined into a single factor for analysis, hereafter referred to as “other pollens”.

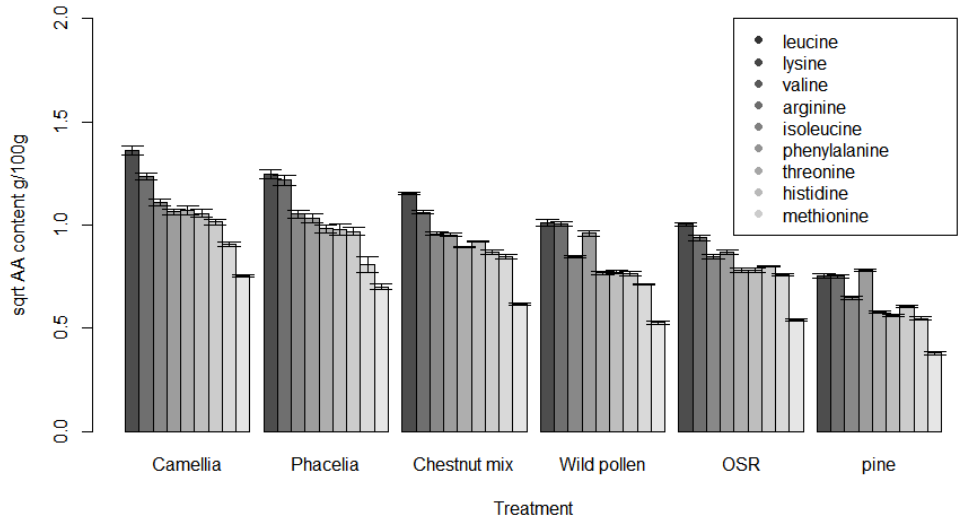


Figure 4.2. The levels of nine essential amino acids (AA) contained in the six pollen mixes used in treatments. Mean (± S.E.) of assessments (g/100g) of three samples from each treatment.

No effect of treatment on larval development time between egg hatch and pupation ($H = 11.06$, d.f. = 5, $p = 0.05$) or any interaction between treatment and time ($H = 9.69$, d.f. = 29, $p = 0.084$) was recorded. Similarly no significant differences were identified between individual components of larval development time (date of egg hatch, commencement of larval stages or date of cocoon completion).

Larval survival of *O. bicornis* (defined as survival from egg hatch to successful pupation) differed significantly between the pollen diets (Kaplan–Meier analysis: log-rank test, $x2 = 34.94$, d.f. = 2, $p < 0.001$; Figure 4.3). Significantly lower survival was recorded when larvae were offered pure pine pollen when compared to all other treatments ($Z = -5.36$, d.f. = 2, $p < 0.001$), with highest survival rates occurring when wild collected pollen was offered ($Z = -5.36$, d.f. = 2, $p < 0.001$).

Pupal weight at cocoon formation was found to be independent of bee sex, or pollen treatment ($F = 0.081$, d.f. = 4, 31, $p = 0.987$). All pupae survived to eclosion in all treatments.

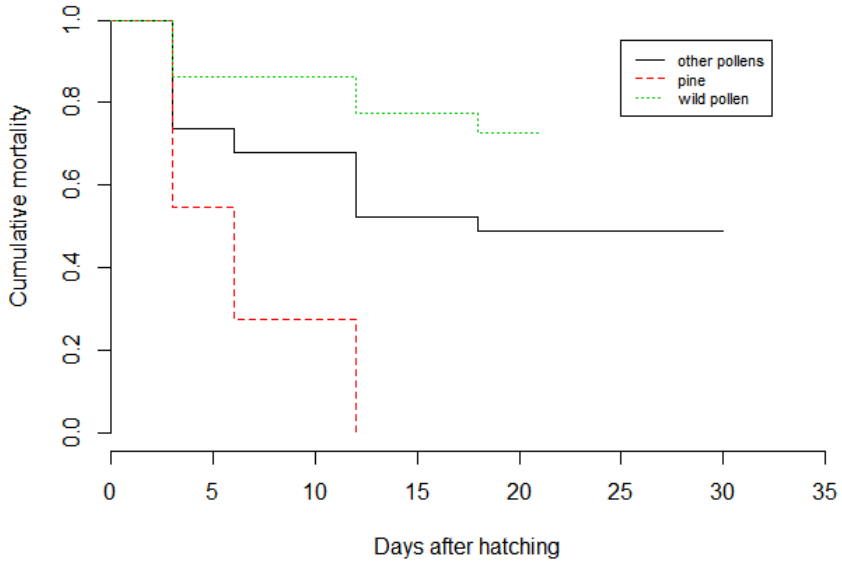


Figure 4.3. Cumulative survival of *Osmia bicornis* larvae when offered six different pollen diets. Mortality did not vary significantly between *Camellia*, *Phacelia*, OSR and Chestnut pollen treatments and were combined into a single factor ("Other pollens") for analysis.

4.5 Discussion

The wild pollen mix collected by adult *O. bicornis* foraging at the Shrewsbury field site was associated with the highest rate of larval survival to pupation (Figure 4.3). Extensive floral surveys conducted at the Shrewsbury field site, indicate a high degree of pollen selection by adult female *O. bicornis* when collecting larval resources (Chapter 3). The field site was botanically rich, and the high larval performance when compared to the artificially restricted man-made diets may indicate that the selection was, at least in part, related to nutrition quality, and that foraging females may be able to identify pollens, and pollen mixtures, favourable to successful larval development.

The importance of increasing flowering plant diversity when designing and incorporating pollinator-promoting habitats into landscapes is widely accepted (Hannon & Sisk, 2009; Holzschuh *et al*., 2012; Sidhu & Joshi, 2016). In the present study however, *O. bicornis* larval survival was similar when offered pure OSR, *Phacelia*, or *Camellia* pollens, and the chestnut dominated pollen mix. The latter displayed similar levels of species diversity to the wild collected pollen (Table 4.1),

but supported poorer survival. The current work may therefore support the proposal that, in addition to diverse plant resources, the inclusion of carefully selected individual keystone species is also important (Paine, 1969; Mills *et al*., 1993) and can promote the optimisation by foraging females of the nutritional composition of larval pollen diet. The critical nutritional components of pollen, however, need to be more fully elucidated because the relationship between larval performance and the amino acid content of diets was not straightforward in the present study. Keystone species promoting different groups of bees are also likely to vary. For example, in Chapter 5, the bumblebee, *Bombus terrestris* was offered the same pollens as used in the present study and exhibited improved colony performance when the pure *Camellia* and chestnut dominated mixes were offered to foraging workers compared to when pure oilseed rape pollen was available. The species composition of managed pollinator habitats should account for these varying requirements.

Overall total protein, total non-EAA and total EAA content varied consistently between the pollen resources offered to larvae in this study. The highest contents of all three nutritional components were recorded in the *Camellia* and *Phacelia* treatments, and the lowest in the pine pollen treatment. The chestnut dominated pollen mix also had consistently higher concentrations than the wild collected pollen mix. The poorest performance of *O. bicornis* larvae was recorded when they were fed with pine pollen leading to none surviving to pupation. The highest survival rates were however, recorded for larvae offered the wild collected pollen. It is possible that, provided the minimum required levels of protein/amino acids are available, assessment of their total content does not offer a reliable method of distinguishing between the relative nutritional values of different larval diets. More accurate predictions of performance may require more detailed consideration of specific amino acids and nutritional factors other than proteins. There are, however, parallels between the results for *O. bicornis* reported here and studies focussing on proteins in the diets of larval honeybees and these are considered briefly in the following paragraph.

More extensive work has been conducted on honeybees and it has been suggested that levels of ten specific amino acids are critical for successful development of their larvae (DeGroot, 1953; Cook *et al*., 2003; Nicolson, 2011). Individual levels of nine of the EAAs thought to be critical for honeybees (excluding tryptophan) were assessed in the present study. As was found for total amino acid content, the highest levels of all nine were recorded in *Camellia* and *Phacelia* pollen, with the

wild collected pollen (which was associated with the highest larval performance) having intermediate levels, while the pine pollen contained the lowest quantities. The quantities of isoleucine, leucine and valine, the three EAAs required in highest quantities by honey bees were amongst the five with the highest quantities in the pollen diets used in this study, with leucine present in the highest levels of all. Pine pollen contained significantly lower levels of all three of these EAAs compared with other pollens, while the highest levels were again found in *Camellia* and *Phacelia* pollen diets. Thus, with the exception of the low performance recorded for larvae fed on pine pollen, assessment of amino acid levels alone was not strongly associated with larval survival in this study, supporting the need for holistic studies of the combined effect of multiple nutritional factors.

Such a holistic overview may incorporate a range of other non-protein nutrients which have been implicated as important nutritional components for bees, such as carbohydrates, lipids, minerals, starch, sterols and vitamins, all of which can limit honeybee larval performance (Day *et al*., 1990; Cook *et al*., 2003). Inclusion of such components is supported by previous work which suggests that *Bombus* spp. can identify pollen sources with higher protein to lipid ratios (Vaudo *et al.,* 2016) enabling them to differentiate between pollens of different chemical compositions and quality as they forage. The mechanisms underpinning such nutritional discrimination are, however, not fully understood although chemo-tactile senses are thought to be important (Ruedenauer *et al.,* 2015). In some plant groups grain size may also be correlated with nutritional quality (Roulston *et al.,* 2000) offering another potential selection factor (Roulston *et al.,* 2000; Nicholls & Hempel de Ibarra, 2017), and nutrient extractions from the protoplasm of pollen grains (or subsequent assimilation) can be affected by the pollenkitt layer (Peng et al., 1985; Suarez-Cervera *et al*. 1994; Dobson & Peng, 1997; Williams, 2003). Pollen selection by foragers may also be affected by non-nutritional factors such as toxic secondary metabolites. For example, alkaloids which have been shown to affect survival of larval and adult honeybees and inhibit the development in the larvae of the solitary bee *O. bicornis* (Hitchcock, 1959; Detzel & Wink, 1993; Kevan & Ebert, 2005; London-Shafir *et al*., 2008; Kempf *et al.* 2010; de Mesquita *et al,.* 2010; de Assis Junior *et al.,* 2011; Reinhard, 2011; Sedivy, *et al*., 2011; Sedivy, *et al*., 2012; Gosselin *et al.,* 2013).

In conclusion, pollen species selection by foraging female *O. bicornis* may be important for provision of optimal larval nutritional resources. This study indicates

that low total essential and non-essential amino acid content, and low levels of each of nine individual essential amino acids in pollen offered to larvae, are all associated with increased mortality. It also suggests however, that provided minimum required levels of amino acids are available, one or more of a range of other nutritional factors may also contribute to variations in larval performance when offered different pollen diets. Further studies of the combined effect of multiple nutritional factors are therefore required before comprehensive conclusions can be drawn on the importance of pollen selection, or the plant species profiles in a landscape that promote the development of large and stable solitary bee populations.

4.6 Acknowledgements

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Chapter 5: Improving pollen nutritional quality reduces time to nest initiation in *Bombus terrestris audax* micro-colonies

5.1 Abstract

It has been hypothesised that amino acid content of the pollen in larval diets is a primary driver of colony success in polylectic bees. Pollen diversity in larval diets is thought to increase the potential for essential amino acids being present in sufficient quantities together with other essential nutritional components.

This study investigated the effect of five pollen diets (including single and multi-pollen mixes, three with varying proportions of the relatively unfavoured *Brassica napus* pollen) on the development of *Bombus terrestris audax* micro-colonies to test the hypothesis that colony performance is in part defined by the essential amino acid and total protein content of the diet.

The performance of micro colonies provided with the different diets was assessed by recording colony biomass gain, time of initiation of nest building, the number of brood produced (eggs, small and large larvae, pupae and drones), and nectar and pollen consumption.

Colony biomass gain varied between diets. Stronger colony performance correlated with higher levels of nine essential amino acids (leucine, lysine, valine, arginine, isoleucine, phenylalanine, threonine, histidine, methionine) but there was no consistent relationship between the diversity (number) of pollen species contained in a diet and biomass gain.

The poorest performance was recorded in micro-colonies offered the pure oilseed rape pollen diet, the treatment with the lowest levels of all nine amino acids. Performance variation related to the proportion of oilseed rape pollen in the diet suggested that eggs were produced and nest building initiated later in those with high levels of this pollen, reflecting the lower amino acid content.

The results are discussed in relation to previous studies and the selection of plant species for use in habitats designed to promote bee populations.

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Key Words: *Bombus terrestris audax*, nutrition, amino acids, pollen mixing, colony performance

5.2 Introduction

Bumblebees (*Bombus* spp.), are a key group of wild pollinators; displaying high pollination efficiency, and the ability to forage on a wide variety of flowers and plants (Reynolds & Fenster, 2008). Foraging behaviour in *Bombus* spp. is highly selective and they utilize a sub-set of the flower species available (Harmon-Threatt *et al*., 2017). It has been suggested that amino acid, lipids, vitamins and mineral content of pollen are important parameters affecting flowers selection (Moller, 1995; Robertson *et al.*, 1999; Nicolson, 2011; Willmer, 2011). For example, Kitaoka and Nieh (2009) showed that when the protein concentration in pollen was doubled, successful foraging attempts were increased by three-fold. Protein concentration is especially important for bumblebees as they respond to protein-rich pollens, compared to honeybees in which respond more strongly to the quantity of resources available (Leonhardt & Blüthgen, 2012; Brunet *et al*., 2015).

Individual foragers in the buff tailed bumblebee *Bombus terrestris (*L) maintain a degree of floral consistency (Raine and Chittka, 2005; Goulson, 2009), while differences in preferences between foragers result in the colony as a whole utilizing a wider range of plant species (Free 1970). Using a diverse pollen diet for larval provisioning has been reported to result in stronger colony development in this species (Baloglu & Gurel, 2015). The importance of pollen diversity for pollinators is widely recognized (Free, 1970; Carvell *et al*., 2006a; Carvell *et al*., 2006b) and many

recent habitat management schemes have focused on increasing floral diversity to enhance pollinator populations (Girard *et al.*, 2012). In the UK, environmental stewardship schemes promote a range of species mixes and sowing options to enhance botanical diversity of arable land (e.g. Carvell *et al.*, 2007), but it has been suggested that further work is required to ensure that their impact on wild bee colonies is optimized (Albrecht *et al.*, 2007). Field studies have aimed to establish bumblebee preferences by analysing the species composition of pollen loads collected during foraging, which often contain multiple species of pollen (Cresswell & Robertson, 1994; Carvell *et al.*, 2006b; Jha *et al.*, 2013; Somme *et al.*, 2015; Kriesell *et al.,* 2017). These studies may, however, have limited generality because they are necessarily restricted to specific geographic locations. Pollen provisions collected from bees on foraging flights, will be affected both by the prevalence of plant species growing within the bees foraging range, and resource competition (Kleijn & Raemakers, 2008), and will thus be subject to spatial and temporal variation (Carvell *et al.,* 2006a; Leonhardt & Blüthgen, 2012). For example, Carvell *et al.* (2007) compared different arable margin management options used under the UK Environmental Stewardship Scheme, and reported that of the six options investigated, legume-based flower mixes were the most attractive to bumblebees. In a second study in which florally rich habitats containing 30 species of flowering plants were offered to bumblebees (including legumes), Jha *et al.* (2013) found that bumblebee pollen loads were not dominated by Fabaceae (Legume). Thus, the outcomes of such field comparisons can be difficult to interpret.

Ruedenauer *at al.* (2015) demonstrated that bumblebees use olfactory cues to distinguish between different pollens but can only discriminate between food varying in nutrient content after antennal contact, concluding that chemotactile senses are essential for regulation of nutrient intake. Changes in chemo or gustatory receptor sensitivity based on haemolymph concentration of nutrients after consumption, and leading to post-ingestive behavioural responses have also been suggested. (Vaudo *et al.,* 2016). As bumblebees are not thought to employ intra-colonial feedback mechanisms individual workers may utilise such mechanisms to optimise nutrient collection rather than foraging opportunistically, leading to the apparently variable pollen preferences recorded at different sites (Kriesell *et al.,* 2017). If individual workers can assess the nutritional quality of pollen, then collection from different plant species may enhance individual and colony fitness through provision of improved larval nutritional resources. Mass flowering crops, combined with

floristically poor margins, may limit the ability of bumblebees to forage optimally and result in larval diets that are deficient in essential nutrients (Moerman *at al.,* 2017).

The ratio and level of the major nutritional components of pollen, including proteins, amino acids, lipids (including phytosterols), carbohydrates, vitamins, carotenoids and flavonoids are related to its nutritional value for bumblebees (Vanderplanck *et al.,* 2014; Somme *et al.,* 2015). Pollen can vary both quantitatively in terms of total protein and lipid concentration, and qualitatively in terms of sterol and amino acid profiles (Moerman *et al.,* 2016) but most studies have focussed on protein and amino acid content (Kriesell *et al.,* 2017). Amino acid composition is thought to be a better determinate of pollen quality for bees than total protein content; for example, *Eucalyptus* spp. pollen has a high protein content but is deficient in the essential amino acid isoleucine, reducing its nutritional value (Nicolson, 2011). Kriesell *et al.* (2017) compared pollen mixes collected by foraging bumblebees and reported that despite differences in pollen species collected, the essential amino acid profile of their diets remained constant.

Assessing the role of selective foraging on colony success relies on an understanding of the effect of pollen diet on bumblebee colony performance. Colony development and brood production, bee physiology and immune system function has been widely investigated (Dance *et al.,* 2017), with colony fitness being assessed using parameters such as egg production, larval weight, larval ejection, adult body size, adult mortality and longevity, and the number of active workers exiting nests (Regali & Rasmont, 1995; Tasei & Aupinel, 2008a; Kitaoka & Nieh, 2009; Vanderplanck *et al.,* 2014; Kriesell *et al.,* 2017).

Colony responses to defined pollen diets may be investigated using laboratory based micro-colony experiments to identify response parameters that can subsequently be verified under field conditions (Génissel *et al.*, 2002). Tasei and Aupinel (2008b) compared micro-colonies with entire colonies of *B. terrestris*, reporting comparable performance assessments when the same diet was fed. Micro-colony studies conducted by Génissel *et al.* (2002) and Vanderplanck *et al.* (2014) indicated that mixed pollens were more favourable than the mono-species diets tested. Moerman *et al.* (2017) suggested that the amino acid content of pollen is one of the primary drivers of colony success, with pollen diversity being important as it increases the potential for essential amino acids being present in the pollen mix collected by bumblebees, and can also increase the likelihood of other essential nutritional components being included. A range of studies comparing the impact of

different pollen diets on colony performance are required, however, to allow nutritional components of diet to be linked with specific biological mechanisms that lead to observed colony level outcomes.

This study investigates the effect of five defined pollen diets on the development of *B. terrestris audax* micro-colonies to test the hypothesis that colony performance is defined, in part, by the essential amino acid content of the diet.

5.3 Materials and methods

5.3.1 Micro-colony establishment

Queenless *B. terrestris audax* micro-colonies were established using bees taken from stock colonies (each consisting of approximately 60 worker bees) sourced commercially from Agralan Ltd., Swindon, UK (originating from Biobest®, Belgium). Prior to use, colonies were fed on Biobest standard pollen mix (Biobest®) and *ad libitum* proprietary liquid sugar solution, and maintained for a 7 day acclimation period in a CE room at 27°C, 65% RH, with an 8:16 Light-Dark cycle (Yoon *et al.*, 2002; Amin *et al.*, 2007).

The micro-colony arenas were modified from those used by Elston *et al*. (2013) and consisted of 500 ml open-topped plastic containers (11 cm diameter × 7 cm deep), closed with muslin mesh. The base of each cage was lined with filter paper to reduce condensation, and a small ball of cotton wool was added to encourage nest building.

Artificial nectar solution (honey solution: 60 %, w/v Rowse Pure Honey and water) was offered ad libitum to micro-colonies in lidded plastic feeding tubes (length = 10cm, Φ = 1cm, with an upward-facing feeding hole (Φ = 2mm) pierced at one end) inserted at a 30° angle through a hole in the side of the colony cages. Pollen was offered to the colony using a similar tube (except that a 10 x 20 mm feeding trough was cut at one end of the tube instead of the feeding hole) inserted horizontally into the colony cage so that it was separated by 180° in each direction from the nectar tube.

Feeding tubes were weighed, re-filled and re weighed at 2 day intervals ensuring that a minimum of 2g of pollen and 8mls nectar where available throughout the

experiment. Three worker bees were transferred from stock colonies to each micro-colony cage at the start of the experiment, and bees that remained inactive for 1 hour after transfer were replaced.

5.3.2 Treatments

One of five different commercially sourced pollens or pollen mixes were offered in different treatments. The control treatment was fed with the Biobest standard pollen mix (standard pollen mix) that had been used to feed the stock colony (henceforth referred to as “Standard pollen mix”). Experimental treatments included a commercially- sourced Organic Chestnut Pollen mix (TOCA®, Spain; “Chestnut pollen mix”), Pure *Camellia* pollen (Simianshan®, China; “Camellia”), pure oilseed rape pollen (Simianshan®; China; “OSR”), and a mixture of 50% Biobest standard pollen mix and 50% OSR pollen (Standard pollen/OSR

5.3.3 Palynological analysis

To investigate pollen purity and composition a palynological analysis of each pollen treatment was conducted. Stained slide preparations were made from each of three random samples taken from each homogenised treatment; 0.1ml of 50% isopropanol was dropped onto a blank microscope slide, then a sample of pollen was transferred to the liquid, spread evenly across the slide using a micro-pestle before being heat fixed to the slide. The slide was washed with 100% isopropanol to remove remnants of the lipid coating of the grains in preparation for staining, and dried on a hotplate at 50ºC until the excess had evaporated. To stain the samples a piece of approximately 2 x 2mm standard safranin glycerol-gelatin (Brunel Microscopes Ltd., UK) was placed over the residual pollen. When the gelatin had melted and dried, a cover slip was placed over the stained pollen sample and the edges sealed using clear varnish.

Pollen identification was carried out at 400× magnification using a Microtec compound microscope (TEC Microscopes Ltd., UK). A minimum of 50 grains were selected at random from each slide, identified to at least genus (Sawyer & Pickford, 1981) and the percentage contribution of each genus/species to the sample was determined. Pollen grains that could not be identified were recorded as ‘unknown’.

5.3.4 Amino acid analysis

Sub-samples of the homogenised pollen mixes used in each treatment where retained and analysed to determine amino acid content by Alta Bioscience Ltd (Redditch, UK), according to European Pharmacopoeia methodology (https://www.edqm.eu/en/european-pharmacopoeia-ph-eur-9th-edition); this is an ISO 17025:2005-accredited method and the limit of quantitation was 5 nmol. The proteins and/or peptides within the sample are broken down by acid hydrolysis into their individual amino acids, the final extract is therefore a sum of amino acids which were free in solution and those which were previously incorporated into proteins. The amino acids within the hydrosylate are then separated using a sodium citrate buffer system prior to detection. Tryptophan and cysteine/cystine are usually lost during acid hydrolysis. Creatine and creatinine cannot be analysed using this method. The results were presented in two groups, essential amino acids which are either not produced or produced in insufficient amounts by the body (and must be obtained from the diet), and non-essential amino acids that are produced by the body but can be supplemented by the diet.

5.3.5 Assessments

5.3.5.1 Nectar and pollen consumption: Consumption of nectar and pollen by each micro-colony was calculated from the difference between feeder weight at the start and end of each 2-day period and expressed as mean consumption (g) per bee (taking account of recorded mortality).

5.3.5.2 Mortality: Mortality (if any) in each micro-colony was recorded at the end of each 2-day period and dead bees were removed but not replaced.

5.3.5.3 Nest building: Each micro-colony was observed at the end of each 2-day assessment period and the first nest building activity (either wax cell or honey pot construction) recorded.

5.3.5.4 Final micro-colony performance: After the 37-day experimental period, all micro-colonies were euthanized by placing the colony cage in a -20°C freezer for between 24 and 26 hours. Nests (including all the wax material and the brood

inside) were weighed and dissected, and the number of eggs, small larvae (<0.8 cm across when curled), large larvae and pupae were recorded. The number of drones produced were counted and weighed. The sum of the drone weight and nest weight (including immature bees) was recorded as 'colony biomass gain'.

5.3.6 Statistical analysis

Statistical analysis was conducted using R Studio 0.99.903 (RStudio Team, 2015), and the following packages; multcomp (Hothorn *et al.,* 2008), ggplot2 (Wickham, 2009) and lsmeans (Lenth, 2016). All data were checked for normality and Log or sqrt transformations applied where necessary. Factor reduction was conducted allowing for the removal of non-significant terms and interactions in order to reach the minimum adequate model for all statistical tests conducted as described by Crawley (2013). During factor reduction, analysis of variance (ANOVA) between models was conducted to verify that the validity of the statistical model was not affected, following normal practice.

5.3.6.1 Pollen amino acid composition: Total amino acid content of each pollen treatment (g/100g) was normalised using sqrt transformation and subjected to ANOVA with Tukey post-hoc test to confirm where significant differences occurred between treatments. The analysis was repeated for total non-essential amino acids (NAA) and total essential amino acids (EAA) only.

5.3.6.2 Nectar and pollen consumption data: The effects of treatments on nectar and pollen consumption were analysed using repeated measures analysis of covariance (ANCOVA). Tukey's post-hoc test was used to confirm where significant differences occurred.

5.3.6.3 Nest initiation: Time before nest initiation (first nest building activity) was compared between treatments using generalized linear (GLM) with binomial error structure.

5.3.6.4 Worker mortality and brood production data: The number of dead workers, eggs, larvae (both small larvae and large), pupae and drones present in each treatment were compared between treatments using GLM with Poisson error distribution, and quasi-Poisson error distribution where data was over-dispersed.

5.3.6.5 Colony biomass gain: The weight gain of the colony in each treatment was analysed using repeated measure analysis of variance. Turkey's post-hoc test was used to confirm where significant differences occurred.

5.4 Results

5.4.1 Palynological analysis of commercially sourced pollen

Palynological analysis showed that two of the three commercially sourced single species pollen samples used in the experimental treatments, Camellia and OSR, contained only the species noted on the product label (Table 5.1). Sweet chestnut pollen represented 65% of the grains identified from the pollen mix marketed as "Organic Chestnut Pollen", with the remainder including a large (>7%) proportion of each of three other genera/species (*Prunus* spp., *Lotus corniculatus* (Birds foot trefoil), *Brassica napus* (Oilseed Rape). These four pollen species contributed more than 99% of the pollen grains in the Chestnut pollen mix treatment and also appeared in the commercially sourced Standard pollen mix which included eight different species (Table 5.1).

5.4.2 Pollen amino acid composition

5.4.2.1 Total amino acid content: There was a significant difference between the total amino content of treatments ($F = 126.3$, d.f. = 4,10, $p < 0.001$; Figure 5.1a). Tukey post-hoc tests confirmed that the total amino acid content of the Camellia treatment was higher than in all other treatments ($p < 0.001$). The OSR treatment had a significantly lower total amino acid content than all other treatments ($p < 0.001$), but the Chestnut pollen mix, Standard pollen mix and Standard pollen/OSR pollen mix did not vary from one another ($p > 0.05$).

5.4.2.2 Total non-essential amino acids: A significant difference between the total NAA content of treatments was identified ($F = 94.95$, d.f. = 4, 10, $p < 0.001$). Camellia pollen had higher levels of NAA than all other treatments ($p < 0.001$), and OSR lower levels than both the Chestnut pollen mix and the Standard pollen mix treatments ($p < 0.001$).

5.4.2.3 Total essential amino acids: Significant differences were also recorded between the total EAA content of the pollen treatments ($F = 112.7$, d.f. = 4, 10, $p < 0.001$; Figure 5.1b). Tukey post-hoc tests confirmed that Camellia pollen had higher levels of EAA than all other treatments ($p < 0.001$), with OSR having lower levels than the other treatments ($p < 0.001$). The Standard pollen mix had higher levels of EAA than the Chestnut pollen mix ($p < 0.05$) and the Standard pollen/ OSR mix ($p < 0.001$).

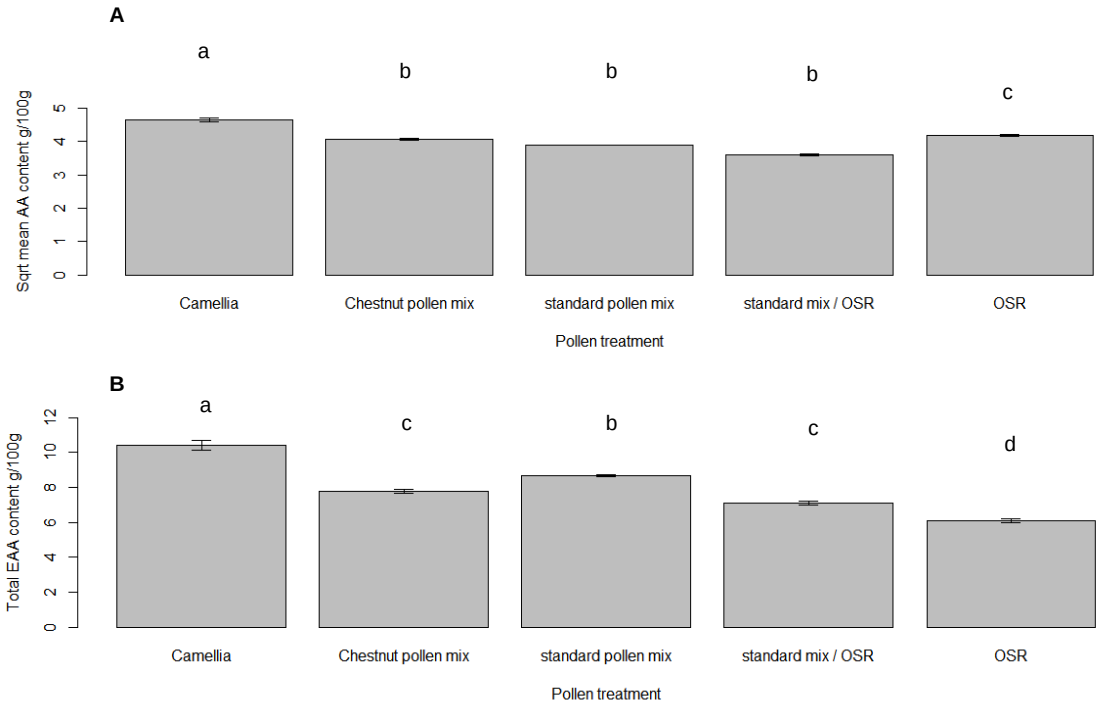

Figure 5.1. Mean (± S.E.) (A) Total mean amino acid (AA) content (g/100g) or (B) Total essential amino acid (EAA) used in treatments. Mean (± S.E.) of assessments (g/100g) of three samples from each treatment. Bars with the same letter are not significantly different ($p > 0.05$).

Table 5.1. Palynological analysis of the commercially sourced pollens used in the experimental treatments. Proportion of pollen grains that were identified to each constituent species and mean percentage of each species present.

Commercial Name	**Content Pollen**	**Prop of Pollen Grains**			**%**
		Smpl 1	**Smpl 2**	**Smpl 3**	**Mean**

Standard Pollen Mix	*Taraxacum officinale* (Dandelion)	0.16	0.13	0.19	16.3
	Pinus spp.	0.07	0.10	0.0	5.7
	Brassica napus (Oilseed rape)	0.22	0.33	0.28	27.6
	Prunus spp.	0.22	0.05	0.16	14.2
	Castanea sativa (Sweet chestnut)	0.05	0.05	0.05	5.2
	Salix spp.	0.11	0.26	0.19	18.8
	Lotus corniculatus (Birds foot trefoil)	0.05	0.0	0.07	4.2
	Ranunculus repens (Creeping buttercup)	0.11	0.08	0.05	8.1
Camellia	*Camellia* spp.	1.0	1.0	1.0	100.0
Chestnut Pollen mix	*Castanea sativa* (Sweet chestnut)	65.5%			
	Prunus spp.	17.2%			
	Lotus corniculatus (Birds foot trefoil)	9.5%			
	Brassica napus (Oilseed rape)	7.0%			
	Unknown	0.9%			
OSR	*Brassica napus* (Oilseed rape)	100.0%			

5.4.2.4 Individual essential amino acids: All assumptions of normality were met. There was a statistically significant interaction between treatment and individual EAAs ($F = 13.77$, d.f. = 32, 90, $p < 0.001$). Tukey post-hoc tests confirmed that significant differences in levels of individual EAAs occurred between treatments (Figure 5.2).

5.4.2.5 Leucine: Camellia pollen contained higher levels of leucine when compared to all other treatments ($p < 0.001$). The Chestnut pollen mix and the Standard pollen

mix both had higher levels than the Standard pollen / OSR mix ($p < 0.05$, $p < 0.05$) and OSR ($p < 0.001$, $p < 0.05$), but did not vary from each other ($p > 0.05$). Finally the Standard pollen/OSR mix had higher levels than OSR ($p < 0.001$).

5.4.2.6 Lysine: Camellia pollen and the Standard pollen mix had higher levels of lysine when compared to all other treatments ($p < 0.001$). The Chestnut pollen mix contained higher levels than the Standard pollen/OSR mix ($p < 0.05$) and OSR ($p < 0.001$).

5.4.2.7 Valine: Camellia pollen had higher levels of valine ($p < 0.001$) and OSR lower levels than all other treatments ($p < 0.01$).

5.4.2.8 Arginine: Higher levels of arginine were recorded in the Camellia pollen treatment when compared to all other treatments ($p < 0.001$). The Chestnut pollen mix and Standard pollen mix both had higher levels than were found in OSR ($p < 0.001$), but did not vary from each other ($p > 0.05$).

5.4.2.9 Isoleucine: Camellia pollen had higher levels of isoleucine compared to all other treatments ($p < 0.001$). The Chestnut pollen mix and the Standard pollen mix both had higher levels than OSR ($p < 0.001$), but did not vary from one another ($p > 0.05$). The Standard pollen/OSR mix also had higher levels than OSR ($p < 0.05$).

5.4.2.10 Phenylalanine: Camellia pollen had higher levels of phenylalanine compared to all other treatments ($p < 0.001$). The Chestnut and the Standard pollen mix had higher levels than were recorded in OSR ($p < 0.001$, $p < 0.001$), with the Standard pollen mix also having higher levels than then Standard pollen / OSR mix ($p < 0.001$).

5.4.2.11 Threonine: The Standard pollen mix and Camellia pollen had higher levels of threonine compared to all other treatments ($p < 0.001$).

5.4.2.12 Histidine: OSR contained lower levels of histidine than were recorded in Camellia, the Chestnut pollen mix or the Standard pollen mix ($p < 0.05$).

5.4.2.13 Methionine: Camellia pollen had higher levels of methionine compared all other treatments ($p < 0.001$), with the exception of the Standard pollen mix ($p > 0.05$). The Standard pollen mix was found to contain higher levels than were recorded in OSR ($p < 0.01$).

5.4.3 Consumption of honey solution

Data describing honey solution consumption were normalized using square root transformation. Repeated measure ANOVA showed that consumption per bee varied over time ($F = 14.547$, d.f. = 1, 943, $p < 0.001$), but was not affected by treatment ($F = 1.969$, d.f. = 4, 74, $p = 0.108$).

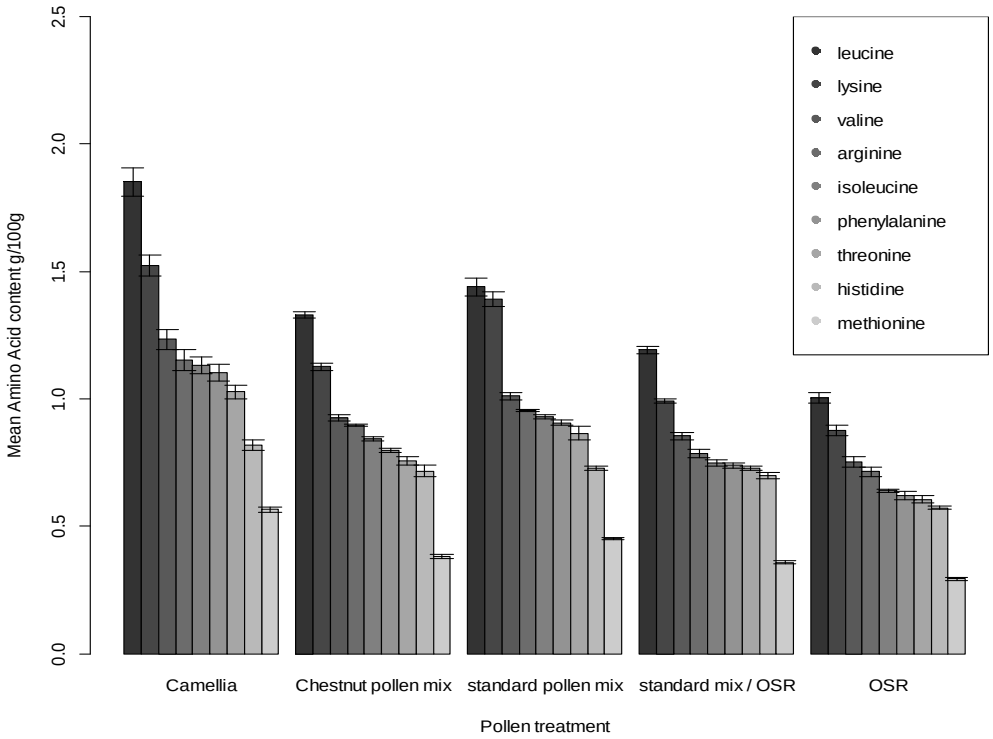

Figure 5.2. The levels of nine essential amino acids contained in the five pollen mixes used in treatments. Mean (± S.E.) of assessments (g/100g) of three samples from each treatment.

5.4.4 Pollen consumption

Pollen consumption was normalized by log transformation. Repeated measure ANOVA indicated that both treatment ($F = 12.107$, d.f. = 4, 69, $p < 0.001$) and time (day; $F = 15.550$, d.f. = 1, 1085, $p < 0.001$) significantly affected pollen consumption per bee (Figure 5.3). No interaction between treatment and day was found ($F = 2.334$, d.f. = 4, 1338, $p = 0.054$). Post-hoc t-test analysis indicated that a significantly higher weight of pollen was consumed when bees were offered the

Standard pollen or Chestnut pollen mixes than when the Standard pollen/OSR, Camellia or OSR pollen were available (Table 5.2)

5.4.5 Worker mortality

Mortality of workers was low (6.3%) and GLM with Poisson error structure found no significant differences between treatments ($p > 0.05$).

Table 5.2. Post-hoc t-test analysis of weight of pollen consumed by micro-colonies offered different pollen mixes.

T- test results

	Standard pollen	Chestnut	Camellia	OSR
Standard pollen mix	-	-	-	-
Chestnut pollen mix	$p > 0.5$	-	-	-
Camellia	**$p < 0.01$**	**$p < 0.001$**	-	-
OSR	**$p < 0.001$**	**$p < 0.001$**	$p > 0.05$	-
Standard mix / OSR	**$p < 0.001$**	**$p < 0.001$**	$p > 0.05$	$p > 0.05$

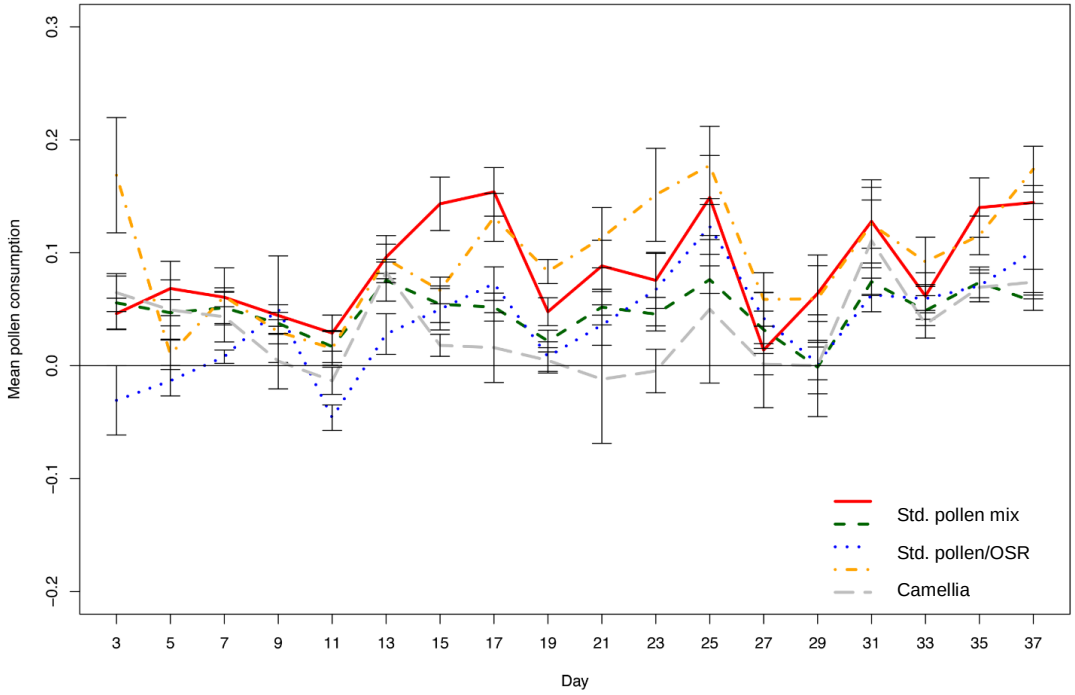

Figure 5.3. Mean (± S.E.) pollen collected per bee (g) during the 37 days of the experiment when micro-colonies were offered different pollen mixes.

5.4.6 Micro-colony biomass gain

Micro-colony biomass gain during the 37 days of the experiment varied significantly between treatments ($F = 5.811$, d.f. = 4, 74, $p < 0.001$) (Figure 5.4). ANOVA and Tukey post-hoc analyses confirmed that lower colony biomass was recorded in the pure OSR pollen and Standard pollen/OSR mix when compared to that recorded in the Chestnut pollen treatment which attained the highest biomass gain ($p < 0.01$, $p < 0.01$).

5.4.7 Components of biomass gain

5.4.7.1 Initiation of nest building: The day on which nest building commenced in micro-colonies subjected to different treatments varied significantly (Figure 5.5). During the creation of the minimum adequate model no interaction between day and treatment was found and so was removed from the model, although overall a GLM with binomial error structure showed that the proportion of nesting micro-colonies increased with time ($z = 14.848$, d.f. = .1416, $p < 0.001$) All other treatments were

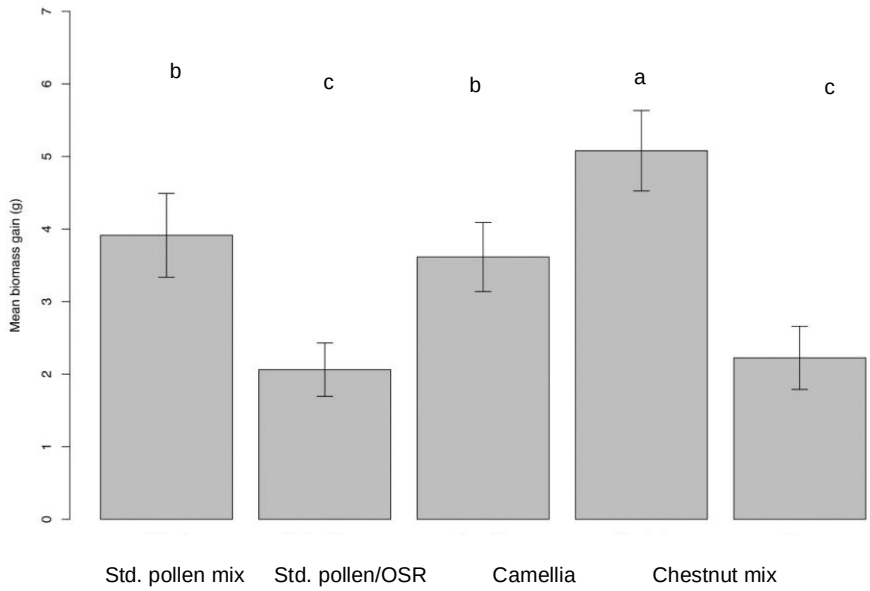

Figure 5.4. Mean (± S.E.) biomass gain (g) of micro-colonies (day 37 of the experiment) in treatments offered five different commercially sourced pollen mixes. Bars with the same letter are not significantly different (p >0.05).

found to differ significantly from the Standard pollen mix, with the standard pollen/OSR mix and pure OSR pollen displaying later nest initiation ($z = -4.131$, d.f. = 1416, $p < 0.001$; $z = -3.057$, d.f. = 1416, $p = 0.002$) Micro-colonies offered the Camellia or Chestnut pollen treatments commenced nest building earlier than those offered the Standard pollen mix ($z = 4.075$, d.f. = 1416, $p < 0.001$; $z = 2.287$, d.f. = 1416, $p = 0.022$).

5.4.7.2 Brood production: All brood data were found to be over-dispersed and was analysed using a GLM with quasi-poisson error structure. When nests were dissected at the end of the experiment (day 37) significantly more eggs were recorded in the treatment that was offered the Standard pollen/OSR mix (in which later nest initiation had been recorded), than in all other treatments (Figure 5.6a; $t = 3.641$, d.f. = 77, $p < 0.001$).

The total number of larvae (small + large larvae) found in the micro-colonies was lower in the Standard pollen/OSR mix treatment (Figure 5.6b; $t = -2.458$, d.f. = 77, $p = 0.016$) than in the other treatments. In addition, significantly fewer small larvae, were recorded in the Standard pollen/OSR mix treatment than in other treatments on day 37 (Figure 5.6c; $t=-2.337$, d.f. = 77, $p = 0.022$).

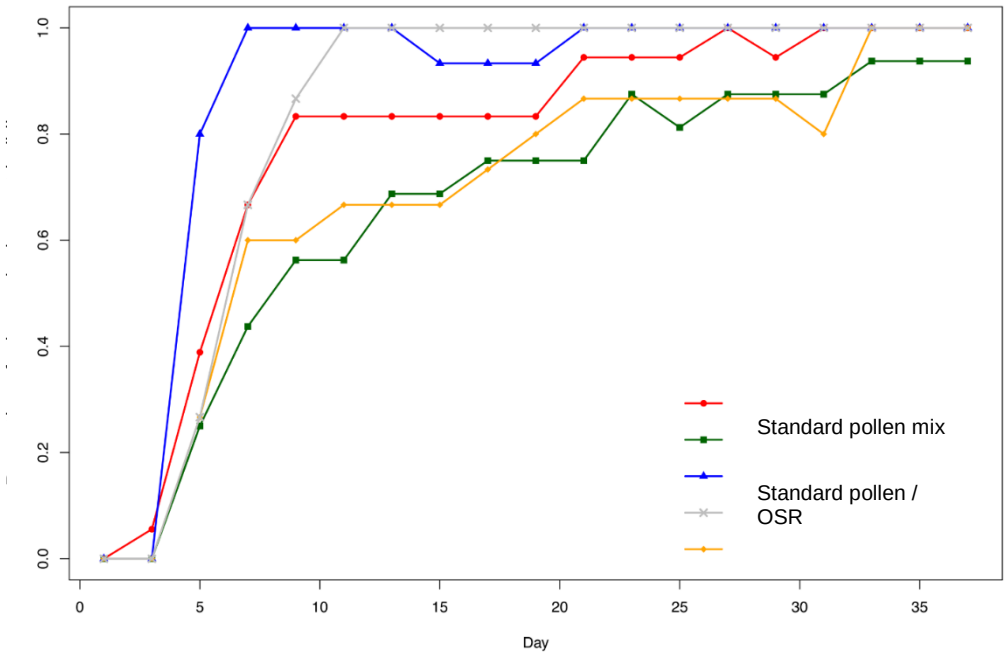

Figure 5.5. Proportion of micro-colonies displaying nest building activity on sequential assessment days, when offered different pollen mixes. Reduced proportions in adjacent assessment days occurred when small numbers of nests were initiated on feeding tubes and destroyed when the tube was removed for weighing/refilling.

Very low numbers of both pupae and drones were recorded in all treatments, and no significant differences were found between treatments in either case. Combining these data with that for larger larvae to investigate the impact of treatment on production of “older brood” in the experiment, significantly fewer older brood (total number of large larvae, pupae and drones) were found in nests from both the Standard pollen/OSR mix treatment (Figure 5.6d; $t = -2.701$, d.f. = 76, $p = 0.008$) and the treatment fed pure OSR pollen ($t = -2.542$, d.f. = 76, $p = 0.013$), the two treatments displaying the latest nest initiation

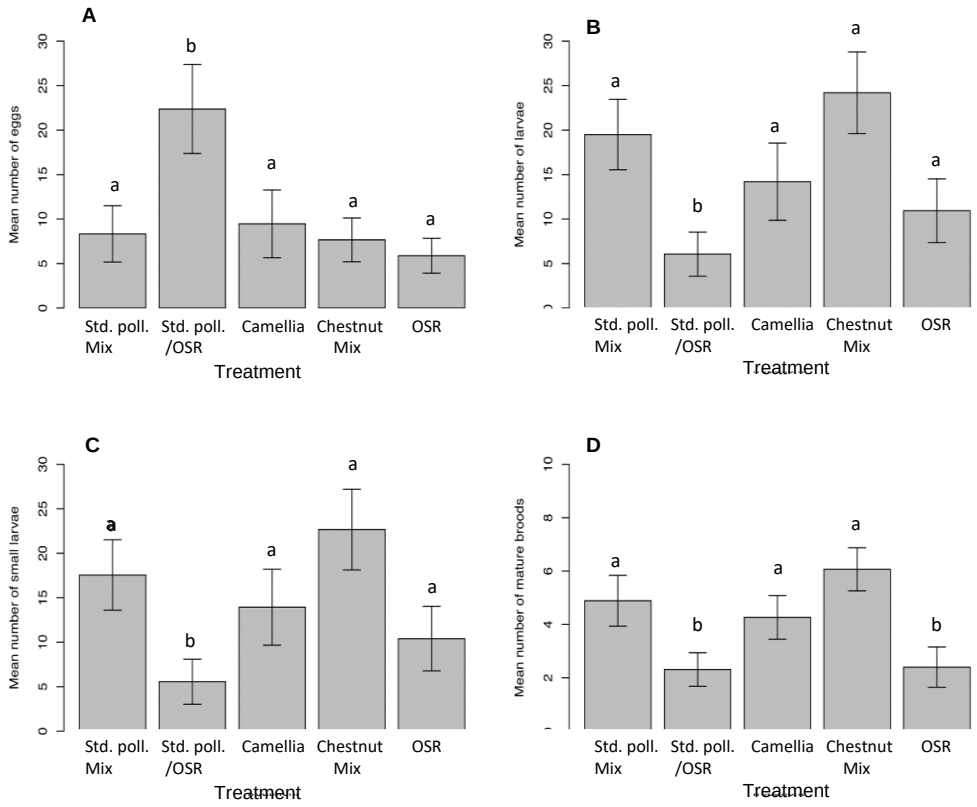

Figure 5.6. Mean (± S.E.) number of brood per micro-colony recorded (day 37 of the experiment) in treatments offered different commercially sourced pollen mixtures. Mean number of: A = of eggs, B = total larvae (small + Large), C = small larvae, and D = older "brood" (including large larvae, pupae and drones). Error bars show ±1 standard error of the mean, calculated from linear models. Bars with the same letter are not significantly different (p >0.05).

5.5 Discussion

Different species of pollen are known to vary in both physical structure and chemical composition (Roulston & Cane, 2000) and previous work has suggested that larval diets that contain a range of pollen species are more favourable for bumblebee colony development than mono-species diets (Génissel *et al.*, 2002; Vanderplanck *et al.*, 2014). Moerman *et al.* (2017), however, proposed that pollen nutrients better explain differences in colony performance than pollen diversity and that the amino acid content of pollen is one of the primary drivers of colony success. Analysing pollen loads collected by foraging workers of five different species of *Bombus* spp., Kriesell *et al*. (2017) reported that although the individual pollen species collected

varied widely in their amino acid content, lower variability in the essential amino acid content of the overall pollen loads were recorded, suggesting selective foraging may result in improved nutritional quality of diets fed to larvae. In the present study we investigated the impact on micro-colony performance of both single species pollen and diverse pollen mixes which contained significantly different amino acid profiles, when offered as food to queenless *B. terrestris* micro-colonies.

Consistent responses to different pollen diets were recorded when components of biomass gain were investigated. All diets offered were consumed and successful nest construction and brood production was recorded in all treatments. Low mortality of worker bees (6.3%) was recorded across all treatments, honey solution consumption did not differ between treatments, and both were similar to levels recorded in other studies (Elston *et al*., 2013; Thompson *et al*., 2014) implying that all diets offered at least the minimum required levels of nutrition for colony growth. Pollen consumption, however, varied significantly with both time and treatment, possibly a response to nutrient content.

Micro-colonies offered the Chestnut pollen mix (containing 67.3% sweet chestnut with the remaining portion dominated by three other species) achieved the highest colony biomass gain during the 37 days of the experiment. The lowest biomass gains were recorded from those micro-colonies fed on either pure OSR pollen, or the Standard pollen mix combined in equal proportions with OSR pollen (the mix containing eight different species with 65% of pollen grains being OSR). Micro-colonies fed with the Standard pollen mix on its own (containing the same 8 pollen species with OSR contributing 28% of the pollen grains) recorded a mean biomass gain that was not significantly lower than the Chestnut pollen mix. Thus, although the highest growth rate was associated with a diverse pollen source, no simple correlation between diverse pollen diets and biomass gain was identified in this study. Instead, evidence was obtained that when diets contained high proportions of OSR pollen there was a depression of biomass gain. Thus species composition, as well as diversity may be important.

There were significant differences in the timing of nest initiation (and associated egg production) following establishment of micro-colonies. Colonies offered a diet of the Chestnut pollen mix or pure *Camellia* pollen commenced nest building activities earlier than those offered the Standard pollen mix. Micro-colonies offered either a pure OSR pollen diet, or the Standard pollen mix/ OSR (containing about 65% OSR pollen), took significantly longer to initiate nest building than those offered the

Standard pollen mix on its own. This may reflect that egg production in holometabolous insects is a nutrient limited process reliant on sufficient resources being available (Wheeler, 1996). Hoover e*t al.* (2006) demonstrated that both larval and adult honey bees offered high protein diets had the highest levels of ovary development, but that adult nutrition had a greater influence on fecundity. Delays in nest initiation recorded in this study, associated with diets containing higher proportions of oilseed rape pollen, may therefore reflect delays in ovary development and egg laying. Although bumblebee nests are initiated by the queen in spring, nest enlargement is subsequently undertaken by other castes (Michener, 2007), and further work is required to determine whether the results of this study are predictive of queen-right colonies. Responsiveness of nest building is important as it can promote synchrony with periods during which optimal floral resources are available in the field (Geib et al 2015).

Brood recorded at the end of the experiment also varied between the treatments. Significantly more eggs, but fewer larvae, were recorded in the treatment that was offered the Standard pollen/OSR mix, than in other treatments. This is expected, based on the observation of later initiation of nest building in this treatment and associated delays in egg laying, and a smaller proportion of eggs having hatched by day 37. It is also in agreement with the results from micro-colonies offered pollen with a high proportion of OSR, where significantly fewer older brood (total number of large larvae, pupae and drones) were found compared to micro-colonies offered pollen mixes with lower levels of OSR pollen. Colony biomass was also significantly higher in the Chestnut pollen mix treatment with the earliest nest building activity initiation.

The focus of previous micro-colony studies of the effect of nutrition on colony development has often been on larvae, which are frequently considered to be the most sensitive stage, and potential effects on nest building activity are rarely considered (Genissel *et al*., 2002). Many micro-colony studies of nutrition have terminated experiments much earlier than the current work (12-14d after the first eggs were laid), and data were not collected on later colony development (Tasei & Aupinel 2008b; Moerman *et al.*, 2015). Published work that has maintained micro-colonies for longer periods of time, has also encountered both oophagy and larval ejection, with associated difficulties when interpreting results (Génissel *et al.*, 2002). This study indicates that assessment of colony success should not rely on the presence of larvae alone, but should take into account a range of other factors. The

biomass gain results in this study reflected the variation between treatments in the number of older brood (large larvae, pupae and drones) in nests, suggesting that total biomass gain may be a comprehensive parameter to measure the overall brood production of the colony.

Previous studies have indicated that poly-floral pollen is more likely to be beneficial for both honeybee and bumblebee colony health and reproductive success, suggesting that species-rich habitats such as gardens offer better resources than conventional farmland which often contain significantly lower floral diversity (Goulson *et al.*, 2002; Huang, 2012; Dance *et al.*, 2017). This is thought to result from the nutritional limitation of mono-species pollens, whereas poly-floral pollens have multiple contents that may be nutritionally-complimentary to each other. Such theory has been widely accepted and diversity has been a key factor when creating and promoting pollinator-friendly land use, such as in environmental stewardship schemes. This study provides information regarding potential criteria that might be considered when selecting plants for use in such pollinator promoting habitats.

Camellia pollen was tested as a mono-species treatment, and scored well in comparison with the other species mixes tested, supporting previous work that non-native ornamental species can be a valuable resource (Gunarsson & Federsel, 2014). Indeed, gardens with a diversity of exotics can provide a stronghold for bumblebees (Goulson *et al.*, 2010). Similarly, the Chestnut pollen mix supports the view that well-planned woodlands and wild woodlands may also provide a source of bumblebee nutrition (Osborne *et al.*, 2008). It is often suggested that OSR is a major pollen source for pollinators in agricultural environments (Stanley *et al.* 2013; Carruthers *et al.* 2017). Comparisons in this study clearly show however, mixes containing higher levels of oilseed rape pollen were associated with lower colony performance, casting doubt on this conclusion.

A recent study of bumblebee larval development concluded that the amino acid content within the pollen is a key factor determining performance of bee colonies (Moerman *et al*., 2017). If the essential amino acid(s), or combination of amino acids and other important nutritional components can be established, then selection of plants used in habitats designed to promote pollinators can be assisted by chemical analysis of candidate pollens or directed breeding of plants that are rich in certain amino acids. The results presented here (particularly those involving OSR) support the conclusion that pollen chemistry may be important for definition of pollinator-plant interactions. Insufficient information on the chemical composition of bee

collected pollen, its impact on developing bee larvae, and mechanisms of pollen digestion by consumers, make identification of key chemical components difficult, which in turn limits generalizations on plant taxa that contain favourable or unfavourable chemical properties (Roulston & Cane, 2000; Eckhard *et al*., 2014).

Although a range of essential nutrients for honey bees have been identified (carbohydrates, lipids, protein, sterols, vitamins, minerals, and starch; Day *et al*., 1990), amino acid composition is often used to assess nutritional quality (Cook *et al*., 2003). Early studies suggested arginine, leucine, isoleucine, lysine, tryptophan, methionine, phenylalanine, histidine, valine, and threonine are essential for honeybees (DeGroot, 1953). Of these, leucine, isoleucine and valine were needed in largest quantities; tryptophan, methionine and histidine in lower quantities. With the exception of tryptophan, significant differences between treatments in the levels of all these amino acids were recorded in the current bumblebee study. The lowest levels of each was found in the pure oilseed rape pollen, which was associated with the lowest micro-colony biomass gain, and significantly higher levels were detected in the pollens offered to the highest performing colonies (Chestnut pollen mix, Camelia pollen, Standard pollen mix). The five essential amino acids that were present at the highest levels in diets used in the bumblebee experiment included leucine, isoleucine and valine.

Cook *et al*. (2003) compared two pollen species as diets for honey bees (oilseed rape and *Vicia fabae*) concluding that oilseed rape, being richer in valine, leucine and isoleucine, was of greater nutritional quality than *V. fabae*, and that this was consistent with foraging preferences being based on pollen nutritional quality. Bumblebee micro-colonies performed least well when fed on oilseed rape pollen in the current study, it is notable that these three essential amino acids (leucine, isoleucine and valine) were present in lower quantities than in the other pollen diets investigated. Thus, the conclusion that the level of valine, leucine and isoleucine are important in bee diets is supported by evidence from this study of bumblebees. Further support is gained from the results of comparisons of colony and individual performance when three diets each containing a different proportion of oilseed rape pollen were offered to the bumblebee micro-colonies. Those offered the diet containing the highest levels of these amino acids performed significantly better,, suggesting that polylectic bees such as bumblebees can address nutritional deficiencies of some pollen species by collecting multiple species. Bumblebees frequently exploit flowers from several plant species in single foraging flights

(Leonhardt & Blüthgen, 2012; Goulson, 2009), and pollen profiles of up to 2-8 species have been recorded in pollen loads taken from *Bombus lucorum* and *Bombus pascuorum* (Free, 1970). Faecal analysis of *Bombus* spp. larvae has also indicated that up to 96% of individuals had consumed between 2-5 species of pollen (Brian, 1951). It has been proposed that bumblebee foragers may be able to assess protein content of pollen, as they display preferences for plants offering high-protein pollen (Leonhardt & Blüthgen, 2012).

Further evidence of the nutritional importance of the amino acid content of pollen was obtained from poor brood-rearing performance recorded when honey bees were offered dandelion pollen (*Taraxacum officinale)* low in tryptophan phenylalanine and arginine. Supplementing arginine (but not tryptophan or phenylalanine) levels of dandelion pollen was reported to increase brood rearing success (Herbert *et al*., 1970), although the finding was not replicated in a later study using a different rearing technique (Loper & Berdel, 1980). Pollen from *T. officinale* has also been shown to be low in leucine, and, in relation to honey bee requirements, valine and isoleucine, leading to the suggestion that the observed poor brood rearing may be the result of multi-amino acid deficiency (Loper & Cohen, 1987).

In conclusion, pollen diversity in diets and amino acid content are important drivers of bumble bee colony success. Collection of pollen from different sources by foragers may optimise larval nutrition. This study has identified timing of nest building activity and its relationship with brood production as an important parameter contributing to micro-colony success. Future work should concentrate on analysis of key nutritional components of pollen which may be common to favoured pollen mixes. This will support the selection of plant species to be used in stewardship schemes designed to increase bumble bee abundance. The findings of micro-colony experiments suggest amino acid composition may be one key factor governing the performance of bumblebee colonies in the field, but they can only be used to define parameters for further investigation using queen-right colonies in the natural environment. The importance of field studies is emphasised because some plants utilise a variety of morphological floral traits to restrict exploitation by some pollinator species; thereby balancing effective pollination and excessive pollen removal (Harder & Barclay, 1994; Műller, 1995; Thorp, 2000; Schlindwein *et al*., 2005; Műller *et al*., 2006; Westerkamp & Claβen-Bockhoff, 2007). When, as in this study, pollen is disassociated from flowers the impact of such floral traits are eliminated, enabling

pollen mediated factors to be more closely studied, but floral traits will also determine bumblebee foraging behaviour. Field work investigating the impact of the presence of a range of plant species on nest building activity as an aspect of colony development will therefore be required.

Chapter 6: Behavioural responses of *Bombus terrestris audax* encountering neonicotinoid insecticide residues in nectar: mechanisms of initial avoidance

6.1 Abstract

The behavioural responses of *Bombus terrestris* to nectar spiked with insecticides were investigated. In no-choice arena tests nectar substitute (sucrose) with 5 or 10 µg L^{-1} imidacloprid, clothianidin or thiamethoxam resulted in a fully reversible reduction in nectar consumption. No reduction in consumption occurred when concentrations of 1 µg L^{-1} were offered. In arena choice tests in which treated and untreated nectar was offered without an associated visual cure, worker bees could not distinguish between untreated nectar and that spiked with an insecticide.

In a third experiment workers emerging from a queen-right colony in flight cages were offered a choice of thiamethoxam treated (5µg L^{-1}) and untreated nectar substitute associated with visual cue (colour of artificial flowers). The number of visits by foraging bees was affected by flower colour, but not the insecticide contamination status of nectar. After settling on a flower insecticide contamination did not affect the number of feeding sessions that were initiated suggesting that the bees did not learn either preference or avoidance of the flowers with treated nectar prior to landing. Instead a significant response occurred during feeding that resulted in a shortening of the feeding session. At the concentration tested (5µg L^{-1}) the effect was subtle so that the overall level of nectar consumed from contaminated flowers was unaffected. The results are discussed in relation to possible mechanisms leading to these findings which may be behavioural or metabolically (e.g. upregulation of enzymes responsible for detoxification) based.

Key Words: Neonicotinoids, Imidacloprid, Thiamethoxam, Clothianidin, *Bombus terrestris*, Antifeedant, Nectar

6.2 Introduction

Pollinators, including bumblebees, provide a vital ecosystem service, but have shown significant decline over the past 60 years (Vanbergen *et al*., 2013; Ollerton *et al*., 2014; Goulson *et al*., 2015). Several stressors have been implicated as drivers of this decline, including habitat loss, loss of floral diversity in key landscapes, predators, parasites, disease and pesticides (Vanbergen *et al*., 2013; Ollerton *et al*., 2014; Goulson *et al*., 2015), although debate regarding their relative importance continues.

Agricultural pesticides have frequently been implicated as a significant cause of pollinator decline, with neonicotinoid insecticides (systemic acetylcholine receptor agonists) becoming a focus of concern following large scale incidents linked to honey bee losses in Germany, Italy and Slovenia (Blacquiere *et al*., 2012; Goulson, 2013; Godfray *et al*., 2014; Godfray *et al*., 2015; Walters, 2013). These incidents were thought to result from dust generation during drilling of seed treated with clothianidin and technical modifications to application equipment have been introduced to address the causes.

These incidents were followed by extensive research into the impact of neonicotinoid use on pollinators, particularly bees. Due to their systemic activity, risk of exposure through translocation of the insecticides to pollen and nectar and subsequent consumption by pollinators became a focus of research, with most attention directed towards three active ingredients imidacloprid, clothianidin and thiamethoxam (Mommaerts *et al*., 2010; Walters, 2016). These insecticides were known to be present at very low levels in pollen and nectar from commercial field (EFSA, 2013a; EFSA, 2013b; EFSA, 2013c), but whether such exposure resulted in significant risk was debated as it was suggested that the dose delivered was too low to stimulate either acute or chronic lethal or sub-lethal responses (Carreck & Ratnieks, 2014). This may in part explain why risks predicted in the laboratory and identified in some field experiments (Rundlof *et al*., 2015; Whitehorn *et al*., 2012; Woodcock *et al*., 2017) have not been confirmed in other extensive field studies (Cutler & Scott-Dupree, 2014; Cutler *et al*., 2014; Godfray *et al*., 2015). Despite the conflicting evidence, approval for thiamethoxam-treated oilseed rape was suspended in 2012 (Campbell, 2013), with a 2-year Europe wide ban on the use of imidacloprid, clothianidin and thiamethoxam as seed treatments on bee-attractive

crops introduced in 2013 (EC, 2013), followed in 2018 by a total ban of the outdoor use of these active ingredients (EC 2018a; EC 2018b; EC 2018c).

Research into the impact of the three active ingredients on bumblebee pollen and nectar feeding may indicate that nutrient optimisation could be affected. Selective foraging by bumblebees through which pollen or nectar from less commonly available plant species are preferentially utilised as nutrient sources can be linked to improved individual or colony level performance (Kriesell *et al*., 2017). Presence of neonicotinoid residues in nectar has, however, been shown to influence feeding behaviour, which may affect the utilisation of favoured plant species and lead to sub-optimal foraging. Dose-dependent reductions in feeding by both bumblebees and *Apis* bees offered sugar solution spiked with imidacloprid, clothianidin or thiamethoxam have been reported (Alaux *et al.*, 2010; Cresswell *et al*., 2014; Thompson *et al*., 2014). The effect of imidacloprid residues on bumblebee feeding is known to be reversible and delays before complete recovery reflect the clearance rate of ingested imidacloprid in bumblebees (Cresswell *et al*., 2014). Thompson *et al.* (2014) have suggested that some characteristics of the recovery response (absence of significant mortality and increased intake of the follow-on untreated sucrose) indicated that it was a reversible antifeedant response. No work investigating the effect of these active ingredients in pollen has been reported.

Responses of free-flying *Bombus terrestris audax* workers to insecticide contaminated sucrose were investigated in choice experiments in which bees were offered imidacloprid, clothianidin and thiamethoxam at various concentrations in sucrose solution (Kessler *et al*., 2015). The study tested the proposal that bumblebees could detect and avoid insecticide spiked sucrose, arriving at the apparently contradictory conclusions that for imidacloprid and thiamethoxam they could not detect the active ingredient, consumed less contaminated nectar, but nonetheless foraged preferentially on treated nectar.

Arce *et al.* (2018) investigated *B. terrestris audax* foraging in laboratory flight cages for a period of 10 days, on an array of identical sucrose feeders containing two different concentrations of thiamethoxam or untreated nectar in an attempt to mimic pesticide exposure in the wild. No visual cue was associated with feeders to distinguish those containing different levels of neonicotinoid. At the start of the period the proportion of visits to pesticide spiked feeders was lower than to feeders offering untreated nectar, suggesting an avoidance response and supports this aspect of the findings of the above studies. The proportion of visits to thiamethoxam

spiked feeders however, increased with time at the lower of the two concentrations investigated but not at the higher concentration, resulting in greater consumption of pesticide-laced sucrose relative to untreated sources. The study concluded that worker bumblebees can detect thiamethoxam and alter their behaviour to continue feeding on it, but offered no explanation regarding why the bees changed from initially avoiding consumption of the toxin to displaying no avoidance behaviour over time.

Where selective foraging on different food plants is undertaken to optimise quality of nutritional provision for both individuals and colonies, contamination of key source plants with potential toxins such as insecticides and associated avoidance may lead to a lowering of colony performance. In some cases, however, such toxins may be detected at levels that are too low to have significant negative effects, imposing an ecological cost on the colony if higher quality nutrition is not exploited due to the response. In such cases behavioural adaptations that allow continued utilisation of the favoured nutritional source when insecticide content was sufficiently low would be advantageous. This may require sequential decision making with cues identifying a favoured flower species governing visitation, followed by a second decision on whether to feed depending on detection of the pesticide concentration.

The current study investigates the behavioural mechanisms associated with avoidance of thiamethoxam, testing the hypothesis that separate, sequential decisions relating to selection of the food plant and whether to commence extended feeding are made, using visual cues (such as flower colour) for the former, and chemical cues allowing detection of insecticide contamination of nectar for the latter.

6.3 Materials and methods

Bombus terrestris audax stock colonies were sourced from Agralan Ltd, Swindon, UK (originating from Biobest, Belgium), and maintained in a controlled environment (CE) plant growth room (Fitotron) set at 21 ± 2°C, and 60% RH, with a 8 h light:16h dark (8L:16D) light cycle, for a minimum of 72 hours prior to use in experiments. Experiments were conducted in the same room, allowing bees to acclimatise to experimental conditions. Each colony consisted of a queen and approximately 60 worker bees. Mixed source pollen (Agralan Ltd.) was provided to each colony at 2-day intervals together with ad libitum proprietary liquid sugar solution feed (Biobest).

Prior to transfer to experimental arenas individual workers were collected from stock colonies by allowing them to walk through an exit aperture in the colony cage into a 10ml plastic tube, before being weighed. This avoided the need to anaesthetise worker bees prior to experiments.

6.3.1 Insecticides and nectar substitute

Imidacloprid, clothianidin and thiamethoxam (as Pestanal analytical standards (>99% purity)) were sourced from Sigma-Aldrich (UK). A stock solution of each insecticide was created in acetone as described by Thompson *et al.* (2014). A 30% w/v sucrose solution that reflected sugar concentrations in oilseed rape nectar (henceforth referred to as 'nectar substitute') was prepared in deionised water, and the stock solution was diluted using the nectar substitute to produce the required concentrations.

6.3.2 Experiment 1 - Consumption of nectar substitute spiked with neonicotinoid insecticides and post-exposure recovery (no-choice tests)

Individual forage arenas (IFA) were constructed using a design modified from the cages used by Elston *et al.* (2013) and Thompson *et al*. (2014). Each consisted of a circular 500 mL open topped clear plastic container (11 cm diameter x 7 cm deep) closed with 40% cotton/60% nylon mesh secured using an elastic band. A lidded feeding tube (length = 10cm, Φ = 1cm), with an upward-facing feeding hole (Φ = 2mm) pierced at one end was inserted at a 30° angle through a hole in the side of the IFA. To reduce build-up of condensation the bottom of the arena was lined with filter paper.

Bees used in the experiment were sourced from three stock colonies, with an equal number of bees taken from each colony and systematic allocation to treatment. A single worker bee was introduced into the IFA and offered either insecticide-free nectar substitute, or nectar substitute spiked with insecticide residue.

6.3.2.1: Treatments and assessments: Feeding tubes were filled with nectar substitute spiked with one of three field realistic concentrations (EFSA, 2012) of imidacloprid (1µg L^{-1}; 5µg L^{-1}; or 10µg L^{-1}) and weighed. At 24h intervals thereafter

tubes were removed, re-weighed to determine the mass of sucrose solution consumed, and replaced with another (pre-weighed) feeding tube. Following an initial four-day period during which bees were offered nectar substitute spiked with insecticides, they were fed for a further 4 days with insecticide-free nectar substitute. In controls bees were offered untreated nectar substitute throughout the 8 day period of the experiment. The experiment was conducted in a CE room at 21 ± 2°C, 60% RH and 8L:16D, and treatments and controls were replicated 20 times.

Mortality was recorded at 24 h intervals throughout the experiment. After the full eight day experimental period, surviving bees were euthanized by freezing then immediately weighed; bees that died during the 8-day period were weighed upon discovery.

The experiment was repeated using two other neonicotinoid insecticides, thiamethoxam and clothianidin, offered to bees at the same concentrations used for imidacloprid.

6.3.2.2 Statistical analysis: Statistical analysis was conducted using R Studio 0.99.903 (RStudio Team, 2015), with and the following packages; multcomp (Hothorn *et al.,* 2008), ggplot2 (Wickham, 2009) and plyr (Wickham, 2011). All data was tested for normality and appropriate transformations applied where necessary. Data describing consumption of nectar substitute was subjected to repeated measures ANOVA with day as the repeated measure and concentration as the treatment factor. For the days on which time point analysis detected significant effects of concentration on consumption of nectar substitute, multiple Tukey comparison models were used to characterise the differences between treatments.

Data describing mortality were analysed using a generalised linear model (GLM) with binomial error structure to investigate the effects of treatment on mortality. Where the residual deviance was greater than the degrees of freedom a quasi-binomial error structure was adopted.

6.3.3 Experiment 2 - Consumption of nectar substitute spiked with neonicotinoid insecticides (choice tests)

To investigate the responses of worker bees to insecticide contaminated nectar substitute when they had simultaneous access to insecticide free sucrose the design

of the IFA was modified by the addition of a second feeding tube positioned on the opposite side of the arena at (at 180º to the first). All other aspects of the IFA design were as described for Experiment 1.

The experiment consisted of three treatments and a control. In each treatment, one of the feeding tubes contained nectar substitute spiked with a known concentration of imidacloprid, while the second tube contained insecticide free nectar substitute. Three concentrations of imidacloprid tested were selected, two spanning the range of concentrations recorded in nectar taken from commercial oilseed rape fields in Europe (1µg L^{-1} and 10µg L^{-1}; EFSA 2013a), and one that exceeded the highest levels recorded in commercial fields (100µg L^{-1}). In controls, both tubes contained insecticide free standard sucrose. There were ten replicates of each treatment and the control.

Bees used in the experiment were sourced from three stock colonies, with an equal number of bees taken from each colony and systematic allocation to treatment. A single worker bee was released into the IFA and allowed to feed for 5 consecutive days during which period the weight of nectar substitute consumed from each feeding tube was recorded daily using the procedure described for Experiment 1. Total mortality was also recorded at the end of the experiment. After five days, survivors were euthanized and weighed. All other aspects of the experimental design and assessments were similar to those in the no-choice experiment.

The procedure was repeated using two other neonicotinoid insecticides, clothianidin and thiamethoxam, both offered to bees at the same concentrations (EFSA 2013b; EFSA 2013c). Each of the three experiments investigating individual active ingredients was replicated 12 times, and the experiment was run twice (described as “experimental run” in the results section).

6.3.3.1: Statistical analysis: To investigate feeding preference, the proportion of the total nectar substitute consumption (expressed as g consumed/g bee) that was taken from feeding tubes containing contaminated nectar was calculated. For the untreated controls feeding tubes were randomly labelled as “a” or “b” and the proportion of nectar taken from tube “a” was used in the analysis. Proportion consumption was analysed using GLM with binary error structure, using R Studio 0.99.903 (RStudio Team, 2015). Where residual deviance was greater than degrees of freedom a quasi-binomial error structure was adopted.

Data describing bee mortality were analysed using GLM with binomial error structure to investigate the effects of mortality. Where the residual deviance was greater than the degrees of freedom a quasi-binomial error structure was adopted. During creation of all models experimental run was found to be non-significant and thus removed as a factor.

6.3.4 Experiment 3 - Neonicotinoid avoidance - feeding response to thiamethoxam when associated with a visual cue

In this experiment the foraging behaviour of bees was observed within flight cages each containing a bumblebee colony. Colonies were offered nectar substitute feeding tubes placed within artificial flower feeding stations of two different colours.

6.3.4.1 Flight cages and feeding stations: Flight cages consisted of clear Perspex boxes (120 x 60 cm base, 120 cm high), from which two side walls had been removed and replaced with white nylon mesh (0.5mm). Access to the cage was facilitated by a hinged hatch (50 x 35 cm) in the front wall, with a netted access sleeve on either side of the hatch.

Feeding stations consisted of a single lidded feeding tube (described above), inserted at a downward 30º angle into a slot at the top of a 20 cm high wooden stand. A 110mm diameter disk of coloured (yellow or blue) card with a small circular hole cut in the centre was slid onto the feeding tube to simulate the visual cue provided by flowers.

Bee activity in each flight cage was recorded using a video camera (SONY HDR-CX240E Handycam, SONY, Toyko, Japan) trained to record both visits to each feeding station, and the display of a digital clock mounted on the cage.

6.3.4.2 Bombus terrestris conditioning: To condition colonies to feeding from the artificial flower feeding stations, stock colonies of *B. terrestris* housed in the original nest box in which they were supplied and provided with 20g of dried mixed pollen (Agralan Ltd.) were placed individually in flight cages set up in a CE room. The CE room was set at 21°C, 60% RH and 8L:16D with an artificial dawn and dusk. The sucrose solution feeders attached to the nest box were capped to prevent access by the bees. Two (one yellow and one blue) artificial flower feeding stations filled with nectar substitute, providing the only sources of sucrose available to the colony, were

positioned equidistant from the entrance to the nest box before it was opened to allow the workers to forage freely for 72h. The feeding tubes and coloured card in each artificial flower were refilled/replaced, and the colonies given a further 10g of pollen, at 24-hour intervals during the artificial dawn.

6.3.4.3 Treatments: This experiment investigated whether worker bees could detect thiamethoxam spiked nectar substitute, can associate it with a visual cue provided by the artificial flower, and either avoid or be attracted to the insecticide residue. Following the 72h conditioning period and during the artificial dawn, the feeding stations were replaced in each cage. Replacement stations offered one of two treatments or the control:

> Control: Untreated nectar substitute was offered in both blue and yellow feeding stations.
>
> Treatment 1: A pairing in which thiamethoxam spiked (5µg L^{-1}) nectar substitute was offered in blue flowers and untreated nectar substitute in yellow flowers.
>
> Treatment 2: A pairing in which thiamethoxam spiked (5µg L^{-1}) nectar substitute was offered in yellow flowers and untreated nectar substitute in in blue flowers.

Each treatment and the control were replicated 10 times. Treatments and controls were arranged in the CE room in a randomized blocked design to support statistical analysis.

6.3.4.4 Assessments: The experiment was run for three days and data describing nectar substitute consumption over the whole of this period was recorded and analysed. Time constraints resulted in analysis of specific characteristics of foraging behaviour only being extracted from video recordings made during the active foraging period of day 2. Video records for days 1 and 3 are stored in the Harper Adams University Centre for Integrated Pest Management (CIPM) archives for future analysis. Active foraging period was defined as the 8-hour daylight period (09:00-17:00). Assessments of a series of factors were made:

Consumption of nectar substitute and flower colour: The weight of nectar substitute (g) consumed from each feeding tube/flower was recorded for each of the three days of the experiment.

Total number of visits to flowers (day 2 only): The number of flower visits during each 8 hour active foraging period was recorded and the data expressed as the total number of visits h^{-1} to the individual flowers in each treatment.

Total length of flower visits (day 2 only): The total time spent by bees visiting each artificial flower in a treatment (i.e. in contact with the flower and with wings motionless) was recorded. Visits by different bees were combined and expressed as the total accumulated time visiting each individual flower during each hour of photophase.

Mean length of flower visits (day 2 only): The mean length of flower visits by individual bees to each artificial flower in each treatment was recorded during each hour of photophase.

Number of feeding sessions (day 2 only): The number of feeding sessions on each flower (defined as the extension of the proboscis into the feeding tube of the artificial flowers), was assessed during each hour of photophase.

Total time spent feeding (day 2 only): The combined length of feeding sessions by individual bees during each hour of photophase was calculated and expressed as total feeding time h^{-1}.

Mean length of feeding sessions (day 2 only): The mean length of feeding sessions by individual bees to each artificial flower in each treatment was recorded during each hour of photophase.

6.3.4.5 Statistical analysis: Consumption of nectar substitute and flower colour: Data were subjected to repeated measures ANOVA with time (day) as the repeated measure and treatment (flower colour/insecticide presence) as the treatment factor. Post-hoc Tukey tests were used to identify significant differences.

Total number of visits to flowers: As residual deviance was found to be greater than the degrees of freedom data were analysed using a GLM with quasi-poisson error structure.

Total length of flower visits and mean length of flower visits: Following log transformation to normalise the residuals for data were subjected to ANOVA and post-hoc Tukey tests to characterise significant differences.

Number of feeding sessions: Data were analysed using GLM with quasi-poisson error structure to account for over dispersion.

Total time spent feeding: Data was normalised using log transformation and subjected to ANOVA.

Mean length of feeding sessions: Following log transformation to normalise the data the mean time spent feeding was subjected to an ANOVA and post-hoc Tukey tests to characterise where significant differences lay.

6.4 Results

6.4.1 Experiment 1 - Consumption of nectar substitute spiked with neonicotinoid insecticides and post-exposure recovery (no-choice tests)

Consumption by worker bumblebees of artificial nectar solution contaminated (spiked with insecticide residue) with imidacloprid, clothianidin or thiamethoxam (at three different concentrations) was recorded for four days. The bees were subsequently offered uncontaminated nectar solution during the “recovery phase” of the experiment and consumption assessed for a further four days.

6.4.1.1 Imidacloprid:

Days 1-4: Repeated measures ANOVA of data collected during days 1-4 showed a significant difference in sucrose consumption between both treatment (concentration of imidacloprid solution; $F = 27.094$, d.f. = 3,248, $p < 0.001$) and days ($F = 4.218$, d.f. = 3,248, $p < 0.001$). A significant interaction also occurred between treatment and day ($F = 5.544$, d.f. = 9,248, $p < 0.001$).

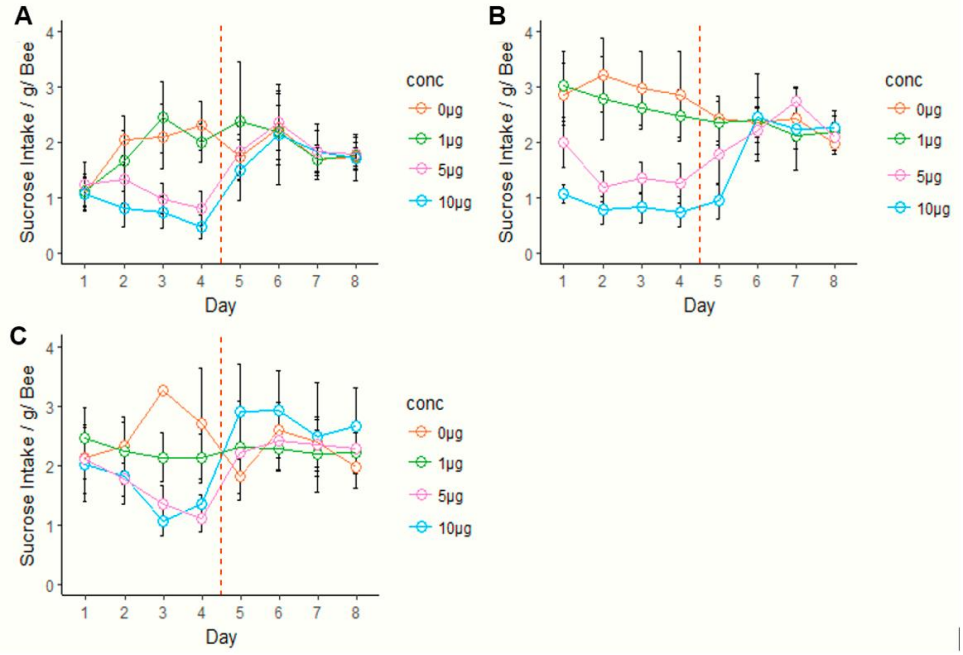

Figure 6.1. Mean (± S.E.) daily consumption of artificial nectar solution (sucrose) contaminated with (A) imidacloprid, (B) clothianidin, and (C) thiamethoxam at three different concentrations (1 µg L^{-1}, 5 µg L^{-1}, 10 µg L^{-1}), and of an untreated control nectar solution. Data is expressed as g consumed per gram of bee to take account of variable size of bees. Vertical broken lines = day on which contaminated artificial nectar solution was replaced by untreated solution.

Time point analysis by day showed that no significant differences ($p > 0.05$) occurred on day 1 between the consumption of artificial nectar solutions containing any of the three concentrations of imidacloprid or the untreated control (Figure 6.1a). There was a significant difference in nectar consumption between treatments and the control on days 2 ($F = 5.642$, d.f. = 3,70, $p = 0.002$), 3 ($F = 12.761$, d.f. = 3,62, $p < 0.001$) and 4 ($F = 28.678$, d.f. = 3,56, $p < 0.001$).

Multiple comparison models for each day on which significant differences in artificial nectar solution occurred between treatments (Table 6.1) indicate that, compared with the untreated control) significantly lower consumption of artificial nectar solution contaminated with 5 µg L^{-1} or 10 µg L^{-1} imidacloprid was recorded. No difference was found between the untreated control and nectar contaminated with 1 µg L^{-1} of the active ingredient.

Days 5-8: Results of repeated measures ANOVA on data from days 5-8 of the experiment found day to be significant (F =3.827, d.f. = 3,198, p = 0.011). Treatment (concentration of imidacloprid solution) was not significant (F = 0.671, d.f. = 3,198, p = 0.57), and no interaction between treatment and day was found (F = 0.717, d.f. = 9,198, p = 0.69). Time point analysis by day confirmed that no significant differences

Table 6.1. Results of multiple comparison models investigating differences between the volume of artificial nectar solution consumed by bees (on days 1-4 of the experiment) when it is contaminated with different concentrations of imidacloprid, compared with untreated control nectar. Day = day of the experiment; only days on which significant differences between untreated controls and at least one insecticide contaminated treatment occurred are reported.

Imidacloprid concentration in nectar	**Day 2**		**Day 3**		**Day 4**	
	t	p	t	p	t	p
1 µg L^{-1}	1.243	0.22	-1.071	0.29	1.349	0.18
5 µg L^{-1}	2.309	0.02	3.417	0.001	6.478	<0.001
10 µg L^{-1}	3.959	<0.001	4.046	<0.001	7.745	<0.001

had been found between treatments on any day, indicating that an immediate recovery in nectar consumption by the bees occurred when uncontaminated nectar was offered. Although no significant differences between consumption of contaminated artificial nectar solution and untreated control nectar were recorded on any days over the period, greater variability was noted on day 5.

Cumulative mortality over the full 8 day period of the experiment was low. During creation of the minimum adequate model and factor reduction the GLM with binomial error structure showed that mortality was found to vary independently of all explanatory variables. Thus no effect of treatment on mortality was recorded.

6.4.1.2 Clothianidin:

Days 1-4: Repeated measures ANOVA of data collected between days 1-4 showed a significant difference in the consumption of artificial nectar solution in different treatments (concentration of clothianidin solution; $F = 56.230$, d.f. = 3,243, $p < 0.001$; Figure 6.1b). Day was however, not significant ($F = 1.766$, d.f. = 3,243, $p = 0.15$), and no interaction between concentration and day was found ($F = 0.762$, d.f. = 9,243, $p = 0.65$).

Time point analysis found that nectar consumption was significantly different between treatments on day 1 ($F = 15.316$, d.f. = 3,75, $p < 0.001$; Figure 1b), day 2 ($F = 18.190$, d.f. = 3,68, $p < 0.001$), day 3 ($F = 17.131$, d.f. = 3,62, $p < 0.001$) and day 4 ($F = 8.596$, d.f. = 3,53, $p < 0.001$). Multiple comparison models comparing nectar consumption in each treatment with that in untreated controls (Table 6.2) were run independently for each day. No significant differences in consumption were found between untreated controls and the treatment with nectar containing 1 µg/L clothianidin between days 1 and 4 of the experiment. Significantly lower consumption of nectar containing 5 µg/L or 10 µg/L occurred when compared to untreated controls on all days over the time period

Days 5-8: Repeated measures ANOVA for days 5-8 of the experiment found day to be significant ($F = 2.753$, d.f. = 3,193, $p < 0.05$). Treatment (concentration of clothianidin solution) was not significant ($F = 0.390$, d.f. = 3,193, $p = 0.760$), but an interaction between concentration and day was found ($F = 3.686$, d.f. = 9,193, $p < 0.001$).

Time point analysis by day highlighted a significant difference between treatments on day 5 ($F = 6.056$, d.f. = 3, 53, $p < 0.01$), and a multiple comparison model was run to determine where this interaction lies (Table 6.3). No significant difference was found between the consumption of nectar solution by bees that had previously (during days 1-4 of the experiment) been offered nectar contaminated with 1 µg L^{-1} clothianidin, and the untreated control ($t = 0.318$, $p < 0.05$). Significantly lower nectar solution consumption was recorded on day 5 of the experiment in both the 5 µg L^{-1} ($t = 2.140$, $p < 0.05$) and 10 µg L^{-1} ($t = 3.819$, $p < 0.001$) treatments than in the untreated control, but in both cases consumption recovered to similar levels to that in controls on all days thereafter (days 6-8 inclusive).

Cumulative mortality over the full 8 day period of the experiment was low in the untreated controls, or 1 and 5 µg L^{-1} treatments, but 35% mortality was recorded in the 10 µg L^{-1} treatment. During factor reduction the GLM with quasi- binomial error structure showed that significantly higher mortality occurred in the 10 µg L^{-1} than in the untreated control, 1 and 5 µg L^{-1} treatments (t=4.054, d.f.=78, p<0.001).

Table 6.2. Results of multiple comparison models investigating differences between the volume of artificial nectar solution consumed by bees when it is contaminated with different concentrations of clothianidin, compared with untreated control nectar (days 1-4 of the experiment). Day = day of the experiment; only days on which significant differences between untreated controls and at least one insecticide contaminated treatment occurred are reported.

Clothiandin concentration in nectar	**Day 1**		**Day 2**		**Day 3**		**Day 4**	
	t	p	t	p	t	p	t	p
1 µg L^{-1}	-0.460	0.65	1.143	0.26	1.211	0.23	1.034	0.31
5 µg L^{-1}	2.664	<0.01	5.273	<0.001	4.978	<0.001	3.886	<0.001
10 µg L^{-1}	5.535	<0.001	6.089	<0.001	6.018	<0.001	3.929	<0.001

6.4.1.3 Thiamethoxam:

Days 1-4: Repeated measures ANOVA for days 1-4 showed that significant differences in the amount of artificial nectar solution consumed between treatment (concentration of thiamethoxam; F =14.510, d.f. = 3,257, p <0.001; Figure 6.1c). There was an interaction between concentration and day (F = 2.929, d.f. = 9,257, p <0.001), but day was not found to be significant (F = 1.341, d.f. = 3, 257, p = 0.262).

Time-point analysis by day indicated that a significant difference in nectar consumption occurred between treatments on days 3 (F = 9.625, d.f. = 3, 66, p <0.001) and 4 (F = 9.025, d.f. = 3, 63, p <0.001) of the experiment. Multiple comparison models were run for each day on which significance was found to characterise this interaction (Table 6.3). In the 1 µg L^{-1} treatment a lower consumption of nectar solution than in the untreated control was recorded on day 3 of the experiment but a similar significant difference did not occur on day 4. In

treatments with higher concentrations of thiamethoxam in the nectar (5 µg L^{-1} and 10 µg L^{-1}), significantly lower consumption of nectar was recorded on both days.

Table 6.3. Results of multiple comparison models investigating differences between the volume of artificial nectar solution consumed by bees when it is contaminated with different concentrations of thiamethoxam, compared with untreated control nectar (days 1-4 of the experiment). Day = day of the experiment; only days on which significant differences between untreated controls and at least one insecticide contaminated treatment occurred are reported.

Thiamethoxam concentration in nectar	**Day 3**		**Day 4**	
	t	p	t	p
1 µg L^{-1}	2.564	<0.05	1.785	>0.05
5 µg L^{-1}	4.321	<0.001	4.700	<0.001
10 µg L^{-1}	4.859	<0.001	3.839	<0.001

Days 5-8: Results of repeated measures ANOVA for days 5-8 found treatment to be non-significant ($F = 2.280$, d.f = 3,237, $p = 0.08$). Day was not significant ($F = 0.889$, d.f. = 3, 237, $p = 0.45$), and no interaction between concentration and day was found ($F = 0.654$, d.f. = 9, 237, $p = 0.75$). Thus, no significance differences between consumption of nectar in different treatments containing variable concentrations of thiamethoxam occurred.

Cumulative mortality over the full 8 day period of the experiment was low. During creation of the minimum adequate model and factor reduction, mortality was found to vary independently of all explanatory variables. Thus no effect of treatment on mortality was found.

6.4.2 Experiment 2 - Consumption of nectar substitute spiked with neonicotinoid insecticides (choice tests)

The results of the no-choice tests in experiment 1 show that reversible depression of consumption occurred when bumblebees were offered artificial nectar solution contaminated (at field realistic levels) with the three active ingredients tested. The underpinning mechanism resulting in this finding was not, however, investigated. The outcome may have resulted from detection and avoidance of contamination, or sub-lethal toxicity which temporarily prevented feeding, or other mechanisms. To further investigate the hypothesis that the bees could detect and avoid contaminated nectar, a second experiment was conducted in which bees were released into IFAs containing two identical feeding tubes, one containing contaminated nectar and the second untreated nectar. In controls both tubes contained untreated nectar. The proportion of the nectar consumed on each day of the experiment that was taken from each feeding tube was calculated, and avoidance of contaminated nectar defined as a consistently lower proportion of nectar being taken from the tube containing treated nectar.

During factor reduction of the minimum adequate model, the GLM with binary error structure showed that the proportion of sucrose consumed varied independently of all recorded explanatory variables (experimental run, active ingredient, insecticide concentration in sucrose solution, and day). Therefore, no significant differences in consumption of treated or untreated artificial nectar was recorded on any day of the experiment, or for any of the three active ingredients tested (imidacloprid, clothianidin, thiamethoxam; Figure 6.2), suggesting an inability to distinguish between tubes containing contaminated and uncontaminated nectar.

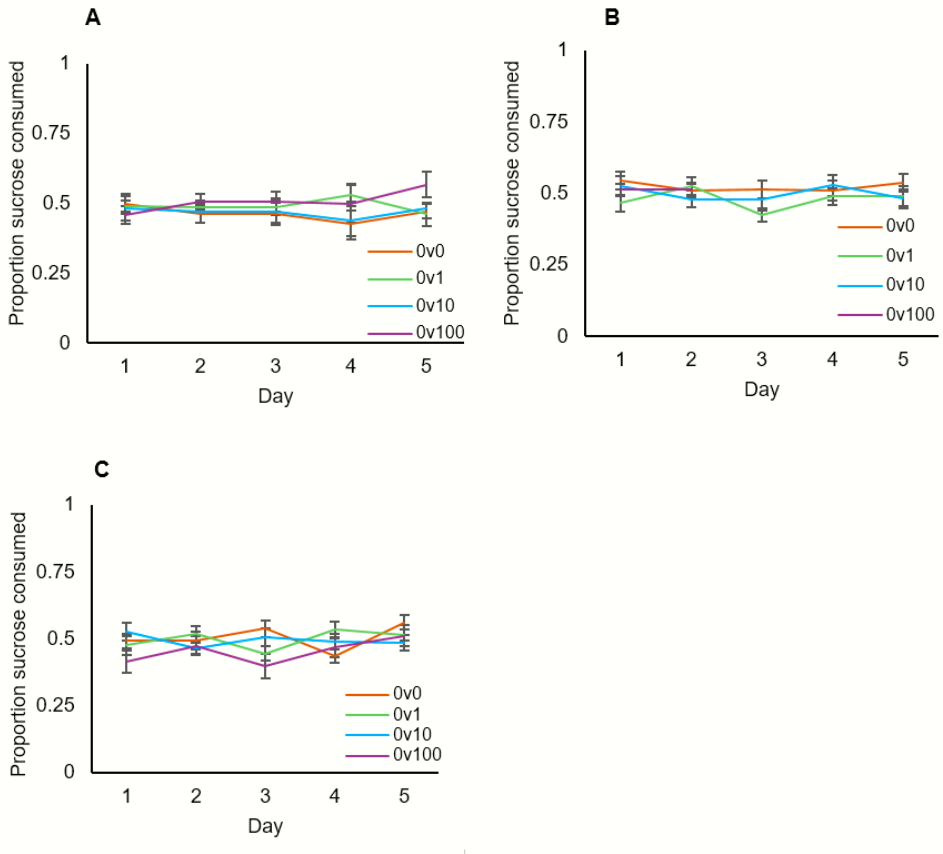

Figure 6.2. Consumption of artificial nectar substitute (sucrose) in individual foraging arenas offering bumblebees a choice of untreated nectar solution or nectar contaminated with field realistic and supra field-realistic concentrations of (A) imidacloprid; (B) clothianidin; (C) thiamethoxam. Graphs show mean (± S.E.) proportion of total daily consumption (assessed as g consumed g^{-1} bee) that was taken from the feeding tube containing insecticide contaminated nectar. 0v1 = bees were offered a choice of nectar with 1 µg L^{-1} insecticide or untreated nectar; 0v10 = 10 µg L^{-1} insecticide or untreated nectar; 0v100 = 100 µg L^{-1} insecticide or untreated nectar. 0v0 = control (untreated nectar was offered in both feeding tubes).

Total mortality of bees at the end of the experiment was low in all treatments in which bees were exposed to either imidacloprid or thiamethoxam. During creation of the minimum adequate model using data generated in the investigation of imidacloprid and factor reduction, mortality was found to vary independently of all explanatory variables. Thus no effect of treatment on mortality was recorded. Similarly no effect of treatment occurred when bees were exposed to thiamethoxam.

Mortality of bees after the 5 days of the experiment investigating clothianidin was also low in all treatments, except that offering a choice of nectar substitute containing 100 µg L^{-1} clothianidin or untreated nectar. In both experimental runs, mortality in this treatment was 100% and thus was removed from analysis. During creation of the minimum adequate model and factor reduction, mortality for the remaining treatments was found to vary independently of all explanatory variables.

6.4.3 Experiment 3 - Feeding response to nectar containing thiamethoxam when it is associated with a visual cue

The results of the choice test do not provide evidence that contamination with pesticides affects the selection of artificial nectar solution by feeding bumblebees. Selection of nectar sources (flowers) by foraging bumblebees in the natural environment is, however, mediated through a range of physical and chemical signals. As insecticide contaminated and untreated nectar was offered to bees in identical feeding tubes in the choice experiments, it is possible that the lack of visual stimuli associated with contaminated nectar source prevented effective selection. In experiment 3, nectar contamination was associated with the colour of artificial flowers. These experiments were conducted using a single active ingredient, thiamethoxam.

6.4.3.1 Sucrose consumption and flower colour: During the creation of the minimum adequate model of the ANOVA, factor levels of treatment and day were found to be non-significant and thus removed. As presence of insecticide (treatment) did not affect response to flower colour the data was combined in subsequent analysis and the results showed that sucrose consumption varied as a function of flower colour ($F = 5.084$, d.f. = 1, 178, $p = 0.03$). Post-hoc Tukey tests found that a consistently higher level of sucrose consumption was recorded when it was offered in blue flowers than in yellow flowers, irrespective of whether it contained thiamethoxam or was insecticide-free. (Figure 6.3; $p = 0.03$).

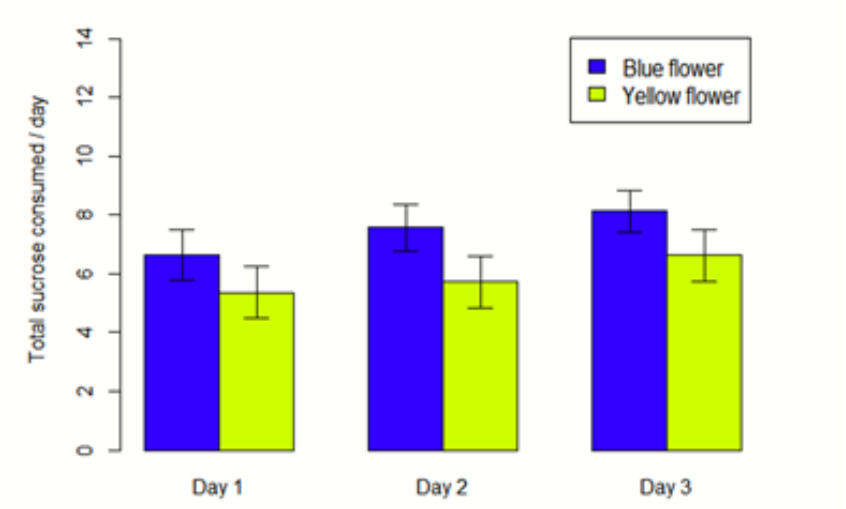

Figure 6.3. Mean total (± S.E.; g day^{-1}) artificial nectar (sucrose) solution consumed in each of three days when offered to *Bombus terrestris audax* in blue (blue shading) and yellow (yellow shading) flowers.

6.4.3.2 Bee behaviour - Number of visits to flowers: Due to time constraints, data was extracted from the video recordings taken during the second day of the experiment only. The behavioural characteristics investigated were assessed separately for each hour during the photophase period (09:00-17:00) for use in the analysis.

The effect of flower colour and nectar contamination (thiamethoxam) status on the total number of visits (visit defined as the bee being in contact with the flower and with its wings motionless) to flowers were analysed using a GLM with quasi-poisson error structure as residual deviance was found to be greater than the degrees of freedom. Treatment was found to be non-significant and was therefore removed from the analysis. The number of visits to blue flowers was significantly higher than to yellow flowers irrespective of insecticide contamination status (Figure 6.4a; t= -2.247, d.f. = 238, p = 0.026).

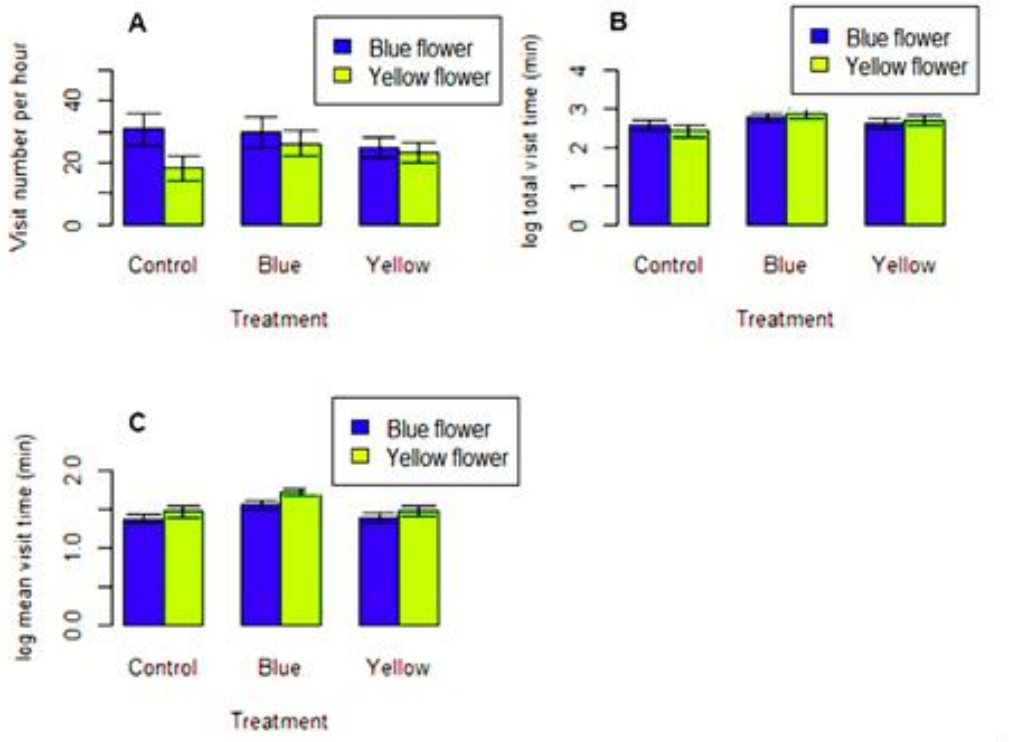

Figure 6.4. Bumblebee visitation to blue and yellow flowers (indicated by colour of bar). (A) Mean ± S.E. number of visits h^{-1}; (B) Total time (mins h^{-1}) ± S.E. spent visiting flowers; (C) Mean ± S.E. time of individual visits (mins). Flowers were presented in pairs in treatments defined on the abscissa legend as: “Blue” = nectar substitute associated with the blue flower contaminated with thiamethoxam and yellow flower untreated; “Yellow” = nectar substitute in yellow flower contaminated with thiamethoxam and blue flower untreated; “Control” = both flowers untreated. In each case: the blue shaded bar = blue flower; yellow shaded = yellow flowers.

6.4.3.3 Bee behaviour - Total length of flower visits: Length of flower visit was defined as the period over which a bee was in contact with a flower with its wings motionless. Following log transformation to normalise the residuals for ANOVA, during the creation of the minimum adequate model no effect of flower colour on total length of visit was observed and was thus the factor was removed from the analysis. This resulted in the analysis comparing the total length of time spent on flowers (irrespective of colour) in three categories, experimental pairings in which the blue flower was contaminated with thiamethoxam but not the yellow; the yellow flower was contaminated and not the blue; and the untreated control pairings.

ANOVA showed that mean total time spent visiting flowers varied as a function of insecticide treatment in the pairing ($F = 4.392$, d.f. = 2,190, $p = 0.014$). Post-hoc Tukey tests revealed that the total time spent by individual bees when visiting flowers in pairings where the blue flower was contaminated was significantly higher than in both the untreated control pairings ($p < 0.05$), or pairings where the yellow flower was contaminated ($p < 0.05$). Total length of visits in the pairing with yellow contaminated flowers and the untreated controls did not vary ($p = 0.92$).

These results may indicate that rapid sub-lethal poisoning occurs when the bees feed on contaminated nectar, reducing activity and resulting in longer delays before they take flight. The differences between total length of visit in blue contaminated and yellow contaminated pairings may then be affected by the innate preference for blue flowers, which the above analysis shows results in a greater visitation rate to blue flowers thus increasing the potential for sub-lethal poisoning and associate delays.

6.4.3.4 Bee behaviour - Mean length of flower visits: Following log transformation to normalise the data, ANOVA showed that the mean length of flower visits varied as a function of treatment ($F = 11.65$, d.f. = 2, 190, $p < 0.001$).

Post-hoc Tukey tests revealed that the mean length of visits all visits (i.e. to both blue and yellow flowers) in the pairing where the blue flower was contaminated were significantly longer than in both the control pairing ($p < 0.001$) or the pairing where the yellow flower was contaminated ($p < 0.001$). The mean length of flower visits in the control pairing and the pairing where the yellow flower was contaminated did not vary ($p = 0.8$). Thus, the analysis of mean visit time supports the assertion that bees that fed on contaminated nectar, display reduced activity resulting in longer delays before they take flight.

6.4.3.5 Bee behaviour - Number of feeding sessions: The number of feeding sessions (defined as the extension of the proboscis into the feeding tube of the artificial flowers) was analysed using GLM with quasi-poisson error structure to account for over dispersion. The number of individual feeding sessions was found independent of flower colour and treatment. Thus, no effect of either variable on the propensity of bees to feed when they alighted on the flowers was found (Figure 6.5).

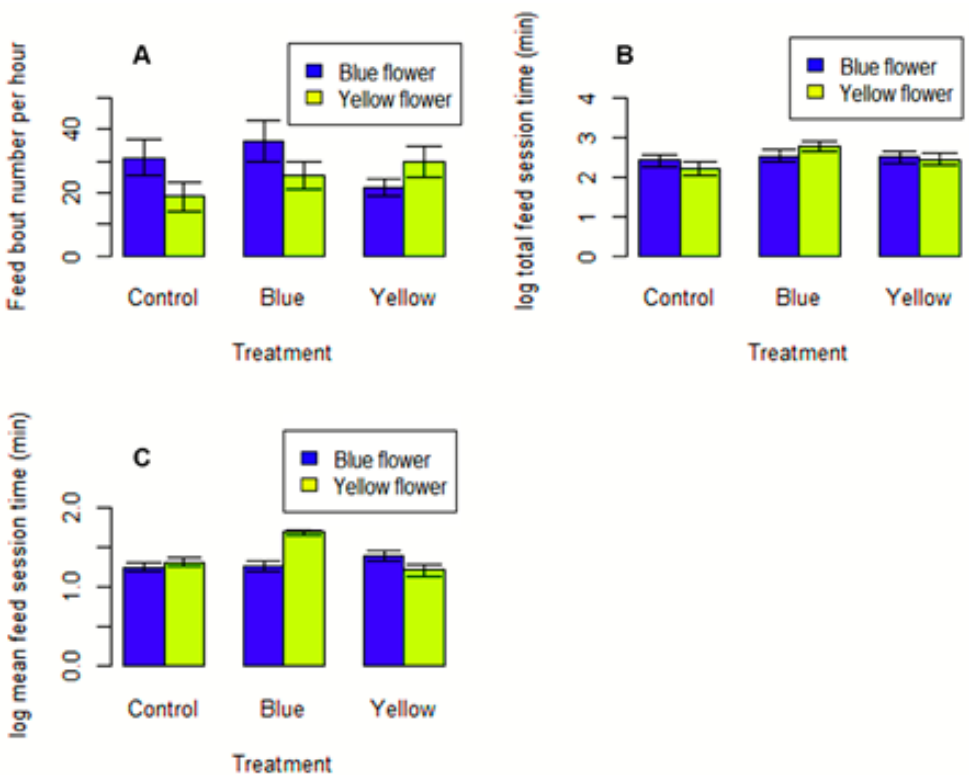

Figure 6.5. Bumblebee feeding on blue and yellow flowers. (A) Mean ± S.E. number of feeding sessions h^{-1}; (B) Total time ± S.E. (mins/ h^{-1}) spent feeding on nectar substitute; (C) Mean ± S.E. time of individual feeding sessions (mins). Flowers were presented in pairs in treatments defined on the abscissa legend as: “Blue” = nectar substitute associated with the blue flower contaminated with thiamethoxam and yellow flower untreated; “Yellow” = nectar substitute in yellow flower contaminated with thiamethoxam and blue flower untreated; “Control” = both flowers untreated. In each case: the blue shaded bar = blue flower; yellow shaded = yellow flowers.

6.4.3.6 Bee behaviour - Total time spent feeding: Total feeding time was defined as the total time in which bees had extended their proboscis into a feeding tube. Data was normalised using log transformation. ANOVA, and factor reduction, resulted in the analysis comparing the total length of time spent feeding on flowers (irrespective of colour) in three categories: experimental pairings in which the blue flower was contaminated with thiamethoxam but not the yellow; the yellow flower was contaminated and not the blue; and the untreated control pairings. The outcome showed total feeding time to be independent of treatment and flower colour. Thus,

no effect of either variable on the total time spent feeding while bees were standing on the flowers was detected (Figure 6.5).

6.4.3.7 Bee behaviour - Mean time spent feeding: Following log transformation to normalise the data the mean time spent feeding was subjected to an ANOVA. During the creation of the minimum adequate model an interaction was found between treatment and flower colour ($F = 12.250$, d.f. = 2, 168, $p < 0.001$).

Post-hoc Tukey tests showed that in the pairing in which the blue flower contained contaminated artificial nectar, average feeding time was significantly shorter than that on the uncontaminated yellow flower ($p < 0.001$), despite the established preference of the bees for blue flowers. In the pairing in which contaminated yellow flowers were compared with uncontaminated blue flowers, the feeding time on yellow flowers was shorter, supporting the conclusion that presence of thiamethoxam resulted in shorter feeding session irrespective of flower colour ($p < 0.05$). As toxic effects of thiamethoxam do not become evident during the short time period of an individual feeding session, this result suggests that the presence of thiamethoxam in nectar may deter feeding in *B. terrestris*.

This conclusion is supported by the observation that in the pairing where a contaminated blue flower was offered simultaneously to an untreated yellow flower, mean feeding time on the yellow flower was significantly longer than that on the yellow flower in the control pairing where neither flower colour offered contaminated nectar ($p < 0.01$). This may be the result of active feeding on the blue flower being suppressed by the presence of thiamethoxam in the nectar, and increased feeding on non-contaminated sources. This strategy would maintain the overall intake of nectar by the bee in the short term, resulting in there being no difference in overall total feeding time in treatments where an uncontaminated source was available, as found above. If this is the case then it would be expected that total feeding time on blue flowers would also be longer than in the pairing in which the yellow flower was contaminated, as was recorded ($p < 0.001$).

6.5 Discussion

This study used field realistic exposure rates to test the hypothesis that nectar foraging by worker bumblebees will be affected by the presence of selected

neonicotinoid insecticide residues in nectar at concentrations that mirror field realistic exposures (IPBES, 2017) in commercial crops in the UK. Most previous laboratory feeding and behaviour studies have been conducted with honeybees and have focussed on no-choice systems (reviewed by Godfray *et al*., 2014; 2015; Pisa *et al.,* 2014). To better understand the risk posed by pesticides present in nectar to pollinators, choice tests are required to determine if pollinators can detect toxins (Tiedeken *et al*., 2014) and, if they do so, whether they modify their behaviour when exposed.

Kessler *et al.* (2015) investigated responses of *B. terrestris audax* workers to insecticide contaminated sucrose solution using a choice test in bees were offered imidacloprid, clothianidin and thiamethoxam at various concentrations in sucrose solution. They concluded that the bees were unable to detect the insecticide residues in the sucrose solution, yet preferentially fed on nectar contaminated with imidacloprid and thiamethoxam (but not clothianidin, the metabolic breakdown product of thiamethoxam). The bees were offered treated and untreated nectar in identical feeding stations under conditions of complete darkness, and as bumblebees utilise olfactory or visual cues when selecting nectar sources under natural conditions, further work was required to elucidate the mechanism by which they responded to the presence of insecticide contamination. A similar investigation by Arce *et al*. (2018) was conducted in the light, but used identical feeders thus again providing no cue for the bees to associate with treatment.

The methodology for the initial experiment in the current study expands and develops previous work investigating the ability of *B. terrestris* to respond to neonicotinoid insecticide residues in nectar by adjusting consumption rate (Thompson *et al*., 2014). At field realistic concentrations, the bees were shown to reduce consumption (and therefore exposure) to insecticide residues in sucrose solutions, but when offered untreated sucrose consumption rates rapidly recovered. The new work was conducted with no visual cues that could be associated with insecticide contamination, in order to provide a baseline to aid interpretation of later experiments.

The results of this no-choice study agree with those of Thompson *et al.* (2014), demonstrating feeding suppression in response to higher levels of contamination. No evidence was recorded for increased feeding on nectar substitute containing imidacloprid and thiamethoxam compared with untreated controls, as may have occurred if the bees fed preferentially on nectar contaminated with these active

ingredients as suggested by Kessler *et al*. (2015). Instead, following a short period of exposure significant reductions in consumption where observed for all three neonicotinoids in the 5µg L^{-1} and 10µg L^{-1} treatments. Even after a longer period of exposure to contaminated nectar substitute (4 days) than was used by Thompson *et al*. (2014), a full recovery of feeding was recorded in all treatments within a maximum period of 2 days after uncontaminated sucrose was supplied. Although anti-feeding responses were recorded at 5µg L^{-1} and 10µg L^{-1} these levels fall at the high end of a field realistic range in nectar following seed treatment (IPBES, 2017) and at 1µg L^{-1} there was no significant effect when compared to the control. As this was a no-choice study, however, it cannot be used to identify whether these effects were due to behavioural avoidance of contaminated nectar or to sub-lethal toxic effects that suppress feeding.

The second experiment in the current study was a choice test investigating the effect of neonicotinoid residues in nectar substitute on feeding preference when a source of untreated nectar was also available. The work was conducted over an extended period of time (5 days compared with the 24 hours utilised by Kessler *et al*. (2015)) and in a standard light cycle frequently used in bumblebee experiments, but as in the earlier work individual bumblebees removed from colonies before the experiment were utilised and no visual cue was provided distinguishing treated and untreated nectar sources. The results under these conditions, do not support the conclusions drawn by Kessler *et al*. (2015). Following an analysis of the proportion of total intake of nectar that came from the treated and untreated sources (thus accounting for effects of feeding reduction at higher insecticide concentrations), the results for all three of the neonicotinoids, and all three concentrations tested (1, 10 and 100 µg L^{-1}) showed a lack of either preferential feeding or avoidance of contaminated nectar sources. This suggests that either the bees cannot detect the contaminated nectar, i.e. there is no volatile or non-volatile chemical cue, or immediate sub-lethal effect, or they cannot associate cues with a specific feeder. Selection of nectar sources by bumble bees in the natural environment is thought to be mediated by both physical (e.g. colour) and chemical (e.g. odour) signals (Heinrich, 1976; Jakobsen *et al*., 1995; Gumbert, 2000; Tiedeken *et al*., 2014). Thus, the lack of a visual cue in this experiment may have prevented the bees from being able to associate effects with a particular source. In addition, these studies have been conducted on individual bees and it is unclear how this effect on individuals may be carried forward to the colony level.

The third experiment aimed to assess the effect of contaminated sucrose in the presence of a cue (colour) on bees foraging for a colony. This approach also addresses the constraints of a more recently published paper by Arce *et al*. (2018) in which two concentrations of insecticide residue and untreated controls were offered simultaneously to bees foraging from a colony. No visual cue was however, available to distinguish treatments, the colony was contained in a box that was only opened for foraging for 6 hours per day, assessments were only made during 3 of those 6 hours, and no food was made available to the bees for the other 18 hours. The photoperiod and temperature were not reported in the earlier study and so could not be replicated in the current work. Based on the proportion of visits to feeders their results suggested initial avoidance of feeding on sucrose solution containing 2 µg L^{-1} of thiamethoxam, but not when it contained 11 µg L^{-1}, but there was no effect on time spent feeding. Over time, however, an acquired preference for feeding on sucrose treated with thiamethoxam in the 2 µg L^{-1} feeders (based on an increase in the proportion of all visits by an individual of 1% per day), but not to the 11 µg L^{-1} feeders, was observed. Total volume consumed over 10 days was 28% and 26% greater respectively for the two insecticide concentrations. These results suggest that bees could both detect and preferentially feed on thiamethoxam, but only on feeders containing thiamethoxam at the 2 µg L^{-1}concentration.

Data describing nectar consumption and number of visits to flowers emerging from our study demonstrated a preference for blue flowers irrespective of whether or not the flowers offered contaminated nectar substitute. This preference of bumble bees for blue flowers has previously been reported (Műller, 1881; Gumbert, 2000) and demonstrated that it was important to control for colour in the analysis of results.

The total length of time spent visiting flowers (irrespective of colour) was higher in pairings where blue flowers were contaminated with thiamethoxam, than where yellow flowers were contaminated or in untreated controls. This may be explained by the occurrence of antifeedant responses following exposure to low levels of thiamethoxam, such as sublethal poisoning, repulsion or others (Thompson *et al*., 2014). Such antifeedance would explain the reversible reduction in consumption of nectar substitute recorded in the no-choice IFA experiment, and if triggered when bees fed on contaminated nectar in the flight cages, factors such as sublethal poisoning would reduce activity and result in longer delays before take-off. The difference between the total length of time spent visiting flowers in the pairings where blue flowers were contaminated and those with yellow contaminated flowers

would then be promoted by the innate preference for blue flowers that results in a higher number of visits to the blue colour. This interpretation relies on the mean length of individual visits also being longer in pairings where blue flowers offered contaminated nectar, which was confirmed by the analysis. Such an antifeedant response immediately after initial exposure to thiamethoxam contaminated sucrose solution was also observed by Arce *et al*. (2018), supporting this conclusion.

Once the bees had landed on the flower, colour ceased to have an effect on active feeding. If however, antifeedant responses to contaminated nectar occur, then it is likely that if they take effect sufficiently quickly, they would result in early termination of individual feeding sessions of bees. This was confirmed in the current work, in which pairings where the blue flower offered contaminated nectar the mean feeding time was significantly shorter than that on the yellow uncontaminated flowers. In the reverse pairings where the yellow flower was associated with contaminated nectar the feeding time was shorter than recorded on the untreated blue flower.

Thus, the observed reduction in the mean feeding time but increased visit time for the blue contaminated flowers suggests that a response occurred during feeding such that feeding ceased, overall activity was reduced leading to a lengthening of the duration of each visit. The mechanism underpinning this outcome may, however, rely on detection of the insecticide in the nectar and not be explained by sublethal toxicity alone. Although the time delay before sublethal effects of thiamethoxam on bumblebees become apparent is likely to be short due to its rapid uptake, significant adverse effects, e.g. wing block, in honeybees fed high doses of thiamethoxam treated sucrose (11 mg L^{-1}) have been shown to only occur within an average of 2 minutes after consumption (Girolami *et al.*, 2009). In this study average feeding session duration on contaminated nectar substitute were in the order to 1-1.5 minutes suggesting that sublethal toxicity alone may not explain the early termination of feeding.

Bumblebees, as in other generalist bees, have low sensitivity to the presence of toxins in nectar (Tiedeken *et al*., 2014), and it has been demonstrated that *B. terrestris* do not detect thiamethoxam via the sensilla on their mouthparts (Kessler *et al*., 2015). The potential for indirect association between the colour of a flower and an effect may be analogous to the proboscis extension reflex used to assess learning and memory in insects. Laloi *et al*. (1999) reported that, compared with honeybees, *B. terrestris* required a far higher concentration of sugar reward (75% sucrose compared to 20%) and twice as long training period (6 seconds exposure to

scent followed by 6 seconds sucrose reward) to learn the responses. Even under these conditions a relatively low proportion (32%) of individuals responded after 10 training sessions to a sugar reward (Sandoz *et al*., 1995). Such slow development of learned responses may be reflected in the relatively small differences in feeding times reported in this study.

In summary, when offered nectar substitute contaminated with three concentrations of imidacloprid, clothianidin or thiamethoxam for 4 days in no-choice arena experiments, individual *B. terrestris audax* workers displayed a reversible suppression of nectar consumption. When offered a choice of treated and untreated nectar substitute contained in identical feeders, however, the bees showed neither avoidance of, nor preference for, the contaminated nectar. In a third experiment workers emerging from a queen-right colony were offered the choice of thiamethoxam treated and untreated nectar substitute associated with visual cue (colour of artificial flowers). Insecticide contamination did not affect either the number of visits to flowers, or the number of feeding sessions that were initiated suggesting that the bees did not learn either preference or avoidance of the flowers with treated nectar substitute prior to landing. Instead a response occurred during feeding resulting in a shortening of the feeding session, but at the concentration tested (5µg L^{-1}) the effect was subtle so that the overall level of nectar consumed from contaminated flowers was unaffected. Further work is required to confirm the mechanism leading to these findings which may be behavioural or metabolically (e.g. upregulation of enzymes responsible for detoxification) based.

Thus, the hypothesis that separate (sequential) decisions relating to selection of the food plant and whether to commence extended feeding are made, is supported by the results of this work. Visual cues (others were not tested) were used when selecting flowers for feeding, and it is likely chemical cues allowing detection of insecticide contamination of nectar for the latter.

Chapter 7: General discussion

Wild pollinators are both popular with the general public and recognised as providing an important ecosystem service to agriculture (James & Pitts-Singer, 2008; Goulson *et al*., 2011; Kirk & Howes, 2012). They have been adopted as model species for investigation of the impacts of changes in environment, land use, climate and farming systems (with an emphasis on the impacts of certain pesticides in the latter case). Many species are thought to be in decline and their aesthetic and financial value has triggered much research in recent years (Potts *et al.,* 2010; Winfree *et al.,* 2011). Although sections of the research community have different motivations, ranging from a pure conservation ethic (Goulson *et al*., 2011; Kennedy *et al.,* 2013; Wood *et al.,* 2015) to a more utilitarian view of pollinators as ecosystem service providers to agriculture (Brown *et al*., 2016; Di Pasquale *et al*., 2016; Mallinger *et al.,* 2017), interests converge on a number of key questions relating to how vegetation in agro-ecological systems may best be managed to support and enhance pollinators; be they wild or managed populations. Critical to answering these questions is an improved understanding of a) the ecological mechanisms by which managed bees may enhance crop production, b) the drivers of selectivity of foraging for pollen exhibited by bees in agroecological landscapes, c) the importance of the nutritional components of pollen to larval development, and its importance as a contributor to foraging behaviour, and d) the response of bees to nectar and pollen contaminated with systemic pesticides. The current research programme has addressed these core questions. The results may be used to illuminate certain aspects of current debate where uncertainty exists as to the causes of pollinator decline, enhance the design of interventions in agricultural systems (including via environmental stewardship schemes), identify strategies to improve the effectiveness of managed populations as ecosystem service providers to agriculture, and signpost avenues for further fruitful research. Two species with contrasting ecologies were selected as 'models' for use in a series of laboratory and field studies; *Osmia bicornis* and *Bombus terrestris*.

Osmia bicornis is a common species of solitary bee in many parts of Europe, including the UK and has shown potential as a managed pollinator in a range of cropping systems including apples, strawberries and *Prunus* fruit (Hansted *et al*., 2014; Sedivy & Dorn, 2014). Commercial uptake and exploitation is currently limited, although some success has been recorded, for example in Poland and Germany

(Lane, 1979; Wilkaniec & Warakomska, 1992). The species is active in Europe from April onwards (Raw, 1972), but commercial rearing techniques are now available which provide adults for release in earlier flowering orchard crops such as cherries, at a time when few alternative wild pollinators are available (Gruber *et al*., 2011). Alternative forage is, however, often limited in cherry orchards during the flowering period and the impact of restricted pollen sources (largely to a single plant species) on retention and efficiency of this polylectic solitary bee has been questioned by commercial producers.

Pollination by *O. bicornis* has been shown to increase quality of fruit crops, particularly in cropping systems that require full pollination of multiple achenes. (Kuhn & Ambrose, 1984; Wilkaniec & Radajewska, 1996; Klatt *et al*., 2014). Such improvements to fruit quality have also been reported for some *Bombus* species, supporting the successful commercial use of several subspecies of *Bombus terrestris* in European crops, including *B. terrestris audax* in the UK. The effective pollination is to the result of mechanical deposition of pollen on the stigma, with *Bombus* and many solitary bees utilising buzz pollination, which results in increased pollen deposition. Some studies also suggest that buzz pollination further stimulates formation of pollen tubes, thus increasing fertilisation potential, in comparison with honeybees (Lane, 1979).

7.1 *Osmia bicornis* as a pollinator in low diversity agroecological systems

Within the UK, use of *O. bicornis* for commercial pollination services is mostly limited to apple orchards and strawberry crops, however there has been recent interest in its use in early flowering sweet cherry orchards. Both yield and fruit quality determine the value of sweet cherry crops, with premium prices realized when well established minimum quality requirements for fresh consumption of produce are met or exceeded. Produce used in processing attract lower returns. Retail standards have been established for produce intended for fresh consumption which set required ranges for fruit size, sugar content, firmness and colour (Sainsbury's Supermarkets Ltd., 2015).

Consistency of fruit quality is key driver of price, and as crop husbandry techniques for achieving the maximum yield potential of modern cherry varieties are well established, the development of growing systems which promote consistency of fruit

quality at harvest are a primary objective of growers (Andrew Hunt, Hope Farms Ltd., pers com.). Currently this is achieved by pickers making multiple passes through the crop during the period of fruit ripening, adding to production costs. Systems that synchronise ripening will increase the commercial viability of the orchard.

Most UK cherry orchards utilise mixed variety planting systems including self-fertile and self-sterile varieties which flower in late March or early April. As rapid ovule degeneration occurs during flowering, self-fertile varieties benefit from supplementary pollination by insects which leads to increased pollen deposition and has been linked to increased yield (Parrie & Lang, 1992). Early pollination affects fruit set, and a reduction in fruit quality at harvest has been reported when flowers are pollinated towards the end of the flowering period (Mayer *et al*., 1987; Ughini & Roversi, 1993). Pollinators therefore play an important role in determining both yield and quality of produce (Lane, 1979; Delaplane *et al*., 2000) but only a restricted range are active during the early flowering period of cherry. Of these, it has been suggested that *Osmia* spp. can make an important contribution to fruit set, increasing yield even when honey bee colonies are also foraging in the orchard (Kirk & Howes, 2012; Holzschuh *et al*., 2012; Guler & Dikmen, 2013).

In the current study, consistent yield responses (measured as total fruit number) were not associated with *O. bicornis* supplemented treatments. Following fruit set, however, commencement of fruit growth occurred earlier in treatments where wild pollinators were supplemented with *O. bicornis* than in those where pollination relied on naturally occurring pollinators only. Pollination could only commence when flowers opened, which occurred at the same time in each treatment, thus the earlier mean time for commencement of fruit growth in the *O. bicornis* supplemented treatment suggests that pollination/fertilisation was completed within a shorter time period when the bees were present. The resultant earlier completion of pollination may result in avoidance of fruit quality penalties associated with pollination occurring towards the end of the flowering period (Mayer *et al.*, 1987; Ughini & Roversi, 1993) and in particular to increased consistency of fruit size at harvest.

Evidence of the predicted increase in fruit quality was collected. Fruit from trees pollinated by wild pollinators supplemented by *O. bicornis*, and by naturally occurring pollinators only, both met the minimum quality requirements for fresh consumption of fruit (fruit size, sugar content, firmness and colour). In both years of the study, fruit weight and width were higher in the *O. bicornis* supplemented

treatment. In addition, some evidence of increased uniformity of fruit quality at harvest was recorded in 2016, the year in which largest differences between treatments in the length of the pollination window occurred, as would be expected if improved synchrony in fruit growth had resulted. Both fruit width and sugar content were found to be significantly more variable in the wild pollinator treatment when compared to the fruit from the *O. bicornis* supplemented treatment.

Thus the study yielded evidence that supplementing wild pollinators with commercially reared *O. bicornis* in cherry orchards can result in improved synchrony of fruit development leading to improved scoring of key quality parameters in commercial assessments and increased consistency of fruit quality at harvest. Further work is required to establish whether the quality improvements reported are sufficiently large and consistent to make exploitation of *O. bicornis* in sweet cherry production commercially viable. In addition, more research is needed to investigate yield and quality responses in both self-fertile and non-self-fertile *P. avium* varieties to support cost benefit analyses of its commercial use, and to enable comparisons with alternative managed pollinators. The effect of pollination treatment on shelf life of the crop, a key characteristic for both growers and retailers, also warrants investigation.

Flowering of sweet cherry trees (March-early April) occurs slightly earlier than when naturally occurring *O. bicornis* become active, thus its efficacy as a managed pollinator may be reduced compared to within other cropping systems (Raw, 1972). Species of solitary bees that are active earlier in the year, such as the blue mason bee (*Osmia caerulescens*; Bosch & Kemp, 2001), or some *Andrena* species, may be more effective pollinators for UK sweet cherry crops. Some of these alternatives can, however, be more difficult to manage commercially. For example, in US alfalfa production, *Nomia melanderi* has been shown to provide effective pollination but its use is restricted to specific soil types due to difficulties relating to the husbandry of this soil dwelling species (Cane, 2008). In such cases conservation biocontrol approaches encouraging development of larger wild populations rather than reliance on introduction of managed bees, may be a preferred option. These may include changes to orchard husbandry practices and establishment of off crop habitats, to provide environmental conditions that favour the required pollinator(s).

In the cherry orchard production system studied, trees were covered with open ended polythene tunnels with rows separated by a 2m grass strip with occasional herbaceous flora including *Taraxacum* and *Ranunculus* spp. Normal commercial

husbandry practice included opening the sides of tunnels during spring and summer, allowing access for pollinating insects during the flowering period, but at other sites the tunnels remain closed in spring to protect developing fruits from adverse weather. Thus although the study simulates the closed system of production, bees would be able to forage more widely on surrounding floral resources when tunnels are opened in the “open” system, potentially altering retention within the orchard. To establish the risk of losing potential *O. bicornis* pollinators from such production systems, a greater understanding is required of selective foraging on favoured plant species and the distances bees will fly in search of sources of the preferred pollen(s).

Although the poor coincidence of the emergence of *O. bicornis* in spring and the flowering period of cherry trees may limit its use in cherry orchards, improved understanding of adult foraging behaviour (including selective foraging) and the impact on developing larvae of the pollen species collected as nutritional resources, would also provide a basis from which to develop the conservation biocontrol techniques referred to above to promote natural populations of the bees for pollination of a range of crops.

7.2 Selective foraging and utilisation of flower species in an agricultural environment

The importance of pollination services for both the naturally occurring flora and crops in mixed lowland or pastoral systems is well understood (Morandin & Winston, 2006; Breeze *et al*., 2014), although the effects of pollination deficiency are arguably less apparent than in some orchard systems (Lane,1979; Bosch *et al*., 2006). Support for wild pollinators is a target of current agri-environment schemes – both because of their commercial value but also because of their wide appeal to the general public and recognition as indicators of environmental quality. From both perspectives, understanding of the nutritional requirements, and foraging behaviour of wild pollinators is important in the design of interventions to support these species.

The field experiment conducted as part of this research programme (Chapter 3) compared the use by *O. bicornis* of floral resources offered by three habitat types established under current agri-environment schemes on a mixed lowland/pastoral

farm. Using the number of nest cells built and resourced as an indicator of population increase,a significant impact of habitat type on nest cell construction was recorded. The greatest number of cells were constructed within boxes positioned within the nectar-rich (NS) and wild bird seed mix (WBS) agri-environment options. This illustrates that current agri-environment schemes can be useful as sources of pollen for *O. bicornis* and may facilitate population growth (although resources prevented monitoring of cocoons to emergence). It was, however, notable that the greatest number of cells was produced in the box located within the wild bird seed mix, rather than the nectar and pollen strip. This was unexpected because the latter was specifically designed to support pollinators. Many of the plant species contained in the pollinator-focussed nectar-rich mix were however, late flowering and thus more useful in the support of *Bombus* and honey bees, rather than early season emerging *Apidae* and other pollinators. Early emerging species, such as *Osmia*, may therefore benefit from a different mix of species, including early flowering plants, in seed mixes designed to support pollinator communities. The investigation of foraging in Chapter 3 not only provides insights into those plant species which were favoured by *Osmia*, but also highlights limitations imposed by their spatial distribution.

Ruddle *et al*. (2018) have shown that *Osmia* will fly over 1km from their nests to collect pollen, although there was an energetic cost resulting in fewer cells being constructed. In the present study, few cells were constructed in the boxes placed along the woodland edge despite these being within 150m of the nectar rich and wild bird seed mix plots. Thus, it would appear that energetic costs, among other factors, such as microclimate and shading, may have limited the success of bees released in these boxes – or perhaps that the long distance foraging flights reported by Ruddle *et al*. (2018) are an exception necessitated by low availability of suitable pollen resources. Limited quantitative information on the typical foraging range of *Osmia* is available, but a distance of 150m is generally thought to be typical (Bosch & Vicens, 2002; Gathmann & Tscharntke, 2002; Gruber *et al*., 2011). The present study, in which positive effects of floristically enriched vegetation were only observed in nest cells associated with the enriched habitats, supports the view that *Osmia* predominantly forages close to the nest. This has implications for the design of agri-environment schemes; wild pollinators will be supported more effectively if floristic enhancement is provided on a field-by-field basis. This is likely to entail changes in farming systems that allow wild flowering plants to persist more generally (e.g. within hedges, or in closely associated but spatially discontinuous habitat

patches), rather than only within bespoke off-crop plots. Considering the limited flight range of *Osmia*, wild bee pollination services may be most effective on farms with small fields (Isaacs & Kirk, 2010), and conversely crops in large field systems may not be able to completely rely on wild bees (Klein *et al*., 2012). This conclusion may also apply to other bee groups (Garibaldi *et al*., 2016).

An opportunity to improve the support for *O. bicornis* population development offered by agri-environment schemes may emerge from evidence of selective foraging by this species. A variety of agricultural weeds, shrubs and trees were utilised by *O. bicornis* in this study including *Vicia* spp., buttercups (*Ranunculus* spp.), *Acer* spp*., Quercus* spp*., Salix* spp. and members of the *Prunus* family. Many of these species are not included in current stewardship options designed to support pollinators. Despite the diversity of flowers available in the landscape, approximately only 20 species (out of 50+ available) were utilised and there was evidence of very strong positive selection for many of these; nest cell pollen provisions at each sample date typically consisted of 2-4 species. This result may allow for the identification of “keystone” species. Support for these specific plant species, in addition to diversity of floral resources, may further enhance *O. bicornis* populations (alongside other pollinator groups) and provide an opportunity to more effectively tailor agri-environment payments.

The drivers of selective foraging in *O bicornis* warrant further investigation. Flower species which are chosen may possess pollen with greater nutritional value (improving support to developing larvae) or may represent lower foraging costs to adult females, when compared with alternative sources available. Pollen mixing may also occur in response to the presence of natural and manmade harmful toxins (Jurgens & Dotterel, 2004; Park *et al*., 2015). For example, the pollen of the *Ranunculus* family has been shown to contain high levels of ranunculin, which can result in bee mortality but in Chapter 4, *O. bicornis* larvae performed well when provided with pollen resources containing significant proportions of *Ranunculus* pollen (60%). The inclusion of apparently “toxic” pollen in foraged resources has also been reported by other studies and mixing of multiple pollen species is thought to enable other nutritional components to be exploited without detriment to the larva (Bergstrom *et al.*, 1995; Jurgens & Dotterel, 2004). Other natural toxins in pollen may include harmful secondary metabolites, such as alkaloids which have been shown to affect survival of larval and adult honeybees and inhibit the development in the larvae of the solitary bee *O. bicornis* (Hitchcock, 1959; Detzel & Wink, 1993;

Kevan & Ebert, 2005; Kempf *et al.,* 2010; de Mesquita *et al.,* 2010; Sedivy *et al*., 2011, 2012; Gosselin *et al.*, 2013).

Amino acid content of the pollen provisions taken from nest cells constructed in the field experiment (Chapter 3) was undertaken to investigate the nutritional value of pollens collected by foraging *O. bicornis*. Other components of nutrition could not be investigated due to financial constraints. Nine individual essential amino acids that are recognised to be important for honey bees (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine; DeGroot, 1953) were analysed separately and no significant differences in their levels in pollen provisions were found between habitats (NS, WBS or grass). The lack of significant differences in the levels of individual amino acid content in larval resources foraged from the different habitats, together with evidence of selective foraging by *O. bicornis* (Holzschuh *et al*., 2013), may indicate that the bees collect mixed pollen resources in order to maintain a minimum amino acid content. In unenriched habitats this may result in the need to travel further distances during foraging which, exacerbated problems associated with the lower floral density and resulted in the lower number of nest cells recorded in the study. The NS habitats are designed to offer increased nectar resources for pollinators but are not designed around pollen availability or quality. Differences between nest cell numbers in WBS and NS habitats (WBS < NS), therefore, may also be explained by longer foraging distances required to optimise nest provisions. Lowest total protein, essential amino acid (EAA) and non-essential amino acid (NAA) were consistently recorded in the florally unenhanced grass habitat, which appears contradictory to the results for individual amino acid content. The differences are explained, however, by only nine of the 20 amino acids that are potentially present being individually assessed.

Pollen provides most of the essential nutritional requirements of bees including protein, carbohydrates, lipids (including sterols), vitamins, minerals and starch. For example, evidence suggests sterols are key in hormone synthesis as precursors of moulting hormones and cell membrane gene expression (Vaudo *et al*., 2015). Most studies have been conducted on honey bees and focus on amino acid composition which is considered an important indicator of nutritional value (Day *et al*., 1990; Cook *et al*., 2003). Previous studies of bumblebees have, however, shown selective foraging in response to protein carbohydrate ratios (Vaudo *et al.,* 2016), thus pollen selection may be driven by a wider range of nutritional components than amino acids alone. The variability of the results in this study may therefore reflect the need

for further work investigating the influence of a combination of factors on forage selection. Furthermore, identification of the amino acid profiles of individual pollen species will provide information to allow comparison of amino acids in pollens that were and were not selected, and whether this amino acid selectivity is an artefact of conservative values of such amino acids found in nature rather than selectivity.

One flower-based strategy that may increase pollinators in the agricultural landscape involves mass-flowering crops that attract pollinators to the area and may benefit a growing pollinator population by providing a pulse of resources (Le Féon *et al*., 2013). There is however, the concern that mass-flowering crops can dilute wild bee populations, or there could be competition between crop flowers and concurrently blooming wildflowers (Holzschuh *et al*., 2008). Also, after a one-time pulse, resources may not be available to support the bee community during the rest of the season. In cases of mass-flowering crops, additional floral resources should be available and must compliment the crop to be available before and after the crop bloom to extend the full foraging season of the pollinator community (Menz *et al*., 2011). These issues appear particularly pertinent to the use, and support, of *Osmia* in cherry orchards (addressed in Chapter 2). Pollinators of early, short blooming trees may need more floral supplements than the pollinators of longer flowering summer crops, such as annual vegetables. Any mismatch in complimentary composition of timing of wildflower availability and crop bloom period may not be effective in benefitting wild pollinator communities associated with a mass flowering crop (Ritz *et al*., 2013). The availability of alternative forage species may also facilitate pollen mixing as a strategy to reduce the impacts of pesticides and naturally occurring toxins in pollen. Field research suggests that the negative effects of pesticides on pollinators can be mitigated in landscapes where flower rich habitats act as potential buffer zones (Park *et al*., 2015).

7.3 Pollen profiles of nutritional resources and larval fitness

The selection of keystone plant species for agri-environment schemes providing florally enriched habitats for pollinators not only requires an understanding of selective foraging but also the relationship between larval nutritional resources and larval fitness. Solitary bees, like other Apiformes, utilise both pollen and nectar as nutritional resources (DeGroot, 1953; Dobson & Peng, 1997) and many flowering plants have developed adaptations that improve the efficiency with which bees

distribute their pollen grains to enhance the potential for fertilisation. These include a range of mechanisms for attracting bees or ensuring efficient pollen transfer from flower to forager (Michener, 2007), or for deterring visits from species that are less efficient pollinators (Adler, 2000). Foraging for pollen by the bees themselves is energetically expensive, and only small amounts are collected from individual floral visits (Müller & Kuhlmann, 2008). Thus as solitary bees are not associated with a colony in which many workers contribute to larval provisioning, development of strong local populations relies on a consistently high level of foraging efficiency by individual adults. This level of efficiency has been supported by the evolution of specialised morphological and behavioural adaptations to exploit the resource effectively (Thorp, 2000).

In addition to effective foraging, floral resources are recognised as a significant factor of pollinator dynamics (Sagili & Pankiw, 2007; Potts *et al.,* 2010; Eckhardt *et al.,* 2014) and are of particular significance for bee species, in which both adults and larvae are dependent on flowers for nutrition (Michener, 2007; Goulson, 2010). In agricultural environments, limitations in floral resources significantly affect bee species diversity (Jauker *et al.,* 2012; Eckhardt *et al.,* 2014), but can be mitigated or minimised through the provision of off-crop habitats, such as those in Chapter 3, offering increased floral diversity (Jauker *et al.,* 2012; Eckhardt *et al.,* 2014). The optimisation of the floral composition of these habitats is however, currently limited by a lack of knowledge on the physiological effects of specific nutritional components on pollinators at both the individual and colony level.

Pollen is required for development of ovaries in females and provisioning of larval offspring (Biliñski & Teper, 2004; Goulson, 2010). Nectar is utilised as a carbohydrate fuel source in order to sustain flight during foraging trips (Tasei & Aupinel, 2008). In honey bees and bumblebees pollen is key to colony development and offspring mass (Ribeiro, 1994; Sagili & Pankiw, 2007; Jauker *et al.*, 2012; Eckhardt *et al.*, 2014), and pollen quality has been linked to antimicrobial immune response (Moret & Schmid-Hempel, 2004; Mapalad *et al.*, 2008), and the detoxification of pesticides (Alaux *et al*., 2010b). Most work investigating pollen quality has been directed towards the design of artificial diets for supplementary feeding of honeybees and there has been limited biochemical analysis of pollen beyond crude protein/lipid analysis and (at the commencement of this research programme) little work on pollen utilisation by bumblebees or solitary bees (Cook *et al.*, 2003; Sagili & Pankiw, 2007; James & Pitts-Singer, 2008).

The pollen produced by different plant species varies significantly in its nutritional components (Roulston & Cane, 2000), and within an individual species in relation to multiple abiotic factors (Suarez-Cervera *et al*., 1994; Dobson & Peng, 1997). Pollen provides protein, amino acids, fatty acids, lipids, vitamins and minerals for pollinators. Bees have specific amino acid requirements and ten amino acids are thought to be essential for the growth of honey bees. Deficiencies of any of these amino acids in pollen can have serious consequences; e.g. bees fed a diet of dandelion pollen, low in arginine, were shown to be unable to rear brood until their diet was supplemented. Fatty acids are also important for bee nutrition and health: some are essential nutrients, whilst others have antimicrobial activity. Some reports suggest that bees actively select pollen with high levels of unsaturated fatty acids (Manning, 2001). In addition to oleic acid (C18:1), minimum levels of linoleic (C18:2) and linolenic acids (C18:3) appear to be required; low levels cause failure of pupal and adult ecdysis, deformed adults, slow growth and decreased adult fecundity. However, pollen high in oleic acid severely reduces the life span of honey bees. Enhanced oleic acid content in seeds of oilseed rape varieties has been an important recent breeding objective and the levels of unsaturated fatty acids have been reduced for human health benefits and for oxidative stability, and this has generated high oleic acid content by reducing erucic and linolenic acid contents. More detailed understanding of the chemical composition of bee collected pollens, and the combined impact of all the nutritional components on both individual or colony level fitness, however, awaits further study (Roulston & Cane 2000; Eckhart *et al*., 2014). Ultimately such understanding may, in combination with analysis of the levels of key components present in the pollen of candidate plant species, offer the opportunity to inform the selection (and optimisation) of species mixes used in man-made florally enriched habitats such as those described in Chapter 3.

If a lack of pollen diversity is contributing to the declining bee health recorded during the last 60 years due to nutritional imbalances, as suggested by several authors, the introduction of a range of sources into an agricultural landscape (such as the habitats investigated in Chapter 3) could help to ameliorate any nutritional deficiencies. In designing such florally enriched habitats, both the nutritional quality and temporal constancy of pollen sources need to be taken into account when selecting plant species to include. Any recommendations on plant species should also consider the effects of larval or adult nutrition on pesticide susceptibility (Alaux *et al*., 2010a; Mao *et al*., 2013).

At the commencement of this research programme limited evidence of the ability of solitary bees to assess pollen quality was available, but some *Bombus* species had been shown to preferentially select high protein pollens (Leonhardt & Bleuthgen, 2012). *O. bicornis* is a polylectic species that provides larvae with nutritional resources consisting of mixtures of pollen species (Cane & Sipes, 2006; Műller & Kuhlmann, 2008). This may result in more effective exploitation of spatially and temporally variable plant communities, increasing floral resource utilisation (and quantity of larval provisions; Williams & Tepedino, 2003), allowing the utilisation of pollens low in particular nutritional components such as amino acids, and buffering pollens with harmful secondary metabolites (Budde & Lunau, 2007; Eckhart *et al.,* 2014). The selective foraging described in Chapter 3 may be based on real-time evaluation of pollen quality, and have resulted from co-evolution of the bee with multiple plant species.

Work conducted under this research programme (Chapters 4 and 5) investigated the hypothesis that amino acid content of the pollen in larval diets is a driver of individual or colony success in polylectic bees. Pollen diversity in larval diets is thought to increase the potential for essential amino acids being present in sufficient quantities together with other essential nutritional components (Génissel *et al.*, 2002; Vanderplanck *et al.*, 2014). *O. bicornis* larvae were offered one of four diets containing a single pollen species, and two with a mixture of pollen species (one of which was collected by naturally foraging bees). Significantly lower survival to pupation was recorded for larvae fed *P. spinosa* pollen than for any other diet tested, with the highest survival associated with the pollen mix collected by naturally foraging *O. bicornis*. No clear relationship occurred between survival and larval diets containing single or multiple pollen species. The levels of each of nine individual EAA, and of total protein and total EAA or NAA were lower in the *P. spinosa* diet than in all others investigated. With the exception of the *P. spinosa* diet, *h*owever, the pollen mix collected by naturally foraging *O. bicornis* was not found to contain higher levels of any of the individual amino acids or of total protein, EAA or NAA, when compared to other larval resources investigated. The *O. bicornis* collected diet contained 60% *Ranunculus repens*, a pollen noted for high levels of the bee toxin ranunculin (see section 7.2), without apparent detrimental effects on survival.

The detailed nutritional requirements of solitary bees differ to those of *Apis* and *Bombus* bees (Leonhardt & Blüthgen, 2012; Kriesell *et al*., 2017). Solitary bee larval resources are also collected by a single adult female and fed to individual larvae,

whereas foraging is shared between multiple workers in colony forming bees. The impact of different pollen diets on colony-level performance were investigated using *B. terrestris audax* micro-colonies. Five pollen diets (including single and multi-pollen mixes, three with varying proportions of the relatively disfavoured oilseed rape (*Brassica napus*) pollen were investigated. Overall colony biomass gain varied between diets, with stronger colony performance correlated with those containing higher levels of nine essential amino acids (leucine, lysine, valine, arginine, isoleucine, phenylalanine, threonine, histidine, methionine), but there was no consistent relationship between the diversity (number) of pollen species contained in a diet and biomass gain. The poorest performance was recorded in micro-colonies offered the pure oilseed rape pollen diet, the treatment with the lowest levels of all nine amino acids. When oilseed rape was mixed with other pollens, performance was related to the proportion of oilseed rape in the diet; eggs were produced and nest building initiated later in those with a high proportion of oilseed rape pollen (reflecting the lower amino acid content).

The experiments investigating both solitary bees and bumblebees suggest that survival or colony development were influenced by the pollen species profile contained in larval diets. No consistent differences in survival of solitary bee larvae between diets containing either single pollen species or multiple pollens were recorded. Poor survival of solitary bee larvae, however, was associated with the pollen diet containing the lowest total protein, and total essential and non-essential amino acid content. When variable but higher levels of amino acids or protein were present in other pollen diets tested, survival was not found to differ, suggesting that provided a minimum threshold level was attained, differences between survival may have been the result of other (perhaps nutritional) factors which were not assessed. Evidence for greater sensitivity to amino acid content of pollen diets was obtained in bumblebee micro-colonies. The proposal that there is a requirement for a minimum protein or amino acid content was supported, but performance could also be correlated with levels of protein or amino acids above this minimum level.

The results of this study support the suggestion that pollen nutrients better explain individual or colony performance than pollen diversity alone (Moerman *et al.*, 2017). Foraging for pollen is energetically expensive (see section 7.3), and only small amounts are collected from individual floral visits (Müller & Kuhlmann, 2008). In habitats offering less favoured floral resources, pollen mixing in larval nutritional resources may enable pollen species that are widely available but lacking in one or

more essential nutritional components to be utilised without detriment to larval performance (Génissel *et al.*, 2002; Vanderplanck *et al.*, 2014; Moerman *et al.*, 2017). The current study provided evidence that the effect of low levels of amino acids in pure oilseed rape pollen diets could be addressed by addition of other pollen species, although plant defence chemicals, e.g. flavonoids (Serra-Bonvehí *et al.,* 2001), cannot be ruled out. Pollen mixing may therefore offer a strategy for more effective utilisation of floral resources available in a local habitat, reducing the need to make inefficient longer distance foraging flights. Other nutritional components, however, may also be important for larval or colony performance, and thus influence selective foraging in bees. In addition, pollen grains are extremely resilient to degradation and the pollenkitt layer may interfere with nutrient extraction, further complicating pollen selection (Peng *et al*., 1985; Suarez-Cervera *et al*., 1994; Dobson & Peng 1997). Further work investigating the effect and interactions between such components is required to provide a more holistic understanding.

In solitary bees populations each individual acts as a single reproductive unit without the benefit of a colony to support reproduction. Foraging for pollen is thought to be directed towards plant species offering higher nutritional rewards, while avoiding competition and unnecessary energy expenditure. This may reinforce niche separation between solitary bee species to avoid competition, and result in species specialisations to increase the nutrients extracted from difficult to digest pollens or biological activity to deal with pollens high in harmful secondary metabolites (Roulston & Cane, 2000; Praz *et al*., 2008; Sedivy *et al*., 2011).

7.4 The effect of insecticide contamination on the utilisation of pollen and nectar

If selective foraging by bees optimises nutritional resources provided for developing larvae within the constraints imposed by the range of plant species growing within their foraging range (and associated frequency of flower sources), then other factors that affect flower selection may result in poorer larval and colony performance. It is well established that behavioural avoidance responses following contact with insecticide residues affect foraging behaviour and consumption of prey in several predatory insects (e.g. Singh *et al*., 2001; Singh *et al*., 2004), and thus contamination of pollen or nectar by pesticides, including systemic insecticides, may also disrupt selective foraging in bees.

Sustained reductions of nectar consumption by *B. terrestris* have been reported when bees are offered nectar spiked with imidacloprid, clothianidin or thiamethoxam at concentrations that have been reported in commercial fields (EFSA, 2012) in controlled experiments (Cresswell *et al*., 2014; Thompson *et al*., 2014). The response was reversible, with rapid resumption of increased consumption rates occurring when untreated nectar was offered following exposure to imidacloprid, but no published results on recovery of bees exposed to the other two active ingredients were available. Low mortality was recorded for all three active ingredients, investigated in the current work suggesting that reduced sucrose consumption conferred a degree of protection from toxicity but the work did not support identification of the underlying mechanism. Feeding suppression may have resulted from reversible sub-lethal poisoning, or reversible antifeedant responses,

The current study confirmed and extended these findings, showing that full recovery of consumption levels of *B.* terrestris also occurred when untreated sucrose was offered to bees following exposure to both clothianidin and thiamethoxam. In a choice test in which treated and untreated sucrose was offered simultaneously in identical feeders, the proposal that the bees can detect and avoid the insecticides was tested. No preference for either was found but the lack of a cue that allowed sources of treated and untreated sucrose to be distinguished may have affected the outcome. Selection of nectar sources by bumble bees in the natural environment is thought to be mediated by both physical (e.g. colour) and chemical (e.g. odour) signals (Heinrich, 1976; Jakobsen *et al*., 1995). If an olfactory signal was present the bees did not appear to respond to it, but the lack of a visual cue may have prevented them from being able to associate effects with a particular source. Further these experiments were conducted using individual bees and it is unclear how this effect on individuals may be carried forward to the colony level.

The proposal that free flying foraging *B. terrestris* can detect and avoid sucrose solution spiked with imidacloprid, clothianidin and thiamethoxam has been investigated in the laboratory. The study arrived at the apparently contradictory conclusions that for sucrose contaminated with imidacloprid and thiamethoxam, bees could not detect the active ingredient, consumed less contaminated nectar, but nonetheless foraged preferentially on treated nectar (Kessler *et al*., 2015). Arce *et al.* (2018) also investigated free flying *B. terrestris* foraging in laboratory flight cages for a period of 10 days, on an array of identical sucrose feeders containing two different (field realistic (EFSA, 2012)) concentrations of thiamethoxam or untreated

nectar. At the start of the flight period, avoidance of pesticide treated sucrose was recorded supporting the findings of the above studies in this respect. Visits to thiamethoxam spiked feeders increased with time, however, at the lower of the two concentrations investigated but not at the higher concentration, resulting in greater consumption of pesticide-laced sucrose relative to untreated sources. The study concluded that worker bumblebees can detect thiamethoxam and alter their behaviour to continue feeding on it, but offered no explanation regarding why the bees changed from initially avoiding consumption of the toxin to displaying no avoidance behaviour over time. The experimental design of both studies of free flying bees again suffered from no visual cue being associated with feeders to distinguish those containing different levels of neonicotinoid, and the work was conducted in the dark in one of them (Kessler *et al*., 2015).

The current study investigated the cues that are utilised in decision making on selection of flowers to visit, commencement of feeding on nectar resources, and feeding for extended time periods, to identify potential mechanisms of initial avoidance behaviour. Responses of foraging free flying *B. terrestris* worker bees emerging from queen right colonies to feeders containing untreated sucrose (nectar substitute), or sucrose containing field realistic concentration of thiamethoxam were recorded and analysed. Treated and untreated feeders were associated with distinctive visual cues (flower colour) and bees were offered the two feeders simultaneously and prior to the introduction of insecticide treated nectar substitute were conditioned to foraging from flowers of both colours.

The study suggested that the bees utilised colour cues when selecting flowers to visit during foraging, with blue flowers visited more frequently than yellow during the course of the experiment, even when identical nectar rewards were offered in both. No evidence of insecticide contamination status influencing choice of flower to visit was obtained.

Commencement of feeding was not related to either flower colour or contamination status (at the concentration used in this study) of nectar substitute, suggesting that colour attracts the bee to the flower but has no further role after landing. The contamination status of the nectar is not determined before ingestion, possibly reflecting the low sensitivity bumblebees to the presence of toxins in nectar (Tiedeken *et al*., 2014). Initiation of a feeding session may be related to the sugar reward in the nectar, although from learning and retention experiments it is known

that *B. terrestris* requires a considerably higher concentration of sugar and longer training periods to learn the responses than honey bees (Laloi *et al*., 1999).

After feeding has commenced the length of the feeding session is reduced by the presence of insecticide (at the 5 µg L^{-1} concentration used). It was concluded that this may be the result of detection of the contamination and associated antifeedant responses leading to early termination of individual feeding sessions of bees. If this is the case, then response time would have to be rapid if toxic effects of thiamethoxam (which is absorbed rapidly) were to be avoided. Significant adverse effects, e.g. wing block, in honeybees fed high doses of thiamethoxam treated sucrose (11 mg L^{-1}) occur within an average of 2 minutes after consumption (Girolami *et al.*, 2009). In this study mean feeding session duration on contaminated nectar substitute was approximately 1-1.5 minutes suggesting that sublethal toxicity alone may not explain the early termination of feeding.

In summary the study concluded that sequential decisions relating to selection of the food plant and whether to commence extended feeding are made. Visual cues (others were not tested) were used when selecting flowers for feeding, and it is likely chemical cues, allowing detection of insecticide contamination of nectar, were used to determine length of feeding session. The design of future studies should be informed by these findings to enable interpretation of results to be based on potential biological mechanisms.

It is well established that the effect of insecticides on wild bee communities can be buffered by increasing the proportion of natural habitat in the surrounding landscape (Park *et al*., 2015). The current research programme has confirmed that utilisation of flower-rich habitats in agricultural landscapes by the solitary bee *O. bicornis* results in the construction of increased numbers of nest cells, and has linked selective foraging on the flower species available within these habitats with the provision of higher value nutritional resources for larvae. The importance of pollen species profiles in larval resources was also demonstrated in colony forming bees such as *B. terrestris*. The presence of systemic insecticides in favoured flowers, however, may disrupt foraging and result in sub-optimal selection of pollen sources, particularly if they include flowering crops. In this research programme the presence at field realistic rates of three neonicotinoid insecticides was shown to reduce consumption of nectar substitute by foraging workers but mortality was not higher than was found when bees had access to untreated control nectar (although 100% mortality was recorded when supra-field realistic concentrations of insecticide was

present), The bumblebees did not react to nectar substitute spiked with insecticides prior to landing, making it unlikely that initial selection of flowers during foraging would be affected in the field. The propensity to start feeding was also not affected by nectar contamination status, but length of individual feeding sessions were significantly shorter when contaminated nectar was offered, a response that may protect the bee from toxicity. This outcome suggested that physical contact with contaminated nectar was required before the insecticide was detected and thus at field realistic concentrations any volatiles emitted were not used as a cue. After feeding more time was spent resting on contaminated flowers, potentially reducing foraging efficiency.

Variable levels of insecticide have been detected in nectar sampled from either European field crops, or from off crop plants (EFSA, 2012; Botias *et al*., 2016), ranging from those eliciting no response, to those at which sub-lethal responses were beginning to be observed in this research programme. This may partly explain the very different responses reported following bee exposure to neonicotinoids in commercial crops, with some authors recording no impact at either individual or colony levels while others note detrimental effects (Gill *et al*., 2012; Henry *et al*., 2012; Pilling *et al*., 2013; Cutler & Scott-Dupree, 2014; Cutler *et al*., 2014; Rundlof *et al*., 2015).

7.5 Conclusions

This study investigated five primary hypotheses relating to the provision of pollination services in agroecosystems by wild (non-*Apis*) bees. The research programme commenced with a study of the efficacy of a common solitary bee species in the UK (*O. bicornis*) as an early season pollinator of sweet cherries grown in monoculture (where limited alternative forage was available). The study moved on to investigate whether provision of florally diverse habitats near monoculture crops benefited local pollinator populations through increased nest cell construction and provisioning (thus increased number of larvae). Linked to this, the potential that selective foraging by adults resulted in larval provisions with more favourable nutritional characteristics (e.g. higher levels of key amino acids), achieved either by utilising single species offering pollen displaying optimal nutritional characteristics or by constructing pollen species mixes, was tested. As the outcomes indicated that foraging was non-random and a low number of pollen species were selected for use

when adults were provisioning larval cells, the performance of *O. bicornis* larvae when offered a range (wild collected and experimental) of pollen mixes as food was investigated.

Foraging is energetically expensive and utilising only a sub-set of the plant species (pollens) available within foraging range may limit population size. Individual solitary bees collect all the provisions required for their own larvae, whilst colony forming bees share foraging activities between multiple workers. Whether this results in colony development in eusocial bees being more responsive to selected pollen mixes that offer improved nutrition was investigated using *B. terrestris* micro-colonies. If selective provisioning results in improved larval diets, then this may be disrupted if bees can detect and are deterred from feeding on favoured plants when they are contaminated with insecticides. The study concluded with an investigation of the ability of *B. terrestris* workers to detect the presence of systemic insecticide in nectar, and behavioural mechanisms by which this is achieved.

The outcomes of the work enabled conclusions to be drawn on each of the five primary hypotheses defined in Chapter 1. Although no clear yield responses were recorded, the study found that the release of *O. bicornis* in cherry orchard plots significantly increased the quality of fruit produced by shortening the pollination window, resulting in greater size and uniformity compared to pollination by wild insects alone. To obtain the premium prices offered for cherries marketed for fresh consumption retailers require a high level of consistency between fruit as well as produce being within a strict size range. Thus the first of the five primary hypotheses defined in Chapter 1 (Supplementing pollination of *O. bicornis* will increase the quality and yield of fruit in commercial cherry orchards) was partially supported.

The results of field studies presented in Chapter 3 indicted that a larger number of *O. bicornis* nest cells were produced when adult bees were released from nest boxes set in florally enriched habitats within agroecosystems when compared to unenriched areas. In addition, palynological analysis of provisions in larval cells indicated that selective foraging was undertaken by adults, with flowers of some commonly available plant species not utilised as pollen sources, whilst favoured pollen sources included some less common species. Linked to these outcomes, a laboratory study was undertaken investigating the development and survival of *O. bicornis* larvae offered pollen diets collected by naturally foraging adults or other experimental diets containing either single or multiple pollen species (Chapter 4). Although there was no evidence of differences between developmental rates of

larvae fed different diets, survival to pupation was significantly higher in those larvae provided with the pollen mix collected by naturally foraging adults than the experimental diets. Amongst the experimental diets larvae offered pure *Pinus spinosa* pollen showed a significantly higher mortality rate than in other treatments. Amino acid analysis indicated that significantly lower levels of amino acids (both total levels and levels of individual compounds) were found in *P. spinosa* pollen than in all other diets. Highest levels were not recorded in the naturally foraged pollen mix, however, potentially indicating that a minimum ("threshold") level of amino acids was required by the larvae but evidence of differentially better performance was not recorded above this level. Analysis of nest cell provisions collected from the field work described in Chapter 3 indicated that the amino acid content of all samples exceeded that in *P. spinosa* pollen used in the laboratory experiment. The work described in these two chapters, therefore, did not disprove the second primary hypothesis defined in Chapter 1 (*O. bicornis* can identify favourable characteristics (amino acids) of pollens from different plant species, and optimise larval provisions in nest cells by selective utilisation of a few plant species from the diverse range available), although it does not suggest a mechanism by which the bees might identify amino acid content of pollen (or definitive evidence that it occurs), or consider other nutritional components or chemical defence compounds of pollen that may affect selection/utilisation. The work also provides evidence partially supporting the third hypothesis (performance (development and survival) of individual *O. bicornis* larvae is determined by pollen species profile and associated amino acid content of wild collected and experimental pollen diets), with poor survival associated with a diet of *P. spinosa* pollen.

In work conducted on *B. terrestris* in Chapter 5, micro-colony experiments indicated that overall colony performance (increase in biomass) and components of performance (timing of nest initiation, and related life stage structure of the micro-colony at termination) could be associated with the pollen diet that the bees were offered. Amino acid content of the diets were more closely associated with colony performance parameters than was the case for solitary bee larval survival. The results, therefore, supported the fourth primary hypothesis (Colony performance of *B. terrestris* is determined by pollen species profile and associated amino acid content of experimental pollen diets).

Experiments in Chapter 6 investigated responses of *B. terrestris* to insecticide contaminated nectar. A clear fully reversible reduction in feeding when insecticides

were present in nectar at field realistic concentrations was demonstrated. Behavioural responses were identified that were associated with colour cues affecting flower selection and commencement of nectar feeding, together with potential chemical cues (detection of the insecticide) linked to early termination of the feeding sessions and thus reduced consumption (hence avoidance) of contaminated food. Thus the final primary hypothesis defined in Chapter 1 (*B. terrestris* displays behavioural mechanisms associated with visual and chemical cues that lead to avoidance of neonicotinoid contaminated food) was supported

8. References

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