A University of Sussex PhD thesis

Available online via Sussex Research Online:

http://sro.sussex.ac.uk/

This thesis is protected by copyright which belongs to the author.

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the Author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the Author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Please visit Sussex Research Online for more information and further details

Flowers for health: the importance of flower diversity and composition for maintaining the health and disease resistance of bumblebee pollinators

Joanne D. Carnell

Submitted in accordance with the requirements for the
degree of Doctoral Philosophy
University of Sussex

September 2019

Table of Contents

Declaration

I confirm that the work submitted here is my own, except that which has formed part of jointly authored submissions and publications as detailed below. I confirm the appropriate credit has been given within the thesis where references have been made to the work of others. This thesis has not been and will not be submitted to another institution for the award of any other degree.

Chapter 2 contains work from a jointly authored publication:

Trialling techniques for rearing long-tongued bumblebees under laboratory conditions

Joanne D. Carnell, Sam Page, Dave Goulson & William O. H. Hughes

Author contribution are as follows: All authors conceived the research; WOHH and JDC designed the experiment; JDC performed experiments and analysis; WOHH and JDC wrote the paper.

Chapter 3 contains work from a jointly authored publication:

Effect of diet on incipient colony success for two long-tongued bumblebee species in the laboratory

Joanne D. Carnell, Rosaline A. Hulse, Sam Page, Dave Goulson & William O. H. Hughes

Author contribution are as follows: JDC, SP, DG and WOHH conceived the research; WOHH and JDC designed the experiment; JDC and RAH collected bees, JDC carried out the experiment, JDC and WOHH performed analyses, JDC wrote the paper.

Chapter 4 contains work from a jointly authored publication:

Habitat type affects the prevalence of bumblebee pathogens in gardens and farmland, but not rates of transmission

Joanne D. Carnell, Sam Page, Dave Goulson & William O. H. Hughes

Author contribution are as follows: All authors conceived the research; WOHH and JDC designed the experiment; JDC collected and analysed the data; JDC wrote the paper.

Chapter 5 contains work from a jointly authored publication:

Characterising floral resource availability, bumblebee health and pathogen prevalence in three important UK habitats

Joanne D. Carnell, Rosaline A. Hulse, Sam Page, Dave Goulson & William O. H. Hughes Author contribution are as follows: JDC, SP, DG and WOHH conceived the research; WOHH and JDC designed the experiment; JDC collected the data, JDC and RAH carried out molecular screening, JDC performed analyses, JDC wrote the paper.

Acknowledgements

Undertaking this PhD has been a life-changing experience that would not be possible without the support and guidance I received from the people around me. First and foremost, thank you to my family. My mum, who has always believed in me. You gave me the confidence to chase this dream, and are already backing me for the next one! My dad, for founding my love of science, and my sister, for being perpetually cheery and enduring every single one of my attempts to teach you about bees and flowers. I am sorry but it's still not going to stop.

A huge thank you to my supervisor Bill Hughes. I will always be grateful to you for this opportunity. The last four years have been immeasurably important to me and I thank you for your guidance throughout, for always listening and supporting me when I needed it most. I would also thank every member of the Hughes and Goulson labs I've met during my time at Sussex, in particular, Rosaline Hulse. I cannot and would not want to imagine this experience without you. I thank you for your love, friendship and courage in the face of every bee-related catastrophe we've withstood together.

Abstract

Bumblebees are important pollinating insects for many crops and wild flowering plants. Due to multiple factors that include agricultural intensification, many populations have experienced severe declines and several species are now listed in the UK Biodiversity Action Plan. Bumblebees rely exclusively on nutrients derived from pollen and nectar, and nutrition is crucial for the development and activation of the immune system. Four microbial pathogens are known to infect bumblebees, and previous work has shown a variety of interactive effects between host nutritional status and pathogen epidemiology. Of the studies that investigated nutritional immunology in bumblebees, almost all have been carried on a limited range of common species because others, notably long-tonged species, have been difficult to rear in captivity. As a result, we lack knowledge of the nutritional needs of many declining species. Here, I test methods for rearing two long-tongued species in captivity and investigate the effect of diet on incipient colony development. A new technique to encourage oviposition and brood care was trialled successfully, and I observed interspecific differences between bumblebee queens on each diet. I also find evidence to support the idea that the nutritional content of pollen, not only plant species diversity, determines bumblebee health. In the wild, floral resources play an important role in regulating bumblebee populations and that of their pathogens. The abundance, diversity and composition of floral resources vary dramatically across the landscape, but studies investigating the effects of floristic composition on host-pathogen dynamics in bumblebees remain scarce. I compare floral resource availability, bee health and pathogen prevalence across three important UK habitats: farmland, gardens and nature reserves. I found that gardens contained the greatest species richness of flowers and had the largest, healthiest bees, despite increased parasitism. Farmland consistently provided the least floral resources, but habitats were complementary to each other in resource provision. I observed interspecific differences in bee health across habitats and report on the prevalence of bumblebee pathogens *C. bombi, N. bombi* and *N. ceranae.* Floral resources have a substantial effect on bumblebee health and pathogen dynamics, but these effects appear to vary between species. To support taxonomically diverse bumblebee communities, it is essential that the nutritional needs of a wider range of bumblebee species are considered.

List of Figures

List of Tables

Chapter 1: General Introduction

1.1 Pollination

Animal-mediated pollination is one of the most important mutualisms that exists on our planet. Pollination services contribute towards the botanical diversity of the terrestrial world and are central to all nutrient and energy cycles, playing a vital role in the maintenance of ecosystem health and food security (Vanbergen *et al.* 2013). Approximately one in six of all described species on the planet are flowering plants, and approximately 86% of these are pollinated by insects and other animals (Ollerton *et al.* 2011). Pollination is the process of moving pollen from the anther of one flower to the stigma of another, resulting in fertilisation (Proctor *et al.* 1973). The flower initiates and controls much of this process, from the production, receipt and protection of male gametes, to the choice of inbreeding or outbreeding (Willmer 2011). However, because both plants and gametes are non-motile, pollen must be transferred by a third-party. Insects are the largest group of animal pollinators and the vast majority of these belong to the four largest insect orders: the flies (Diptera), butterflies and moths (Lepidoptera), beetles (Coleoptera), and bees, wasps and ants (Hymenoptera). It is generally accepted that bees (Hymenoptera: Apoidea) are the primary and most efficient pollinators in most of the ecosystems they inhabit, while the importance of the others varies between habitats and plant species (Kevan *et al.* 1983, Neff *et al.* 1993, Wardhaugh 2015).

Nearly half of the 159 recognised Diptera families are recorded as flower visitors or pollinators (Kearns 2001, Wardhaugh 2015). They pollinate more than 100 cultivated species, including leek, cassava and apples (Hansen *et al.* 1983, Clement *et al.* 2007, Mitra *et al.* 2007). Most visit flowers for their nectar, although the hoverflies (Syrphidae) are an important group of pollen-feeders (Holloway 1976, Krenn *et al.* 2005). Until quite recently, non-Syrphidae species were a neglected group in pollination studies and their contribution to pollination services underestimated, however new research has found no difference in pollen acquisition between the Syrphidae and non-Syrphidae groups (Orford *et al.* 2015). Indeed, flies in the Muscidae family have recently been found to be the key pollinators in the High Arctic (Tiusanen *et al.* 2016).

Historically, Lepidoptera were pollen-feeders before transitioning with the evolution of nectaries, suggested by the archaic pollen-feeding species from the families Micropterigidae and Heterobathmiidae (Krenn 2010). Now, most species are nectar-feeders as adults. Within this order, butterflies are generally considered to be poor pollinators, described as nectar-thieves or parasitic (Wiklund *et al.* 1979, Venales *et al.* 1985). Tropical butterflies, which fly further between plants than temperate species (25-75 m verses 1-10 m), are the most efficient of the group (Shreeve 1981, Murawski *et al.* 1986). In pollination ecology, moths are generally similar to butterflies in their pollination efficiency. The exception to this are hawkmoths (Sphingidae), which are widely considered to be the most effective Lepidopteran pollinators, having good endothermic abilities which allow them to forage in cooler conditions and cover greater distances between flowers (up to 400 m) (Dorsett 1962, Brantjes 1973, Willmer 2011).

The Coleoptera are frequent flower visitors, feeding on both pollen and nectar (Labandeira 1998, Lundgren *et al.* 2011). They are particularly common in tropical forest but are only considered major pollinators for a relatively small number of plants in approximately 25 families, although it is thought this number is underestimated (Kevan *et al.* 1983, Bernhardt 2000, Wardhaugh 2015, Wardhaugh *et al.* 2015). The most important pollinating families are the soldier beetles (Cantharidae) and longhorn beetles (Cerambycidae). An estimated 184 angiosperm species are pollinated exclusively by beetles and 100 others rely on them substantially (Bernhardt 2000).

Most Hymenoptera are nectar-feeders, although the foraging ecology of many species has yet to be investigated (Kevan *et al.* 1983, Kato *et al.* 1993, Jervis 1998). Wasp pollination occurs most notably in figs and orchids, including those which mimic the scent of female wasps to attract males (Schiestl *et al.* 1999, Jersáková *et al.* 2006). Pollination by ants is rare, in part because their size limits their ability to move gametes between different plants, but also because they produce antibiotic secretions that can damage or destroy pollen (Beattie *et al.* 1984, Dutton *et al.* 2012, Wardhaugh 2015). Reported cases of pollination occur between the orchid *Leporella fimbriata* and winged males of the ant *Myrmecia urens* (Peakall *et al.* 1987). Bees (Apoidea) are the dominant pollinators in most ecosystems (see Wardaugh *et al.*, 2015). Worldwide, there are approximately 20,000 species and virtually all of these are pollinators (Naumann 1991).

1.2 Bumblebee ecology

Bumblebees (Apoidea: *Bombus*) are large, hairy bees with over 250 species worldwide and 38 currently recognised subgenera (Cameron *et al.* 2007) (Fig. 1.1). The genus contains both social and cleptoparasitic species (referred to as cuckoo bees), which parasitise one or several related host species (Falk 2015). Bumblebees are common in temperate habitats as well as Neotropical ones (Sladen 1912, Abrahamovich *et al.* 2002). They have achieved partial endothermy, allowing them to stay active in cold weather too extreme for other bees (Heinrich 2004). As a result, they occupy some of the coldest regions inhabited by insects, including the Alps, the Pyrenees and the Arctic Circle (Pradervand *et al.* 2011, Iserbyt *et al.* 2012, Martinet *et al.* 2015).

Most subgenera fall into two distinct clades: the short-faced and long-faced, which broadly correspond to variations in facial morphology and tongue length (Cameron *et al.* 2007) (Fig. 1.1a-b). Generally speaking, species in the short-faced clade are short-tongued, and species in the long-faced clade are long-tongued. Tongue (proboscis) length in bees (measured as the full length of the glossa and prementum) varies widely, from 0.76 mm in *Hylaeus* (Colletidae) to 14.6 mm in *Bombus* (Hanski 1982, Cariveau *et al.* 2016). In bumblebees, there is an overlap between the groups: short-tongued species have a proboscis length of ~8-10 mm and long-tongued bumblebees of ~9-14.6 mm (Hanski 1982).

Bumblebee species are considered to be similar in their morphology and ecology, however subtle differences in life history traits between short-tongued and long-tongued species have been reported since the early 1900s (Sladen 1912, Frison 1917, Rau 1941, Griffin *et al.* 1990, Williams 1994, Fandiño 2007). In the UK, most short-tongued queens emerge from hibernation in early spring, from February to April and long-tongued queens emerge from March to late May. All queens search for a nesting site, which is usually underground, often in old rodent burrows or in dense vegetation at the surface (Goulson 2010), and pollen collection begins as soon as a nest has been established (Evans *et al.* 2007). Short-tongued species will rely heavily on dandelions and spring-flowering plants (such as sallows and *Prunus*) to provision their first brood, while long-tongued species mostly use legumes, such as peas and vetches (Falk 2015).

Two larval feeding strategies occur, distinguishable by the composition of the larval diet (solid or liquid) and how larvae access food (Fig. 1.2). Most short-tongued species for which larval feeding has been observed, are classed as pollen storers. Eggs are laid within a constructed pollen cell and the cell wall is pulled over to enclose the egg (Hobbs 1964a). Pollen and nectar are stored separately in specialised cells before each larva is fed individually by workers on a regurgitated liquid mix containing pollen, nectar and glandular secretions. Additional pollen is packed under cell. Feeding usually occurs regularly throughout larval development and the cell is opened and closed for each feeding (Hobbs 1964a, Pereboom 2000, Den Boer *et al.* 2006).

Long-tongued species often use a second strategy, in which eggs are laid directly onto a mass of pollen that the larvae consume in its solid form, and which workers periodically supplement with fresh pollen (Hobbs 1964a, Pereboom *et al.* 2003). Species using this strategy are known as pocket makers. It is important to note that the distinction between pocket-makers and pollen-storers is not a rigid one and there are exceptions across the clades. For example, there are two species in the short-faced clade that are known to use the pocket-making strategy: *B. alpinus* (*Alpinobombus*) and *B. wurfleni* (*Alpigenobombus*) (Loken 1961, Hobbs 1964b, Loken 1973, Sakagami 1976), and there are three species of pollen-storer known in the long-faced clade: *B. atratus* and *B. bellicosus* (*Fervidobombus dahlbomii* group) and *B. fervidus* (*Fervidobombus*) (Sakagami *et al.* 1967, Ito *et al.* 1984, Salvarrey *et al.* 2013). However, the feeding strategies of a great many species have not been observed or reported. Indeed, no studies could be found describing the nest architecture or feeding strategy of species in the many short-faced or long-faced subgenera (Fig. 1.1).

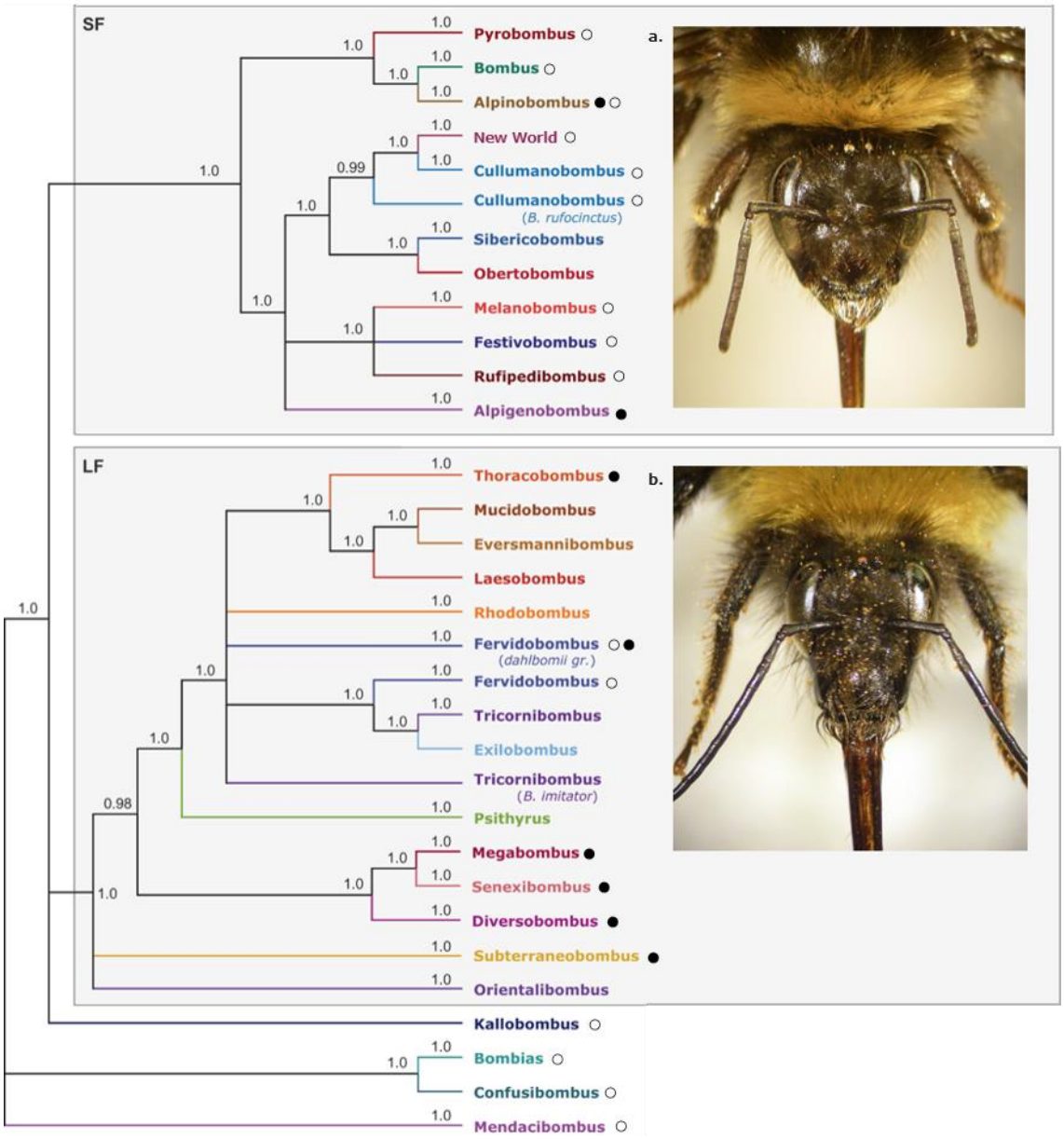


Figure 1.1 *Bombus* phylogeny showing subgeneric relationships with strong support. Values on branches are Bayesian posterior probability values (PP = 0.95). Abbreviations: SF=short-faced clade, LF=long-faced clade. From Cameron *et al.* 2007. Subgenera represented with ○ are known to contain pollen-storing species (see references in Table 1.1), and subgenera with ● are known to contain pocket maker species (Plath 1927, Weyrauch 1934, Loken 1961, Hobbs 1964b, Sakagami *et al.* 1967, Laroca 1972, Loken 1973, Sakagami 1976, Ito *et al.* 1984, Cameron 1989, Hoffmann *et al.* 2004, Peeters 2012, Salvarrey *et al.* 2013). Images show broad differences in facial morphology between clades: a. *B. lucorum* (*Bombus*), and b. *B. hortorum* (*Megabombus*). Photos by Steve Falk.

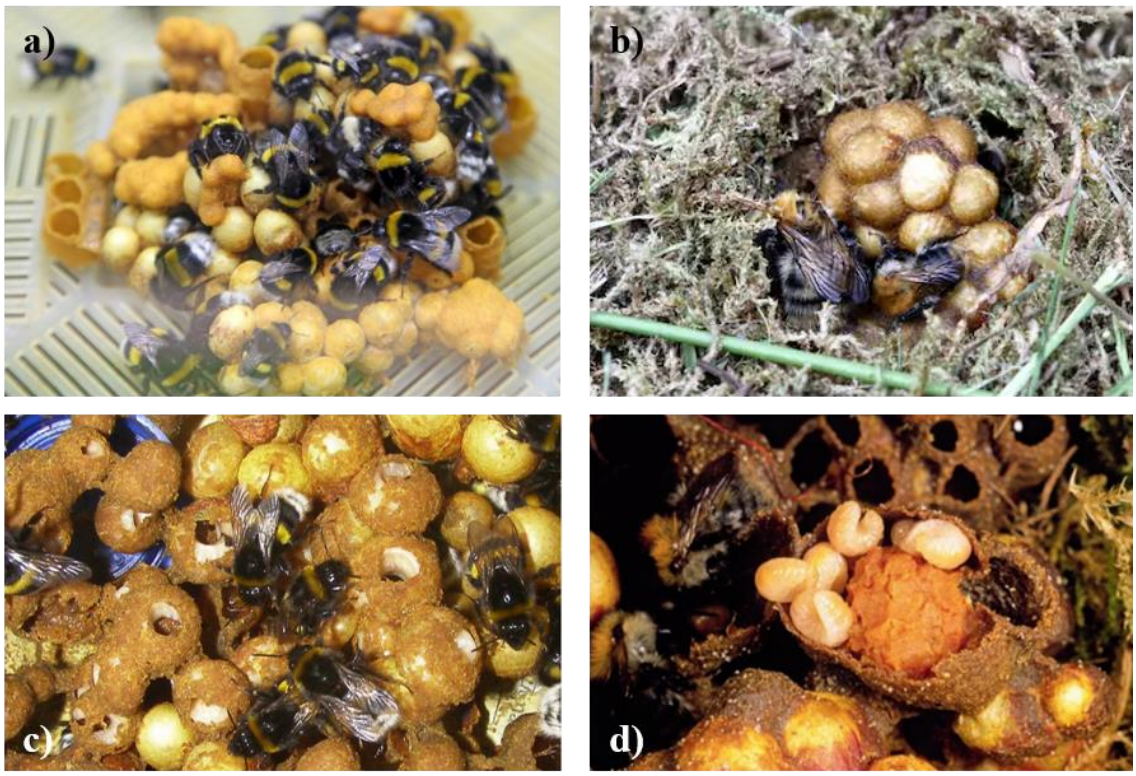


Figure 1.2 Bumblebee nests (a-b) and larval feeding strategies (c-d) used by pollen storer and pocket maker bumblebee species: a) a commercially produced colony of the pollen storer *B. terrestris* in an artificial nest box. Brood cells and food containers are constructed from pollen. Small, dark cells contain eggs and young larvae; b) a wild colony of the pocket maker *B. pascuorum*, depicting queen and worker. Developing larvae are contained inside the darkened cells. Both images show pupae; the cocoons are largest and palest cells; c) a laboratory-reared *B. terrestris* colony depicting the openings in the top of the brood casing through which developing larvae are fed a liquid mixture of pollen and nectar (Ptáček 2008); d) a wild nest of *B. pascuorum*. Queens lay eggs directly onto pollen 'pockets' – a mixture of pollen and nectar – which larvae consume in its solid form (Peeters 2012).

Queens produce workers that forage for the colony and support her in brood care. Only when resources are sufficient will she switch to queen and male production (Goulson 2010). In the UK, many early-emerging short-tongued species are bivoltine (having two generations), and winter-active *B. terrestris* can be trivoltine (three generations) (Falk 2015). Cuckoo queens kill or subdue social queens soon after nest establishment and utilise the nest for the rearing of their own reproductives (Thorp *et al.* 1983, Lhomme *et al.* 2013).

1.3 Bumblebee adaptions for pollination

For all bumblebee species, both the adults and larvae rely entirely on the nectar and pollen derived from floral resources (Michener 2007, Danforth *et al.* 2013). Most species show a high degree of diet elasticity and are capable of utilising multiple plant species across different families (Brian 1957, Hobbs 1962). Bumblebees carry out an extremely high number of flower visits per individual, which has driven adaptation and allowed them to access floral resources from a variety of flower structures (Hobbs *et al.* 1961, Williams *et al.* 1998). These adaptations include sonication, whereby a visiting bee grasps and vibrates its body against the hypanthial cup (a fusion of anthers and style), using thoracic wing muscles, resulting in the ejection of pollen from pores in the anthers (Harder *et al.* 1994, Willmer 2011). Often termed 'buzz-pollination', the vibration must be of suitable frequency for pollen release and is required by 6-8% of flowering plants (Buchmann 1983).

Like most bees, bumblebees have specialised body parts on which they can carry pollen. Bumblebees have a pollen basket located on each of the hind tibia, which is exclusively found on social species (Thorp 1979). Passive pollen collection also occurs as pollen attaches to branched hairs (Willmer 2011). The elongation of mouthparts allows long-tongued species to collect nectar from flowers with deep corollas, which many other pollinating insects, including honey bees, cannot access (Holm 1966). On average, bumblebees will forage on flowers that are marginally shorter than their tongue length (Brian 1957, Prŷs-Jones *et al.* 1991, Willmer 2011).

1.4 Population trends and declines

Declines in insect abundance and diversity have been monitored for many years but recently have received significant attention, particularly amongst the general public (Pyle *et al.* 1981, Hallmann *et al.* 2017, Forister *et al.* 2019, Simmons *et al.* 2019). However, there is disagreement regarding the accuracy of studies describing the decline of pollinators and other insects and whether this decline has been overestimated (Ghazoul 2005, Schowalter *et al.* 2019, Saunders *et al.* 2020). Broadly speaking, amongst insect pollinators there is evidence of decline in both abundance and diversity across multiple spatial scales. Most evidence for this comes from bees, including wild bees in Europe,

South America, Asia, South Africa and North America (Biesmeijer *et al.* 2006, Pauw 2007, Williams *et al.* 2009b, Cameron *et al.* 2011, Burkle *et al.* 2013, Nieto *et al.* 2014), and honey bees throughout North America and Europe (see Potts *et al.* 2010). Bumblebees have undergone population changes and range shifts throughout the world but baseline data is often unavailable so it is difficult to assess the extent, or direction, of these changes (Grixti *et al.* 2009, Williams *et al.* 2009). Hoverflies and wasps are severely understudied throughout the world. Declines in hoverfly diversity have been recorded in the Netherlands and Britain, and declines in diversity have been recorded for aculeate wasps in Britain (Biesmeijer *et al.* 2006, Ollerton *et al.* 2014, Ollerton 2017). Many Lepidopteran species have shown significant losses and reduced distributions in Britain and Europe (Thomas *et al.* 2004, Fox 2013, Nilsson *et al.* 2013), and north America (Warren *et al.* 2001, Wepprich *et al.* 2019).

Bumblebees rely intrinsically on floral resource abundance and so historical records of loss can be compared with changes in floral resource availability. For example, using a combination of vegetation surveys and plant nectar profiles, a study found significant losses in nectar availability in England and Wales between the 1930s and the 1970s (Baude *et al.* 2016). This corresponds to post-World War agricultural intensification, which is widely considered to be the most significant driver of biodiversity loss in Britain and corresponds to widespread aculeate bee extinction (Ollerton *et al.* 2014, Baude *et al.* 2016). As well as contributing to the spread of pests and pathogens, agricultural intensification has dramatically reduced the abundance and diversity of flowering plant species (Biesmeijer *et al.* 2006, Carvell *et al.* 2006, Winfree *et al.* 2011, Senapathi *et al.* 2015). Farming practices once supported bumblebee populations through the use of wildflower-rich hay meadows kept as fodder for livestock. After the Second World War they were utilised for crop production or replaced with rye grass monocultures for silage (Goulson 2010). In just over 50 years, 90% of unimproved lowland grassland was lost in the UK (Fuller 1987, Howard *et al.* 2003). The abundance of plant species characteristic of these habitats in turn declined substantially (Carvell *et al.* 2006, Kleijn *et al.* 2008). Land use change has produced landscapes in which floral resources are highly disparate over space and time (Carvell *et al.* 2006, Roulston *et al.* 2011, Kallioniemi *et al.* 2017). This exposes bumblebees to food-stress, often throughout their geographic range (Grixti *et al.* 2009, Williams *et al.* 2009, Senapathi *et al.* 2015).

The response of bumblebees to these changes varies between species. Three have become extinct in Britain since the 1940s, including *B. pomorum*, *B. cullumanus*, and *B. subterraneus*, although there have been attempts to reintroduce the latter (Alford 1975, Williams 1982, Edwards *et al.* 2004, Gammans 2013, Falk 2015). Several species have shown severe declines and range contractions, including *B. humilis, B. muscuorum, B. distinguendus, B. ruderarius, B. sylvarum* and *B. ruderatus*, which are BAP-listed species. Others, including *B. terrestris* and *B. lapidarius*, have shown range expansions (MacDonald 2001). *B. hypnorum* is a new species in Britain, first observed in 2001 (Goulson *et al.* 2001). Various theories have been suggested for these differential responses. Comparative analyses currently suggest that decline susceptibility is associated with i) species that have small climatic ranges and show strong climatic specialisation, ii) populations of species at their range edges, and iii) late-emerging species (Goulson *et al.* 2004, Williams 2005, Williams *et al.* 2009, Williams *et al.* 2009a). The latter includes many long-tongued bees that rely heavily on Fabaceae (the pea family), which is a dominant plant group in unimproved lowland grassland (Carvell *et al.* 2006).

1.5 Domestication and research

Bumblebees have become valuable model organisms for scientific research and are reared commercially for pollination worldwide. The first recorded attempts to rear bumblebees in captivity were in 1912 (Lindhard 1912, Sladen 1912). Since then, captive rearing has been tested on variety of species (Table 1.1), though with varying degrees of success (Hasselrot 1952, Lhomme *et al.* 2013). These rearing trials suggest different species vary in their sensitivity to captive conditions. For the efficiency of research and commercialisation, efforts to optimise captive rearing focused on those species which were more resilient to captivity and could reliably produce colonies (Velthuis *et al.* 2006).

Worldwide, there are currently five species of two subgenera that are reared commercially. These, particularly *B. terrestris* and *B. impatiens*, are also the focus of most scientific research. The mutualism between *B. terrestris* and its pathogen *Crithidia bombi*, for example, is a widely used model in parasitology (Schmid-Hempel 1998). It is common to use model organisms to understand the biology of other species and biological

systems. Species such as *Escherichia coli*, *Drosophila melanogaster* and *Caenorhabditis elegans* are easy to produce and maintain, and commonly used in molecular biology (Ankeny *et al.* 2011). In other cases, species are chosen because it is not possible to study many of their relatives in captivity. For example, the catshark *Scyliorhinus canicula* and the bullhead shark *Heterodontus portusjacksoni* are useful model organisms in elasmobranch research because they are small enough to be reared in captivity (Ellis *et al.* 1997, Daly *et al.* 2017, Janse *et al.* 2017).

Table 1.1 Bumblebee species that have been reared in captivity, with varying success. Captive rearing of species in **bold** have been optimised for commercial purposes (reviewed in Velthuis and Van Doorn 2006).

	Subgenus	Species	Rearing trials
Short-faced clade	*Pryobombus*	***B. impatiens***	-
		B. hypnorum	Hasselrot (1952)
		B. pratorum	Manino *et al.* (1994)
		B. vagans	Plowright and Jay (1966)
		B. perplexus	Plowright and Jay (1966)
		B. ternarius	Plowright and Jay (1966)
	Bombus	***B. terrestris***	-
		B. lucorum	-
		B. ignites	-
		B. occidentalis	-
		B. terricola	Plowright and Jay (1966)
		B. cryptarum	Bučánková et al. (2014)
	Cullumanobombus	*B. rufocinctus*	Plowright and Jay (1966)
	Melanobombus	*B. lapidarius*	Sladen (1912), Bučánková and Ptáček (2012)
	Rufipedibombus	*B. eximius*	Chiang *et al.* (2009)
Long-faced clade	*Thoracobombus*	*B. pascuorum*	Ptáček *et al.* (2015)
		B. sylvarum	Ptáček *et al.* (2015)
		B. ruderarius	Ptáček *et al.* (2015)
		B. humilis	Manino *et al.* (1994)
	Fervidobombus	*B. atratus*	Pomeroy and Plowright (1980), Almanza *et al.* (2006), Salvarrey *et al.* (2013)
		B. fervidus	Plowright and Jay (1966), Plath (1923)
		B. pensylvanicus	Plowright and Jay (1966)
		B. bellicosus	Salvarrey *et al.* (2013)
	Psithyrus	*B. sylvestris*	Lhomme *et al.* (2013)
		B. vestalis	Alford (1970a), Alford (1970b)
		B. campestris	Fisher (1988), Lhomme *et al.* (2013)
		B. bohemicus	Fisher (1988)
	Megabombus	*B. hortorum*	Manino *et al.* (1994), Griffin *et al*, (1990), Bučánková and Ptáček (2012)
		B. ruderatus	Pomeroy and Plowright (1980)
	Subterraneobombus	*B. subterraneous*	Howlett 2009, Griffin *et al*, (1990)
		B. borealis	Plowright and Jay (1966)
	Bombias	*B. uricomus*	Plowright and Jay (1966)
		B. nevadensis	Plowright and Jay (1966)

Unfortunately, as a result of only utilising resilient bumblebee species that seem unaffected by captivity, we lack similar experience rearing others (Ptáček *et al.* 2015). The species for which we have most experience in captive rearing are short-tongued species and all of them are pollen-storers. Pollen-storers readily accept pollen placed in their nest box and so feeding colonies in captivity is straightforward (Bučánková *et al.* 2014). Long-tongued species pocket-makers do not do this because workers would normally transfer pollen straight to the pockets of the brood cells (Griffin *et al.* 1990, Ptacek 2001).

Early attempts to rear long-tongued species in captivity focus on logistics such as nest box material and either did not report the success of queens or colonies, or only stated that eggs were laid (Plowright *et al.* 1966, Alford 1970a, Alford 1970b, Pomeroy *et al.* 1980, Manino *et al.* 1994). Between 2000 and 2015, rearing trials using several long-tongued bumblebee species were published demonstrating rearing colonies was possible (Ptácek *et al.* 2000, Bučánková *et al.* 2012, Ptáček *et al.* 2015). A variety of techniques to rear *B. hortorum, B. pascuorum, B. sylvarum, B. humilis* and *B. ruderarius* were trialled. This includes pairing queens to encourage one to become dominant and begin egg-laying, while the submissive queen was removed and re-paired. Queens were also given donor cocoons from *B. terrestris*. These methods were successful in encouraging queens to lay eggs, but larval care was a problem; many were ejected or never fed. Successful colonies that produced reproductives were those allowed to forage naturally outside (Ptáček *et al.* 2015). For many of the species listed in Table 1.1 that are not commercially produced, there are only one or two published studies recording attempts at rearing in captivity.

Despite significant effort developing and optimising the commercial rearing process, for both the purposes of crop pollination and scientific research, many bumblebee subgenera have been scarcely utilised. In the Fabaceae family alone, there are nine crops that benefit at least somewhat from bumblebees for pollination (Corbet *et al.* 1991, Delaplane *et al.* 2000). For alfalfa (*Medicago sativa*), sweet clover (*Melilotus* spp.) and clover (*Trifolium* spp.), bumblebee pollinators are essential, and the species best adapted for their pollination are long-tongued species. Beyond agriculture, the use of *B. terrestris* and *B. impatiens* as model species, representing only two of the 38 bumblebee subgenera, and

only one clade, may severely limit our knowledge on the scale of differences in behaviour and biology bumblebees exhibit, and how they respond to environmental change.

1.6 Nutrition and flower choice

1.6.1 Insect nutrition

Nutrition can broadly be defined as the acquisition and assimilation of energy and nutrients for growth, health and function (Watts *et al.* 2012). The study of nutrition in insects has been established for several decades (House 1961, Dadd 1973, Friend *et al.* 1982, Simpson *et al.* 1995). However, a great deal of this research has been based on a few dozen species (Hanife 2006). As a result, it can be difficult to predict how nutritional limitation will affect rare or declining species whose nutritional requirements have not been studied. It is thought most insect groups have broadly similar nutritional requirements, however their diets are remarkably varied (Dadd 1985, Slansky *et al.* 1987). They may feed on solids or liquids, plant material or other animals (Dow 1986, Klowden 2013) (Figure 1.3). Diet may also vary between the larval and adult stage, particularly amongst holometabolous insects such as bees (Chown *et al.* 2004).

1.6.2 Pollination syndromes

Pollinators may feed on pollen, nectar, or a combination of the two, and the diversity of floral displays to attract pollinators is one of the most striking elements of angiosperm radiation. Plants use advertisements such as floral colour, floral patterns and olfactory signals, and the rewards of pollen and nectar. Because pollinators vary in their nutritional requirements and sensory ecology, the floral displays used to attract different species or taxa vary (Chittka *et al.* 2001, Raguso 2008, Reisenman *et al.* 2008, Schaefer *et al.* 2009). The convergent evolution of floral traits that exploit the sensory capabilities and preferences of particular pollinators are known as pollination syndromes (Willmer, 2011) For the pollinator, flower preferences are dependent on innate and learned behavioural responses (Lunau *et al.* 1995, Praz *et al.* 2008, Schiestl *et al.* 2013). Flowers exhibiting the bird pollination syndrome, ornithophily, for example, are characterised by scentless red or orange flowers, while the syndrome for bats, chiropterophily, involves dull and pale flowers with a strong fruity or fermenting scent (Cox 1984, Tschapka *et al.* 2002). Flowers classed under the bee pollination syndrome, melittophily, are often red, purple, blue, white or yellow, with moderate and often sweet scents (Westerkamp 1996).

Pollination syndromes also describe the timing of anthesis (flowering), the presence of nectar guides, flower shape, nectar volume and concentration, and the exposure or concealment of nectar sites (reviewed in Willmer, 2011).

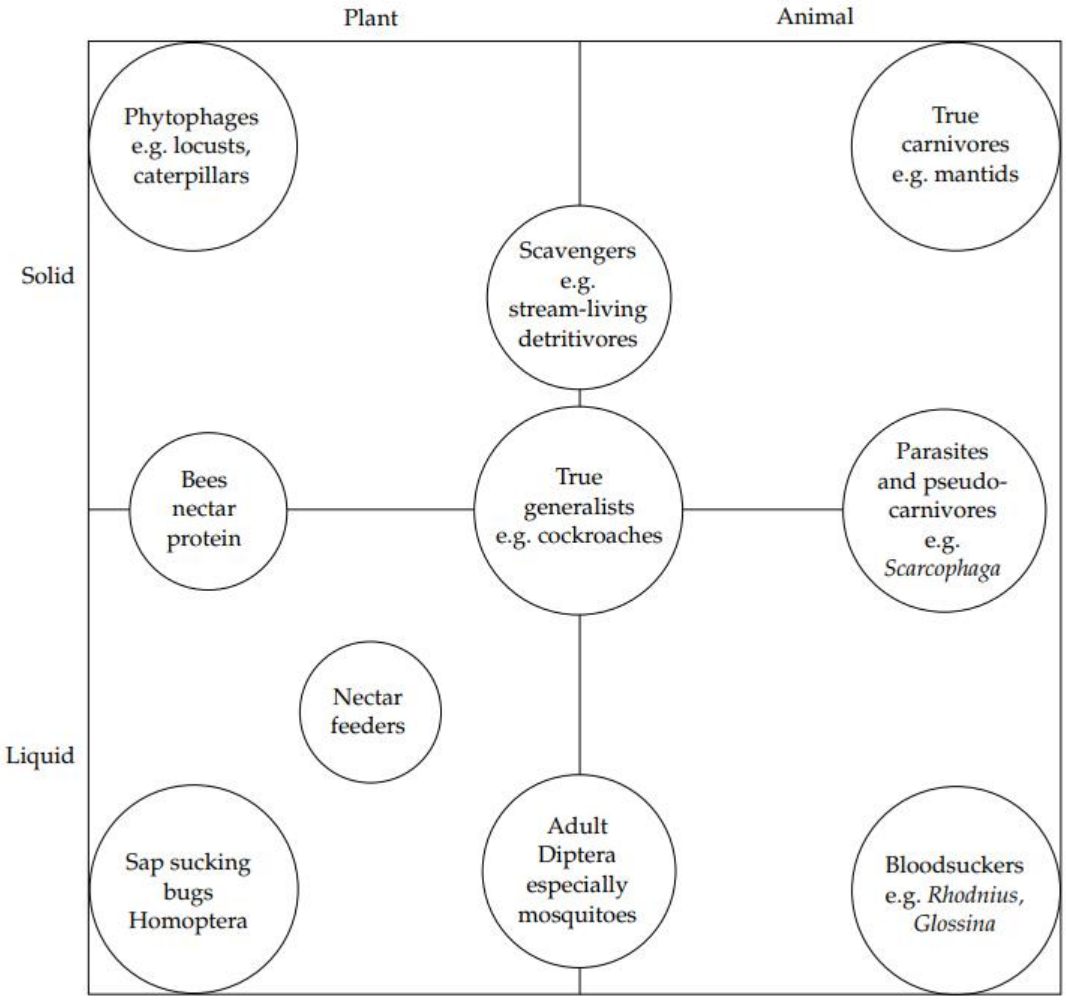


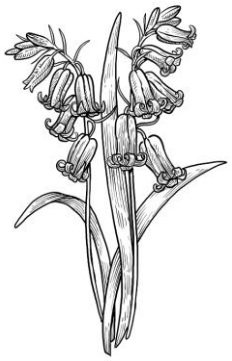
Figure 1.3 Classification of insect diets based on solid/liquid and plant/animal feeding strategies. From Dow (1986).

1.6.3 Bumblebee flower preferences

For social bumblebee species, gathering sufficient quantities of pollen and nectar is essential for the maintenance and growth of both individuals and the colony. Nectar is an energy source that all adults feed on, while pollen is collected to provision the brood (Roulston *et al.* 2000). Three bumblebee species can be classed as oligolectic, relying solely on one plant species or genera, in at least part of their full range; *B. consobrinus* and *B. gerstaeckeri* on *Aconitum* spp. and *B. brodmannicus* on *Cerinthe* spp. (Loken 1973, Rasmont *et al.* 1988, Konovalova 2007). All remaining species, including all of those in the UK, are dietary generalists, foraging flexibly on a wide range of species from many plant groups (Brian 1957, Hobbs 1962, Willmer 2011). However, there are many species that are behaviourally or physiologically biased towards particular plant groups, for which tongue length is critical (Kleijn *et al.* 2008, Kämper *et al.* 2016). Long-tongued bumblebees visit flowers that are correspondingly deep; manoeuvring mouthparts to feed from shallow flowers is likely to be problematic (Brian 1957, Barrow *et al.* 1984, Prŷs-Jones *et al.* 1991). As a result, long-tongued species share a close association with plant families that have deep flowers, including Fabaceae and Lamiaceae (the mint family), although most will forage widely on species within that family (Falk 2015). Short-tongued bumblebees favour open, bowl-shaped flowers but can rob deep flowers by puncturing the base of the corolla with their mandibles (Willmer 2011).

The first flower visits of naïve workers are often long and inefficient (Laverty 1980, Lunau 1990), after which they learn quickly and develop strong flower constancy, collecting pollen and/or nectar usually from one particular plant species (Goulson 2010). Some foragers are lifetime specialists of either pollen or nectar collection, but the majority of workers only show short-term specialisation (Russell *et al.* 2017). The decision of whether to collect pollen or nectar is likely to reflect resource stocks in the colony, but pollen collection is less likely to be carried out in wet weather, when manipulating it into the pollen basket may be difficult (Peat *et al.* 2005). Plant species visited by bumblebees are typically blue, pink, purple or yellow, and are often large and vary in structure (Willmer 2011; Table 1.2)

Table 1.2 Typical bumblebee flowers in Europe and North America. Adapted from Willmer 2011. Terms: zygomorphic: flowers with bilateral symmetry; keeled flowers: flowers in which the lower two petals are fused, forming a boat-shaped keel containing anthers and stigma; brush-blossom: inflorescence with small, densely packed flowers that form a flat or dome-like surface; radial: a circular flower with many axes of symmetry through the centre. Many long-tongued bumblebee species are known to prefer zygomorphic keeled flowers, particularly Fabaceae.

	Tubular flowers	**Zygomorphic keeled flowers**	**Brush-blossoms**	**Buzz-pollinated flowers**
Typical flower				Varies; often radial and open or bell-shaped
Families	Scrophulariaceae, Boraginaceae, Lamiaceae, Gentianaceae	Fabaceae, Fumariaceae	Dipsacaceae, Asteraceae	Papaveraceae, Solanaceae, Ericaceae
Example species	 Bluebell  Gentian	 Clover  Gorse	 Scabious  Thistle	 Poppy  Bittersweet

1.6.4 Bumblebee nutrition

Bees rely on the acquisition of pollen and nectar to meet their nutritional demands. They must consume sufficient quantities of food to cover their energetic costs, but beyond this basic calorific intake, they must also obtain a diverse range of macro and micronutrients to support the health and immune functioning of themselves and the larvae they feed (Genissel *et al.* 2002, Riddell *et al.* 2006, Tasei *et al.* 2008, Brunner *et al.* 2014). The nutrients contained in pollen and nectar are often highly variable and uncoupled (Roulston *et al.* 2000, Campos *et al.* 2008). The ability of bees to identify nutritional resources, is therefore affected by their ability to sense the quality of their food. It is generally accepted that they are capable of assessing nectar quality (Marden 1984, Hanley *et al.* 2003). The chief nutritional component of nectar is carbohydrate. This constitutes the largest proportion of an adult workers diet. Bumblebees require an almost constant supply of sugar (~45 mg day^{-1} in *B. terrestris*); approximately 150 times as much carbohydrate as protein (derived from pollen) (Stabler *et al.* 2015). Accordingly, bees possess a significant number of genes encoding carbohydrate-metabolising enzymes (Kunieda *et al.* 2006, Stabler *et al.* 2015). Across plant taxa, nectar carbohydrates vary in quantity (10-75%) and type; the predominant sugars being glucose, fructose and sucrose, with 11 other minor sugars present (Nicolson *et al.* 2007, Willmer 2011). Flowers with more concentrated nectar are preferred (Cnaani *et al.* 2006, Konzmann *et al.* 2014).

As well as sugars, nectar also contains non-protein amino acids (NPAAs), lipids, secondary metabolites and fatty acids (Nepi *et al.* 2007). The second most abundant component after sugars are amino acids, found in almost all plant nectar (Baker *et al.* 1973). NPAAs occur in low, highly variable concentrations (Gardener *et al.* 2001, Willmer 2011), and these include amino acids that have previously been found to be essential to honey bee health and development (de Groot 1953). For example, glycine and proline are associated with improved learning performance in honey bees, while taurine is known to be important for flight muscle development (Whitton *et al.* 1987, Kim *et al.* 2000).

There is some debate over the ability of bees to assess pollen quality and thus regulate their protein intake (Hanley *et al.* 2008, Konzmann *et al.* 2014, Vanderplanck *et al.* 2014). Pollen grains have a resistive wall of sporopollenin (Wiermann *et al.* 1992), which may prevent the detection of nutrients inside. Despite this, there is evidence of bees selecting

higher quality pollens (Hanley *et al.* 2008, Leonhardt *et al.* 2012, Ruedenauer *et al.* 2016). Proposed mechanisms include mechanically breaking open the pollen grains (Dobson *et al.* 1997), using molecular indictors of quality present in the pollenkitt (Dobson *et al.* 2000, Wacht *et al.* 2000), and learning a combination of visual, tactile, olfactory and gustatory cues associated with the feeding experience (Konzmann *et al.* 2014, Jones *et al.* 2015). Irrespective of the mechanism, the various haemolymph receptors and signalling pathways sensitive to nutrient levels may allow bees to respond accordingly to achieve their optimum nutritional status (Simpson *et al.* 2009, Kapahi *et al.* 2010).

Total protein content in pollen ranges from 2.5-61% (dry mass) across taxa (Buchmann 1986, Roulston *et al.* 2000), and protein-rich diets are also correlated with increased longevity and body size, improved ovary development and larval growth (Knox *et al.* 1971, Tasei *et al.* 2008, Quezada-Euán *et al.* 2011). Pollen stress in larvae is known to lengthen their developmental time and reduce size as adults (Sutcliffe *et al.* 1990). As well as protein, pollen contains numerous other compounds including sterols, lipids, vitamins, minerals and secondary metabolites that are all necessary for development and reproduction in bumblebees (Genissel *et al.* 2002, Tasei *et al.* 2008, Vanderplanck *et al.* 2014, Stabler *et al.* 2015).

Because pollen and nectar vary in widely in their biochemical components, bumblebee colonies benefit from a diverse diet in which pollen and nectar are sourced from variety of plant species (Vaudo *et al.* 2015). The importance of diet diversity is investigated in bumblebees often using monofloral and polyfloral pollen mixes. *B. terrestris* colonies fed on polyfloral diets are generally more successful, with bees showing improved health and increased egg production (Tasei *et al.* 2008, Baloglu *et al.* 2015, Moerman *et al.* 2015). Experimental studies have also shown that *B. terrestris* larvae, which are particularly sensitive to pollen, have higher growth rates and enhanced larval resistance to parasites when reared on polyfloral diets (Tasei *et al.* 2008, Baloglu *et al.* 2015). These studies have also identified specific pollens harmful to bumblebees. Compositae species such as *Taraxacum* and *Helianthus* are associated with reduced larval production (Genissel *et al.* 2002, Tasei *et al.* 2008). For some of these pollens, bees can reduce or negate their harmful effects by foraging on a range of other species (Giacomini *et al.* 2018, LoCascio *et al.* 2019, McAulay *et al.* 2019). These studies suggest that polyfloral mixes are

superior, however their benefits come down to the specific pollens they contain. In a more recent study it was demonstrated that a monofloral diet could be as good as polyfloral one if it contains all the necessary nutrients (Moerman *et al.* 2017). This has also been shown in honey bees (Di Pasquale *et al.* 2013). However, only one study has compared the effect of different pollen diets on different bumblebee species. Three bumblebee species (*B. terrestris, B, hypnorum* and *B. pratorum*) were fed on one of three distinct pollen diets (*Cistus, Erica* and *Salix*). Researchers found interspecific differences in bees' response to the different pollens. Brood mass was higher in micro-colonies of *B. terrestris* and *B. hypnorum* fed on *Salix* pollen, compared to those fed on *Cistus,* while the opposite was true for *B. pratorum* (Moerman *et al.* 2016).

1.6.5 Nutrition and colony fitness

Many social organisms exhibit collective behaviours in the acquisition of nutrients. Group foraging requires individuals to exploit multiple and complimentary food resources in order to collect the necessary resources for all colony members including themselves, non-foraging workers, the queen/s and larvae; each of which may have quite different nutritional needs (Cassill *et al.* 1999, Simpson *et al.* 2012, Lihoreau *et al.* 2018). Ants are known to adjust their foraging behaviour to achieve the colony's protein to carbohydrate intake target (Dussutour *et al.* 2009, Cook *et al.* 2010), while honey bee colonies restricted to nutritionally limited pollen will allocate more workers to forage for complimentary resources (Hendriksma *et al.* 2016).

The abundance and quality of resources collected by an individual can have cumulative effects on colony fitness. In *B. impatiens* colonies placed across three different habitats, the intake rate of nutrients (protein, lipids and carbohydrates) was strongly linked with colony growth and fitness (Vaudo *et al.* 2018). The timing of abundant resources is important. Westphal *et al* (2009) found that while mass flowering events of oilseed rape improved early colony growth, it had no effect on sexual reproduction.

1.7 Bumblebee pathogens

Pathogens are essential components of healthy ecosystems and the interactions with their hosts have significant effects on their population dynamics (Henson *et al.* 2009, Arbetman *et al.* 2017). There are four known microbial pathogens of bumblebees and all can be transmitted via the faecal-oral route (Durrer *et al.* 1994, Imhoof *et al.* 1999). When reporting the prevalence of these pathogens, it is important to note that many studies assume presence to mean infection (reviewed in Brown 2017). As will be discussed later in this chapter, the prevalence of bumblebee pathogens in the environment could be quite high. Ingestion of a small number of cells may not be enough to elicit an infection and so often bumblebee prevalence data, while derived from the bee, may only describe the prevalence of pathogens that bees encounter, and the not the prevalence of infections (Blaker *et al.* 2014).

Crithidia bombi is a well-studied trypanosome and ubiquitous pathogen of adult bumblebees (Lipa 1980, Folly *et al.* 2017). Following ingestion, it attaches and develops primarily in the hindgut, but cells have also been observed in almost every abdominal organ, including the fat body (Macfarlane *et al.* 1995, Schmid-Hempel 2001). Maturation time is fast and infective cells are released two to five days later (Logan *et al.* 2005). *C. bombi* can overwinter with a queen, after which she is heavily infected and her survival and colony-founding success is significantly lower than uninfected queens (Brown *et al.* 2003a). The pathogen also readily infects workers (Durrer *et al.* 1994, Graystock *et al.* 2015). *C. bombi's* prevalence in bumblebees typically ranges between 30-50%, accumulating in the colony and peaking at the end of the foraging season, when up to 100% of bees may be infected (Shykoff *et al.* 1991, Imhoof *et al.* 1998). As a trypanosome, it is characteristically subtle in its effects and is considered to have low virulence, though its effects are context-dependent (Imhoof *et al.* 1998, Brown *et al.* 2000). Infected workers exhibit lower foraging rates, are slower to process floral information and take longer to handle flowers (Gegear *et al.* 2005, Otterstatter *et al.* 2005, Gegear *et al.* 2006).

Two single-cell microsporidia are known to infect bumblebees. Like *C. bombi*, *Nosema bombi* is ubiquitous. It infects both adults and larvae (Schmid-Hempel *et al.* 1998, Rutrecht *et al.* 2007), and is capable of vertical transovarian transmission (transmission

from parent to offspring through the ovaries) (Rutrecht *et al.* 2008). Following ingestion, spores germinate in the cell layer of the gut epithelium (McIvor *et al.* 1995). The primary site of infection is midgut and Malpighian tubules. However, spores have also been observed in the thorax muscles, fat body, nerve tissue, ovaries and testes (McIvor *et al.* 1995, Fries *et al.* 2001, Otti *et al.* 2007). Maturation time is slow and new spores may not appear in the faeces for up to 3 weeks following infection (McIvor *et al.* 1995). Its prevalence is generally low (<5%) (Shykoff *et al.* 1991, Durrer *et al.* 1995, Whitehorn *et al.* 2010, Graystock *et al.* 2014, Jones *et al.* 2014), but has been found to infect 56% of *B. terrestris* in urban areas (Goulson *et al.* 2012). Without determining if these data are confounded by sampling nestmates whose infection status would not be independent of one another, it is difficult to know if this increase accurately describes pathogen prevalence in urban areas (Goulson *et al.* 2012). *N. bombi's* effects on bumblebee varies substantially, from negligible to severe, causing reduced lifespan, larval deformities and reduced fecundity (van den Eijnde *et al.* 1993, McIvor *et al.* 1995, Schmid-Hempel *et al.* 1998, Otti *et al.* 2007). This is likely to be influenced by *N. bombi's* development time in the host, specifically, infections which originate at the larval stage are more virulent than those that begin in adults (Rutrecht *et al.* 2007).

N. ceranae is a pathogen of honey bees but has more recently been found to infect bumblebees. This was initially shown in commercially produced colonies which are fed honey bee collected pollen, in which *N. ceranae* was being transmitted (Graystock *et al.* 2015). Subsequent field studies that have explored its prevalence near apiaries and in urban areas, where beekeeping is frequent, confirm its presence, which can be as high as 44% (Williams *et al.* 1991, Goulson *et al.* 2012, Alton K 2013, Graystock *et al.* 2014, Stange *et al.* 2017). However, large-scale studies exploring its occurrence in wild populations are still lacking. Based on existing data its prevalence seems to be low (7%) (Furst *et al.* 2014), however its virulence and risk to wild bumblebees is unknown (Brown 2017).

1.7.1 Transmission and immune response

While there is evidence of direct vertical transmission in *N. bombi*, most pathogen infections in bumblebees are likely to occur horizontally between nestmates, before spreading to neighbouring colonies (Otterstatter *et al.* 2007; Fig. 1.4). Exactly how this occurs, and at what rate, across the landscape is not known (Figueroa *et al.* 2019).

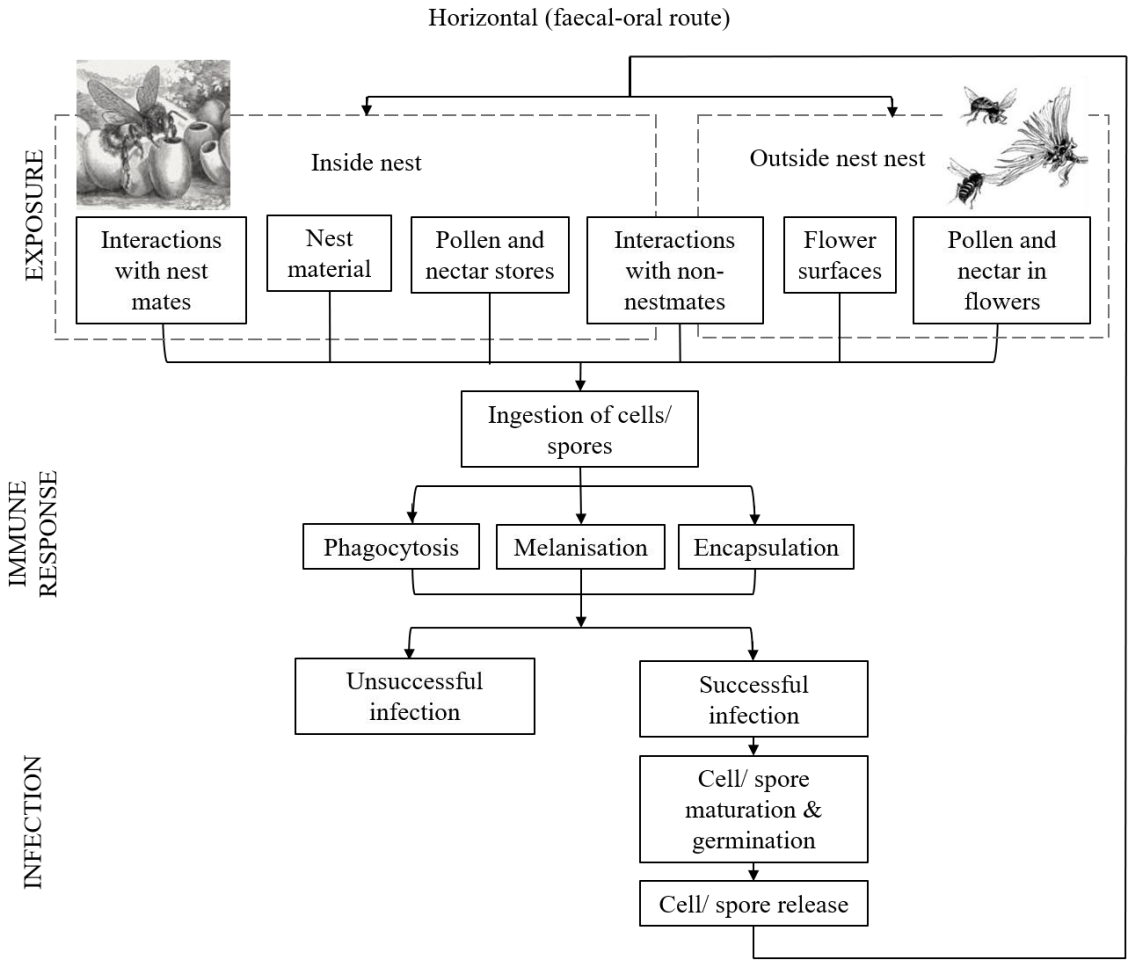


Figure 1.4 Horizontal pathogen transmission and immune response in bumblebees with a focus on opportunities for pathogen exposure inside and outside the nest.

Flowers are important ecological intermediaries for many pathogenic organisms (McArt *et al.* 2014) and all of the microbial pathogens known to infect bumblebees can be horizontally transmitted by their shared use. In a landmark study more than 20 years ago it was found that a *B. terrestris* worker could become infected with *C. bombi* by foraging on a flower previously visited by an infected bee (Durrer *et al.* 1994). More recently this has shown to be the case for all known bumblebee pathogens, as well as RNA viruses (Singh *et al.* 2010, Graystock *et al.* 2015). This occurs as bumblebees feed, mix pollen and defecate on flowers as they forage (Figueroa *et al.* 2019). Flowers act as transmission hubs for many plant pathogens, including fungi, bacteria, viruses and nectar yeasts, all of which can be vectored by pollinators. Of the microorganisms found on flowers that are hosted by animals, remarkably few are known (reviewed in McArt *et al.* 2014). These

include pathogenic microbes such as *Ascosphaera* spp. and *Aspergillus flavus* (Fungi, Ascomycota), the honey bee pathogen *N. apis*, *Spiroplasma apis* (Bacteria, Mollicutes), and the viruses: Deformed wing virus, Black queen cell virus, Israeli acute paralysis virus, and the Kashmir bee virus. Other microbes can be commensal, including the ascomycetous yeasts (Fungi, Ascomycota), Lactobacillales and Acetobacteraceae (Batra *et al.* 1973, McFrederick *et al.* 2012, Vásquez *et al.* 2012, McArt *et al.* 2014).

Pathogen survival on flowers varies depending on the plant species, its structure and biochemistry, and where the cells or spores have been deposited (Figueroa *et al.* 2019). McArt *et al.* (2014) described four floral traits that could influence the transmission of animal pathogens between plants: 1) floral attractiveness of uninoculated plants, 2) pathogen acquisition and viability in flowers, 3) floral attractiveness of inoculated plants, and 4) pathogen acquisition and establishment in hosts upon visiting inoculated flowers. It is probable that complex flowers or those offering rewards requiring prolonged handling times are more likely to acquire pathogen cells/spores (Appendix 7). Following deposition, cells and spores must not only survive but remain viable. A recent study investigated variation in transmission of *C. bombi* to *B. impatiens* workers through shared flower use in three plant species, finding variations in flower structure affected transmission, with plant species differing fourfold in the abundance of pathogen cells establishing in the host (Adler *et al.* 2018). *C. bombi* cannot survive long outside its host; it desiccates under UV light and after 45 minutes exposed in a laboratory its infectivity declines by 10-90% (Schmid-Hempel *et al.* 1999, Otterstatter *et al.* 2008, Figueroa *et al.* 2019). In *Brassica rapa* (oilseed rape) nectar, cell survival drops to 15% after 85 minutes (Otterstatter *et al.* 2008). Low sugar concentrations are beneficial for growth, but too high (80-100% vol/vol) can kill the cells (Cisarovsky *et al.* 2014, Palmer-Young 2017). The *Nosema* species are also vulnerable to UV light, but have a chitinous cell wall, which may offer them more protection inside flowers (Maddox *et al.* 1996, Malone *et al.* 2001, Li *et al.* 2003, van Der Steen 2008, Fenoy *et al.* 2009). It has been suggested that bumblebees may be sensitive to pathogen-mediated changes in nectar chemistry. For example, workers of *B. terrestris* were observed to avoid flowers which had been inoculated with *C. bombi* (Fouks *et al.* 2011)

Given the prevalence of *C. bombi* in particular, and the opportunities for transmission, it seems likely that its cells, whether viable or not, would be widely distributed on flowers

in the landscape (Durrer *et al.* 1994, Graystock *et al.* 2015, Figueroa *et al.* 2019). Based on estimates of the rate of *C. bombi* decay on flowers and rates of deposition, Otterstatter and Thompson (2008) estimated that in a course of 2 hours, bumblebees come into contact with over 60, 000 *C. bombi* cells on flowers, and a total of $4.23x10^4$ *C. bombi* cells were deposited per day at flowers. They noted that given the wide variety of infection intensities observed across infected colonies, pathogen prevalence in faeces was likely to vary widely. Furthermore, these estimations do not account for the abundance of other flower visitors that could ingest or move the cells, or other environmental conditions such as weather. None of the pathogens have been detected in wild plants (Cisarovsky *et al.* 2014), and so the proportion of pathogen cells on flowers that are viable, or the number of flower visits an uninfected bumblebees would have to make in order to ingest enough to become infected, is not known (see Appendix 8). As a result, the extent to which pathogen transmission relies on shared flowers remains unclear, but may be particularly important in determining how host-pathogen dynamics vary between floristically diverse landscapes (Cisarovsky *et al.* 2014). These estimates also illustrate the difficulties in projecting transmission rates of bumblebee pathogens through a floristic landscape. Models must account for the effects of prolonged exposure to a range of biochemical environments, including flowers and the digestive systems of non-host organisms, which might affect pathogen survival and viability. High rates of flower visitation may amplify this, exposing pathogens to an increasing range of non-target taxa.

Most recent studies investigating horizontal host-pathogen dynamics in bumblebees have focused on spillover between managed bees (including honey bees and commercially-produced bumblebees), and wild bees (Colla *et al.* 2006, Otterstatter *et al.* 2008, Murray *et al.* 2013, Graystock *et al.* 2016). Studies exploring the bottom-up effects of floristic composition across landscapes on this dynamic remain scarce. We might expect to see landscapes with more flowers supporting larger pathogen populations, either directly as a result of horizontal transmission, or indirectly via increasing the host population (Anderson *et al.* 1981). A recent study in the Netherlands found that in landscapes with few semi-natural landscape elements (such as forests, heathland and grasslands), the prevalence of pathogens in wild *B. pascuorum* increased with the size of sown wildflower fields (Piot *et al.* 2019). Screening *B. terrestris/ B. lucorum, B. lapidarius* and *B. pascuorum* bees for pathogens suggest urban areas support more pathogens (Goulson *et al.* 2012, Theodorou *et al.* 2016), which could be a result of urban areas being abundant

in flowers (thus increasing the host population and opportunities for horizontal transmission) (McFrederick *et al.* 2006, Osborne *et al.* 2008), or as a result of spillover from urban beekeeping (Williams *et al.* 1991, Alton K 2013, Furst *et al.* 2014),. The studies also show differential effects of urbanisation on pathogen prevalence across species. Goulson *et al.* (2012) found an effect of urbanisation on pathogen prevalence in *B. terrestris*, but not for *B. pascuorum*. Similarly, Theodorou *et al.* (2016) found *C. bombi* prevalence was lower in *B. pascuorum* compared with *B. terrestris* and *B. lapidarius*. *N. bombi* prevalence also varied across bumblebee species, being more common in *B. lapidarius* than *B. terrestris/ lucorum* and *B. pascuorum*. These findings suggest pathogen dynamics are affected by environmental conditions both inside and outside the host (Schmid-Hempel *et al.* 1998, Tay *et al.* 2005, Goulson *et al.* 2012).

Bumblebees have three primary defences to pathogens: phagocytosis, encapsulation and melanisation (Moret *et al.* 2000, Moret *et al.* 2001). The former two are carried out by haemocytes and melanisation utilises the phenoloxidase pathway (Moret *et al.* 2009). This immunological response has become an important model in entomological immunology (Tyler *et al.* 2006), and many of the immunological interactions between European bumblebees and their natural parasites are well studied (Schmid-Hempel 2001, Koch *et al.* 2012).

1.7.2 Interactive effects of nutritional stress and pathogen infection

Nutrition is crucial for immune defence and resistance to pathogens (Ponton *et al.* 2013). The development and activation of the immune system are costly processes that are negatively affected when nutritional limitation forces reallocation of resources away from other biological processes (Moret *et al.* 2000, Smith 2007, Ponton *et al.* 2011). *B. terrestris* workers, for example, have been shown to increase sugar consumption by 7.5% in response to immune stimulation (Tyler *et al.* 2006). Accordingly, pathogens are more virulent in food-stressed hosts (Cunningham-Rundles *et al.* 2005).

There are three known mechanisms by which host nutritional status may affect pathogens, independently or interactively: i) host defence impairment, ii) lack of nutrients for pathogen growth, and iii) changes to the pathogen's environmental conditions in the host (Bundy *et al.* 1987). Host defence impairment has been observed in pollen-starved *B. terrestris* workers that showed reduced immune responsiveness to *C. bombi* infection,

including a failure to upregulate genes (Brunner *et al.* 2014). *C. bombi,* which is often quite benign and can have no effect on worker mortality, is substantially more virulent in food-stressed bees, with starved *B. terrestris* workers had a 50% higher mortality and lower reproductive investment compared with fed bees (Imhoof *et al.* 1998, Brown *et al.* 2000). These latent, or non-apparent infections, are theorised to have evolved - much like vertical transmission and free-living infective stages - to avoid extinction of the pathogen if its access to a host is somehow restricted, for example, by host life-stage (Anderson and May 1981). When bees are starved there are no nutrients moving through their digestive system. Pathogens that derive a large proportion of their nutrition directly from the host develop in accordance with their host's nutritional status (Smith 2007). As such, pollen-starved *B. terrestris* workers host small populations of *C. bombi* with disrupted development (Logan *et al.* 2005), while in honey bees, increasing pollen intake increases the speed of *N. ceranae* development (Porrini *et al.* 2011). Host defence impairment and lack of nutrients for the pathogen may act synergistically. For example, in a study using *B. impatiens* and *C. bombi,* lack of pollen and low nectar sugar reduced pathogen cell counts, but simultaneously reduced bumblebee survival (Conroy *et al.* 2016). The environmental conditions of the host gut may also vary in response to diet through changes in the gut microbiota. Bumblebees have a distinct microbial community that play a role in their immunology (Koch *et al.* 2011) (Appendix 8).

Chemical compounds derived from pollen and nectar can have a strong effect on pathogen virulence. When the effects of plant secondary metabolites were measured in *Crithidia* infected bumblebees, specific alkaloids (anabasine, gelsemine and nicotine), glycosides (amygdalin, acubin and catapol) and the terpenoid thymol strongly reduced infection levels by up to 81% (Richardson *et al.* 2015). Manson et al (2010) also investigated the effects of gelsemine on bumblebees infected with *Crithidia* and found pathogen loads were lower in bees who regularly consumed the compound. That *Crithidia* was unaffected by gelsemine outside the host suggests that while the gut parasite is susceptible to the effects of secondary metabolites, the anti-microbial action is indirect and via the bumblebee (Manson *et al.* 2010). In addition, some studies show that bumblebees self-medicate. *C.bombi*-infected *B. terrestris* workers prefer sugar water containing the alkaloid nicotine, which temporarily delays pathogen development (Baracchi *et al.* 2015). Other secondary metabolites have also been shown to reduce *C. bombi* development (Manson *et al.* 2010, Biller *et al.* 2015, Richardson *et al.* 2015). These studies do not

show secondary metabolite consumption improves survival rates, but these chemicals may still play an important role in alleviating the negative effects of pathogen infection, possibly allowing infected bees more time and energy to forage. So far, no study has empirically tested the effects of host nutrition on *N. bombi*-infected bumblebees.

Unlike *C. bombi* and *N. bombi*, *N. ceranae* is considered to be an emerging infectious disease in bumblebees (Graystock *et al.* 2013a). The effect of host nutrition on *N. ceranae* in bumblebees has not yet been explored. In honey bees, *N. ceranae* infection demands increased energy consumption and when bees are fed *ad libitum*, the pathogen has no effect on survival (Mayack *et al.* 2009, Naug *et al.* 2009). Honey bees reared on polyfloral diets are more resistant to *N. ceranae* infection at both an individual and colony-level (Huang 2012), show increased immune-related enzyme activity (Alaux *et al.* 2010), and increased longevity (Di Pasquale *et al.* 2013). Thymol and resveratrol can reduce *N. ceranae* spore loads in honey bees as well as increase their survival rates (Costa *et al.* 2010). These results demonstrate the importance of diet in determining the interactions between bumblebees and their pathogens.

1.8 Habitat conservation for bumblebees

Bumblebees have naturally exploited a wider range of habitats across a larger geographical area than many other insects. As such, approaches to their conservation can be equally expansive. Conservation schemes that benefit bumblebees are implemented in a variety of habitats, most notably agricultural landscapes, where many species historically thrived (Goulson 2010). More recently, as our understanding of the value of urban greenspace for biodiversity has increased, gardens have also become a target for conservation initiatives. Despite stark differences in the structure and management of these habitats, they each have potential for bumblebee conservation.

In Europe, agri-environment schemes (AES) have been introduced on farmland in response to a loss of biodiversity throughout this landscape. These schemes include the creation, management and restoration of a variety of habitats, including arable margins, hedgerows and traditional orchards. Reports on their success have been mixed and may originate from a failure to distinguish between biodiversity conservation and the support

of ecosystem services (Scheper *et al.* 2013). Studies show conservation schemes tend to support populations of common bumblebees, but often not threatened bumblebee species or other specialist bees (Carvell *et al.* 2015, Wood *et al.* 2015, Wood *et al.* 2017). Some scheme options have been identified that at least somewhat benefit declining species, although there is little evidence to suggest these have any meaningful impact on their populations. In a comparative analysis of options in the scheme, an agricultural legume mix attracted the highest total abundance and diversity of bumblebee species, including two declining species *B. ruderatus* and *B. muscorum* (Carvell *et al.* 2007). In another study, *B. humilis, B. sylvarum* and *B. ruderatus* were observed on field margins sown with a targeted pollen and nectar mix (Pywell *et al.* 2006). The effectiveness of options that increases floral resource abundance seem to rely largely on surrounding landscape characteristics and ongoing habitat management (Carvell *et al.* 2007, Heard *et al.* 2007, Scheper *et al.* 2013, Krimmer *et al.* 2019), and developing flower mixes that coincide with the foraging period of late-emerging species is an ongoing issue (Carvell *et al.* 2007, Pywell *et al.* 2011). In a comparison of nectar supply and demand through the year, researchers identified significant periods of high nectar availability in May and July, as well as periods of low nectar availability they termed 'hunger gaps', which were primarily June, but also in August and September (Timberlake *et al.* 2019).

Wild bumblebees have been shown to benefit from urban and suburban gardens, where floral resources and suitable nesting sites are abundant (Smith *et al.* 2006b, Samnegård *et al.* 2011). Gardens typically contain a mixture of plant species with a variety of geographic origins, and a great deal of conservation and research effort has gone into determining the best plant taxa for biodiverse gardens (Osborne *et al.* 2008, Garbuzov *et al.* 2014, Salisbury *et al.* 2015). Whether a plant is native, near-native or exotic matters little for most bumblebee species, whose full geographic ranges naturally overlap with many species non-native to the UK or Europe (Kendle *et al.* 2000, Hanley *et al.* 2014). Moreover, the chemical constituents valuable to bumblebees are highly conserved within plant genera and families (Roulston *et al.* 2000). Individual habitats can have floral resource gaps through the season, however, multiple habitats can be complimentary in their provision of resources for bumblebees (Samnegård *et al.* 2011, Mandelik *et al.* 2012, Timberlake *et al.* 2019), highlighting the importance of whole-ecosystem approaches for bumblebee conservation.

1.9 Summary and thesis aims

Land use change has resulted in the widespread loss of floral resources and this is likely to have substantial effects on bumblebee health and affect how they respond to infection. The pathogens known to infect bumblebees can be transmitted horizontally via shared flowers and the garden habitat is known to be floristically rich. It is unclear how habitat and floral resource availability affect opportunities for pathogen transmission and pathogen prevalence in the wild, and how this in turn affects bee health. Bumblebee species vary in flower preferences, life history traits and conservation status. Recent work suggests that some species have different nutritional needs (Moerman *et al.* 2016), and therefore may respond differently to biological stressors. In spite of this, laboratory studies investigating nutritional immunology and pathogen dynamics are still almost exclusively carried out on a small handful of species (particularly *B. terrestris* and *B. impatiens*) covering only two subgenera, and which are not in decline. Long-tongued bumblebees are rarely studied, but recent work has made considerable advances (Bučánková *et al.* 2012, Ptáček *et al.* 2015). Despite ongoing difficulties, optimising their rearing should be a priority for future research to inform conservation policy for rare and declining bumblebees.

In this thesis I investigate how floral resources, both in availability and nutritional value, affect bumblebee health across a range of species. To study the effects of diet on long-tongued species, I add to the limited existing knowledge of their rearing using two understudied species, *B. pascuorum* and *B. hortorum* in Chapter 2. In Chapter 3 I compare, for the first time, the effects of nutrition on their reproductive success by feeding queens two distinct pollen diets, and simultaneously trial a new rearing technique based on observations from Chapter 2. Caution is needed when model organisms are relied upon for scientific research and so Chapters 2 and 3 will offer insight into the ecology of new species and highlight the need to utilise a range of species to predict responses to environmental change, particularly where they are threatened in the wild. To investigate the effects of habitat type on bumblebee pathogens, I compare transmission rates and prevalence of *C. bombi, N. bombi* and *N. ceranae* in gardens and farmland in Chapter 4. In Chapter 5, I examine the relationship between habitat type, floral resource availability, bee health and pathogen prevalence. The floristic landscape plays an important role in bumblebee health, however we know very little about how the availability of different

floral resources might affect the abundance of pathogens, both within and across different landscapes. I compare these across three habitats that are important for bumblebee conservation in the UK.

Diet is crucial in determining the health of an organism and a combination of controlled experiments and field sampling is required to understand the effects of diet on an organism's performance. In this thesis, I feed laboratory reared bees different pollen diets and carry out extensive floristic surveys to compare food resource availability across habitats. Measuring the food resource available to flying organisms can be a challenge, particularly when this food may only be confined to small patches in a landscape. To deal with this issue, I use two surveying techniques that collectively describe food availability in the best regions of a site (the most resource-rich regions), and food availability more archetypical to the site (representative regions), therefore allowing estimations of the total food available to flying organisms. Adjusting for foraging distance, this combination of surveying techniques could elucidate resource availability for many flying species.

In addition to bumblebees, solitary bees, stingless bees and honey bees are all reliant on pollen and nectar, while other pollinators such as wasps, flies, butterflies and moths require pollen or nectar during at least part of their lifecycle. For many of these insects our understanding of their nutritional ecology is poor or non-existent, and yet many, like bumblebees, are of conservation concern. The insights this thesis will provide be valuable for our understanding of these other flower-feeding insects.

Nutritional stress, resulting from habitat degradation and other environmental changes, is a major threat for vulnerable animal species. For some, nutritional stress is a result of reduced food quantity (e.g. the defaunation by humans has removed prey species for predators). However, for many it arises from changes in food quality (e.g. a reduction in the diversity of food species available). Because bumblebees have a relatively simple diet (pollen and nectar), they are in many ways good models for investigating the effects of nutritional stress. This thesis will therefore aid in increasing our understanding of the effects that food quality can have on the nutritional health of animals.

Chapter 2: Trialling techniques for rearing long-tongued bumblebees under laboratory conditions

Abstract

Bumblebees are important pollinating insects, but many species have suffered declines over the last century. Long-tongued bumblebees have been identified as particularly at risk, partly due to their more selective diet. Attempts to study these species in captivity have been impeded by stress-induced behaviours which cause queens to kill or abandon their brood. Here we attempt to further develop techniques, using queen pairing and *Bombus terrestris* cocoons, to successfully rear two common long-tongued bumblebee species (*B. pascuorum* and *B. hortorum*) in captivity. Approximately half of queens laid eggs and 29% produced workers. Although challenges remain, there is a great deal to be gained from optimising the captive rearing of these species.

2.1 Introduction

Bumblebees (*Bombus spp.*) are ecologically and economically important pollinating insects, but many species have suffered severe declines in recent decades across Europe and North America (Williams *et al.* 2009, Goulson *et al.* 2015). Nutritional stress, pathogen infection and exposure to pesticides are key drivers in their decline, but these pressures do not act independently of one another and so their effects on bees are not straightforward (Vanbergen *et al.* 2013). Techniques to rear bumblebees in captivity have been developed over the last century (Van den Eijnde *et al.* 1990, Pouvreau 2004, Velthuis *et al.* 2006). However, these experiments have been almost exclusively conducted on short-tongued species (such as *B. terrestris* and *B. impatiens*). In the wild, these species are generally common, characterised by long colony life-cycles and the utilisation of resources from multiple plant groups and habitats (Fussell *et al.* 1992, Goulson *et al.* 2005). While these species are suitable as models for studying some aspects of social insect biology and behaviour, they are not representative of all bumblebee species and differ significantly in their ecological sensitivity and response to stressors (Goulson *et al.* 2005).

A small number of studies have succeeded in rearing bumblebee species other than *B. terrestris* or *B. impatiens* in captivity. Lhomme *et al.* (2013) provided the first precise protocol for rearing two cuckoo species, *B. vestalis* and *B. sylvestris* and Moerman *et al.* (2016) showed differential responses to pollen diet between *B. terrestris, B. pratorum* and *B. hypnorum* micro-colonies reared in captivity. Like *B. terrestris* and *B. impatiens*, these other species also have short-medium tongue lengths (Falk 2015). However, the bumblebees that have declined most severely tend to be the long-tongued species that rely heavily on flowers from the Fabaceae plant family (Goulson *et al.* 2005, Biesmeijer *et al.* 2006). Unlike short-tongued species, many of which have thrived despite land use intensification, long-tongued bumblebees have a restricted range of foodplants, and are from smaller colonies with a smaller foraging range (Goulson *et al.* 2008). As a result, they are more sensitive to local and global pressures (Goulson 2003, Goulson *et al.* 2005). Unfortunately, these species have remained relatively understudied in laboratory conditions due to difficulties of rearing the colonies in captivity. During laboratory rearing trials, queens generally perform stress-induced behaviours (Pomeroy *et al.* 1979, Weidenmüller *et al.* 2002), including failure to settle in nest boxes provided, failure to utilise pollen, neglecting eggs and larvae, and direct ovicide or infanticide, resulting in early colony failure. Rearing conditions, such as nest box design and pollen type, have a substantial effect on the health and reproductive success of bumblebees reared in captivity. There is some evidence to show that giving young, long-tongued colonies access to flowers allows natural foraging and improves colony development (Lhomme *et al.* 2013, Ptáček *et al.* 2015, Moerman *et al.* 2016). Many of the long-tonged bumblebees can be characterised as pocket makers, feeding their larvae a solid rather than liquid diet (Den Boer *et al.* 2006). How this might affect the physiological and behavioural responses of queens, workers and larvae to environmental stressors remains unclear.

The first attempts at long-tongued bee rearing were carried out by Lindhard (1912). At the same time, Sladen (1912) was testing stimuli to encourage oviposition in short-tongued queens. These methods have been combined and developed since although their effects can be highly variable. Stimuli for egg-laying include various interspecies pairings at different life stages, including the provision of cocoons and callow workers. Providing brood has a stimulatory effect on queens of several bumblebee species (Yoneda 2008, Bučánková *et al.* 2010), and cocoons of *B. terrestris* have commonly been used in rearing

trials of long-tongued species (Kwon *et al.* 2003, Bučánková *et al.* 2012, Ptáček *et al.* 2015). Allowing cocoons to hatch and callows to remain in the colony, or adding *B. terrestris* or *Apis mellifera* callows, can also have a stimulatory effect on queen egg-laying and *B. pascuorum* and *B. ruderarius* are at least somewhat encouraged to oviposition by honey bee workers (Ptacek 1983, Ptacek 1985, Ptáček *et al.* 2015). Callows have also been observed caring for the queens own brood; Ptáček *et al.* (2015) observed *B. terrestris* workers feeding *B. pascuorum* and *B. sylvarum* larvae. However, in another trial, *B. hortorum* queens were aggressive towards multiple stimuli and have been observed destroying *B. terrestris* cocoons and killing honey bee workers (Bučánková *et al.* 2012).

The pairing of queens, first tested by Sladen (1912), can also encourage oviposition and brood care later on (Plowright *et al.* 1966, Alford 1975, Duchateau 1985, Ptácek *et al.* 2000, Ptáček *et al.* 2015). This sometimes involves an excluder, which keeps queens physically separated in a shared nest box. Once they are placed together, one female usually becomes dominant and begins egg-laying (Ptáček *et al.* 2015). Ptácek *et al.* (2000) and Ptáček *et al.* (2015) found queen pairing effective for *B. pascuorum* but not for *B. humilis* or *B. ruderarius*, who displayed 'resentful' and aggressive behaviours. Of the three *B. humilis* females they trialled, one broke through the excluder to reach and kill her neighbour. In another study, queens of *B. hortorum* were more likely to establish an egg cell when kept alone (100%, n=9), compared to those who were paired (66%, n=6) or who had been given a honey bee worker (71%, n=7) (Bučánková *et al.* 2012). These various interspecific combinations have therefore yielded contrasting results, from cohabitation to killing, but many of the observations are based on a very small number of individuals, making species-level assessments and progress difficult.

Studying species in captivity is necessary to understand biological and behavioural systems, informing conservation initiatives of wild animals and allowing endangered species to be bred in captivity (Ring *et al.* 1981, Gabriel *et al.* 2007, Sanger *et al.* 2008). Differences in life history traits across species make it difficult to apply our understanding of reliable model species to those at risk in the wild. For bumblebees, utilising a variety of species for research will have significant benefits for the conservation of rare and declining species. Long-tongued bumblebees make up more than half of the bumblebee subgenera, yet very little is known about their biology and behaviour (Cameron *et al.*

2007). Amongst these, pocket makers are one of the most scarcely researched groups, despite many species showing severe declines, including *B. ruderarius*, *B. humilis* and *B. sylvarum* (*Thoracobombus*) (Falk, 2015). Developing methods to rear long-tongued bumblebees in captivity would be of great benefit to research and the conservation of declining species.

In this study we attempt to further develop techniques in long-tongued bumblebee rearing with two common long-tongued species representing two long-tongued subgenera, *B. pascuorum* (*Thoracobombus*) and *B. hortorum (Megabombus*). These pocket makers are amongst the most common and generalist of the long-tongued bumblebees in the UK, making them relatively easy to locate and collect without ethical concerns regarding the use of endangered species. Examining the effects of previously tested methods on these species, we investigate the efficiency of rearing techniques and assess interspecific differences in their responses.

2.2 Materials and methods

Rearing trials took place at the University of Sussex (UK) in 2017 (Fig. 2.1). Queens of *B. pascuorum* and *B. hortorum* were collected from surrounding chalk grasslands in the spring (March to mid-April). Pollen foraging occurs post-nest establishment (Evans *et al.* 2007), and so only queens without pollen in their pollen baskets were collected. Bees were stored in ventilated 5ml eppendorf tubes cooled beside ice packs for transport back to the lab. Queens were then placed in a dark room (30 ± 1^{o}C, 20% RH) in ventilated 15 x 15 x 15 cm plastic boxes. We observed previously that queens failed to thermoregulate brood and so we used the highest temperature described in similar long-tongued rearing trials (Ptáček *et al.* 2015). We did not observe any nest fanning behaviour by queens or workers during the experiment that would have indicated overheating. Queens were provided with a piece of cotton wool to simulate nesting material and 50% (v/v) sugar water (10% fructose, 90% sucrose).

For the first feeding, sugar water was placed onto the floor of the box or pipetted directly to queens if they were lethargic. Thereafter, sugar water was provided in an external feeder *ad libitum* (Fig. 2.1c). Queens were given a fresh 1 g pollen ball every two days.

Pollen balls were made by grinding dry pollen granules to a powder and combining with 50% (v/v) sugar water (10% fructose, 90% sucrose) until a sticky dough was formed. We found that this wet pollen with a high sugar water content was consumed readily by both species. All bees started on mixed *Erica*, *Salix* and *Prunus* pollen (*Pollenergie,* France) and from April a pre-made polyfloral wildflower mix (*mille fleurs, Pollenergie,* France) was added, which became 100% of their food by May to simulate natural conditions for workers. All pollen had been freeze-stored prior to use. Pollen balls were generally placed in the box for bees to collect but attempts were made to feed the first larvae by scattering or gently pressing ground pollen onto the brood casing.

Following Ptacek *et al.* (2015), all *B. pascuorum* queens were paired to prompt one to become dominant and begin egg-laying. We identified dominant individuals as those which spent the most amount of time in the centre of the box and standing on the pollen provided, since this is naturally where they would lay their eggs (Den Boer *et al.* 2006). Some of these queens also exhibited a lengthening of the abdomen, also observed by Ptáček *et al.* (2015). Submissive queens (which tended to be the smaller of the two) were more active and tended to roam the edges of the box. Once identified, submissive females were removed and paired together, prompting another to establish dominance. Paired queens that appeared unsettled or not engaged in egg-laying or brood care were also separated and either re-paired or given their own nest box. The action taken for each queen were based on each queen's dominant/ submissive characteristics and the availability of other single queens available for pairing, and nest boxes (see Supplementary Table 2.1-3 for all pairings and actions). When testing the response of two *B. hortorum* queens being paired, they were highly aggressive, so were separated and no further *B. hortorum* queens were paired together.

For the first 33 days we recorded the progress of queens without additional stimuli. From Day 34, to further stimulate egg-laying and encourage brood-care behaviours, 2-5 day old laboratory-reared *B. terrestris* pupae were introduced to the *B. pascuorum* and *B. hortorum* queens. Three times a week throughout the experiment, we recorded (i) the day of first egg laying, (ii) observable ovicide, larvicide or neglect, (iii) brood pupation, (iv) worker emergence, (v) queen and male production, (vi) aggression between paired queens or queens and *B. terrestris* cocoons/ workers, and (vii) use of cotton wool as nesting material. All efforts were made to minimise disturbance throughout the experiment.

To check for differences in behaviour and reproductive success between species we used Kruskal-Wallis tests for queen survival, the day they first produced eggs or workers, and the number of workers produced. Any pairs of queens that produced workers together were counted as a single queen. Chi-square tests were used to determine if there was a difference between species in whether or not queens produced eggs or workers, and ejected or neglected eggs or larvae.

2.3 Results

A total of sixty-eight queens (11 *B. hortorum* and 57 *B. pascuorum*) were used in the experiment, of which forty queens laid eggs (59%) (Fig. 2.1). Six additional pairs of queens showed close co-operative brood care behaviour with their partner, and as a result it was not possible to tell which queen, if not both, were egg laying (Table 2.1).

There were significant differences between species in whether or not they ejected eggs or larvae, and whether or not they produced workers (χ^2 = 10.6, df = 1, p = 0.001 and χ^2 = 9.9, df = 1, p = 0.0002, respectively). *B. pascuorum* queens were more likely to eject their eggs or larvae (79% of the 34 queens that laid eggs) compared to *B. hortorum* (16% of the 6 queens that laid eggs), and *B. hortorum* queens were nearly three and a half times as likely to produce workers (Table 2.1). There were no significant differences between species in their survival, whether or not they laid eggs, the number of days it took to produce their first egg or worker, the number of workers they produced and whether or not they neglected their eggs or brood (respectively: χ^2 = 0.04, df = 1, p = 0.85*;* χ^2 = 0.09, *p = 0.75;* χ^2 = 0.21, df = 1, *p = 0.64;* χ^2 = 0.71, df = 1, *p = 0.40;* χ^2 = 3.10, df = 1, p = 0.08; χ^2 = 0.95, df = 1 *p = 0.32*).

Survival of queens ranged from 2-178 days (Fig. 2.2), with a 12% mortality rate observed in the first 7 days. Of the 40 queens that laid eggs, two paired *B. pascuorum* queens (BpW and BpM) and one individual *B. hortorum* queen (BhF), did so before the addition of any *B. terrestris* cocoons (Table 2.2). Across both species, 70% of queens ejected eggs and larvae and 33% neglected them. These behaviours were repeatedly observed throughout the experiment. Queens would lay eggs and then either remove them or the developing

larvae, or would fail to feed the larvae so they died in the brood case. This cycle continued even after the first workers were produced. Scattering and pressing additional pollen over the larvae was unsuccessful; the larvae were not observed to feed and the pollen dried around them.

Nine single queens (*B. hortorum* queens BhJ, BhK, BhE, BhD and BhF, and *B. pascuorum* queens BpB and BpAQ) and three pairs of queens (*B. pascuorum* queens BpAL-BpAM, BpBC-BpBK and BpAD-BpAZ) produced pupae, and all pupae successfully eclosed and emerged as workers.

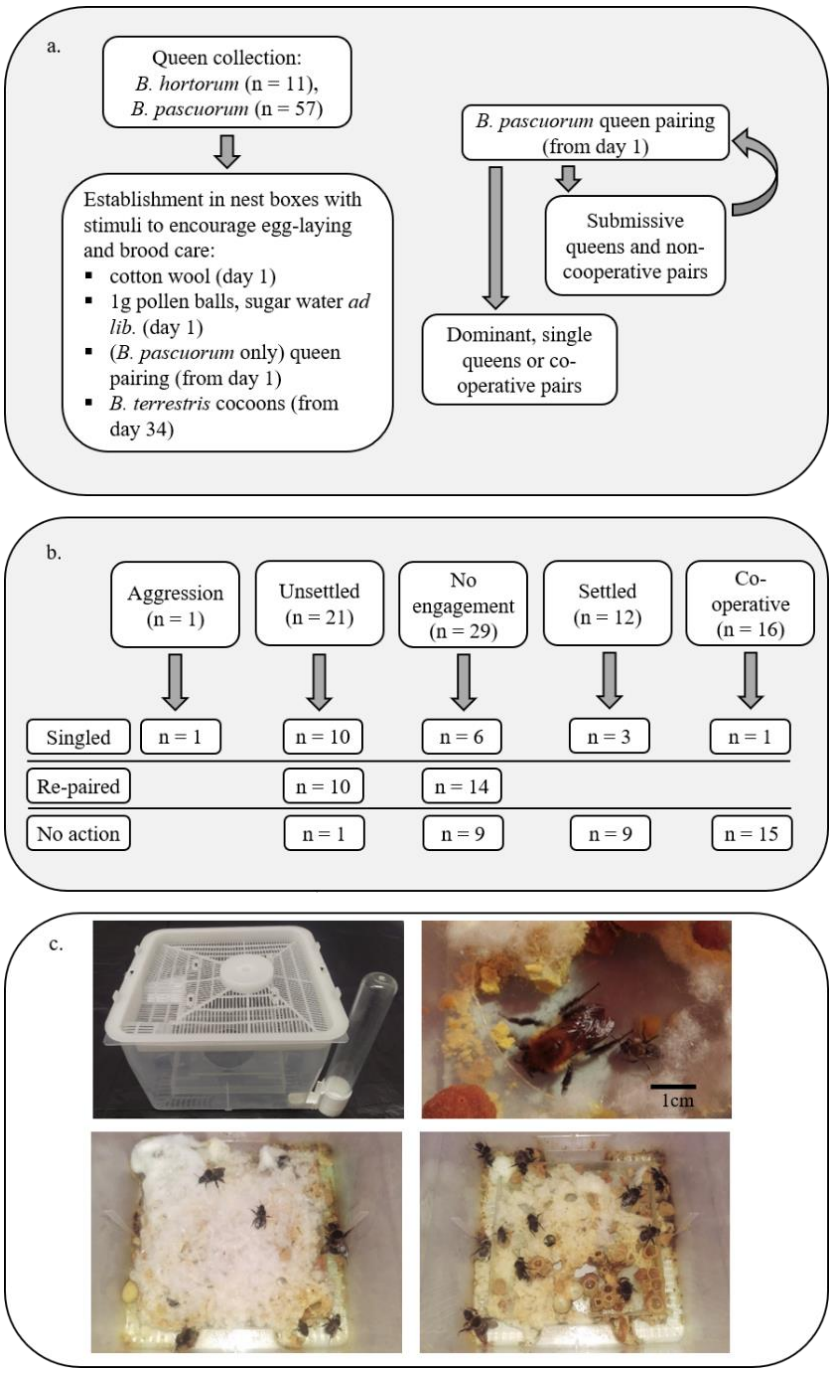


Figure 2.1 Flowchart illustrating the methodology and stimuli used in this study to encourage wild-caught *B. pascuorum* (n = 57) and *B. hortorum* (n = 11) bumblebee queens kept in captivity to produce colonies. *B. pascuorum* queens were all initially paired (79 pairings in total). (b) Flowchart showing the response of *B. pascuorum* queens to pairing (see Supplementary Tables 2.1-3) and the resulting action taken, which was based on each queen's dominant/submissive characteristics and the availability of single queens and nest boxes. (c) Top left to right: Nesting boxes used to rear single or paired long-tongued bumblebee queens; a *B. pascuorum* worker and her queen. Bottom left and right: two colonies of *B. hortorum* utilising cotton wool as nesting and food storage material.

Table 2.1 A summary of colony development, behaviour and longevity of *B. hortorum* and *B. pascuorum* bumblebee queens kept under laboratory conditions. *B. hortorum* queens were kept individuals (n=11). *B. pascuorum* queens were kept in pairs or singly after initial pairing (n=57 queens). Queens were provided with food and sugar water *ad libitum* and stimuli to encourage oviposition and brood-rearing were provided at various points in colony development (Table 2.2).

	B. hortorum queens (n=11)	*B. pascuorum* queens (n=57)
Mean ± SE days survived (queens)	74 ± 20	74 ± 7
Use of cotton wool	91% (10/11)	100% (57/57)
Aggression between queens	-	2% (1/57)
Egglaying	54% (6/11)	60% (34/54)
Unconfirmed egg-laying	-	11% (6/57)
Mean ± SE days to egg-laying	16 ± 2.3	20.6 ± 1.9
Egg/larval ejection	16% (1/6)	79% (27/34)
Larvae neglect	50% (3/6)	29% (10/34)
Pupation	83% (5/6)	24% (8/34)
Production of workers	83% (5/6)	24% (8/34)
Mean ± SE days to first worker	81 ± 9.2	66.4 ± 11.9
Mean ± SE number of workers	4 ± 1.4	3 ± 0.4
Queen production	50% (3/6)	0% (0/34)

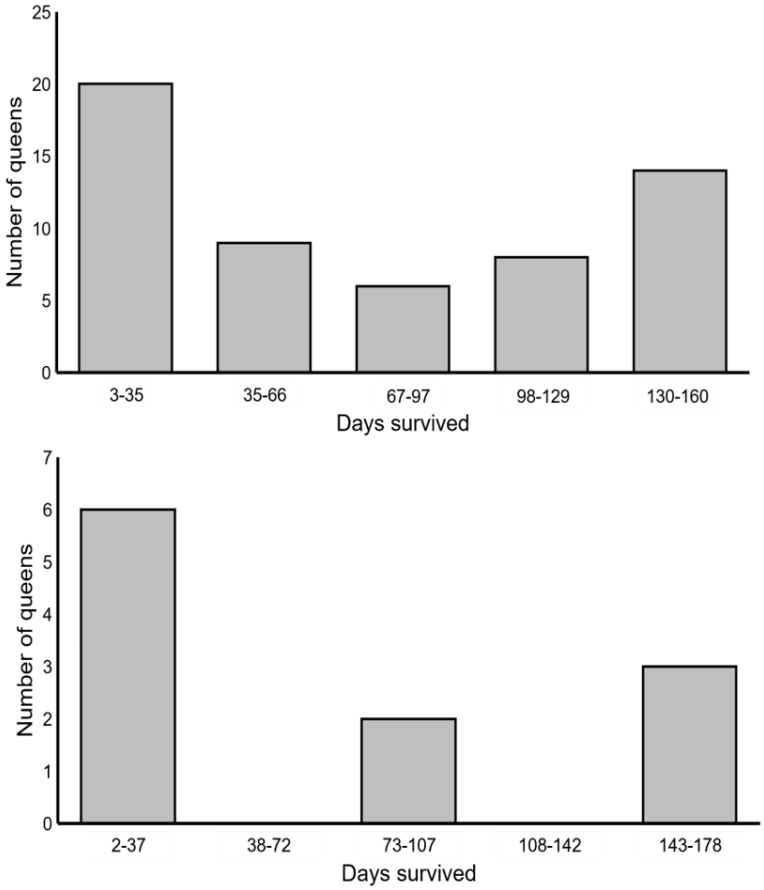


Figure 2.2 Survival of wild-caught a) *B. pascuorum* (n=57) and b) *B. hortorum* (n=11) bumblebee queens reared in captivity to produce colonies. Fourteen percent of queens died in the first 10 days and 76% of queens that survived this period went on to lay eggs.

Table 2.2 Survival and reproductive success (day of first egg, day of first worker and number of offspring produced), of *B. hortorum* and *B. pascuorum* queens kept in laboratory conditions. Egg laying shown in bold denotes co-operation between queens, where it was not possible to tell if one or both queens contributed to egg-laying. *B. pascuorum* queens were all initially paired and based on their behaviour, reproductive success and survival, were either paired (P) or single (S) at different stages of colony production (see Supplementary Tables 2.1-3 for all pairings and justifications for keeping some queens paired and giving some their own nest box). If a *B. pascuorum* queen was paired, the number of pairings she had is shown (P1-3). All queens were initially given cotton wool, pollen and sugar water *ad lib.* at the start of the experiment, then later a *B. terrestris* cocoon to further encourage oviposition and brood care. Response of queens to the resulting *B. terrestris* workers: • *B. terrestris* worker accepted by queen (i.e.: not killed and not avoided), □ Queen actively avoided *B. terrestris* worker until it was removed, ◆Queen killed her *B. terrestris* worker or showed aggression.

Species	Queen	Days survived	Day of first egg	Day of first worker	Day *B. terrestris* cocoon was added	Long-tongued offspring produced		
						workers	males	queens
	BhG	2	-	-	-	-	-	-
	BhB	13	-	-	-	-	-	-
	BhI	17	-	-	-	-	-	-
	BhC	25	13	-	-	-	-	-
Bombus	BhA	29	-	-	-	-	-	-
hortorum	BhH	36	-	-	-	-	-	-
	BhJ	97	16	95	42●	3	-	-
	BhK	105	8	109	41□	1	-	-
	BhE	152	14	72	47□	8	1	-
	BhD	158	22	71	41◆	6	38	4

Cont. over page

Table 2.2. cont.

Species	Queen	Days survived	Day of first egg	Day of first worker	Day *B. terrestris* cocoon added	Long-tongued offspring produced		
						workers	males	queens
	BhF	178	23	58	58☐	1	4	-
	BpAB, BpC	3	-	-	-	-	-	-
	BpD	5	-	-	-	-	-	-
	BpAW	6	-	-	-	-	-	-
	BpG, BpR	7	-	-	-	-	-	-
	BpBD, BpE	8	-	-	-	-	-	-
	BpT	9	-	-	-	-	-	-
	BpF	12	(S) 11	-	-	-	-	-
	BpS	16	-	-	-	-	-	-
	BpQ	17	-	-	-	-	-	-
	BpAJ	20	-	-	-	-	-	-
	BpAA, BpAV	22	-	-	-	-	-	-
	BpAT	23	(P1) 20○	-	(S) 48☐	-	-	-
	BpU	23	-	-	-	-	-	-
	BpAN	24	-	-	-	-	-	-
	BpAK	25	-	-	-	-	-	-
	BpAU	28	(P2) 21○	-	-	-	-	-
	BpAX	47	**(P1) 9**	-	-	-	-	-
	BpBF	50	(P1) 14	-	(P1) 48☐	-	-	-
	BpBE	52	**(P1) 14**	-	(P1) 48☐	-	-	-
	BpAY	60	**(P1) 46**○	-	(P1) 41☐	-	-	-
	BpAZ	61	(P1) 13○	(P1) 64	(P1) 53●	3	-	-
	BpBB	62	(P1) 20○	-	(P1) 56☐	-	-	-
	BpAL	65	(P1) 16○	(P1) 64	(P1) 51☐	3	-	-
	BpAM	65	**(P1) 16**○	(P1) 64	(P1) 51☐	3	-	-
Bombus pascuorum	BpAG	69	(P1) 13	-	(S) 48☐	-	-	-
	BpAS	71	(S) 30	-	(P2) 50☐	-	-	-
	BpAE	76	(P1) 13	-	(P1) 59☐	-	-	-
	BpM	77	**(P1) 36**	-	(P1) 34☐	-	-	-
	BpAC	80	(P2)17	-	(S) 59☐	-	-	-
	BpN	82	(P1) 46○	-	(P1) 41☐	-	-	-
	BpW	92	(P1) 36	-	(P1) 34☐	-	-	-
	BpX	103	(S) 22	-	(S) 57☐	-	-	-
	BpB	114	(S) 23	(S) 84	(S) 69☐	3	-	-
	BpAO	117	(P2) 12	-	(S) 35☐	-	-	-
	BpV	119	(P2) 28	-	(S) 45☐	-	-	-
	BpAD	123	(P1) 13○	(P1) 64	(P1) 53●	3	-	-
	BpBC	123	**(P1) 9**○	(P1) 95	(P1) 54☐	1	-	-
	BpK	124	(P1) 9○	(P1) 95	(P1) 54☐	1	-	-
	BpO	126	(S) 16	-	(S) 49☐	-	-	-
	BpAR	129	(P1) 19○	-	(P1) 55☐	-	-	-
	BpL	130	(S) 20	-	(S) 48☐	-	-	-
	BpBA	132	(S) 20○	-	(P1) 56☐	-	-	-
	BpH	132	(P2) 4○	-	(P3) 58☐	-	-	-
	BpP	132	(P1) 5	-	-	-	-	-
	BpJ	133	(P2) 13	-	(P2) 59☐	-	-	-
	BpAQ	136	(P1) 16	(S) 25	(S) 59◆	3	-	-
	BpHI	136	(P1) 30	-	(S) 50☐	-	-	-
	BpAF	140	(S) 13	-	(S) 48☐	-	-	-
	BpI	141	(S) 38	-	(S) 58◆	-	-	-
	BpY	142	(P2) 21	-	(S) 35☐	-	-	-
	BpAP	149	(P1) 19○	-	(P1) 55☐	-	-	-
	BpAH	153	(P2) 28	-	(S) 50☐	-	-	-
	BpZ	160	(S) 55	-	(S) 62☐	-	-	-

2.3.1 Rearing of *B. pascuorum* queens

In the first ten days, 91% of *B. pascuorum* queens performed nest-associated behaviours, including carding with cotton wool, making nectar cups and sitting on pollen (Fig. 2.1c). All 57 queens were paired and a total of 79 pairings (up to three per queen, $\bar{x}$ ± s.e. 1.4 ± 0.08) were made over the course of the experiment (Supplementary Table 2.1-3). In only one case did a *B. pascuorum* queen exhibit aggression, where a previously submissive queen killed her new partner. The duration of the other 78 pairings varied between 1-129 days (32 ± 4.7). The behaviour of paired queens could be categorised as aggressive (n = 1), unsettled (i.e. avoidance, no egg-laying or interruption of egg-laying) (n = 21), not engaged with brood care (n = 29), settled (i.e.: not aggressive or avoidant) (n = 12) or co-operative (jointly caring for brood) (n = 16) (Supplementary Table 2.1-3). Of the 34 queens that were observed to lay eggs, 32 (94%) produced their first eggs before the addition of cocoons, and two (6%) 2-5 days after.

At the point *B. terrestris* cocoons were given to queens (which was determined by the availability in the source colony), 16 of the *B. pascuorum* queens were paired and 20 were single. The cocoons were always ignored, and workers emerged 1-4 days later. Thirty-one queens actively avoided their *B. terrestris* worker until the worker was removed. In eight nests, the *B. terrestris* worker took over and laid its own eggs. When this happened, the *B. pascuorum* queens ceased their cyclic egg-laying and egg or larval ejection, and even after the *B. terrestris* worker was removed, did not resume egg-laying again for some time. Two queens killed their donor *B. terrestris* after emergence, including one that had successfully produced a worker before the addition of the cocoon (BpAQ; 25 days post-capture; Table 2.2). Only one pair of queens accepted their worker and exhibited positive physical contact (BpAD-BpAZ). In the remaining nests, the *B. terrestris* workers stayed around the perimeter of the boxes and we observed very few interactions between them and the *B. pascuorum* queens. We saw no evidence that *B. terrestris* workers assisted the *B. pascuorum* queens in brood care.

For five queens (pairs BpAZ-BpAD and BpAL-BpAM and single queen BpB) the addition of a *B. terrestris* cocoon was followed by the successful production of *B. pascuorum* workers, 11-15 days later. One additional pairing produced their first worker 41 days later (BpK-BpBC).

Successful queens (i.e. those which produced workers) produced up to three small *B. pascuorum* workers each (Fig. 2.1c). No males or new queens were produced. One queen (BpAQ), which had produced a worker before the addition of cocoons, produced another two after the *B. terrestris* worker was removed. One *B. pascuorum* worker laid eggs after the death of the queen. These were neglected and died at the ~L2 larval stage.

2.3.2 Rearing of *B. hortorum* queens

Due to their aggression, *B. hortorum* queens were not paired. All but one of the 11 queens carried out nesting building using the cotton wool (Fig. 2.1c). Fifty-four percent of queens laid eggs, and of these, 83% went on to produce 4 ± 1.4 workers; three of these also produced males (1, 4 and 38 individuals respectively; Table 2.2).

Three queens survived for less than 17 days in captivity and did not lay any eggs in that time (BhG, BhB and BhI; Table 2.2). Of the three queens which survived between 25 and 36 days (BhC, BhA and BhH), only one laid eggs. These were reared up to the L3 larval stage before she ceased brood care and remained relatively still in a corner for ten days before dying. Five queens survived between 97 and 178 days (BhJ, BhK, BhE, BhD and BhF), all successfully producing workers. These queens were first observed with eggs or L1 stage larvae on days 8-23, but did not produce their first workers until, at the earliest, day 58 and at the latest, day 109.

The donor cocoons used to prompt oviposition and brood care in *B. hortorum* were given to all queens that survived to this period (from day 41 post-capture) and produced mixed behavioural responses. Queens were not observed interacting with the cocoons and generally continued their own egg-laying and larval feeding. BhF was given a cocoon on the day her first daughter emerged. Within a week BhF had (for the first time) ejected her remaining larvae. She did not kill or show any aggression to the *B. terrestris* worker but laid new eggs four days later, which later emerged as males. Three other queens responded negatively to the *B. terrestris* worker when it emerged. BhD killed it within a day of emergence and BhK and BhE actively avoided theirs, ceasing all nest-associated behaviours until the workers were removed. BhJ was the only queen whose interactions with her *B. terrestris* worker appeared consistently natural (settled physical contact). No

evidence of *B. terrestris* workers engaging in brood care behaviour when paired with *B. hortorum* queens was observed.

2.4 Discussion

Queens of both species readily laid eggs in the study, with more than three-quarters laying eggs if they survived past day ten. Even *B. hortorum* queens, who were given no initial stimulus beyond nesting material, had generally laid their first eggs approximately 2 weeks after capture. Despite not being a natural pollen choice for long-tongued bumblebees, the diet was sufficiently good for the queens to produce workers (n = 7 single queens and 3 pairs) and reproductives (n = 3). Very few workers were needed for the colony to produce males, demonstrated by a *B. hortorum* colony (queen BhD) which only produced six workers before producing 38 males. Problems arose for both species during larval development, when queens neglected or ejected their young. Although we cannot exclude eggs or larvae being abandoned due to poor health, we suggest this behaviour was more likely a response to stress or a perceived lack of resources (Smith 1985, Parmigiani *et al.* 1994). However, we did not observe any queen consuming their abandoned larvae, or failure of the larvae to feed pollen provided by the queen. Queens of both species were found to commit ovicide and larvicide, and this behaviour did delay worker production noticeably in both species, which has also been observed in trials with *B. lapidarius* (Bučánková *et al.* 2012). Queens of *B. terrestris* may begin laying eggs as little as 2 days after being placed in a nest box, but in sub-optimal temperatures this may take several weeks (Jie *et al.* 2005). Although the time needed to produce *B. pascuorum* and *B. hortorum* colonies in this study was not ecologically realistic or practical for immediate short-term studies, the cyclic egg-laying-ovicide behaviour presented multiple opportunities to encourage larval feeding and care. It may also suggest that, while queens may be ready to lay eggs and start a colony, they may also be particularly sensitive to these and other external conditions (such as disturbance) which interfere with rearing.

The facilities used in this study limited change to temperature and humidity and clearly these are aspects of captive rearing that must be optimised for each species, particularly since this has been demonstrated to delay colony initiation (Jie *et al.* 2005). Differences in natural nesting sites (e.g. above or below ground, nest material), should be considered

in developing optimal conditions for each species. Behaviourally, *B. pascuorum* seemed much less aggravated by the artificial conditions and stimuli provided, while *B. hortorum* queens responded more sensitively and more aggressively if disturbed. This included pollen feeding, during which the lid of the nest box had to be removed. However, given the frequency of ovicide and larvicide in both species, reducing stress post-egg-laying is likely to reduce the time it takes for queens to produce colonies and should be a continuing priority in rearing trials.

The proportion of *B. hortorum* queens used in the experiment that went on to produce workers (45%) is very promising. A much lower success rate was achieved for *B. pascuorum*, with only eight queens (14%) being involved in worker production. This may be improved by giving younger cocoons to queens as soon as they have acclimatised to their nest boxes. This could not be done in our experiment due to availability of cocoons, but other studies have demonstrated that younger cocoons and earlier exposure can prompt queens to settle quicker in their boxes – and ultimately produce workers sooner (Bučánková *et al.* 2012, Ptáček *et al.* 2015). Previous rearing trials have generally assumed that the presence of cocoons prompts queens to engage in natural brood care activities. This could be as a result of the cocoon's scent (Heinrich 1974, Gamboa *et al.* 1987), or slightly higher temperature (Barrow *et al.* 1985). While it is possible the queen might assume the brood are her own, nest intrusions occur naturally in the wild (Lopez-Vaamonde *et al.* 2004, Goulson *et al.* 2018a), and so the cocoons may instead be perceived as evidence of a female competitor being present. This would explain why queens have also been documented destroying cocoons and may stimulate females to assert dominance over the apparent rival via their own egg-laying, essentially stressed into developing a colony. Assuming that this is the case, there must be factors which discourage queens from performing natural rearing behaviours before the addition of cocoons, but which can be at least somewhat overridden when cocoons are added. Given that queens naturally would self-select nesting sites, it is possible that they do not recognise their captive surroundings and that the addition of cocoons functions at the very least as an indicator that the surrounding environment is a suitable nest.

As a result of intermittent feeding by the queen, the long-tongued workers (mainly *B. pascuorum*) that did emerge were small and may have made little contribution to colony development, suggesting queens may need continued support even after their first

workers emerge. We did not screen queens for pathogens and it is possible that parasite infections could have affected their propensity to lay eggs, and the size and health of their larvae. Providing *B. terrestris* cocoons did encourage *B. pascuorum* queens to engage in more larval care (Table 2.2) but had less effect on *B. hortorum* queens. After emergence, most *B. terrestris* workers made little to no physical contact with the queen or her brood, which is in contrast to previous studies in which *B. terrestris* workers actively engage in brood care and larval feeding for *B. pascuorum* and *B. sylvarum* queens (Ptáček *et al.* 2015).

We did observe co-operative brood care between paired *B. pascuorum* queens and up to 17 queens appeared to contribute equally to egg laying and brood care. For *B. pascuorum*, the value of intra- and interspecific pairings clearly needs further investigation.

B. hortorum and *B. pascuorum* are amongst the most generalist of the long-tongued bumblebees. They are common in the UK and share the same association with Fabaceae as those species most in decline, making them suitable models for rearing trials and laboratory experiments. In our study we found that queens of *B. pascuorum* and *B. hortorum* could rear small colonies through to the reproductive stage even when fed pollen they may not naturally collect in the wild. We found that queens lay eggs readily under artificial conditions even without the use of cocoons as stimuli; this might be further improved using CO_2 exposure (Röseler 1985, Tasei 1994). It is also clear that species responded differently to captive conditions, as previously shown in other bumblebee species (Bučánková *et al.* 2012, Ptáček *et al.* 2015, Moerman *et al.* 2016). Repeated egg/larvae abandonment remains a problem. Future trials should test methods to manage queen stress from the initial collection through to colony initiation. Trialling various nest box types and pollen diets will further clarify their nesting preferences (Lhomme *et al.* 2013, Moerman *et al.* 2016). Since queens may respond positively to stress-inducing stimuli due to a perceived competitive pressure, techniques to restrict stressful conditions to the first egg-laying phase might elucidate this. Future research should examine how pollen type and preparation (e.g. water content) affects larval development. Pollen prepared with a higher liquid content than we used here may be more suitable for manual larval feeding.

Although rearing long-tongued bees may require more sensitive, species-specific maintenance in captivity, our results show that it is possible. Continued efforts developing captive rearing techniques may also serve to utilise these species for commercial crop pollination, which is already being developed in the long-tongued *B. atratus* (Almanza *et al.* 2006, Fandiño 2007). Given the ecological implications and conservation status of many long-tongued species, further work to refine the protocols is clearly worthwhile.

Chapter 3: Effect of diet on incipient colony success for two long-tongued bumblebee species in the laboratory

Abstract

Bumblebees (*Bombus* spp.) are ecologically and economically important pollinating insects and nutritional stress is one of the most significant factors causing their decline. Adults and larvae rely solely on the nutrients derived from pollen and nectar, and previous research has highlighted the need to study the nutritional needs of a variety of species as these may vary interspecifically. Here we compare the reproductive success of queens in two species of pocket maker bumblebees (*B. pascuorum* and *B. hortorum*) when fed either a monofloral or polyfloral pollen diet. Our results show that while queens of both species could successfully rear works on either diet, they performed significantly better on the monofloral diet. Our findings support previous work that suggests that the right monofloral diet can be as good as, if not better than, a polyfloral mix. We also observed significant differences between species, demonstrating why we must not rely only on one or two model species to understand the effects of nutritional stress on bumblebee communities.

3.1 Introduction

The study of nutrition in insects has been well established for several decades (House 1961, Dadd 1973, Friend *et al.* 1982, Simpson *et al.* 1995). However, a great deal of this research has been based on a few dozen insects (Hanife 2006). Nutrition, described as the energy and nutrients required by organisms for their growth, maintenance, reproduction and energy, is well known to affect trade-offs on life history traits and can shape life-history evolution across species (Van Noordwijk *et al.* 1986, Hanife 2006, Flatt 2011). Although insects have broadly similar nutritional requirements, their diets can be surprisingly varied (Dadd 1985, Slansky *et al.* 1987). As a result, it can be difficult to predict how nutritional limitation will affect rare or declining species whose nutritional requirements have not been studied.

Bumblebees (*Bombus* spp.) are ecologically and economically important pollinating insects for many wild flowering plants and crops (Klein *et al.* 2007, Ollerton *et al.* 2011,

Willmer 2011, Garratt *et al.* 2014), but many species worldwide are experiencing significant declines (Williams *et al.* 2009, Nieto *et al.* 2014). Bumblebees rely solely on the nutrients derived from pollen and nectar, with pollen providing proteins, polypeptides, free amino acids, lipids and sterols that are required for development, physiology and reproduction (Genissel *et al.* 2002, Fliszkiewicz *et al.* 2007, Tasei *et al.* 2008, Cardoza *et al.* 2012, Vanderplanck *et al.* 2014). The types, quantities and concentrations of these pollen constituents varies widely amongst plant groups (Roulston *et al.* 2000, Cardoza *et al.* 2012). While most bumblebee species collect pollen from a variety of flower species to cover their nutritional needs (Kämper *et al.* 2016), many species exhibit morphological and behavioural biases towards particular plant groups (Goulson *et al.* 2005, Kleijn *et al.* 2008, Roger *et al.* 2017a). Increasing evidence suggests that adults, even without feedback from larvae, can identify high-quality pollen and forage selectively on nutritionally-rich floral resources to achieve a nutritional optimum (Dobson *et al.* 2000, Hanley *et al.* 2008, Ruedenauer *et al.* 2015).

A combination of selective foraging, interspecific pollen sourcing and pollen mixing (Somme *et al.* 2015), allows bees to reap benefits that a single pollen diet may not provide. For example, sunflower (*Helianthus annus*) pollen reduces worker longevity when consumed on its own, but when it is consumed in a polyfloral mix, it does not have this effect and in fact can be beneficial by reducing pathogen infection (Giacomini *et al.* 2018, LoCascio *et al.* 2019, McAulay *et al.* 2019). Testing single and polyfloral pollen mixes demonstrate that diet can have extreme effects on bumblebee reproductive success (Tasei *et al.* 2008, Vanderplanck *et al.* 2014, Baloglu *et al.* 2015, Moerman *et al.* 2015). While polyfloral mixes have been shown to improve reproductive performance (Tasei *et al.* 2008), this is ultimately down to the specific pollens used. Moerman *et al.* (2017) demonstrated that diet suitability has less to do with interspecific plant diversity and more to do with the nutritional composition of the pollen meeting the specific resource requirements of the bee. These requirements are likely to vary between species, reproductive state and energetic demands (Vaudo *et al.* 2015).

Investigations into the effect of diet on reproductive success of bumblebees are almost exclusively carried out on generalist species such as *B. terrestris* (*Bombus*) and *B. impatiens* (*Pyrobombus*), that are not generally suffering population declines or nutritional stress (Goulson *et al.* 2005), and which are confined to just two of the currently

38 recognised subgenera and both within the short-tongued clade (Cameron *et al.* 2007). However, Moerman *et al.* (2016) demonstrated that these species are not representative of all bumblebees in their nutritional requirements. In a comparative assay using three pollens, workers of *B. hypnorum* and *B. pratorum* not only performed worse than *B. terrestris*, regardless of pollen diet, but each species also responded differently to each pollen. Non-*B. terrestris/B. impatiens* queens or incipient colonies are seldom studied, and yet nutrition plays a particularly important role during colony establishment; queen bumblebees must collect adequate quantities of pollen to meet not only their own physiological needs, but that of her first brood as well. Proteins, sterols and lipids are required for ovary maturation (Vogt *et al.* 1998, Aupinel *et al.* 2000, Tanaka *et al.* 2019), and for brood development (Tasei *et al.* 2008), while increased food intake in the early stages of development increases ongoing colony growth (Herrmann *et al.* 2007, Westphal *et al.* 2009, Carvell *et al.* 2011). It is perhaps not surprising that wild queens at this stage have also been recorded choosing high-quality pollen sources over low (Moquet *et al.* 2015), and this behaviour might be more common amongst more specialist species.

These results illustrate that while the bumblebees typically used as models in scientific investigation provide important insight into their physiology and behaviour, the results are not representative of all species or all bees within those species. To understand how pollen diet affects bees both intra- and interspecifically, it is necessary to investigate different species and colonies at different developmental stages in experimental trials.

A large group often neglected in experimental work are long-tongued species known as pocket makers. Unlike short-tongued, pollen storer bumblebee species that feed their larvae a regurgitated liquid mix of nectar, pollen and glandular secretions, long-tongued bumblebees lay their eggs directly onto pollen stores, which the larvae consume in its solid form (Pereboom 2000, Den Boer *et al.* 2006). Long-tongued bumblebees are understudied because they have been very difficult to keep in captivity, but recent developments now make rearing long-tonged bumblebees in the lab possible Bučánková *et al.* 2012, Ptáček *et al.* 2015, Chapter 2). Long-tongued bumblebee species are often more selective in their diet than short-tongued species, and many rely heavily on pollen from the Fabaceae plant group (Goulson 2003). Since both adult and larval feeding is somewhat different in these species to *B. terrestris* and *B. impatiens* model species, it is

likely they will respond differently to pollen diets if they are less tolerant of toxic plant chemicals, or if their fundamental nutritional requirements differ.

Here we investigate for the first time the nutritional requirements of long-tongued bumblebee incipient colonies. We used *B. pascuorum* (*Thoracobombus*) and *B. hortorum* (*Megabombus*), we compare reproductive success and colony development of incipient colonies fed either a monofloral diet or a polyfloral pollen mix. Our aim was to test the incipient colony success of these species with two pollen diets that were as different as possible in their species diversity. We hypothesised that queens of both species would perform better on the polyfloral mix, in which they can utilise the nutrients from a range of plant species. The pocket maker species used in this study are amongst the most common and generalist of the long-tongued bumblebees in the UK, representing two understudied subgenera and being ecologically similar to many species of conservation concern, thus making them excellent models for investigating the biology of long-tongued bumblebees.

3.2 Materials and methods

Sixty-two *B. pascuorum* queens and 20 *B. hortorum* queens were collected between March and April 2018 from woodland and chalk downland in East Sussex (UK). Pollen collection occurs after queens have established a nest (Evans *et al.* 2007), and so only queens without pollen were collected. Queens were placed into individual, ventilated 15 x 15 x 15cm plastic boxes in a dark room (30^{o}C, 20% rh). Each queen was housed separately and provided with 50% (v/v) sugar water (10% fructose, 90% sucrose) *ad libitum*. Queens were assigned randomly to one of two pollen diets: a monofloral hawthorn (*Crataegus monogyna*) pollen, or a polyfloral mix consisting of 75% pre-mixed wildflower, 15% heather (*Erica sp.*) and 10% hawthorn pollens. All pollen was obtained from *Pollenergie* (France). Commercially available pollen such as these is collected by honey bees and so is unlikely to contain pollen from plants favoured by long-tongued bumblebees. We therefore hypothesised that queens would perform poorly on monofloral pollen, but that the polyfloral mix might contain sufficient diversity to meet the nutritional needs of the bees. Pollen was ground to a powder and combined with 50% (v/v) sugar water (10% fructose, 90% sucrose) to form a sticky dough. Every two days they were

given a 1/g pollen ball, which increased to 2/g as offspring were produced. To induce nesting behaviours, all queens were given a 3 x 3cm piece of cotton wool, which the queens readily used as nesting material. To encourage egg-laying and brood rearing, queens were given a *B. terrestris* cocoon (> 48 hours old), from day two. The callow *B. terrestris* worker was removed as soon as it eclosed to avoid the negative effects on the queen, that can otherwise occur (Bučánková *et al.* 2012, Ptáček *et al.* 2015, Chapter 2). Cocoons were provided repeatedly until the queen had her own pupae.

It is generally accepted that stress caused by disturbance elicits defensive behaviours in bumblebees that are likely to inhibit oviposition and brood care (Kirchner *et al.* 1999, Bučánková *et al.* 2012). Disturbance of the nest boxes was therefore kept to a minimum to reduce this. To measure colony development, five variables that could be taken without interfering with the contents of the nest box, were recorded every two days: the proportion of queens that produced (i) eggs, (ii) third stage (L3) larvae, and (iii) workers; and (iv) the number of weeks to the first L3 larvae, and (v) the number of workers produced by the end of the experiment (14 weeks).

The effects of bee species and diet on the colony variables were investigated using generalised linear models. Model distributions and link functions were chosen based on data distributions and AIC values. A binomial distribution and logit link function was used for the proportion of queens that produced eggs, L3 larvae and workers. The effects on the number of weeks to first L3 larvae and the number of workers produced at the end of 14 weeks were analysed using a Poisson distribution and log link function.

3.3 Results

Queens in both treatment groups fed on the pollen provided. All queens that survived past Week 1 (n = 81), showed nesting behaviour by carding the cotton wool, so that it either covered their nest box or formed part of their brood structures. The single queen that showed no nesting behaviour and died on Day 2 is excluded from further analysis. Over the 14 weeks of the study, 40 of the *B. pascuorum* queens (66%) and 12 of the *B. hortorum* queens (60%) laid eggs (Table 3.1). All but one of the egg-laying queens also successfully reared their brood to the L3 stage, which was reached between Weeks 2 and 12, and 21

of these (33% *B. pascuorum* and 67% *B. hortorum*) produced up to 5 workers ($\bar{x}$ ± s.e. = 2.1 ± 0.3; Fig. 3.1). Offspring mortality ranged from 2% between egg and L3 stage, and 41% between L3 and worker. No dead pupae were observed. Following an infestation of wax moth, no further progress was expected, and the experiment was terminated at 14 weeks. No reproductives were produced during this period.

3.3.1 The effect of diet and species on early-stage colony development

There was a significant interaction between the effects of diet and bee species on the proportion of queens that produced eggs and L3 larvae, and the number of weeks before the first L3 larvae were produced (respectively, χ^2 = 4.02, df = 1, p = 0.045; χ^2 = 4.58, df = 1, p = 0.032; χ^2 = 4.44, df = 1 p = 0.035). Seventy-two percent of queens on the monofloral pollen laid eggs (14.5 ± 6.5 eggs per queen), compared to 56% on the polyfloral diet (11.5 ± 7.5 eggs per queen; Fig. 3.1), and only one queen which was fed the monofloral diet did not also produce L3 larvae (14 ± 6.5 L3 larvae per queen). On average, 66% of *B. pascuorum* queens and 60% of *B. hortorum* queens laid eggs and for both species the first L3 larvae generally appeared in Week 6 (respectively after, 6.05 ± 0.41 and 6.41 ± 0.96 weeks). Overall, queens reared on the polyfloral mix produced L3 larvae a little earlier than those on the monofloral diet (respectively after 5.9 ± 0.53 and 6.4 ± 0.54 weeks), however this effect varied between species (Table 3.1).

3.3.2 The effect of diet and species on late-stage colony development

Based on the queens which produced L3 larvae (n = 51), there was a significant interaction between diet and species on the proportion of queens that produced workers (χ^2 = 3.87, df = 1, p = 0.049). Thirty-three percent of *B. pascuorum* queens produced workers (6 ± 6.5 workers per queen), compared to 67% of *B. hortorum* queens (all of which produced 4 workers each). There were no significant effects of species or diet on the number of workers produced (respectively, χ^2 = 0.15, df = 1, p = 0.70, χ^2 = 0.012, df = 1, p = 0.91).

Table 3.1 Incipient colony success of wild-caught *B. hortorum* (n = 20) and *B. pascuorum* (n = 62) bumblebee queens in the laboratory over a 14-week period. Bees were fed either a monofloral hawthorn diet or polyfloral mixed wildflower diet. Colony success was measured as whether queens laid eggs, reared third-stage (L3) larvae, produced workers, and the number of workers produced. Sample sizes for queens producing L3 larvae and workers are the total queens achieving the previous measure of success. No gynes or males were produced within the 14 week period.

Measure of queen success	**Diet**	***B. pascuorum***	***B. hortorum***
Proportion of queens that produced eggs	Hawthorn	68% (21/31)	89% (8/9)
	Wildflower	63% (19/30)	36% (4/11)
Proportion of queens that reared L3 larvae	Hawthorn	95% (20/21)	100% (8/8)
	Wildflower	100% (19/19)	100% (4/4)
Mean (± s.e.) no. of weeks until L3 larvae were produced	Hawthorn	5.9 ± 0.62	7.5 ± 1.05
	Wildflower	6.2 ± 0.54	4.25 ± 1.60
Proportion of L3-producing queens that produced workers	Hawthorn	35% (7/20)	50% (4/8)
	Wildflower	32% (6/19)	100% (4/4)
Mean (± s.e.) no. workers produced	Hawthorn	2.14 ± 0.60	2 ± 0.41
	Wildflower	1.83 ± 0.65	2.5 ± 0.96

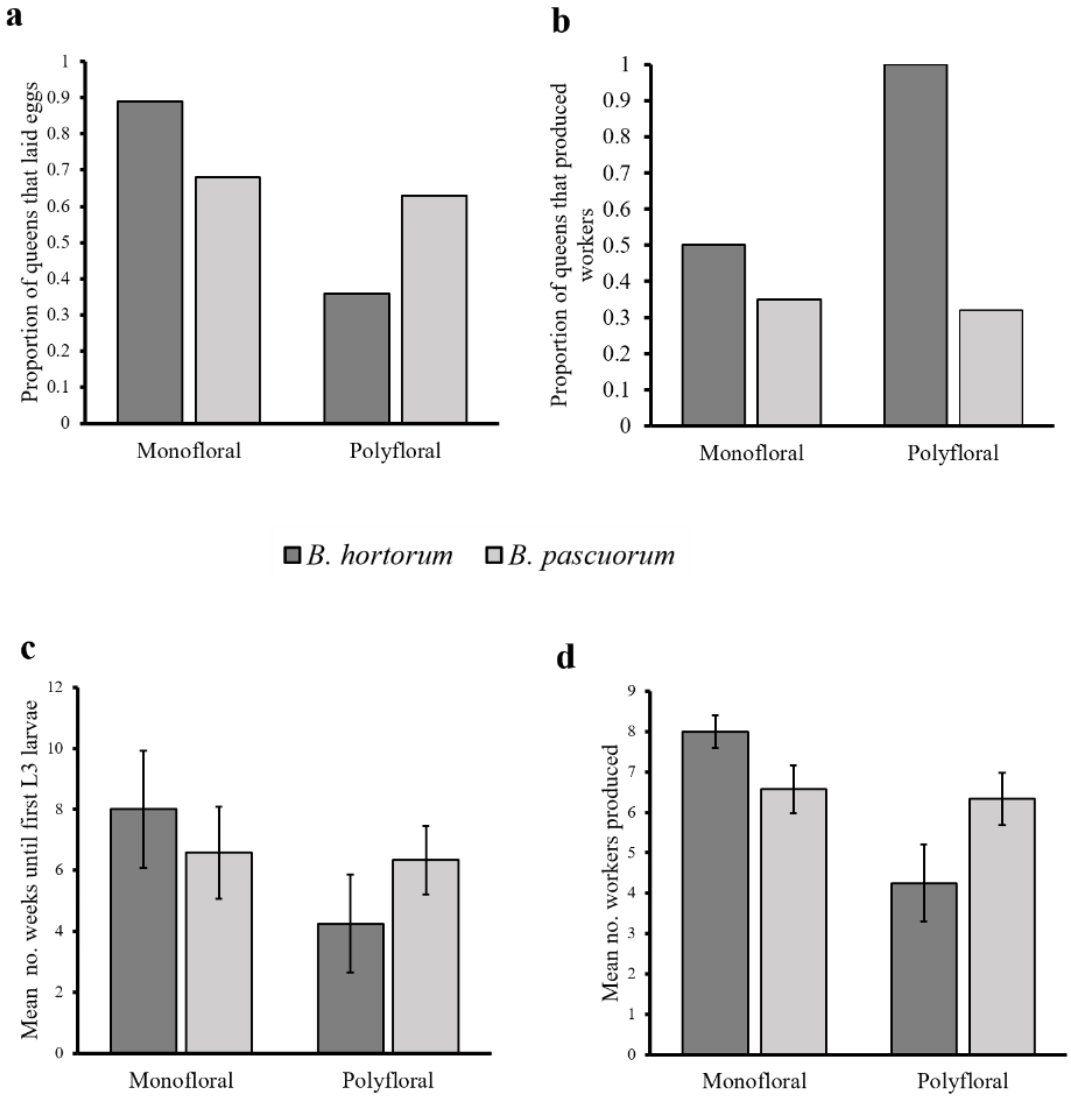


Figure 3.1 Effect of diet on incipient colony success of wild-caught *B. hortorum* (n = 20; dark grey) and *B. pascuorum* (n = 61; light grey) bumblebee queens in the laboratory measured by (a) proportion of queens that laid eggs (n = 52), (b) proportion of queens that reared third-stage larvae (L3) to workers (n = 21), (c) mean (± se) number of weeks before the first L3 larvae were produced, and (d) mean (± se) number of workers produced. Queens were randomly assigned a diet treatment of either monofloral hawthorn pollen or a polyfloral wildflower pollen mix. Only one egg-laying queen (a *B. pascuorum* on the hawthorn diet) did not produce L3 larvae (see Supplementary Fig. 3.1).

3.4 Discussion

We found that queens of *B. pascuorum* and *B. hortorum* can survive and successfully rear workers on both monofloral hawthorn pollen and the polyfloral mix provided. A wax moth infestation developed towards the end of the experiment but despite this, and the additional stress it may have imposed on queens, the proportion of queens that laid eggs was amongst the highest observed in these species when reared in captivity (Bučánková *et al.* 2012, Ptáček *et al.* 2015, Chapter 2). Very few workers are required for queens to switch to male production (Chapter 2), so it is quite likely that in the absence of wax moths this would have occurred.

Perhaps surprisingly, queens fed the monofloral pollen (hawthorn) were significantly more likely to lay eggs than those on the polyfloral mix. Hawthorn is not a natural foraging choice for queens of these species; its open-bowl flowers appear in May and are usually pollinated by flies and small bees (Corbet 2006, García *et al.* 2007), whereas *B. pascuorum* and *B. hortorum* start emerging in March (Falk 2015), and favour plants with flowers a little deeper than their tongue length (~10-13 mm) (Brian 1957, Barrow *et al.* 1984, Prŷs-Jones *et al.* 1991). Despite this and across all treatments, offspring mortality between egg and L3 stage was very low, demonstrating both monofloral hawthorn and the polyfloral pollen mix were adequate for early-stage larval development. Interestingly, a much higher mortality was observed between the L3 and worker stage, which could be pinpointed to the L3 and L4 stages because we observed no dead pupae. This suggests that in the critical period just before pupation (Plowright *et al.* 1977), larvae may be more sensitive to quantitative and/or qualitative deficiencies in pollen or other stresses. Some may have died of starvation if the queen failed to replenish the pollen pockets and the effect of this on the larvae may only be visible when they have the highest resource demands (Plowright *et al.* 1977). We did not find any evidence that the pollen delayed larval growth, as has previously been reported (Genissel *et al.* 2002). However, we intentionally avoided any methods of data collection that might have increased queen stress and interfered with colony production. More intricate measures of long-tongued bumblebee species will be possible with practice and when increasingly intensive experiments can be carried out. The failure of queens to feed their larvae is a significant hinderance to pocket maker rearing and future work should attempt to elucidate precisely

when queens are most sensitive to external conditions, and how this could be mitigated to encourage egg-laying and ongoing brood care.

Queens of *B. pascuorum* and *B. hortorum* varied in their response to the pollen diets during the experiment. Egg-laying success was more affected by diet for *B. hortorum* queens, with twice as many laying eggs when they were fed on the monofloral diet compared to the polyfloral diet, whereas *B. pascuorum* queens varied very little across treatments in egg-laying success. There was no difference between the species in success from egg to the L3 stage, with almost all queens that obtaining L3 larvae, but there was a significant difference between species in success from L3 to the adult worker stage, with a significantly greater proportion of the *B. hortorum* queens successfully rearing workers. No dead pupae were observed so this suggests a species difference arose at the L3/L4 stage which may be a critical period of sensitivity to nutritional stress (Sutcliffe *et al.* 1990). Qualitative differences between species could explain the variation in worker production if the pollens were nutritionally more suitable for the *B. hortorum* larvae, or if they contained compounds harmful to *B. pascuorum*. Regardless of the mechanism, the results suggest fundamental differences in the nutritional requirements of these bees. This highlights how essential it is to consider the needs of individual species to fully understand how wild bee communities are affected by nutritional stress.

In previous experimental trials using donor cocoons for long-tongued bee rearing, avoidance and aggression has often been observed between queens and the emerging workers, sometimes leading to a cessation of queen reproduction (Bučánková *et al.* 2012, Ptáček *et al.* 2015, Chapter 2). We removed donor callows as soon as they emerged and relied only on the cocoons themselves to encourage egg-laying and brood care. Queens should identify these as foreign (Heinrich 1974, Gamboa *et al.* 1987), and perceive a rival queen is present (Lopez-Vaamonde *et al.* 2004, Goulson *et al.* 2018b). This should encourage her to out-compete it producing her own colony. We found that repeated cocoon-exposure was indeed an effective method to trigger oviposition, which is particularly useful for bumblebee species unsuited to interspecific cohabitation.

Nutrition clearly plays an important role in queen reproductive success during colony establishment and our results support previous work suggesting that the right monofloral diet can be as good as, if not better than, a polyfloral mix (Moerman *et al.* 2017). Our

results also suggest that different bumblebee species have different nutritional requirements, demonstrating the value of studying a range of bumblebee species to fully understand the effects of biological stressors such as nutritional stress. Despite a serious wax moth infestation towards the end of the experiment, our queens achieved amongst the highest success rate of oviposition recorded in long-tongued bumblebees reared in the laboratory. Challenges remain, including the need for more detailed measurements of the effects of diet in pocket maker species that doesn't disrupt colony progress. Future work should focus on comparing long-tongued species with reliable models such as *B. terrestris*, which could not be done in our experiment due to space restrictions. The results here and those drawn from Chapter 2 show that species show differential responses to captivity and it is important that future studies attempt to test the effects of capture, transit, diet and the conditions of captive nesting separately on different species. Honey bee colonies that are transported over long distances for pollination exhibit higher stress levels than stationary ones (Simone-Finstrom *et al.* 2016) and it is possible that chronic effects of capture, transport and handling may also affect bumblebee queens, but this effect may vary between species. The results here show that survival of queens following capture can be relatively high and so it is important that future studies maximise sample size through intensive queen collection. Although biochemical analysis of the pollen diets was not conducted, characterisation of the protein, lipid and amino acid content may elucidate the differential responses we observed. There is a great deal to be gained from studying the effects of nutritional stress on a variety of bumblebee species. Achieving this is key to developing evidence-based conservation management to protect diverse bumblebee communities.

Chapter 4: Habitat type affects the prevalence of bumblebee pathogens in gardens and farmland

Please refer to Appendix 5 for additional notes on this chapter.

Abstract

Bumblebees are ecologically and economically important pollinators that are suffering widespread declines due to multiple interacting factors, including pathogen pressure and lack of floral resources. Gardens are floristically rich habitats and efforts have been made to increase bumblebee-friendly floral resources in gardens to aid pollinator conservation, but the effect of this on host-pathogen dynamics is unknown. The provision of nutrient-rich flowers may support a healthy immune system in bumblebees but may also increase the horizontal transmission of pathogens via shared flower use. Here we compare the prevalence of bumblebee pathogens between gardens and farmland by surveying 27 sites for the pathogens *Crithidia bombi*, *Nosema bombi* and *N. ceranae.* We found that garden sites were significantly more likely to have pathogens than farmland sites, but that within those sites with pathogens, the proportion of bees carrying pathogens did not differ between gardens and farmlands and there was notable discrepancy between *C. bombi* prevalence across and within sites. Further work is needed to explore how floral resource availability affects pathogen transmission and prevalence.

4.1 Introduction

Animal-mediated pollination is required by more than 87% of angiosperms (Ollerton *et al.* 2011), and the conservation of pollinators is essential to ecosystem functioning and crop production (Ollerton *et al.* 2011). Bumblebees (*Bombus* spp.) are highly efficient and abundant pollinators in temperate ecosystems (Willmer 2011, Garratt *et al.* 2014), but many species are experiencing declines worldwide (Biesmeijer *et al.* 2006, Williams *et al.* 2009, Cameron *et al.* 2011). This has been linked to a variety of interacting stresses, including pathogen pressure and the loss of the flower-rich habitats that bumblebees historically thrived in (Potts *et al.* 2010, Goulson *et al.* 2015).

Like most bees, bumblebees cover their energetic costs and rear their offspring exclusively on the resources derived from pollen and nectar (Willmer 2011). The

availability of diverse, abundant and nutritionally rich flowering plant communities is therefore essential for their survival (Vaudo *et al.* 2015). The chemical composition of floral resources and the proteins, lipids and carbohydrates they provide varies widely within and between plant species (Somerville *et al.* 2007, Willmer 2011). Either through direct assessment or indirect inference, bumblebees have the ability to assess pollen quality (Dobson *et al.* 2000, Ruedenauer *et al.* 2015), and many insects regulate their nutritional intake around temporary and contextual optimums which reflect their developmental, immunological and reproductive status (Moret *et al.* 2000, Genissel *et al.* 2002, Behmer 2009, Moquet *et al.* 2015, Kämper *et al.* 2016). Host nutrition modulates resistance and tolerance to pathogen infections through various direct and indirect signalling pathways affected by the ingestion of specific nutrients (Ayres *et al.* 2012, Ponton *et al.* 2013). Receptor molecules, sensitive to both qualitative and quantitative changes in nutrients, allow bees to respond to nutritional deficiencies (Simpson *et al.* 2009, Kapahi *et al.* 2010). Malnourishment reduces immune responsiveness (DeBlock *et al.* 2008, Ayres *et al.* 2009, Medzhitov *et al.* 2012), while a diverse diet improves it (Alaux *et al.* 2010, Roger *et al.* 2017b). To support immunologically-healthy bumblebee populations, a diverse and high-quality pollen supply must be available so bees can achieve their nutritional optimum (Kämper *et al.* 2016, Ruedenauer *et al.* 2016, Vaudo *et al.* 2016), so if they become immunologically challenged, they can compensate for the energetically costly activation of their immune system by increasing their dietary intake (Moret *et al.* 2000).

Most conservation efforts for bumblebees focus on increasing floral resource availability. Amongst these are schemes to increase floral resources in urban and rural gardens and there is a great deal of evidence underpinning the value of gardens for maintaining and enhancing biodiversity (Gaston *et al.* 2005, Smith *et al.* 2006a, Majewska *et al.* 2018). Gardens are unusual habitats, containing vast numbers of flowering and non-flowering plant species, ranging from long-native to truly exotic species as well as hybrids and cultivars, frequently represented by very few individual plants in any one garden (Loram *et al.* 2008). A growing understanding of pollinator importance and decline has renewed interest in increasing the availability of floral resources in the urban environment (Hall *et al.* 2017, Majewska *et al.* 2018, Baldock *et al.* 2019, Turo *et al.* 2019). However, one major consequence of landscape management such as this, where food availability is increased, is the associated pressure exerted on bumblebees by their parasites. All known

microbial pathogens of bumblebees can be transmitted via the faecal-oral route and there is experimental evidence that these pathogens, including *Crithidia bombi*, *Nosema bombi* and *N. ceranae*, can be vectored via shared flowers (Durrer *et al.* 1994, Graystock *et al.* 2015, Piot *et al.* 2019). Infected bees spread disease to their nestmates and then to colonies nearby (Otterstatter *et al.* 2007). Infected or contaminated individuals may deposit spores or cells on flowers through defecation or handling, and if the pathogen survives long enough it could be picked up by other bees. Although we are only just beginning to understand the precise mechanisms mediating this process (Adler *et al.* 2018, Figueroa *et al.* 2019), the potential for between-colony transmission relies upon the availability of flowers that bumblebees forage on. The more flowers an uninfected bee visits, the greater its risk of coming into contact with pathogens and the greater the chance new infections will occur.

In Europe, the most prevalent pathogen of bumblebees is the trypanosome *Crithidia bombi*, which infects adult bees (Folly *et al.* 2017). When the colony reaches peak activity (usually June-July) the prevalence of *C. bombi* within a colony can be anywhere between 50-100% (Shykoff *et al.* 1991, Durrer *et al.* 1995, Whitehorn *et al.* 2010). Characteristically of a trypanosome, its effects are variable and context-dependent (Schaub 1994), causing increased mortality when the host is food-stressed and often having virtually no effect at low intensities (Brown *et al.* 2000, Brown *et al.* 2003b). *N. bombi* is a microsporidian with a prevalence that is frequently found to be very low (<5%) (Shykoff *et al.* 1991, Durrer *et al.* 1995, Whitehorn *et al.* 2010, Graystock *et al.* 2014, Jones *et al.* 2014), but has also been detected as high as 56% (Goulson *et al.* 2012). Although it too can be transmitted from adult-to-adult, it is better adapted at infecting larvae and young bees (Schmid-Hempel *et al.* 1998, Rutrecht *et al.* 2007). The virulence of *N. bombi* is highly variable, ranging from negligible to severe (Macfarlane *et al.* 1995, McIvor *et al.* 1995, Schmid-Hempel *et al.* 1998, Otti *et al.* 2007). More recent work has shown that bumblebees can also become infected with *N. ceranae*, an emerging microsporidian pathogen previously only associated with honey bees (Graystock *et al.* 2013b, Furst *et al.* 2014). It's prevalence in UK wild bumblebees seems to generally be low (7%) (Furst *et al.* 2014), but can be as high as 44% near honey bee colonies and in urban areas (Goulson *et al.* 2012, Graystock *et al.* 2014). Its epidemiology and risk to wild bumblebees has yet to be shown (Brown 2017).

Here we investigate the prevalence of bumblebee pathogens at gardens and farmland, comparing first whether pathogens were present or not at a site, and second the proportions of bees at sites with pathogens. Our aim was to determine if there was an association between habitat type and pathogen prevalence. Gardens tend to contain more flowers than farmland as a result of their management (Chapter 5). Therefore, we hypothesise that as a result of increased food availability, pathogen prevalence should be higher in gardens where host density – and opportunities for both vertical and horizontal transmission – are greatest.

4.2 Materials and methods

4.2.1 Site selection and bee collection

We sampled 15 non-domestic gardens and 12 farmlands in south-east England (Sussex and Kent), in June-July 2018 (Supplementary Table 4.1; Supplementary Figure 4.1). Non-domestic gardens included private or trust-owned gardens open to the public, such as botanical gardens, and formal and non-formal gardens, where visitors are charged for entry and so there is often a dedicated gardening team. Similar to domestic gardens, they contain a diverse plant community with abundant floral resources for bees (Smith *et al.* 2006b), but are considerably larger, allowing the their floral resources to be more easily compared to other large-scale habitats. Garden sites were typically on the edge of towns or villages. Farmland sites included roadside hedges and public footpaths through farmlands. *B. terrestris* workers are known to forage up to 1.75 km from their nests (Walther-Hellwig *et al.* 2000), so each site was at least 3 km apart to minimise repeated measurement from the same colonies. Floral surveys carried out on four of the garden sites and four of the farmland sites the previous year showed the gardens typically had 3.5 times as many plant species in flower and 2.5 times as many flowers (Chapter 5). After locating patches of flowers, we collected *B. terrestris/ B. lucorum* workers for 30 min, or until 10 workers had been collected. Workers were freeze-killed in the laboratory, the abdomen removed and stored at -20^{o}C for pathogen screening by PCR.

4.2.2 DNA extraction and PCR screening

For pathogen screening, a sample of the Malpighian tubules, fat body, midgut and hindgut were taken from each bee. They were transferred together to 96-well plates containing a digestive solution of STE buffer (100 mM NaCl, 10 mM Tris pH8, 25 mM EDTA and 0.5% SDS), proteinase K (0.1 µg/µl) and 50% Chelex. Negative controls were included on each plate. After 20 min in solution, the samples were homogenised with autoclaved toothpicks and incubated for 6 h at 55ºC and 15 min at 95ºC. Two elution steps were carried out, the first with 1:1 isopropanol and the second with 70% EthOH. After each elution step the samples were centrifuged for 1 h and the supernatant discarded. The resulting DNA pellets were resuspended in molecular grade water and stored at -4ºC until further use. PCR reagents and cycling conditions are shown in Supplementary Table 4.1. Positive controls were included in each PCR and a subset of positive samples were sequenced to confirm the identity of the pathogen.

4.2.3 Statistical analyses

The effect of site type and pathogen species on the prevalence of pathogens were investigated using generalised linear mixed models with a gamma distribution and log link function. We removed nonsignificant interaction terms to obtain the minimum adequate models. Mann-Whitney U tests was used to determine if the number of bees collected in gardens and farmlands in the 30 min sampling period was different and if, within sites where pathogens were recorded, there was a significant difference in the proportion of bees carrying pathogens between gardens and farmlands.

4.3 Results

A total of 167 bumblebees were screened from across the 15 garden and 12 farmland sites. Overall, we detected pathogens at 74% of sites. Most bees (73%) did not carry any of the pathogens we screened for, most of the remainder (24%) carried one pathogen and only 5 bees (3%) carried two. No bees carried all three. *C. bombi* was the most common pathogen, detected at 59% of sites and in 19% of bees. *N. bombi* was detected at 11% of sites and 2% of bees, and *N. ceranae* was detected at 22% of sites and 6% of bees. *C. bombi* and *N. ceranae* were recorded in both gardens and farmlands, while *N. bombi* was only found in gardens.

There was a significant effect of site type and pathogen species on the proportion of sites at which pathogens were detected, (respectively, df = 72, $p = 0.014$; df = 72, $p < 0.001$), and a significant difference in the number of bees that could be collected from gardens and farmlands in the 30-minute sampling period (U = 27.5, z = -3.15 $p = 0.001$). Pathogens occurred at 87% of gardens (n = 13) and 42% of farmlands (n = 5; Fig. 4.1a). At sites where pathogens were found, 61% had carried one pathogen, 33% carried two and 6% carried all three (Fig. 4.1b). In the 30 min sampling period, more bumblebees were found in gardens compared to farmland (Fig. 4.2). In sites where pathogens were detected, there was no significant effect of the interaction between site type and pathogen species and no significant difference in the proportion of bees carrying pathogens (respectively, df = 72, $p = 0.34$; U = 40, z = -1.10, $p = 0.303$).

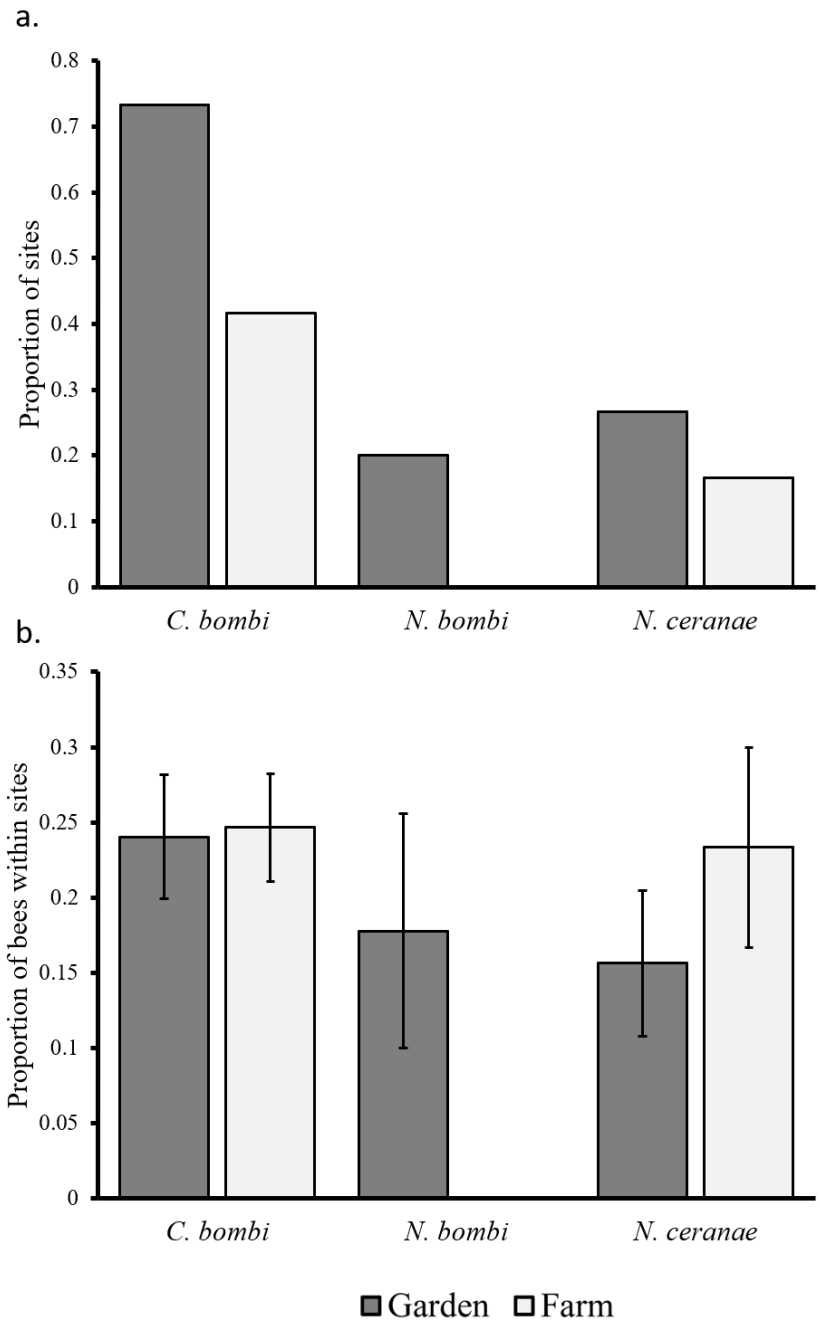


Figure 4.1 The prevalence of bumblebee pathogens in gardens (grey) and farmland (white). a) The proportion of sites where the bumblebee pathogens *C. bombi*, *N. bombi* and *N. ceranae* were recorded. b) Mean ± s.e. proportion of bumblebees within sites that tested positive for pathogens, for sites in which each pathogen was detected. Bumblebees were collected June-July 2018 from non-domestic gardens (n = 15) and farmlands (n = 12) across Sussex and Kent (UK), and screened for pathogens by diagnostic PCR (see Supplementary Table 4.1). *C. bombi* was recorded in 17 sites (12 gardens and 5 farmlands), *N. bombi* in 3 farmlands and *N. ceranae* in 6 sites (4 gardens and 2 farmlands).

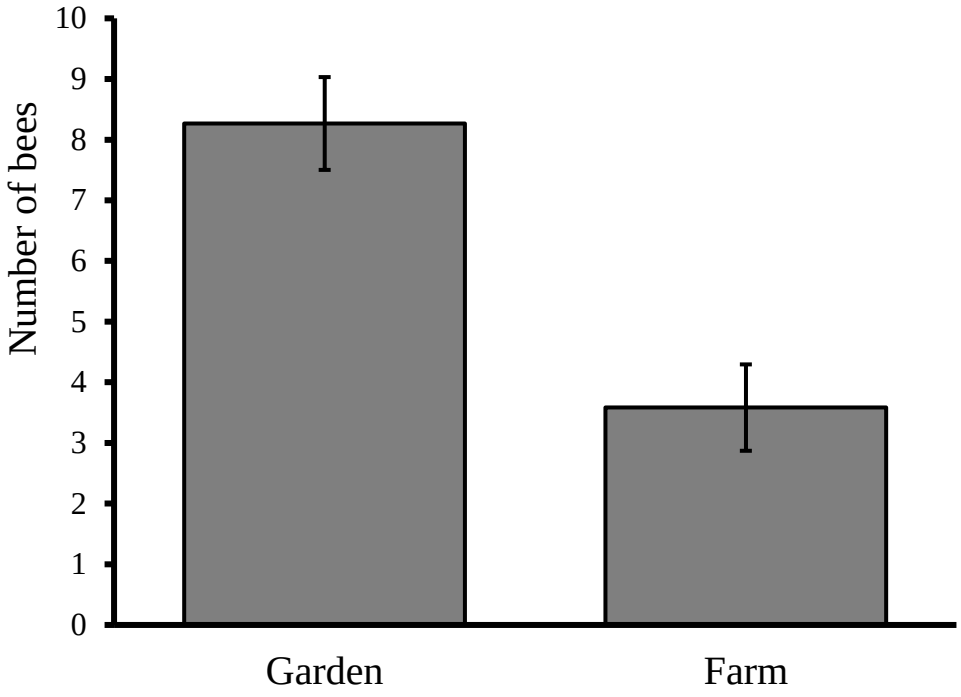


Figure 4.2 Mean ± s.e. number of *B. terrestris/ lucorum* workers collected in June and July 2018 from non-domestic gardens (n = 15) and farmlands (n = 12) in Sussex and Kent (UK). Bumblebees were hand netted on or around flowers and sampling was carried out for 30-minutes, or until 10 workers had been collected.

4.4 Discussion

We found evidence to suggest the prevalence of bumblebee pathogens in the environment is affected by habitat type, which is consistent with previous work (Goulson *et al.* 2012, Theodorou *et al.* 2016). Garden sites were more than twice as likely as farmland sites to have pathogens present and the prevalence of each pathogen varied substantially between and within sites. These data show the prevalence of pathogens that bumblebees encounter in the landscape, a proportion of which will be true infections (Brown 2017).

While the geographical distribution and density of pathogens is irrevocably tied to the geographical distribution and density of the host (Anderson *et al.* 1981), an abundance of flowers facilitates their spread indirectly by supporting bumblebee population growth, and directly by acting as vectors (Durrer *et al.* 1994, Graystock *et al.* 2015, Figueroa *et al.* 2019). In horizontally-transmitted pathogens, transmission dynamics are highly variable and involve many interlinking factors associated with both host and vector species, which can result in fluctuating infection and survival rates (Fontenille *et al.* 2004). That bumblebee pathogens are both vectored by multiple plant species and hosted by multiple bee species may increase this heterogeneity further (Power *et al.* 2008). Although here we did not quantify floral resources, we show in Chapter 5 that in June and July, non-domestic gardens can contain more than four and a half times as many plant species in flower and more than two and a half times as many flowers per unit area, compared to farmlands. In the present study, we found bumblebees were significantly easier to find in gardens, which may have been caused in part by dense aggregations of flowers, but also suggests that gardens support larger bumblebee populations. Interestingly, although pathogens were more likely to occur in gardens, we found no evidence that pathogen transmission was higher in either habitat, since the proportion of bees carrying pathogens at gardens and farmlands was virtually the same.

We observed unexpectedly low prevalence of pathogens within sites based on the habitat and time of year. An unusually low percentage (27%) of bumblebees were positive for even one of the three pathogens tested for. This was most striking for *C. bombi*, which has previously been recorded infecting up to 100% of bees at sites at this time of year (Shykoff *et al.* 1991, Durrer *et al.* 1995, Whitehorn *et al.* 2010). We also found a notable disparity in *C. bombi* prevalence between and within sites. This was particularly evident

in gardens, where the pathogen occurred in more than half of sites, but was carried by less than a quarter of bees within those sites. Since the *Nosema* species have consistently been shown to occur at much low rates than *Crithidia* (Whitehorn *et al.* 2010), it is perhaps surprising they were observed at all. For both *Nosema* species, the proportion of sites where the pathogen occurred was not dissimilar to the proportion of bees carrying the pathogen within those sites (16-25%), and consistent with some previous research (Durrer *et al.* 1995, Furst *et al.* 2014, Jones *et al.* 2014). The year-to-year changes in pathogen epidemiology, and how this affected by ecological and biological factors is still largely unknown (Brown 2017). *C. bombi's* infrequency observed here in bumblebees may suggest an effect of local environmental conditions that do not appear to have affected the *Nosema* species. June 2018 was the third warmest and driest June in a series since 1910 and rainfall was 48% of average (METOffice 2018). Bees were harder to find than we have found at these sites previously (Chapter 5). The interactive effects of nutritional stress and pathogen infection may have increased mortality in *C. bombi*-infected adults (Brown *et al.* 2000, Logan *et al.* 2005, Gegear *et al.* 2006), or reduced the survival of pathogens on flowers. *C. bombi* is a trypanosome, cannot survive long periods outside its hosts and desiccates under UV light (Schmid-Hempel *et al.* 1999, Figueroa *et al.* 2019). This is suggestive of horizontal transmission occurring more between nestmates and less through shared flowers. The *Nosema* species are pathogens of larvae (Schmid-Hempel *et al.* 1998, Rutrecht *et al.* 2007, Eiri *et al.* 2015), and infected individuals may have been buffered from the effects of starvation by the foraging efforts of workers. Bumblebee pathogens derive their nutrients directly from the bumblebee gut and so food-stress in bees may have also reduced transmission rates (Logan *et al.* 2005, Smith 2007). To elucidate the observed patterns of prevalence, more long-term and regular screenings of multiple sites are needed to capture seasonal and long-term changes. As climate change continues to cause extremes in temperature and rainfall, survival assays that test how this affects the transmission of pathogens on flowers should be explored.

How infectious diseases are transmitted through the landscape, and how this varies across habitat types, is poorly understood. We have shown here that gardens are more likely to have bumblebee pathogens than farmland. It should be noted that due to a limited sample size, these results should be treated with caution, particularly amongst farmland where so few bees could be found. Further surveys and screening are required to strengthen these

conclusions. We did not collect data on surrounding landscape features, which may have further elucidated patterns of pathogen prevalence. While pathogens only require their host and a vector to become introduced to a site, actual infections are reliant upon complex, interconnected factors associated with pathogen virulence and infectivity, the abundance and chemistry of flowers, and the health of bumblebees. Climate is likely to have played a role in the small number of pathogens we observed. However, the pathogens were differentially affected by this. Further work is needed to determine if the floral resources in different habitats affect transmission dynamics of bumblebee pathogens, and to quantitatively and qualitatively compare the immunological response of bumblebees to pathogens across habitats.

Chapter 5: Floral resource availability, bumblebee health and pathogen prevalence across habitats

Abstract

Bumblebees (*Bombus* spp.) are important pollinating insects and loss of floral resources has been a major driver in their decline. The nutrients contained in pollen and nectar are essential for bumblebee survival and support them immunologically against pathogens. However, we lack definitive data on how floral resource availability and composition affects bee health and pathogen prevalence across habitats. Here, we compare floral resource availability, bumblebee health and pathogen prevalence across the peak foraging period for three important UK habitats; farmland, gardens and nature reserves. We found that gardens had the greatest species richness of flowers, but were virtually equal to nature reserves in floral unit abundance. Farmland consistently had the poorest floral resource availability. The pathogen *Crithidia bombi* was found in almost half of bumblebees screened and was most common at garden sites, while both the *Nosema bombi* and *N. ceranae* pathogens were much rarer, being found in only 2% of bumblebees. Overall, while bumblebee health varied between species and month over the course of the summer, they were in general larger and healthier in gardens and nature reserves than farmland. Our results show that irrespective of floral resource availability, bumblebee health and pathogen prevalence is affected by additional characteristics of the landscape.

5.1 Introduction

Semi-natural habitats once provided abundant and diverse floral resources for important pollinators such as bumblebees (*Bombus* spp.) (Ollerton *et al.* 2011, Garratt *et al.* 2014), but many have become degraded and fragmented as a result of land-use intensification (Winfree *et al.* 2009). The floral resources available in these habitats play an important role in regulating bumblebee health and population dynamics (Knight *et al.* 2009, Carvell *et al.* 2017). Both adult and larva bumblebees feed solely on the pollen and nectar acquired from flowers (Michener 2007, Danforth *et al.* 2013). While most floral resources provide proteins, lipids, carbohydrates and other important macronutrients, the specific biochemistry of pollen and nectar varies greatly within and between plant groups

(Roulston *et al.* 2000, Somerville *et al.* 2007). This requires bees to forage flexibly to achieve their nutritional optimum (Ruedenauer *et al.* 2016), which varies between species and castes (Moerman *et al.* 2016, Stone 2018). The taxonomic diversity of nutritionally-rich floral resources, rather than simply flower abundance, is therefore integral to conserving a wide range of pollinator species (Vaudo *et al.* 2015, Cane 2016).

Nutrition, the acquisition and assimilation of energy and nutrients (Watts *et al.* 2012), has significant effects on biological processes in bumblebees (Heinrich 1975, Manson *et al.* 2010, McCallum *et al.* 2013, Conroy *et al.* 2016). Anthropogenic change is likely to affect the synergistic interaction between nutritional state and susceptibility to pathogens (Woodard *et al.* 2017). Flowers are known to act as transmission hubs for a range of bee pathogens and commensals (McArt *et al.* 2014) and all known bumblebee pathogens can be vectored on shared flowers (Durrer *et al.* 1994, Graystock *et al.* 2015, Adler *et al.* 2018, Figueroa *et al.* 2019)Furthermore, the nutritional status of bumblebees affects their interactions with pathogens. Nutritional stress reduces their tolerance (Brown *et al.* 2000, Moret *et al.* 2000, Brown *et al.* 2003a, Logan *et al.* 2005, Riddell *et al.* 2006, Brunner *et al.* 2014), while a high quality diet inhibits pathogen development (LoCascio *et al.* 2019). However, we lack definitive data on how anthropogenic change, and the associated shift in floral resource availability, affects bee health and pathogen prevalence comparatively across landscape types. The composition of floral resources varies substantially between major habitats (Baldock *et al.* 2015), and the bottom-up effects of this on disease transmission in pollinators is largely unknown. For example, bumblebees from different habitats are known to carry different gut microbiota, which in turn play a role in immune defence (Bosmans *et al.* 2018).

Bumblebees have three widespread microbial pathogens (*Crithidia bombi, Nosema bombi* and *Apicystis bombi*). More recently, *N. ceranae*, a pathogen of honey bees has also been found capable of infecting bumblebees (Graystock *et al.* 2013b). These pathogens are known to affect pollination efficiency by changing bee behaviour (Gegear *et al.* 2005, Otterstatter *et al.* 2005, Gegear *et al.* 2006), and can reduce survival and fecundity (Schmid-Hempel *et al.* 1998, Brown *et al.* 2003a, Otti *et al.* 2007). As such they can have significant multi-trophic effects (Theodorou *et al.* 2016). Pathogens distribution is intrinsically tied to the distribution of the host (Anderson *et al.* 1981), but flower abundance is likely to also influence bumblebee pathogen prevalence because it will

affect the abundance of the host and opportunities for between-colony transmission. Despite the role flowers play in the transmission of bumblebee pathogens, no study to our knowledge has investigated the relationship between the abundance of flowers and the prevalence of pathogens.

In the UK, significant areas of floristic habitat important for bumblebees have been lost or radically altered in the last century due to agricultural intensification (Carvell *et al.* 2006, Baude *et al.* 2016). Simultaneously, an influx of new plant species from around the world have been incorporated into traditional garden planting and are now a firm fixture of the garden habitat (Smith *et al.* 2006b, Loram *et al.* 2008); many of these are visited by bumblebees (Williams *et al.* 2011, Hanley *et al.* 2014). In a series of comprehensive studies into garden biodiversity, a rich and diverse plant community was found in urban gardens (Smith *et al.* 2006b), but one in which some species were represented by just a single specimen. In contrast, nature reserves are often managed to retain historical habitats, such as those created through sheep grazing. Both habitats types can provide abundant floral resources (Smith *et al.* 2006b, Öckinger *et al.* 2007, Kohler *et al.* 2008, Loram *et al.* 2008), however their plant species composition (and thus their peak flowering period), is likely to differ significantly.

Substantial variations in food availability have arisen as a consequence of land use change. Understanding how these variations affect bumblebees will lead to a greater understanding of their resilience to change and to inform the conservation of more specialist species. Although bumblebee health and pathogen prevalence are irrevocably tied to floral resource availability, remarkably few studies quantify more than one at a time (Gillespie, 2010; Goulson *et al.* 2012). Here, using a combination of extensive floral surveys, molecular screening and multiple health measures, we carry out a systematic comparison of the floral resources in three contrasting habitats: gardens, farmland and nature reserves, to determine their effects on bumblebee health and pathogen prevalence. We predict that pathogen prevalence will be highest where floral resources are most abundant and that this abundance will vary across habitats through the course of the year. Accordingly, where floral resources are most abundant, we expect to see the largest, healthiest bees.

5.2 Materials and methods

5.2.1 Site selection

The study was carried out in Sussex (southern England) in three floristically distinct landscapes: four gardens, four SSSI chalk grassland nature reserves, and four Entry Level Stewardship farmland sites (Supplementary Table 5.1). Because we were interested specifically in how different floral resources affect bee health, and a large number of domestic gardens would have been required to provide a comparable area to farmland and nature reserves, we used non-domestic gardens in this study. This included formal, non-formal and botanical gardens that are large, charge for entry and typically have a dedicated gardening team. Visual surveys confirmed they contained abundant and diverse plant species with a range of geographic origins, like domestic gardens, but considerably larger. Entry Level Stewardship farms are those not implementing any bumblebee or pollinator-focused enhancement.

5.2.2 Floristic surveys

Between May and August 2016, floral resource availability at each site was quantified by recording species richness of plants in flower, and number of flower units. Plants that bumblebees are known not to visit, including grasses and some garden cultivars, were not included. One flower unit was a single flower, or for umbellifers, a single flowering stalk (pedicel), in order to quantify the potential resource provision of each flower. These data were collected using two survey methods for each site. The first was a 200 m transect line designed to intersect with as many sub-habitats as possible (including hedgerows, fields, woodland areas, etc.), and which remained constant throughout the sampling period. Transect surveys were used to assess the floral resources of the habitat as a whole. Plants which were in flower and within 2 m of the transect line were recorded. The second survey was carried out in 40 m^2 flower-rich plots, which were selected based on a visual survey of flower abundance. A different plot was surveyed each month across the sampling period. Plot surveys were used to collect data on the highest quality areas of the habitat at any particular time based on flower abundance. In total, eight floristic surveys (each consisting of both transects and plots) were carried out at each of the twelve sites over the sampling period.

5.2.3 Bee surveys

During each site visit, bumblebee queens, workers and males of *B. terrestris/ B. lucorum, B. pascuorum, B. pratorum, B. hortorum, B. hypnorum* and *B. lapidarius* were hand-netted from the flower-rich plots. We did not attempt to distinguish between *B. terrestris* and *B. lucorum* and hereafter refer to both as *B. terrestris.* Site visits were only carried out on days with no rain or low cloud cover. Collections were carried out for 1 h, or until 40 individuals had been collected in order to minimise the chances that all bees of the same species were nestmates (Darvill *et al.* 2004). All bees were stored initially in aerated 5 ml eppendorfs and following ID confirmation, in 100% ethanol for later analysis.

5.2.4 Pathogen screening and health measures

To screen for pathogens, a sample of the Malpighian tubules, fat body, midgut and hindgut were taken from each bee and transferred to a digestive solution of STE buffer (100 mM NaCl, 10 mM Tris pH8, 25 mM EDTA and 0.5% SDS), proteinase K (0.1 µg/µl) and 50% Chelex. After 20 min, the samples were homogenised using sterile toothpicks and incubated for 6 h at 55^{o}C and 15 min at 95^{o}C. Two elution steps, the first using 1:1 isopropanol and the second using 70% EthOH, were carried out. After each elution the samples were centrifuged for 1 h and the supernatant discarded. The remaining DNA pellets were resuspended in molecular grade water and stored at -4^{o}C until they were used for screening (Supplementary Table 5.2). Negative controls were included in each extraction plate and positive controls in each PCR.

To estimate bee size, we performed linear measurements of marginal cell length on 165 workers selected randomly across habitats and months. Cell length was measured under x20 magnification. On the same workers we carried out a fat content assay using diethyl ether (Brown *et al.* 2000). The abdomen of each bee, devoid of gut and crop, was dried for 4 days at 70^{o}C (after which their weight no longer decreased, so indicating that drying was complete) and weighed. Diethyl ether was added to each abdomen in 5 ml eppendorfs and allowed to sit for 24 h to dissolve lipids. After rinsing in fresh diethyl ether, the abdomens were then dried again for a further 4 days and reweighed to obtain the final dry abdomen weight. The fat content was the difference between the first and second weighing and relative fat content was calculated by dividing fat content by marginal cell length as a proximate for body size.

5.2.5 Statistical analyses

Factors affecting floral resource availability, the prevalence of pathogens and fat content were investigated using generalised linear mixed models with a gamma distribution and log link function, and bee size investigated using a linear mixed model. For each measure of floral resource availability (species richness and floral units), full factorial models included the effect of habitat, survey type and month. For pathogen prevalence, models included the effect of habitat, month, total floral unit abundance and total species richness, and for measures of bee health, the effect of bumblebee species was also investigated. Site was included as a random factor in all models to account for the structured nature of the data. Minimum adequate models were produced by removing non-significant interaction terms.

Based on existing literature, we identified eight plant families known to be important foraging groups for bumblebees and for those that were found in more than one habitat type, compared their floral unit abundance across habitats using a Kruskal Wallis test. A Kruskal Wallis test was also used to compare the mean difference in floral resource availability between plot and transect surveys across each habitat. To compare and group specific site visits across habitats and months based on plant composition, a hierarchical cluster analysis using between-groups linkage based on size difference was used for plant genus clustering and site visit similarity analysis.

5.3 Results

Over the course of the summer, a total of 96 floral surveys were carried out and 903 bumblebees were collected. Flowering plant species richness ranged from 1 to 62 species per site ($\bar{x}$ = 16.4 ± 1.2 s.e.), and number of floral units per site from 40 to 25,711 (4,619 ± 545). Most bees were found to not carry any pathogens (54%) or carried one pathogen species (44%), with only 15 bees (1.7%) carrying two. No bees carried both *Nosema* species. Mean bee size (using marginal cell length) was 2.71 ± 0.02 μm and mean fat content was 0.51 ± 0.07 g.

5.3.1 Floral resources

There were significant interactions between the effects of habitat and survey, habitat and month, and survey and month, on the number floral units (respectively, df = 78, $p = 0.04$, df = 78, $p = 0.002$, df = 78, $p = 0.015$). Floral unit abundance was overall highest in nature reserves and gardens, and lowest on farmland. There was a significant interaction between habitat and survey and a significant effect of month, on plant species richness (respectively, df = 87, $p = 0.04$, df = 87, $p = 0.02$). Total species richness was highest in gardens, on average containing nearly twice as many species as nature reserves, and 3.5 times as many species as farmland. Between the two surveying methods (transect and plot), plot species richness was generally higher across habitats and months and the difference between surveying methods ranged from 0-38 (3.73 ± 1.61) species (Fig. 5.1). The average difference between plot and transect surveys in floral resource availability varied between habitats (Supplementary Fig. 5.1). Farms exhibited the least variation between survey methods, while nature reserves showed the greatest. There was a moderate positive correlation between floral units and species richness ($R^2 = 0.08$, $y = 0.0006x + 13.5$), which was stronger in plot compared to transect surveys (respectively, $R^2 = 0.24$, $y = 0.001x + 12.5$, $R^2 = 0.009$, $y = 0.0002x + 13.7$).

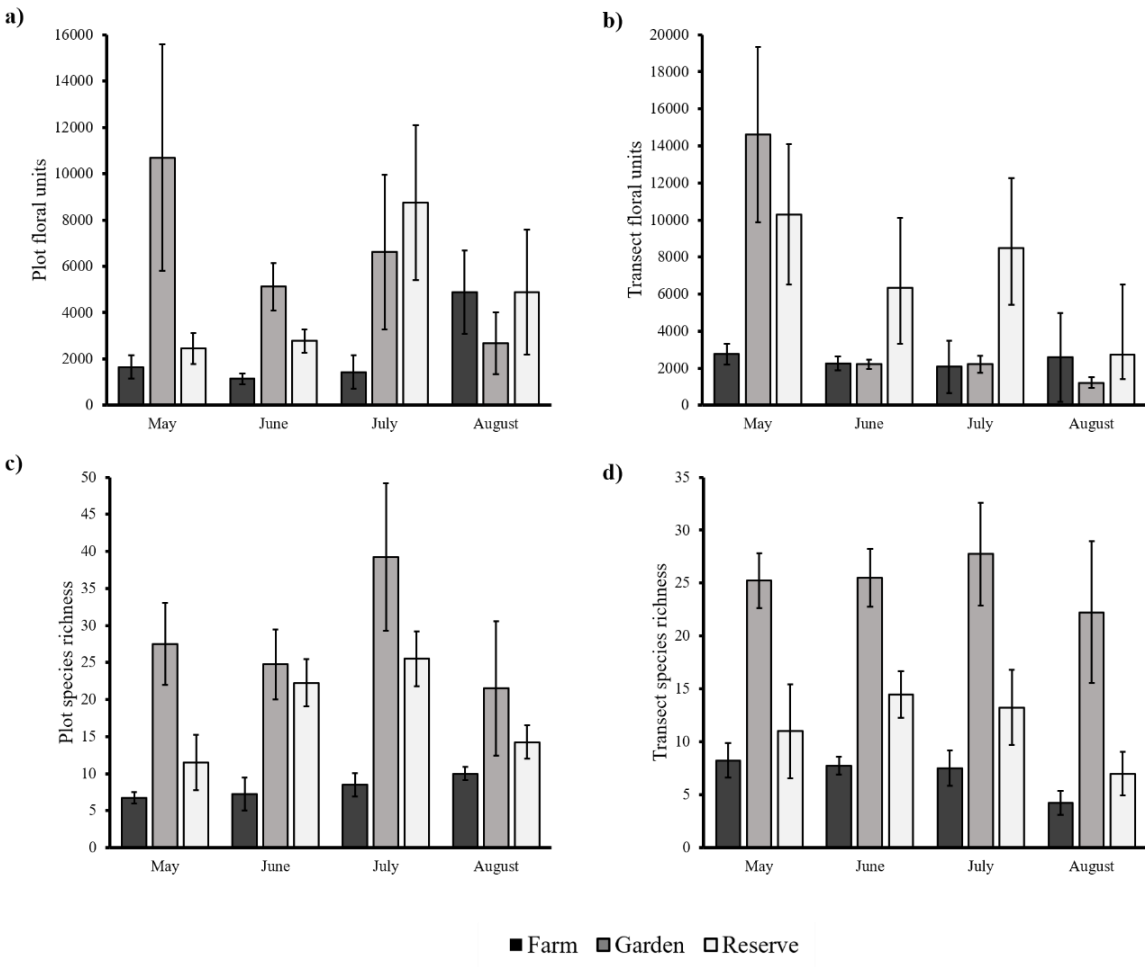


Figure 5.1 Mean (± s.e.) floral resource availability recorded in farmland (n = 4), gardens (n = 4) and nature reserves (n = 4); a-b: Floral units and species richness recorded in 40m^2 flower-rich plot; c-d: Floral units and species richness recorded along 200m transect surveys. Both surveys were carried out on the same day at each site, once a month between May and August 2016. Flower-rich plots were selected each month, targeting areas of high floral abundance, and transect surveys remained consistent through the sampling period. Each site was surveyed once a month between May and August 2016.

A total of 22 plant families were recorded in farmland (6.56 ± 0.35), 74 in gardens (17.75 ± 1.29), and 30 in nature reserves (9.88 ± 0.99). Overall, plant diversity at the family level, represented by species in flower, increased from May (11.25 ± 1.85) to July (13.08 ± 1.99), and fell in August (9.17 ± 1.78). There were significant differences in the floral unit abundance of several plant families across habitats, including Lamiaceae, Asteraceae and Ranunculaceae (respectively, χ^2 = 6.9, df = 2, p = 0.032; χ^2 = 17.6, df = 2, p <0.001; χ^2 = 12.3, df = 2, p = 0.002), but not for Fabaceae, Geraniaceae, Boraginaceae or Scrophulariaceae (χ^2 = 5.0, df = 2, p = 0.08; χ^2 = 1.5, df = 2, p = 0.48; χ^2 = 2.4, df = 2, p = 0.30; χ^2 = 0.04, df = 1, p = 0.85). Lamiaceae floral unit abundance in nature reserves was more than twice as high as in gardens, and four times as high as in farmland (respectively, 482 ± 200, 236 ± 53 and 199 ± 42 floral units). Asteraceae floral unit abundance was substantially higher in nature reserves, compared to gardens and farmland (respectively, 869 ± 189, 294 ± 79 and 254 ± 59 floral units). Six plant families accounted for 50.4% of diversity in gardens (Ranunculaceae, Ericaceae, Geraniaceae, Rosaceae, Fabaceae and Asteraceae), while only three plant families accounted for 50-53% of diversity in farmland and reserves (respectively, Rosaceae, Fabaceae and Asteraceae, and Lamiaceae, Fabaceae and Asteraceae; Fig. 5.2). At the genus level, nature reserves were the most diverse, with six families accounting for 17.5% of diversity (*Prunella, Lotus, Leontodon, Centaurea, Trifolium* and *Ranunculus*), while in farms, only two genera accounted for 17.5% (*Trifolium* and *Ranunculus*). In gardens, three genera accounted for 16% of diversity (*Rosa, Rhododendron* and *Geranium*).

The composition of plant genera across all site visits formed six clusters (Fig. 5.3). All farmland visits were confined to cluster 4, along with six nature reserve visits and two garden visits. Clusters 1-3 were predominantly gardens, consisting of nine garden visits and one nature reserve visit. Clusters 5 and 6 each contained a mix of garden and nature reserve visits, consisting of nine nature reserve visits and five garden visits (Table 5.1; Supplementary Tables 5.3-5.4).

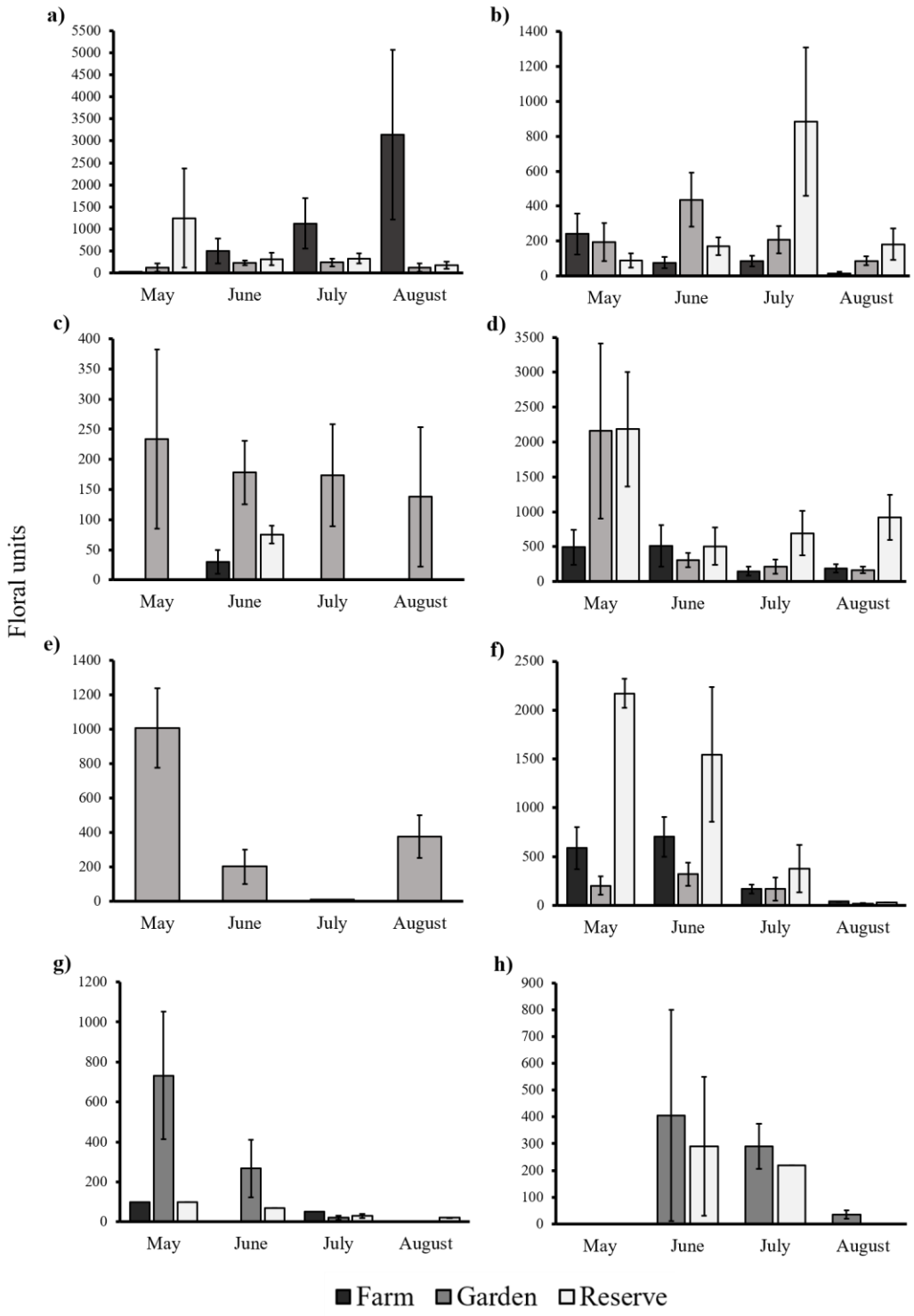


Figure 5.2 Mean (± s.e.) floral unit abundance of four important plant families for bumblebees: a) Fabaceae, b) Lamiaceae, c) Geraniaceae, d) Asteraceae, e) Ericaceae, f) Ranunculaceae, g) Boraginaceae, and h) Scrophulariaceae. Floral unit abundance data were collected from farmland, gardens and nature reserves between May and August 2016. Means are calculated based on summed floral unit abundance of the two surveys carried out during each site visit (flower-rich plot and transect).

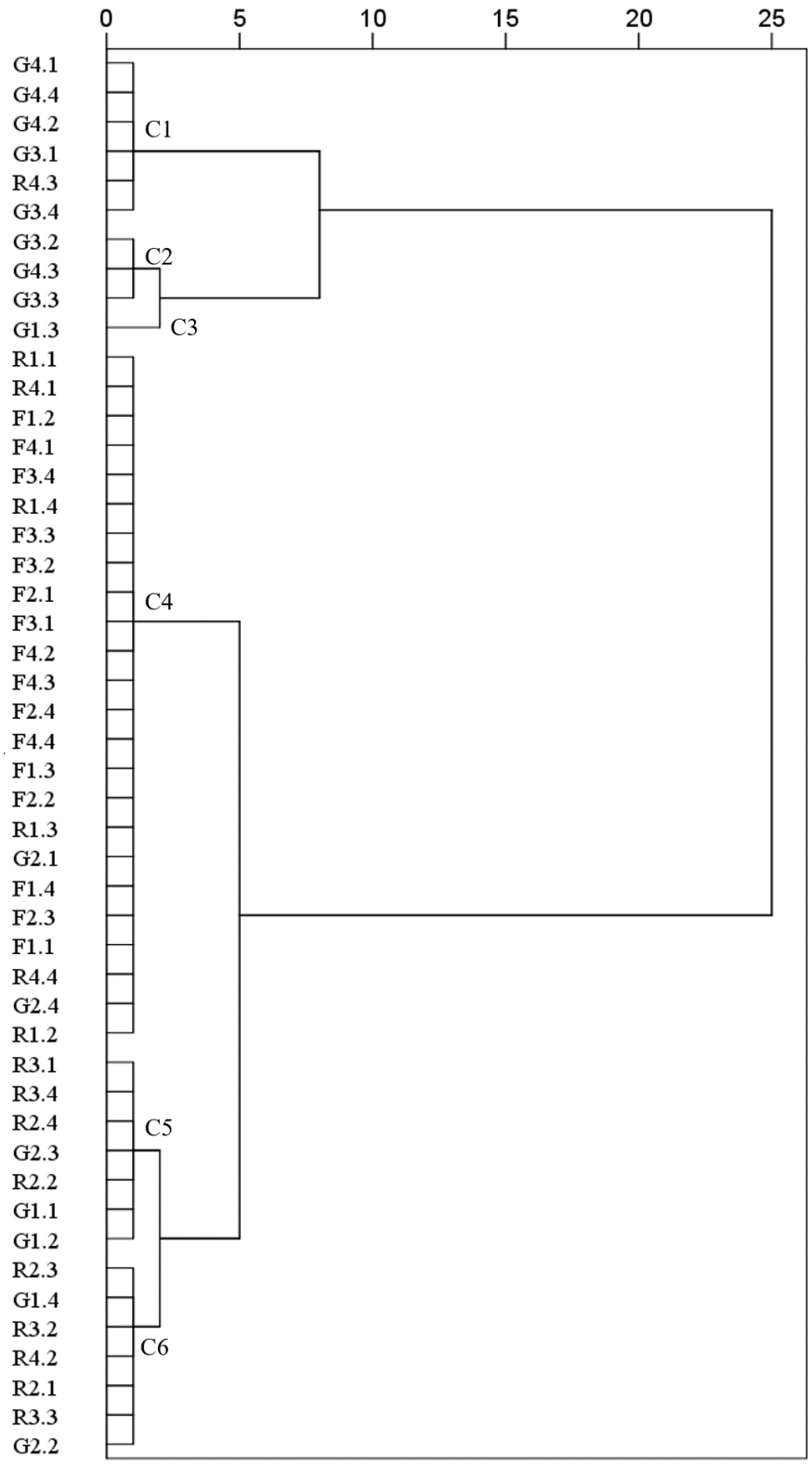


Figure 5.3 Dendrogram showing the similarity between site visits based on the presence plant genera. Each site visits specifies habitat (F = farmland, G = garden and R = reserve) site number (see Supplementary Table 5.1) and month (1 = May, 2 = June, 3 = July, 4 = August). Plant species in flower were recorded in two survey areas per site visit, and each site was visited once a month between May and August 2016.

Table 5.1 Common plant genera occurring in each of the six clusters identified in the cluster analysis (see Fig. 5.3), based on species richness data collected across 48 site visits in farmland, gardens and nature reserves between May and August 2016. Common genera were identified as those with the highest frequency, based on the number of times a species of that genera was recorded. *Cluster 3 contained 54 plant genera each represented by a single species. A full list of plant genera in each cluster is shown in Supplementary Tables 5.3-5.4.

	Cluster	Habitats	Months	Common plant genera	Frequency	Accumulative %
Garden group	1	Garden, reserve	All	*Achillea, Chamaenerion, Eschsholzia, Geranium, Nepeta, Persicaria, Phlox, Salvia*	4	16%
	2	Garden	June, July	*Achillea, Clematis, Geranium, Hydrangea, Persicaria, Phlox, Salvia, Sisyrinchium, Verbena*	3	18%
	3	Garden	July	*		
Mixed group	4	All	All	*Trifolium, Ranunculus, Cirsium, Stellaria, Silene, Lotus, Rubus*	6-15	28%
	5	Garden, reserve	All	*Centaurea, Leontodon, Ranunculus, Rubus, Taraxacum*	4	16%
	6	Garden, reserve	All	*Lotus, Ranunculus, Trifolium, Rubus*	5-6	14%

5.3.2 Pathogen prevalence

There was a significant interaction between the effects of habitat and month on the prevalence of *C. bombi* ($F_{6, 34} = 2.47$, $p = 0.043$), and no significant effect of floral units or species richness (respectively, $F_{1, 34} = 2.38$, $p = 0.13$, $F_{1, 34} = 0.13$, $p = 0.72$). *C. bombi* was the most common, occurring in 100% of sites and 43% of bees (Fig. 5.4a-b). For both *N. bombi* and *N. ceranae*, there was a significant effect of month on pathogen prevalence (respectively, $F_{3, 40} = 3.07$, $p = 0.039$, $F_{3, 40} = 4.10$, $p = 0.013$), but no effect of habitat, floral units or plant species richness ($F_{2, 40} = 0.56$, $p = 0.58$, $F_{1, 40} = 0.36$, $p = 0.55$, $F_{1, 40} = 0.17$, $p = 0.68$; $F_{2, 40} = 1.60$, $p = 0.22$, $F_{1, 40} = 0.04$, $p = 0.84$, $F_{1, 40} = 2.26$, $p = 0.14$). Each *Nosema* species was found in 2% of bees. *N. bombi* was found in 42% of sites and only found in gardens and farmland, and *N. ceranae* was found in 58% of sites and all three habitats (Fig. 5.4c-d). There was no clear relationship between the proportion of bumblebees carrying pathogens and floral resource availability (Fig. 5.5).

5.3.3 Bee health

There were significant interactions between the effects of bumblebee species and habitat and bumblebee species and month on bee size (respectively, $F_{6, 139} = 2.33$, $p = 0.036$, $F_{9, 139} = 2.66$, $p = 0.007$), and no significant effect of floral units or plant species richness ($F_{1, 139} = 0.09$, $p = 0.77$, $F_{1, 139} = 1.22$, $p = 0.27$). Bees were larger in gardens and nature reserves (2.72 ± 0.03 µm, 2.70 ± 0.03 µm), compared to farmland (2.52 ± 0.08 µm). Bumblebee fat content was significantly affected by the interaction between bumblebee species, habitat and month ($F_{33, 121} = 2.19$, $p = 0.001$), but not by floral units or plant species richness ($F_{1, 121} = 0.1$, $p = 0.75$, $F_{1, 121} = 0.98$, $p = 0.32$). Body size and fat content varied across categories of habitat, month and bumblebee species (Fig. 5.6, Fig. 5.7).

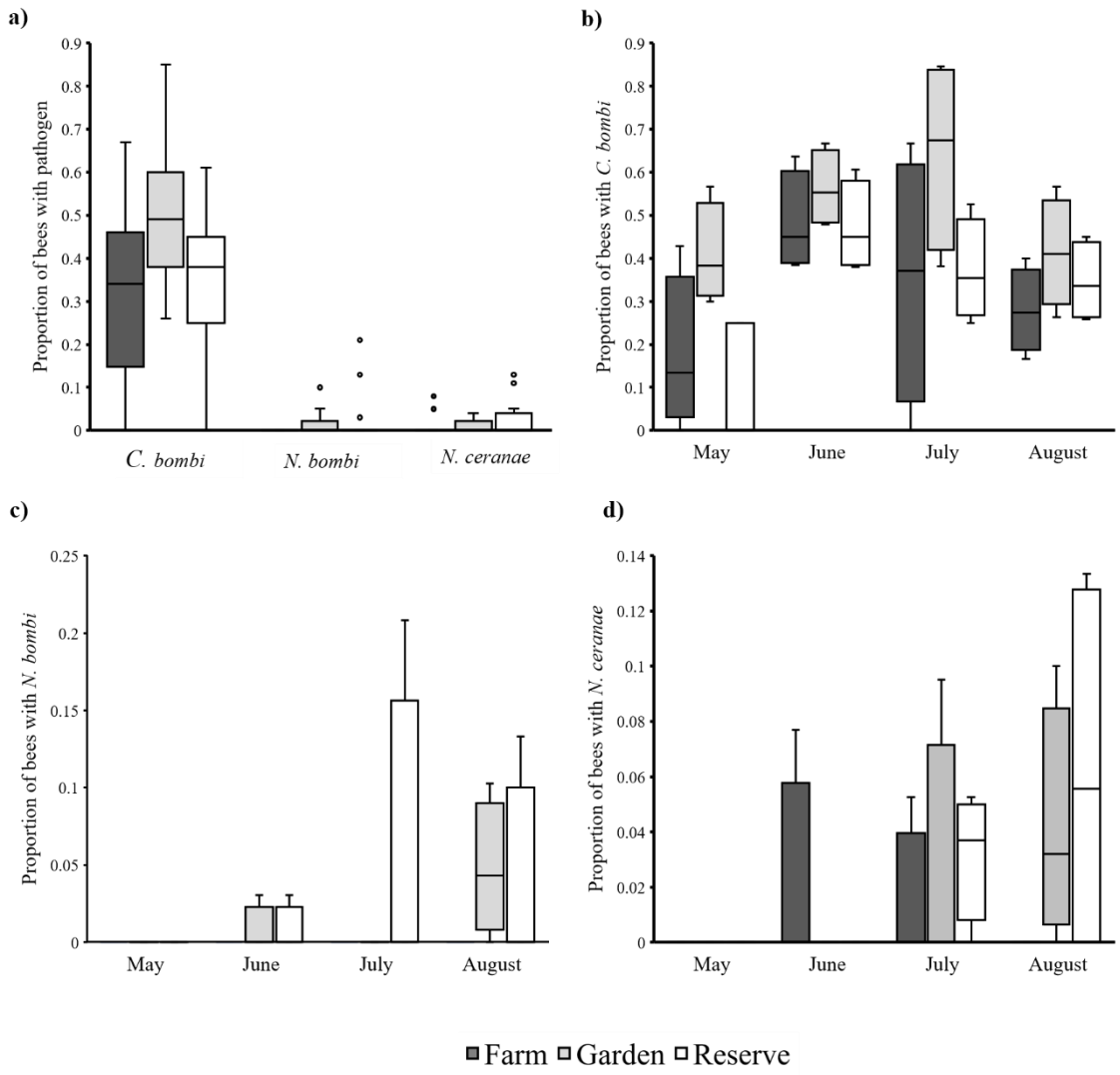


Figure 5.4 Pathogen prevalence amongst bumblebees collected in farmland, gardens and nature reserves. a) Proportion of bumblebees carrying *C. bombi*, *N.bombi* and *N. ceranae*; b-d) Proportion of bumblebees collected from gardens, farmland and nature reserves between May and August 2016 carrying the pathogens *C. bombi*, *N. bombi* and *N. ceranae*. The black bar within the box represents the median value; box boundaries show the interquartile range; whiskers show the maximum and minimum values. Circles show outliers.

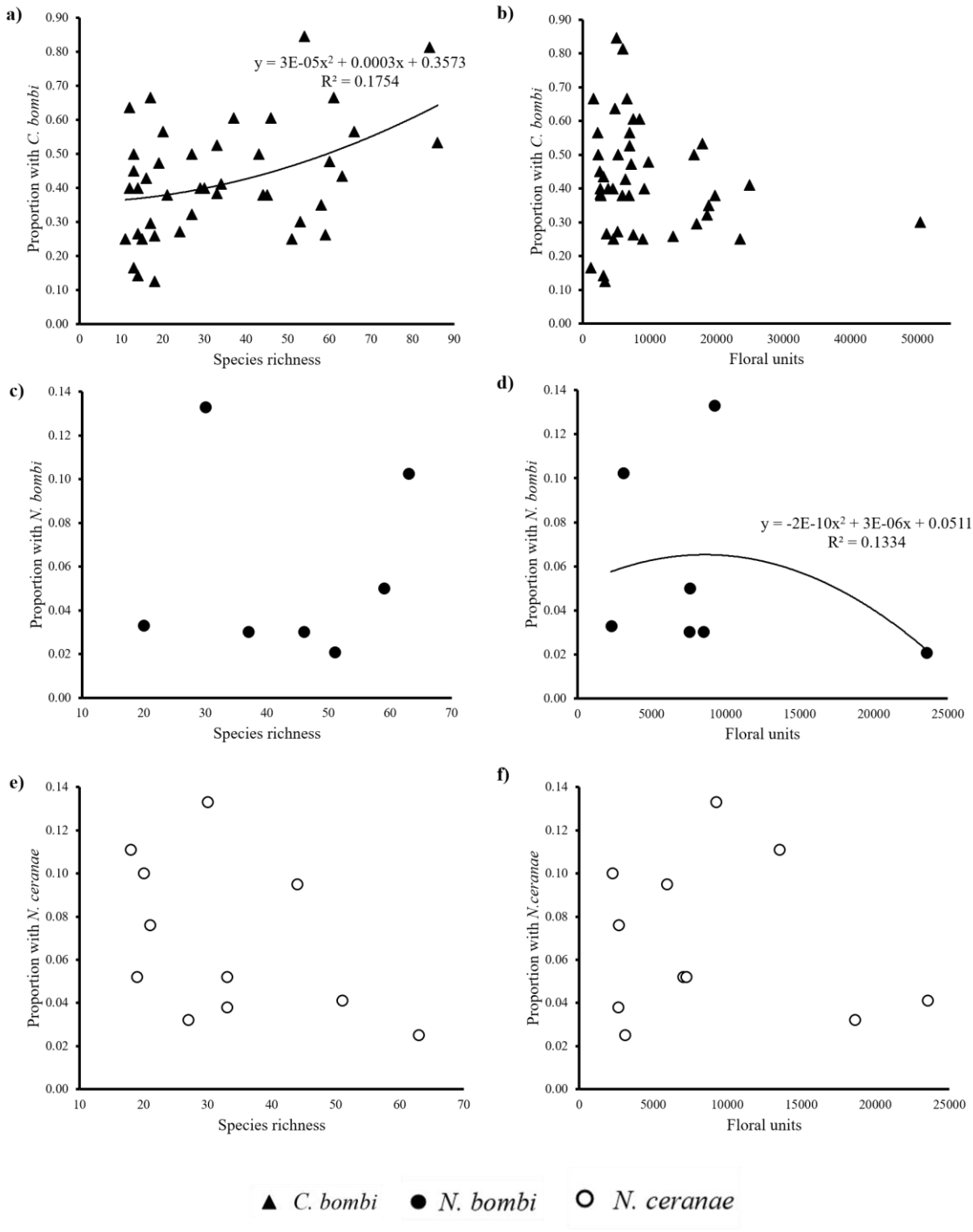


Figure 5.5 Scatterplots showing the relationship between pathogen prevalence and floral resource availability (measured in floral unit abundance and plant species richness) for *C. bombi* (a-b), *N. bombi* (c-d), and *N. ceranae* (e-f). Bumblebees were collected from farmland, gardens or nature reserves between May and August 2016.

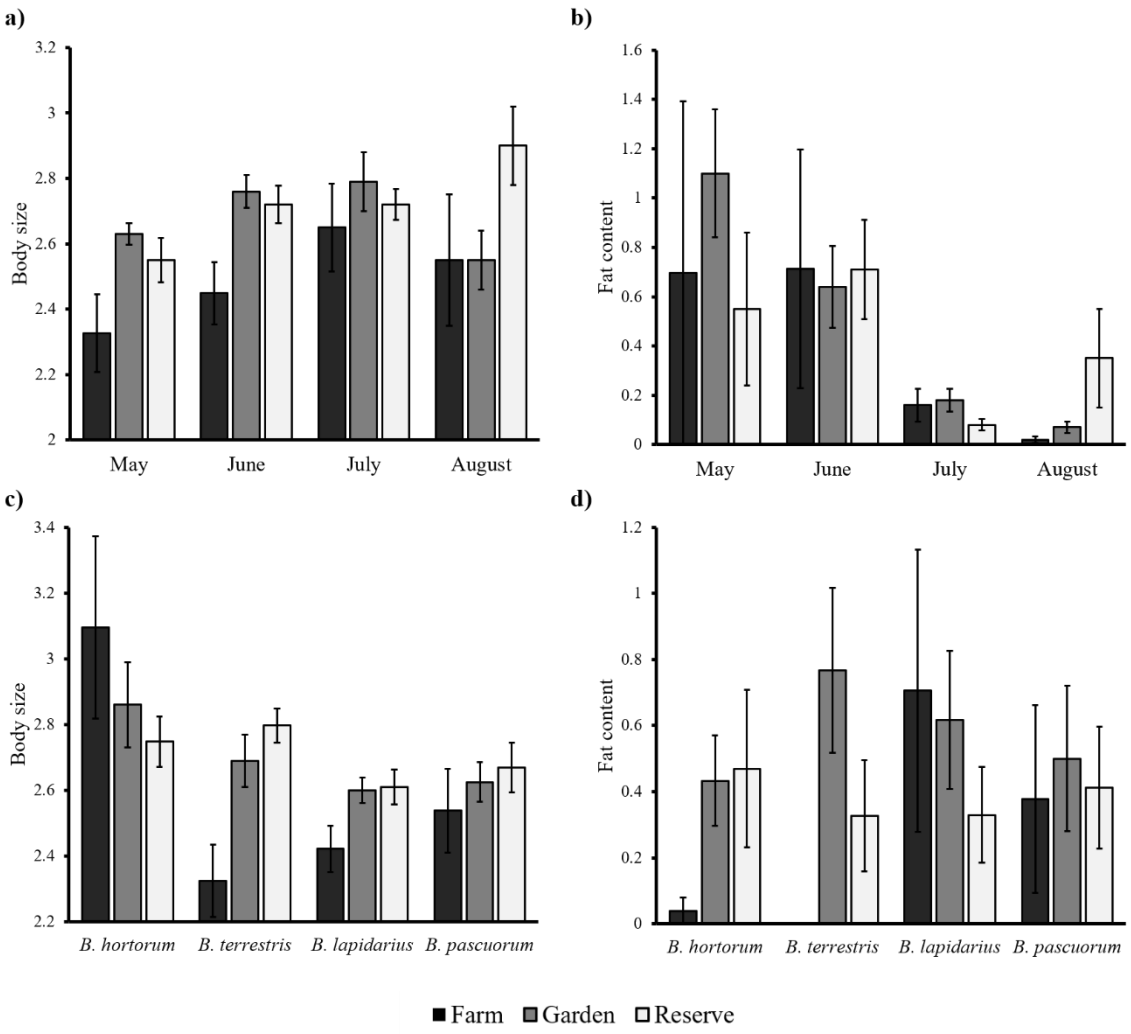


Figure 5.6 Bumblebee health measured across habitat, month and bumblebee species. a) Effect of habitat and month on body size (µm); b) effect of habitat and month on fat content (mg); d) effect of habitat and bumblebee species on body size (µm); d) effect of habitat and bumblebee species on fat content (mg).

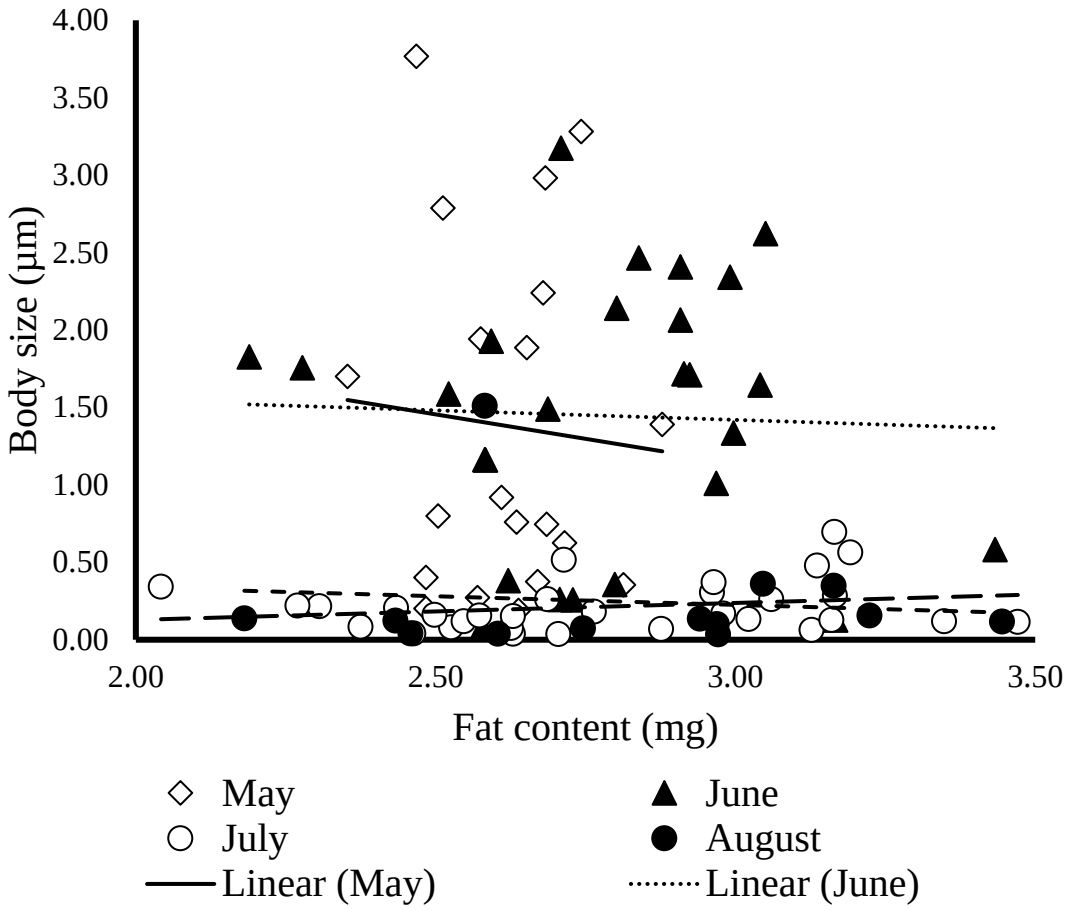


Figure 5.7 Scatterplot of bumblebee health measures: bee size (µm) and absolute fat content (mg) (n = 146). Bumblebees were collected between May and August 2016 from farmland, gardens and nature reserves throughout Sussex, UK. May: $y = -0.6314x + 3.0337$, $R^2 = 0.005$; June: $y = -0.1231x + 1.7886$, $R^2 = 0.0015$; July: $y = 0.1096x - 0.0916$, $R^2 = 0.0536$; August: $y = -0.1113x + 0.559$, $R^2 = 0.0105$.

5.4 Discussion

This is the first study to systematically compare floral resource availability, bumblebee health and pathogen prevalence in replicate farm, garden and nature reserve field sites. The results show that floral resource availability differed substantially between habitats and over the course of the year, both in number of floral units and flower species richness. The prevalence of bumblebee pathogens also varied widely and was overall highest in gardens. Despite this, based on marginal cell length and weight of the fat body, bees were largest and healthiest in gardens.

Previous studies have shown that gardens contain abundant floral resources (Thompson *et al.* 2004, Smith *et al.* 2006b, Loram *et al.* 2008), and the results here show that gardens contain the highest plant species richness. The effect was produced by the large number of non-native species, while floral unit abundance was virtually equal between gardens and reserves. Farmland contained the fewest floral resources per unit area, as has previously been observed (Baldock *et al.* 2015), but did contain a substantial proportion of Scrophulariaceae and, in particular, Fabaceae, which is a particularly important pollen source for long-tongued species (Goulson *et al.* 2008). By identifying similarly important plant groups and mapping their floral unit abundance through the year, we were able to identify both resource gaps and reliable resource pools. For example, while Geraniaceae occurred in all habitat types, it was only found in gardens outside of June, usually abundantly (186.2 ± 50 floral units). Nature reserves were reliable sources for both Lamiaceae and Asteraceae. Some important plant families were excluded from analysis because they could only be found in one habitat; Ericaceae, for example, which was only found in gardens.

By clustering site visits based on genus-level plant richness, we found that while farms were compositionally similar between sites and across months, the composition of gardens and nature reserves was more diverse. The six clusters of flowering plant communities could broadly be assigned one of two groups; the garden cluster group (clusters 1-3) and the mixed cluster group (clusters 4-6). The former was dominated by near-native, non-native and exotic species commonly found in gardens (Kendle *et al.* 2000, Smith *et al.* 2006b), including *Salvia*, *Nepeta*, *Persicaria* and *Hydrangea* (Supplementary Table 5.3). The mixed group contained genera that are ubiquitous across

multiple habitats, such as *Trifolium, Ranunculus, Cirsium* and *Rubus*, but also included many garden (e.g. *Buddleja, Iris, Phlomis* and *Solidago*), heathland (e.g. *Erica, Cytisus, Ulex*) and grassland (e.g. *Lotus, Centaurea, Sanguisorba, Leucanthemum, Lychnis*) genera (Supplementary Table 5.4). Garden sites were not confined to the garden group, demonstrating the variation between gardens and within gardens over time, an effect likely driven by individual garden planting regimes. We found gardens to be vastly more heterogenous than farmland or nature reserves. Due to their size and precise management practises, they could contain several overlapping flowering habitats, including orchards, wildflower meadows, wetland and woodland. Garden 2, for example, was a woodland garden with a ploughed chalk grassland that intersects the garden. This is reflected in the cluster analysis, in which it is more compositionally similar to nature reserves and farmland consistently throughout the sampling period (clusters 4-6). Furthermore, we observed such significant changes in plant composition across months in Garden 1, that it occurred in cluster 1, cluster 3 and cluster 5, depending on the time of year. It is often assumed that gardens in which non-native species and cultivars are abundant are of less value to wildlife, particularly specialist species (Burghardt *et al.* 2010, Salisbury *et al.* 2015). As we show here, it is clearly not so straightforward, and gardens with plants from multiple origins are likely to support a variety of pollinator species at different times of year (Kendle *et al.* 2000, Smith *et al.* 2006b, Lowenstein *et al.* 2019).

Using two survey methods we intended to capture information on each habitats typical and best floral resource availability. Since it is known that there are spatial gaps in floral resource availability on farms (Timberlake *et al.* 2019), and our visual surveys of gardens showed the reverse, we expected a greater discrepancy between plot and transect surveys on farmland in both floral units and species richness data. However, farms showed the most consistency in floral units and species richness between plot and transect surveys while reserves exhibited greater heterogeneity and gardens were intermediate (Supplementary Fig. 5.1). We hypothesise this effect comes down to the floristic homogeneity of farmland and the heterogeneity of gardens and reserves at different spatial scales (Blackstock *et al.* 1999, Loram *et al.* 2008, Bullock *et al.* 2011, Littlewood *et al.* 2012). Gardens had high species richness and abundant flowers within surveys, which often did not vary substantially across the rest of the site, likely as a result of consistent garden management and plant choice for the season. In contrast, nature reserves had low species richness and high flower abundance in plots surveys, which was

not reflected in the transect surveys done on the same day. Based on these results, transect surveys are a suitable means for collecting floral resource data on farms, but are insufficient to capture the full floristic diversity present in gardens and nature reserves. At any point in time, gardens tend to provide abundant, consistent and densely aggregated floral resources. Farms provide very few species, but contain a large proportion of Fabaceae in the landscape, which is important for many bumblebee species (Goulson *et al.* 2005). Nature reserves provide abundant floral resources, but they are patchy in distribution across the habitat.

The prevalence of all pathogens was significantly different across months. We recorded enough *C. bombi* to see that its overall abundance broadly tracked that of bumblebees. Previous studies have shown pathogen prevalence peaks around July and August, concurrent with bumblebee abundance (Shykoff *et al.* 1991, Imhoof *et al.* 1998), and our *Nosema* data support this, however, *C. bombi* prevalence peaked much earlier in June, and steadily decreased through the summer. Even though we controlled for floral resource availability in our analyses, *C. bombi* prevalence was significantly affected by habitat, suggesting other landscape factors affect the prevalence of this pathogen. Previous studies have shown that the benefits bumblebees gain from conservation initiatives on farmland, such as pollen and nectar mixes on field margins, vary with surrounding landscape features (Heard *et al.* 2007, Scheper *et al.* 2013, Krimmer *et al.* 2019). Bumblebee abundance is strongly correlated with flower richness at a given site (Potts *et al.* 2003), but proximity to semi-natural habitats that provide additional food sources and nesting sites also have a positive effect on local populations (Holzschuh *et al.* 2007, Knight *et al.* 2009, Scheper *et al.* 2015), which in turn will affect pathogens. We did not select any sites based on the presence of absence of honey bee hives. One garden had two hives, but we observed honey bees commonly in all of them. Proximity to managed bees had been shown to increase the rates of pathogen presence in wild bees (Colla *et al.* 2006, Murray *et al.* 2013, Graystock *et al.* 2014), presumably through shared use of flowers. However, since these studies assumed presence meant infection, it is difficult to say if further spread occurs and how this may affect bumblebees across different habitats. Recent work has shown that pathogen survival and viability can be compromised by flower morphology, nectar biochemistry and UV exposure (Cisarovsky *et al.* 2014, Palmer-Young *et al.* 2016, Adler *et al.* 2018, Figueroa *et al.* 2019), suggesting pathogen transmission via flowers is often inefficient. Since taxonomical differences (chemistry, shape, habitat) of flowering

plants are predictors of transmission efficiency, and plants have been shown to differ widely in their ability to transmit *C. bombi* to bumblebees (Adler *et al.* 2018, Figueroa *et al.* 2019), it is reasonable to predict that increasing floral abundance would only increase pathogen transmission significantly if the plant species were good vectoring sites.

Worker size and fat content is determined at the larval stage (Bailey 1975, Tasei *et al.* 2008, Quezada-Euán *et al.* 2011). Having controlled for floral resource availability, measures of bee health showed bumblebees overall were largest and healthiest in gardens. We did not collect data on landscape features outside of our study sites, which may have provided further insight; in particular, the availability of surrounding semi-natural habitat may have alluded to supplementary resources bees could utilise (Holzschuh *et al.* 2007, Knight *et al*. 2009, Scheper *et al.* 2015). Interestingly, when pooled across species and months, bees collected in nature reserves were almost as large as garden bees, but had much lower fat content. *B. terrestris*, *B. pascuorum* and *B. lapidarius* were all smaller in farmland, largest on nature reserves and intermediate in gardens, while the opposite was the case for *B. hortorum*, despite fewer replicates on farmland. Differences in fat content between species were more varied but generally decreased for all species through the year, although not proportionally across habitats. In the process of measuring fat content, we observed dried abdomens would begin regaining weight as they absorbed moisture from the air. Accordingly, all measurements were taken as quickly as possible although it may be too crude a measure – particularly for small species. Fat content is known to vary quantitatively and qualitatively, which has implications of for immune system functioning (Moret *et al.* 2000, Arrese *et al.* 2010). Future studies could assess how different bumblebee species vary in their qualitative fat content (including protein analysis) between habitats (Patel 1971, Obenchain *et al.* 1973). Previous studies show a trade-off between energy storage and growth or reproduction in other insects (Forsman *et al.* 1991, Kristensen *et al.* 2011), as well as mammals (Lesage *et al.* 2001). A compromise in the allocation of resources at the larval stage between body size and fat content may explain the broad pattern seen in Figure 5.7. Combining a qualitative analysis of both diet and fat content would greatly benefit future research and conservation policy. We did not carry out an analysis of the pollen carried by the bumblebees in this study (Appendix 6). However, previous studies on the species composition of pollen collected from foraging bees show that bees forage flexibly and pollen diets vary seasonally (Wood *et al.* 2018). A thorough characterisation of floral resources available to bumblebee populations, the

plant species they choose to utilise, and an analysis of the fat body would not only reveal foraging preferences and the nutritional value of different pollens, but could be carried out on a range of pollinating insects to illuminate species that are not meeting their nutritional needs and are therefore at the greatest risk of decline.

Gardens are important sites for biodiversity in the UK and our findings on non-domestic gardens can be reasonably applied to domestic gardens at a street or neighbourhood scale. Investigating the effects of these diverse floristic plant communities on pollinator health and population dynamics on a largescale in neighbourhoods, towns or cities, is often hindered by public interest and site access. In our experience, those running and managing non-domestic gardens are interested in the wildlife they support and are supportive of research. As urbanisation continues to rise, and pollinators become more reliant on the floral resources we plant, the bottom-up effects of plant community composition are clearly worth exploring further.

This is the first study to compare the synergistic effects of habitat and floral resource availability on bumblebee health and pathogen prevalence. Our findings suggest that gardens are comparable to nature reserves in their floral unit abundance, but are significantly more species rich. They host larger, healthier bees but also larger populations of pathogens. It is unlikely that managing agricultural habitats alone will alleviate the effects of reduced floral resource availability on bee population health and a more inclusive approach, in which multiple landscape types are utilised for conservation efforts, is needed.

Chapter 6: General discussion

6.1 Summary

Floral resources play an important role in shaping bumblebee communities by influencing behaviour and population dynamics through a variety of direct and indirect mechanisms (Roulston *et al.* 2011). They provide an essential source of nutrients, thus regulating bumblebee health and immunity, but also provide increasing opportunities for pathogen transmission and establishment in the host (Durrer *et al.* 1994, Graystock *et al.* 2015, Woodard *et al.* 2017). Variations in floral preferences and nutritional biology between bumblebee species suggest they may vary in their tolerance to nutritional stress and pathogen infection (Shykoff *et al.* 1991, Brown *et al.* 2000, Korner *et al.* 2005, Vaudo *et al.* 2015). Previously, research into the importance of nutrition for bumblebees has been almost exclusively confined to a restricted range of species that are easy to study (Schmid-Hempel 1998, Velthuis 2002, Velthuis *et al.* 2006). Using a combination of field surveys and laboratory experiments, I show how floral resources, both in nutrition and availability, affect health and reproductive success across multiple bumblebee species. In addition, I show that flower abundance and plant species richness vary extensively in the landscape and explore the bottom-up effects of floral resource availability on bumblebee health and the prevalence of their pathogens.

6.2 Floral resource availability across habitats

Bumblebees utilise a variety of habitats to collect the resources necessary for their survival. They historically thrived on farmland but increasingly rely on plant communities in other habitats, including semi-natural grasslands and gardens (Osborne *et al.* 2008, Kennedy *et al.* 2013, Baude *et al.* 2016, Hall *et al.* 2017). Using two plant surveying methods and measuring flower abundance, taxonomic diversity and floristic heterogeneity, I found the three habitats varied dramatically in floral resource availability across multiple scales. Gardens were the most floristically diverse and were spatially consistent in this diversity. In terms of flower abundance, gardens contained a substantial proportion of many plant families known to be important for bumblebees, including Geraniaceae, Ranunculaceae and Boraginaceae, and gardens were the only habitat to contain Ericaceae. Perhaps surprisingly, their genus-level diversity revealed considerable

elasticity in plant community 'naturalness', caused by management style of the garden. As a result, some of their plant communities conformed to what was expected of modern gardens; being dominated by non-native and exotic species (Smith *et al.* 2006b). Other communities were compositionally much more similar to semi-natural habitats, and a single garden site could vary across this spectrum through the course of the summer. Nature reserves, defined here as protected semi-natural habitats containing chalk grassland, contained as many flowers as gardens, but were highly heterogenous in resource availability. This was largely a result of topography, management and footfall. Flower-rich areas were usually away from footpaths and often on slopes. Reserves also showed substantial variations in genus-level diversity and contained a large proportion of flowers from the Fabaceae, Asteraceae, Ranunculaceae and Scrophulariaceae families. Farmland was the most floristically homogenous landscape with the lowest flower abundance. The habitat contained the lowest diversity of Fabaceae species and all of those recorded (*Trifolium pratense, T. repens, Medicago lupulina, Lotus corniculatus, Lathryus pratensis* and *Vicia cracca*), were also found in the other habitats. However, more than half of the Fabaceae flowers recorded in the study were on farmland sites. These sites did not have any wildflower strips or wildflower meadows; all data were collected from field edges, tracks and one sheep-grazed pasture. This demonstrates that the farmland habitats have considerable potential for bumblebee conservation based on the existing seedbank.

As landscapes become more urbanised the importance of gardens and their value for conservation increases. The intriguing relationship between floral resources, bumblebees and their pathogens may vary in different habitats where the composition of floral resources – and thus their structural and biochemical complexity – varies widely. Gardens are compositionally unique and structurally complex habitats in which bumblebees' adaptations for minimising the effects of pathogens, and the pathogens' adaptations for maximising infectivity, may not function as they would in more natural landscapes. Bees compensate for seasonal dips in food availability by switching to alternative plant species, often to alternative habitats (Whittington *et al.* 2004, Wood *et al.* 2018). The role gardens play in the nutritional ecology of pollinators is virtually unknown. Beyond studying attractiveness and pollinator preferences, future studies could seek to characterise the nutrients of the floral rewards in common garden plants and map the seasonal variability of high-quality rewards.

6.3 Factors affecting pathogen prevalence

Across each of these habitats, I showed the prevalence of three important bumblebee pathogens. Pathogen transmission is mediated by spatial heterogeneity arising from environmental conditions inside and outside the host (Paull *et al.* 2012). Individual bee and colony health, population density and interspecific diversity of the bumblebee community might all affect pathogen success in the host (Anderson *et al.* 1981, Shykoff *et al.* 1991, Gillespie 2010, Graystock *et al.* 2016). Outside the host, pathogens may be subjected to a variety of biochemical and climatic conditions depending on where they have been deposited, for example, in the nest, in nectar, pollen, or on the surface of a flower) (Adler *et al.* 2018, Figueroa *et al.* 2019). The pathogens of bumblebees are transmitted via the faecal-oral route and so a large proportion of transmission is likely to occur in the nest itself (Schmid-Hempel 1998). The degree to which the pathogens rely on horizontal transmission via shared flowers is unknown, yet without it we should expect host-specific adaptation of pathogen strains, which there does not appear to be (Schmid-Hempel *et al.* 1998, Fries *et al.* 2001). In addition, genetic work has found identical clones of *C. bombi* existing in different species at the same site, demonstrating horizontal transmission does occur (Erler *et al.* 2012). While some biochemical constituents of flowers may be beneficial to pathogens (Palmer-Young 2017), it seems their survival and/or virulence is often negatively affected (Otterstatter *et al.* 2008, Cisarovsky *et al.* 2014, Figueroa *et al.* 2019). In a social organism with minimal sanitary behaviours, horizontal transmission should commonly occur inside the nest where conditions are relatively stable (Yoon *et al.* 2002). Outside the nest, the deposition of pathogens could be nearly as high, but then cells and spores are susceptible to environmental conditions that may reduce their survival and/or virulence, such as UV light, rainfall and plant chemicals (Paull *et al.* 2012, Cisarovsky *et al.* 2014, Figueroa *et al.* 2019). Importantly, these conditions exist irrespective of habitat type. While under controlled conditions it has been shown that pathogens can be transmitted via flowers, in the wild this may be infrequent (Cisarovsky *et al.* 2014). These studies show important advancements have been made in the study of bumblebee pathogen transmission on flowers, but we are still far from being able to make ecologically-realistic predictions concerning the importance of this process in regulating bumblebee populations and affecting the coevolution of bees, pathogens, and flowers. The ability for bees to detect and avoid flowers containing pathogens would be advantageous and could have evolutionary implications for plants,

and yet this area of study is almost completely unexplored (but see Fouks and Lattorff 2011). Greater insight into the transmission of pathogens under field-realistic conditions is needed. A broad census of wild plants - in which viable verses non-viable cells/spores are differentiated - will indicate the prevalence of infective pathogens that bees may encounter. The fate of the infective cells/spores should also be elucidated – are most shed through the faeces or do they accumulate in the forager, or the developing larvae? The role of flowers as transmission hubs for the pathogens of other animals is lacking. Further investigation is required to identify pathogens of other flower-visitors that are vectored in this way.

If horizontal transmission via shared flowers was evolutionarily important for bumblebee pathogens, we might expect to see a relationship between their prevalence and the abundance of flowers. I show that pathogen prevalence varied widely in bumblebees between and within sites, but this variation was not explained by flower abundance or the species richness of the flowering community. Rather, I find evidence that pathogens are affected by environmental conditions and broader characteristics of the landscape, but that these effects are mediated by bumblebees (Anderson *et al.* 1981, Paull *et al.* 2012). Nature reserves and gardens each had two and a half times as many flowers as farms, but *C. bombi* prevalence in farmland and reserves was roughly equal, while in gardens it was approximately 20% higher. In Chapter 4, I found *C. bombi* occurred much less frequently than previously reported. Existing literature shows it reaches peak abundance (up to 100%) at the same time bumblebees reach theirs (typically around July) (Shykoff *et al.* 1991, Imhoof *et al.* 1999). However, in Chapter 4, *C. bombi* was only present in 59% of the 26 sites surveyed and prevalence within sites ranged from 10-50%. A direct comparison of these findings with those of Chapter 5, focusing solely on data collected from the same habitats and months show clear differences in pathogen abundance: in Chapter 5 it was found in 90% of the eight sites and prevalence within sites ranged from 27-85%. As described in Chapter 1, pathogens are more virulent in food-stressed bees (Imhoof *et al.* 1998, Brown *et al.* 2000), and so it is possible food shortages in bumblebees contributed to *C. bombi* occurrence. Specifically, the warm, dry summer may have reduced nectar availability and thus increased the mortality of infected workers, thereby reducing *C. bombi* prevalence (Phillips *et al.* 2018). Alternatively, lack of food in the host may have inhibited pathogen survival in the gut, hindering further transmission (Logan

et al. 2005). Long-term studies of pathogen prevalence are needed to explain such variations.

In Chapter 5, I also found that pathogen prevalence was not explained by floral resource availability, and suggest this is related to broader characteristics of the surrounding landscape. Studies have shown that biodiversity is greater in agricultural landscapes that have semi-natural components and high landscape complexity (Heard *et al.* 2007, Kennedy *et al.* 2013, Lichtenberg *et al.* 2017). Bumblebees are likely to benefit from nearby semi-natural habitat through the acquisition of additional resources. Since bumblebees can forage up to ~2km from their nest, the scale of available resources is clearly an important factor in determining their population size, which will in turn affect pathogen prevalence (Walther-Hellwig *et al.* 2000). Furthermore, one of the consequences of agricultural intensification has been habitat fragmentation, causing reduced genetic diversity. It is already known that bumblebees with low genetic diversity host a higher prevalence of *C. bombi* (Whitehorn *et al.* 2010), and several of the hundreds of *C. bombi* strains identified have differential growth rates across different bumblebee species (Imhoof *et al.* 1998, Popp *et al.* 2011, Salathé *et al.* 2011, Ruiz-González *et al.* 2012). Together, these data suggest landscape features that affect the species richness and abundance of bumblebee communities, in turn can affect their pathogens through a variety of mechanisms. Further work is needed to elucidate the mechanisms that drive colony growth and population viability.

Before *N. ceranae* was detected in commercial colonies in 2003, its importation in probably began in the 1980s (Velthuis *et al.* 2006, Graystock *et al.* 2013b). Since 2003, there have been growing concerns that the pathogen poses a risk to wild bumblebee populations, but there is very little data on its prevalence in wild bumblebee communities in the UK (Graystock *et al.* 2013a, Furst *et al.* 2014). I screened a total of 1070 bumblebees from 31 sites and found on average, less than 3% of bees at each site were carrying the pathogen. Once again, it is important to note that this represents presence, not infection. In a previous study the prevalence of non-viable and low sporulating *N. bombi* was found to be significantly higher than of sporulating infections (Blaker *et al.* 2014), illustrating that one cannot be used as a measure for the other. *N. ceranae* is consumed by wild bumblebees foraging near managed bee colonies, however, there is no evidence as yet to show *N. ceranae* infections spread between wild bumblebees

(Otterstatter *et al.* 2008, Murray *et al.* 2013, Furst *et al.* 2014). Data in this thesis suggest *N. ceranae* is not a widespread pathogen of bumblebees. This is not to say that *N. ceranae* exerts little or no pressure on wild bumblebee populations, since models of pathogenicity predict that infection prevalence is inversely related to virulence (Anderson *et al.* 1981). Without first determining the virulence of *N. ceranae* in bumblebees, the pathogen cannot be assumed to be inconsequential in the regulation of the host population. This should be a priority for future studies.

6.4 Factors affecting bee health

Through the course of the year and across each habitat, bumblebees varied in body size and fat content. While these measures provide a limited view of bee health, they are at least partially determined at the larval stage, and so are useful indicators of both individual and colony health (Bailey 1975, Doums *et al.* 2002, Tasei *et al.* 2008, Quezada-Euán *et al.* 2011). The fat body is an important, multi-functional organ that reflects energy stores, but also plays a role in bumblebee immunity through the synthesis of antibacterial proteins (Doums *et al.* 2002, Korner *et al.* 2005). Overall, bumblebees were largest in gardens and nature reserves, and had the highest fat content in gardens. Similarly with pathogen prevalence, bumblebee health varied beyond what could be explained by variations in floral resource availability across habitats. Fat content decreased dramatically in July and August. This may have only partially been explained by senescence as previous work has shown fat content shows only very small decreases with bumblebee age (Doums *et al.* 2002).

6.5 Species differences

Throughout the chapters in this thesis I found significant differences between bumblebee species in their response to environmental conditions, including diet. In Chapter 3, I showed that both *B. hortorum* and *B. pascuorum* queens were more reproductively successful on a monofloral hawthorn diet compared with a polyfloral mix, but *B. hortorum* was especially sensitive. Despite this, *B. hortorum* queens were twice as likely to successfully produce workers, suggesting they are perhaps more sensitive to diet and less sensitive to captive rearing conditions. In Chapter 5, I found interactive effects of bumblebee species, habitat and month on body size and fat content. *B. terrestris*, *B. pascuorum* and *B. lapidarius* were all smallest on farmland and largest in nature reserves,

which may be explained by the quality and heterogeneity of the habitat type (Heard *et al.* 2007, Kennedy *et al.* 2013, Lichtenberg *et al.* 2017). The opposite was the case for *B. hortorum*, which was largest on farms and yet had the lowest fat content. None of the *B. terrestris* bees from farmland lost any weight during the fat assay, suggesting their fat content was so low on farmland that the change was too small to detect. Conversely, the fat content of *B. lapidarius* was highest on farmland. Previous studies have shown pathogen prevalence varies between bumblebee species (Korner *et al.* 2005, Gillespie 2010). Further interspecific differences are likely to be found in response to different pathogen strains, which have significant effects on host-pathogen dynamics in food-restricted habitats (Popp *et al.* 2011). Overall, these results suggest fundamental differences between bumblebee species in their nutritional requirements and response to environmental pressures. These must be further investigated to inform conservation strategies for declining species. To do so, optimising captive rearing procedures for a variety of species is vital.

6.6 Rearing long-tongued bumblebees

In early rearing trials of bumblebees in captivity, long-tongued species were quickly replaced with more competitive and resilient species, most notably, *B. terrestris* and *B. impatiens* (Velthuis 2002, Velthuis *et al.* 2006). As a result, virtually all our knowledge of bumblebee behaviour and biology is derived from experimental work of just two species. Fortunately, long-tongued bumblebees have not been entirely overlooked. Research conducted on wild populations have added substantially to knowledge of their ecology, conservation schemes aimed at bumblebees usually include Fabaceae-rich mixes or encourage the management or restoration of habitats dominated by legumes, and that many long-tongued species are particularly at risk of decline has been identified (Williams 2005, Ellis *et al.* 2006, Genersch *et al.* 2006, Herrmann *et al.* 2007, Goulson *et al.* 2008). Despite this progress, long-tongued species still rarely feature in laboratory research (Ptácek *et al.* 2000, Bučánková *et al.* 2012, Ptáček *et al.* 2015). Nutritional stress has had dramatic effects on bumblebee populations throughout many parts of the world (Williams *et al.* 2009a, Baude *et al.* 2016), and yet as far as I was able to determine, there have been no studies investigating the nutritional biology of any long-tongued species outside flower preference.

Here, I conducted two rearing studies on two common long-tongued bumblebee species from two subgenera (Cameron *et al.* 2007). It is often not possible to study rare species under laboratory conditions for ethical or practical reasons, however at this stage, with so few bumblebee subgenera represented in experimental work, there is value in studying any species not currently used in mainstream research. *B. pascuorum* and *B. hortorum* are common and queens are relatively easy to obtain. In Chapters 2 and 3, I found queens of both species readily laid eggs in captivity. In Chapter 3, egg-laying almost guaranteed the production of L3 larvae. Importantly, this suggest that despite the pollen used here not being typical of these species' diet, larvae can survive and develop on it. Mortality increased significantly after this period. I suggest this reflects a sensitive stage in larval development just before pupation, where food requirements are highest, which is problematic if the queen is not fully engaging in brood care (Plowright *et al.* 1977). Managing queen stress seems to be the key to long-tongue bee rearing. It is unclear whether queens are sensitive to their surroundings, which may be sub-optimal (e.g. nutrition, nest material, build-up of stress hormone in the rearing room, noise, etc.), or whether the stress caused by capture and transport have long-lasting effects on their reproductive performance (e.g.: damage caused in transit). It is well known that sublethal effects of stress may have profound effects on individual bees and colonies (Moret *et al.* 2000, Desneux *et al.* 2007), and stress induced by capture and captive conditions may be no different. Irrespective of the causes, stress disrupts reproductive behaviour and its management in captive queens remains a priority.

In both chapters I trialled techniques to manage queen stress and encourage egg-laying and brood care. Initially, this involved giving all queens *B. terrestris* cocoons and pairing *B. pascuorum* queens (Ptácek *et al.* 2000, Bučánková *et al.* 2012, Ptáček *et al.* 2015). I found queen pairing was not a straightforward means to make all queens dominant and thus elicit egg-laying, as had previously been described (Ptácek *et al.* 2000, Ptáček *et al.* 2015), nor was it always easy to tell dominant females from submissive ones. In several instances, neither queen was observably dominant and pairs of *B. pascuorum* could rear brood together. The provision of cocoons probably prompts egg-laying and brood care because it replicates a nest invasion in the wild, essentially stressing a queen into action before she is outcompeted (Lopez-Vaamonde *et al.* 2004, Goulson *et al.* 2018a). I altered this technique in the second study; repeatedly exposing queens to cocoons and removing callow workers as they eclosed or shortly after, which showed promise.

It is vital for future work to separate the effects of diet and captivity. This is possible using enclosed outdoors spaces, such as cages or greenhouses. I have shown that *B. pascuorum* and *B. hortorum* can survive and produce colonies on pollen collected by honey bees, but this pollen may not be sufficient for building fat reserves before hibernation, or for the survival of other long-tongued species. If captive rearing is constrained by the nutritional suitability of commercial pollen, an artificial diet must be produced. With an optimised diet, behavioural problems that occur in response to captive can be identified and managed. For this aspect, the effects of capture, transit and maintenance should also be considered and alternatives tested separately.

6.7 Overview

In this thesis I provide insight into the effect of floral resource availability on bumblebee ecology. Diet has important consequences for bumblebee reproductive fitness, but in wild populations, the relationship between floral resource availability and bumblebee health is not straightforward. Rather, it seems that the plant community is part of a range of large-scale landscape characteristics that affect individual and colony health, and the transmission of bumblebee pathogens. These results suggest bumblebees living in landscapes in which nutrients are abundant may be differentially affected by nutritional stress and pathogen pressure than bees living in resource-restricted, homogenous landscapes. Interspecific differences in bumblebee response to land use change demonstrates the importance of understanding the ecology and behaviour of different species and not relying so heavily on highly competitive model species. Identifying how landscape characteristics and host nutritional status affects bee health and immunity will support the development of cost-effective conservation schemes that benefit the range of diverse bumblebee species needed for the maintenance of plant communities and the provision of ecosystem services.

References

Abrahamovich, A. H. and B. Díaz (2002). Bumble bees of the neotropical region (Hymenoptera: Apidae). *Biota Colomb.* **3**(2): 199-214.

Adler, L. S., K. M. Michaud, S. P. Ellner, S. H. McArt, P. C. Stevenson and R. E. Irwin (2018). Disease where you dine: plant species and floral traits associated with pathogen transmission in bumble bees. *Ecology* **99**(11): 2535-2545.

Alaux, C., F. Ducloz, D. Crauser and Y. Le Conte (2010). Diet effects on honeybee immunocompetence. *Biol. Lett.* **6**(4): 562-565.

Alford, D. (1970a). The incipient stages of development of bumblebee colonies. *Insectes Soc.* **17**(1): 1-10.

Alford, D. (1970b). The production of adults in incipient colonies of *Bombus agrorum* (F.)(Hymenoptera: *Bombidae*). *Proc. Royal Entomol. Soc.* **45**(1-3): 6-13.

Alford, D. V. (1975). *Bumblebees*. London, Davis-Poynter.

Almanza, M., D. Rojas, J. Cure and D. Wittmann (2006). Modeling colony dynamics of *Bombus atratus*: factors affecting pollination management of Lulo *Solanum quitoense,* Anais do encontro sobre Abelhas, 850p. CDROM.

Alton K, R. F. (2013). To bee or not to bee. *Biologist* **60**: 12-15.

Anderson, R. M. and R. M. May (1981). The population dynamics of microparasites and their invertebrate hosts. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **291**(1054): 451-524.

Ankeny, R. A. and S. Leonelli (2011). What's so special about model organisms? *Studies in History and Philosophy of Science Part A* **42**(2): 313-323.

Arbetman, M. P., G. Gleiser, C. L. Morales, P. Williams and M. A. Aizen (2017). Global decline of bumblebees is phylogenetically structured and inversely related to species range size and pathogen incidence. *Proc. Royal Soc. B* **284**(1859): 20170204.

Arrese, E. L. and J. L. Soulages (2010). Insect fat body: energy, metabolism, and regulation. *Annu. Rev. Entomol.* **55**: 207-225.

Aupinel, P., A. Genissel, J.-N. Tasei, J. Poncet and S. Gomond (2000). *Collection of spring pollens by Bombus terrestris queens. Assessment of attractiveness and nutritive value of pollen diets*. VIII International Symposium on Pollination-Pollination: Integrator of Crops and Native Plant Systems 561.

Ayres, J. S. and D. S. Schneider (2009). The role of anorexia in resistance and tolerance to infections in *Drosophila*. *PLoS Biology* **7**(7): e1000150.

Ayres, J. S. and D. S. Schneider (2012). Tolerance of infections. *Annu. Rev. Immunol.* **30**: 271-294.

Bailey, E. (1975). Biochemistry of insect flight. *Insect biochemistry and function*, Springer**:** 89-176.

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

Baldock, K. C., M. A. Goddard, D. M. Hicks, W. E. Kunin, N. Mitschunas, H. Morse, L. M. Osgathorpe, S. G. Potts, K. M. Robertson and A. V. Scott (2019). A systems approach reveals urban pollinator hotspots and conservation opportunities. *Nat. Ecol. Evol.* **3**(3): 363.

Baldock, K. C. R., M. A. Goddard, D. M. Hicks, W. E. Kunin, N. Mitschunas, L. M. Osgathorpe, S. G. Potts, K. M. Robertson, A. V. Scott, G. N. Stone, I. P. Vaughan and J. Memmott (2015). Where is the UK's pollinator biodiversity? The importance of urban areas for flower-visiting insects. *Proc. Royal Soc. B* **282**(1803).

Baloglu, G. H. and F. Gurel (2015). The effects of pollen protein content on colony development of the bumblebee, *Bombus terrestris* L. *J Apic Sci* **59**(1): 83-88.

Baracchi, D., M. J. Brown and L. Chittka (2015). Weak and contradictory effects of self-medication with nectar nicotine by parasitized bumblebees. *F1000Res* **4**(73).

Barrow, D. and R. Pickard (1984). Size-related selection of food plants by bumblebees. *Ecol. Entomol.* **9**(4): 369-373.

Barrow, D. and R. Pickard (1985). Larval temperature in brood clumps of *Bombus pascuorum* (Scop.). *J. Apic. Res.* **24**(2): 69-75.

Batra, L. R., S. W. Batra and G. Bohart (1973). The mycoflora of domesticated and wild bees (Apoidea). *Mycopathologia et Mycologia applicata* **49**(1): 13-44.

Baude, M., W. E. Kunin, N. D. Boatman, S. Conyers, N. Davies, M. A. Gillespie, R. D. Morton, S. M. Smart and J. Memmott (2016). Historical nectar assessment reveals the fall and rise of floral resources in Britain. *Nature* **530**(7588): 85-88.

Beattie, A. J., C. Turnbull, R. B. Knox and E. G. Williams (1984). Ant inhibition of pollen function: a possible reason why ant pollination is rare. *Am. J. Bot.* **71**(3): 421-426.

Behmer, S. T. (2009). Insect herbivore nutrient regulation. *Annu. Rev. Entomol.* **54**: 165-187.

Bernhardt, P. (2000). Convergent evolution and adaptive radiation of beetle-pollinated angiosperms. *Plant Syst. Evol.* **222**(1-4): 293-320.

Biesmeijer, J. C., S. P. M. Roberts, M. Reemer, R. Ohlemüller, M. Edwards, T. Peeters, A. P. Schaffers, S. G. Potts, R. Kleukers, C. D. Thomas, J. Settele and W. E. Kunin (2006). Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science* **313**(5785): 351-354.

Biller, O. M., L. S. Adler, R. E. Irwin, C. McAllister and E. C. Palmer-Young (2015). Possible synergistic effects of thymol and nicotine against *Crithidia bombi* parasitism in bumble bees. *PLoS One* **10**(12): e0144668.

Blackstock, T., C. Rimes, D. Stevens, R. Jefferson, H. Robertson, J. Mackintosh and J. Hopkins (1999). The extent of semi-natural grassland communities in lowland England and Wales: a review of conservation surveys 1978-96. *Grass Forage Sci.* **54**(1): 1-18.

Blaker, E. A., J. P. Strange, R. R. James, F. P. Monroy and N. S. Cobb (2014). PCR reveals high prevalence of non/low sporulating *Nosema bombi* (microsporidia) infections in bumble bees (*Bombus*) in Northern Arizona. *J. Invertebr.* **123**: 25-33.

Bosmans, L., M. Pozo, C. Verreth, S. Crauwels, L. Wilberts, I. Sobhy, F. Wäckers, H. Jacquemyn and B. Lievens (2018). Habitat-specific variation in gut microbial communities and pathogen prevalence in bumblebee queens (*Bombus terrestris*). *PLoS One* **13**(10): e0204612.

Brantjes, N. B. M. (1973). Sphingophilous flowers, function of their scent. *Pollination and dispersal*. N. B. M. Brantjes and H. F. Linskens. Nijmegan, Netherlands, University of Nijmegan**:** 27-46.

Brian, A. D. (1957). Differences in the flowers visited by four species of bumble-bees and their causes. *J. Anim. Ecol.* **26**: 71-98.

Brown, M. (2017). Microsporidia: an emerging threat to bumblebees? *Trends Parasitol.* **33**(10): 754-762.

Brown, M., R. Loosli and P. Schmid-Hempel (2000). Condition-dependent expression of virulence in a trypanosome infecting bumblebees. *Oikos* **91**(3): 421-427.

Brown, M., Y. Moret and P. Schmid-Hempel (2003b). Activation of host constitutive immune defence by an intestinal trypanosome parasite of bumble bees. *Parasitology* **126**(03): 253-260.

Brown, M. J., R. Schmid-Hempel and P. Schmid-Hempel (2003a). Strong context-dependent virulence in a host–parasite system: reconciling genetic evidence with theory. *J. Anim. Ecol.* **72**(6): 994-1002.

Brunner, F. S., P. Schmid-Hempel and S. M. Barribeau (2014). Protein-poor diet reduces host-specific immune gene expression in *Bombus terrestris*. *Proc. R. Soc. B* **281**(1786): 20140128.

Bučánková, A., O. Komzáková, T. Cholastová and V. Ptáček (2014). Notes on distribution of *Bombus cryptarum* (Hymenoptera, Apoidea) in Moravian territory (Czech Republic) and its laboratory rearing. *Acta Univ. Agric. et Silvic. Mendel. Brun.* **59**(6): 69-74.

Bučánková, A. and V. Ptáček (2010). Experiences in rearing of *Bombus lucorum L.*(Hymenoptera: Apoidea) in captivity. *Úroda* **12**: 621-624.

Bučánková, A. and V. Ptáček (2012). A test of *Bombus terrestris* cocoon and other common methods for nest initiation in *B. lapidarius* and *B. hortorum*. *J Apic Sci* **56**(2): 37-47.

Buchmann, S. L. (1983). Buzz pollination in angiosperms. *Handbook of experimental pollination biology*. C. Jones, Little, R. New York, Van Nostrand-Rheinhold**:** 73-113.

Buchmann, S. L. (1986). Vibratile pollination in *Solanum* and *Lycopersicon*: a look at pollen chemistry. *Solanaceae: biology and systematics*. D. A. W, Columbia University Press, New York**:** 237-252.

Bullock, J. M., R. G. Jefferson, T. H. Blackstock, R. J. Pakeman, B. A. Emmett, R. J. Pywell, J. P. Grime and J. Silvertown (2011). Semi-natural grasslands. *UK National Ecosystem Assessment*.

Bundy, D. A. and M. H. Golden (1987). The impact of host nutrition on gastrointestinal helminth populations. *Parasitology* **95**(3): 623-635.

Burghardt, K. T., D. W. Tallamy, C. Philips and K. J. Shropshire (2010). Non-native plants reduce abundance, richness, and host specialization in lepidopteran communities. *Ecosphere* **1**(5): 1-22.

Burkle, L. A., J. C. Marlin and T. M. Knight (2013). Plant-pollinator interactions over 120 years: Loss of species, co-occurrence, and function. *Science* **339**(6127): 1611-1615.

Cameron, S. A. (1989). Temporal patterns of division of labor among workers in the primitively eusocial bumble bee, *Bombus griseocollis* (Hymenoptera: Apidae) 1. *Ethology* **80**(1-4): 137-151.

Cameron, S. A., H. Hines and P. Williams (2007). A comprehensive phylogeny of the bumble bees (*Bombus*). *Biol. J. Linn. Soc.* **91**(1): 161-188.

Cameron, S. A., J. D. Lozier, J. P. Strange, J. B. Koch, N. Cordes, L. F. Solter and T. L. Griswold (2011). Patterns of widespread decline in North American bumble bees. *Proc. Natl. Acad. Sci. U.S.A.* **108**(2): 662-667.

Campos, M. G., S. Bogdanov, L. B. de Almeida-Muradian, T. Szczesna, Y. Mancebo, C. Frigerio and F. Ferreira (2008). Pollen composition and standardisation of analytical methods. *J. Apic. Res.* **47**(2): 154-161.

Cane, J. H. (1987). Estimation of bee size using intertegular span (Apoidea). *J. Kans. Entomol. Soc.* **60**(1): 144-147.

Cane, J. H. (2016). Adult pollen diet essential for egg maturation by a solitary *Osmia* bee. *J. Insect Physiol.* **95**: 105-109.

Cardoza, Y. J., G. K. Harris and C. M. Grozinger (2012). Effects of soil quality enhancement on pollinator-plant interactions. *Psyche* **2012**: 8.

Cariveau, D. P., G. K. Nayak, I. Bartomeus, J. Zientek, J. S. Ascher, J. Gibbs and R. Winfree (2016). The allometry of bee proboscis length and its uses in ecology. *PLoS ONE* **11**(3): e0151482.

Carvell, C., A. F. Bourke, S. Dreier, S. N. Freeman, S. Hulmes, W. C. Jordan, J. W. Redhead, S. Sumner, J. Wang and M. S. Heard (2017). Bumblebee family lineage survival is enhanced in high-quality landscapes. *Nature* **543**(7646): 547.

Carvell, C., A. F. G. Bourke, J. L. Osborne and M. S. Heard (2015). Effects of an agri-environment scheme on bumblebee reproduction at local and landscape scales. *Basic Appl. Ecol.* **16**(6): 519-530.

Carvell, C., W. R. Meek, R. F. Pywell, D. Goulson and M. Nowakowski (2007). Comparing the efficacy of agri-environment schemes to enhance bumble bee abundance and diversity on arable field margins. *J. Appl. Ecol.* **44**(1): 29-40.

Carvell, C., J. L. Osborne, A. F. G. Bourke, S. N. Freeman, R. F. Pywell and M. S. Heard (2011). Bumble bee species' responses to a targeted conservation measure depend on landscape context and habitat quality. *Ecol. Appl.* **21**(5): 1760-1771.

Carvell, C., D. B. Roy, S. M. Smart, R. F. Pywell, C. D. Preston and D. Goulson (2006). Declines in forage availability for bumblebees at a national scale. *Biol. Conserv.* **132**(4): 481-489.

Cassill, D. L. and W. R. Tschinkel (1999). Regulation of diet in the fire ant, *Solenopsis invicta*. *J. Insect Behav.* **12**(3): 307-328.

Chiang, C., I. Sung, K. Ho and P. Yang (2009). *Colony development of two bumblebees, Bombus eximius and B. sonani, reared in captivity in a subtropical area of Taiwan (Hymenoptera, Apidae, Bombini)*. 13th Asian Apicultural Association Conference.

Chittka, L., J. Spaethe, A. Schmidt and A. Hickelsberger (2001). Adaptation, constraint, and chance in the evolution of flower color and pollinator color vision. *Cognitive Ecology of Pollination: Animal Behaviour and Floral Evolution*. L. Chittka and J. D. Thomson. the Pitt Building/ Trumpington St/Cambridge CB2 1RP/Cambs, England, Cambridge Univ Press**:** 106-126.

Chown, S. L. and S. Nicolson (2004). *Insect physiological ecology: mechanisms and patterns*, Oxford University Press.

Cisarovsky, G. and P. Schmid-Hempel (2014). Combining laboratory and field approaches to investigate the importance of flower nectar in the horizontal transmission of a bumblebee parasite. *Entomol. Exp. Appl.* **152**(3): 209-215.

Clement, S. L., B. C. Hellier, L. R. Elberson, R. T. Staska and M. A. Evans (2007). Flies (Diptera: Muscidae: Calliphoridae) are efficient pollinators of *Allium ampeloprasum* L. (Alliaceae) in field cages. *J Econ Entomol* **100**(1): 131-135.

Cnaani, J., J. D. Thomson and D. R. Papaj (2006). Flower choice and learning in foraging bumblebees: Effects of variation in nectar volume and concentration. *Ethology* **112**: 278-285.

Colla, S. R., M. C. Otterstatter, R. J. Gegear and J. D. Thomson (2006). Plight of the bumble bee: Pathogen spillover from commercial to wild populations. *Biol. Conserv.* **129**(4): 461-467.

Conroy, T. J., E. C. Palmer-Young, R. E. Irwin and L. S. Adler (2016). Food Limitation Affects Parasite Load and Survival of *Bombus impatiens* (Hymenoptera: Apidae)

Infected With *Crithidia* (Trypanosomatida: Trypanosomatidae). *Environ. Entomol.*: 99.

Cook, S. C., M. D. Eubanks, R. E. Gold and S. T. Behmer (2010). Colony-level macronutrient regulation in ants: mechanisms, hoarding and associated costs. *Animal Behaviour* **79**(2): 429-437.

Corbet, S. A. (2006). A typology of pollination systems: implications for crop management and the conservation of wild plants. *Plant–pollinator interactions: from specialization to generalization*, University of Chicago Press**:** 315-340.

Corbet, S. A., I. H. Williams and J. L. Osborne (1991). Bees and the pollination of crops and wild flowers in the European Community. *Bee World* **72**(2): 47-59.

Costa, C., M. Lodesani and L. Maistrello (2010). Effect of thymol and resveratrol administered with candy or syrup on the development of *Nosema ceranae* (*Apis mellifera*) artificially infected honeybees. *Apidologie* **41**(2): 141-150.

Cox, P. A. (1984). Chiropterophily and ornithophily in *Freycinetia* (Pandanaceae) in Samoa. *Plant Syst. Evol.* **144**(3-4): 277-290.

Cunningham-Rundles, S., D. F. McNeeley and A. Moon (2005). Mechanisms of nutrient modulation of the immune response. *J. Allergy Clin. Immunol.* **115**(6): 1119-1128.

Dadd, R. (1973). Insect nutrition: current developments and metabolic implications. *Annu. Rev. Entomol.* **18**(1): 381-420.

Dadd, R. (1985). Nutrition: organisms. *Comprehensive insect physiology, biochemistry and pharmacology* **4**: 313-390.

Daly, J. and R. Jones (2017). The use of reproductive technologies in breeding programs for elasmobranchs in aquaria. *The Elasmobranch Husbandry Manual II: recent advances in the care of sharks, rays and their relatives. Columbus: Ohio Biological Survey*: 363-374.

Danforth, B. N., S. Cardinal, C. Praz, E. A. Almeida and D. Michez (2013). The impact of molecular data on our understanding of bee phylogeny and evolution. *Annu. Rev. Entomol.* **58**: 57-78.

Darvill, B., M. E. Knight and D. Goulson (2004). Use of genetic markers to quantify bumblebee foraging range and nest density. *Oikos* **107**(3): 471-478.

de Groot, A. P. (1953). *Protein and amino acid requirements of the honeybee (Apis Mellifica L.)*, W. Junk.

DeBlock, M. and R. Stoks (2008). Short-term larval food stress and associated compensatory growth reduce adult immune function in a damselfly. *Ecol. Entomol.* **33**(6): 796-801.

Delaplane, K. S. and D. F. Mayer (2000). *Crop pollination by bees*. New York, CABI Publishing.

Den Boer, S. P. A. and M. Duchateau (2006). A larval hunger signal in the bumblebee *Bombus terrestris*. *Insectes Soc.* **53**(3): 369-373.

Desneux, N., A. Decourtye and J.-M. Delpuech (2007). The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.* **52**(1): 81-106.

Di Pasquale, G., M. Salignon, Y. Le Conte, L. P. Belzunces, A. Decourtye, A. Kretzschmar, S. Suchail, J.-L. Brunet and C. Alaux (2013). Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? *PLoS One* **8**(8): e72016.

Dobson, H. E. M., J. Arroyo, G. Bergström and I. Groth (1997). Interspecific variation in floral fragrances within the genus *Narcissus* (Amaryllidaceae). *Behav. Ecol. Sociobiol.* **25**(8): 685-706.

Dobson, H. E. M. and G. Bergström (2000). The ecology and evolution of pollen odors. *Plant Syst. Evol.* **222**(1-4): 63-87.

Dorsett, D. A. (1962). Preparation for flight by hawk-moths. *Journal of Experimental Biology* **39**(4): 579-588.

Doums, C., Y. Moret, E. Benelli and P. Schmid-Hempel (2002). Senescence of immune defence in *Bombus* workers. *Ecol. Entomol.* **27**(2): 138-144.

Dow, J. (1986). Advances in insect physiology. *Insect midgut function* **19**: 187-328.

Duchateau, M. (1985). Analysis of some methods for rearing bumblebee colonies. *Apidologie* **16**: 225-227.

Durrer, S. and P. Schmid-Hempel (1994). Shared use of flowers leads to horizontal pathogen transmission. *Proc. Royal Soc. B* **258**: 299-302.

Durrer, S. and P. Schmid-Hempel (1995). Parasites and the regional distribution of bumblebee species. *Ecography* **18**(2): 114-122.

Dussutour, A. and S. J. Simpson (2009). Communal nutrition in ants. *Curr. Biol.* **19**(9): 740-744.

Dutton, E. M. and M. E. Frederickson (2012). Why ant pollination is rare: new evidence and implications of the antibiotic hypothesis. *Arthropod-Plant Interactions* **6**(4): 561-569.

Edwards, M. and P. Williams (2004). Where have all the bumblebees gone, and could they ever return? *Br. Wildl.* **15**: 305-312.

Eiri, D. M., G. Suwannapong, M. Endler and J. C. Nieh (2015). *Nosema ceranae* can infect honey bee larvae and reduces subsequent adult longevity. *PLoS One* **10**(5): e0126330.

Ellis, J., M. E. Knight, B. Darvill and D. Goulson (2006). Extremely low effective population sizes, genetic structuring and reduced genetic diversity in a threatened bumblebee species, *Bombus sylvarum* (Hymenoptera: Apidae). *Mol. Ecol.* **15**(14): 4375-4386.

Ellis, J. and S. Shackley (1997). The reproductive biology of *Scyliorhinus canicula* in the Bristol Channel, UK. *J. Fish Biol.* **51**(2): 361-372.

Erler, S., M. Popp, S. Wolf and H. M. G. Lattorff (2012). Sex, horizontal transmission, and multiple hosts prevent local adaptation of *Crithidia bombi*, a parasite of bumblebees (*Bombus* spp.). *Ecol. Evol.* **2**(5): 930-940.

Evans, E., I. Burns and M. Spivak (2007). *Befriending bumble bees: a practical guide to raising local bumble bees*, St. Paul, MN: University of Minnesota Extension Service.

Falk, S. J. (2015). *Field guide to the bees of Great Britain and Ireland*, British Wildlife Publishing.

Fandiño, M. T. A. (2007). *Management of Bombus atratus bumblebees to pollinate lulo (Solanum quitoense L), a native fruit from the Andes of Colombia*, Cuvillier Verlag.

Fenoy, S., C. Rueda, M. Higes, R. Martín-Hernández and C. Del Aguila (2009). High-level resistance of *Nosema ceranae*, a parasite of the honeybee, to temperature and desiccation. *Appl. Environ. Microbiol.* **75**(21): 6886-6889.

Figueroa, L. L., M. Blinder, C. Grincavitch, A. Jelinek, E. K. Mann, L. A. Merva, L. E. Metz, A. Y. Zhao, R. E. Irwin and S. H. McArt (2019). Bee pathogen transmission dynamics: deposition, persistence and acquisition on flowers. *Proc. Royal Soc. B* **286**(1903): 20190603.

Fisher, R. M. (1988). Observations on the behaviours of three European cuckoo bumble bee species (*Psithyrus*). *Insectes Sociaux* **35**(4): 341-354.

Flatt, T. (2011). Survival costs of reproduction in *Drosophila*. *Exp Gerontol* **46**(5): 369-375.

Fliszkiewicz, M. and Z. a. Wilkaniec (2007). Fatty acids and amino acids in the fat body of bumblebee *Bombus terrestris* (L.) in diapausing and non-diapausing queens. *J Apic Sci* **51**: 55-63.

Folly, A. J., H. Koch, P. C. Stevenson and M. J. Brown (2017). Larvae act as a transient transmission hub for the prevalent bumblebee parasite *Crithidia bombi*. *J. Invertebr.* **148**: 81-85.

Fontenille, D. and F. Simard (2004). Unravelling complexities in human malaria transmission dynamics in Africa through a comprehensive knowledge of vector populations. *Comp. Immunol., Microbiol. Infect. Dis.* **27**(5): 357-375.

Forister, M. L., E. M. Pelton and S. H. Black (2019). Declines in insect abundance and diversity: We know enough to act now. *Conserv. Sci. Practice*: e80.

Forsman, A. and L. Lindell (1991). Trade-off between growth and energy storage in male *Vipera berus* (L.) under different prey densities. *Funct. Ecol.*: 717-723.

Fouks, B. and H. M. G. Lattorff (2011). Recognition and avoidance of contaminated flowers by foraging bumblebees (*Bombus terrestris*). *PLoS ONE* **6**(10): e26328.

Fox, R. (2013). The decline of moths in Great Britain: a review of possible causes. *Insect Conservation and Diversity* **6**(1): 5-19.

Friend, W. and R. Dadd (1982). Insect nutrition a comparative perspective. *Adv. Nutr. Res.*, Springer**:** 205-247.

Fries, I., A. De Ruijter, R. J. Paxton, A. J. da Silva, S. B. Slemenda and N. J. Pieniazek (2001). Molecular characterization of *Nosema bombi* (Microsporidia: Nosematidae) and a note on its sites of infection in *Bombus terrestris* (Hymenoptera: Apoidea). *J. Apic. Res.* **40**(3-4): 91-96.

Frison, T. H. (1917). Notes on Bombidae, and on the life history of *Bombus auricomus* Robt. *Ann. Entomol. Soc. Am.* **10**(3): 277-286.

Fuller, R. M. (1987). The changing extent and conservation interest of lowland grasslands in England and Wales: a review of grassland surveys 1930–1984. *Biol. Conserv.* **40**(4): 281-300.

Furst, M. A., D. P. McMahon, J. L. Osborne, R. J. Paxton and M. J. F. Brown (2014). Disease associations between honeybees and bumblebees as a threat to wild pollinators. *Nature* **506**(7488): 364-366.

Fussell, M. and S. A. Corbet (1992). Flower usage by bumblebees: a basis for forage plant management. *J. Appl. Ecol.* **29**: 451-465.

Gabriel, W. N., R. McNuff, S. K. Patel, T. R. Gregory, W. R. Jeck, C. D. Jones and B. Goldstein (2007). The tardigrade *Hypsibius dujardini*, a new model for studying the evolution of development. *Dev. Biol.* **312**(2): 545-559.

Gamboa, G. J., R. L. Foster and K. W. Richards (1987). Intraspecific nest and brood recognition by queens of the bumble bee, *Bombus occidentalis* (Hymenoptera: Apidae). *Can. J. Zool.* **65**(12): 2893-2897.

Gammans, N. (2013). The short-haired bumblebee reintroduction. Project progress report**:** 20.

Garbuzov, M. and F. L. Ratnieks (2014). Listmania: the strengths and weaknesses of lists of garden plants to help pollinators. *Bioscience* **64**(11): 1019-1026.

García, D. and N. P. Chacoff (2007). Scale-dependent effects of habitat fragmentation on hawthorn pollination, frugivory, and seed predation. *Conserv. Biol.* **21**(2): 400-411.

Gardener, M. C. and M. P. Gillman (2001). Analyzing variability in nectar amino acids: composition is less variable than concentration. *J. Chem. Ecol.* **27**(12): 2545-2558.

Garratt, M. P., D. J. Coston, C. Truslove, M. Lappage, C. Polce, R. Dean, J. Biesmeijer and S. G. Potts (2014). The identity of crop pollinators helps target conservation for improved ecosystem services. *Biol. Conserv.* **169**: 128-135.

Gaston, K. J., R. M. Smith, K. Thompson and P. H. Warren (2005). Urban domestic gardens (II): experimental tests of methods for increasing biodiversity. *Biodivers. Conserv.* **14**(2): 395.

Gegear, R. J., M. C. Otterstatter and J. D. Thomson (2005). Does parasitic infection impair the ability of bumblebees to learn flower-handling techniques? *Anim.* **70**(1): 209-215.

Gegear, R. J., M. C. Otterstatter and J. D. Thomson (2006). Bumble-bee foragers infected by a gut parasite have an impaired ability to utilize floral information. *Proc. Royal Soc. B* **273**(1590): 1073-1078.

Genersch, E., C. Yue, I. Fries and J. R. de Miranda (2006). Detection of Deformed wing virus, a honey bee viral pathogen, in bumble bees (*Bombus terrestris* and *Bombus pascuorum*) with wing deformities. *J. Invertebr. Pathol.* **91**(1): 61-63.

Genissel, A., P. Aupinel, C. Bressac, J. N. Tasei and C. Chevrier (2002). Influence of pollen origin on performance of *Bombus terrestris* micro-colonies. *Entomol. Exp. Appl.* **104**(2-3): 329-336.

Ghazoul, J. (2005). Buzziness as usual? Questioning the global pollination crisis. *Trends Ecol. Evol.* **20**(7): 367-373.

Giacomini, J. J., J. Leslie, D. R. Tarpy, E. C. Palmer-Young, R. E. Irwin and L. S. Adler (2018). Medicinal value of sunflower pollen against bee pathogens. *Sci. Rep.* **8**(1): 14394.

Gillespie, S. (2010). Factors affecting parasite prevalence among wild bumblebees. *Ecol. Entomol.* **35**(6): 737-747.

Goulson, D. (2003). *Bumblebees. Behaviour and Ecology*, Oxford University Press.

Goulson, D. (2010). *Bumblebees: Behaviour, Ecology, and Conservation*. Oxford, Oxford University Press.

Goulson, D. and B. Darvill (2004). Niche overlap and diet breadth in bumblebees; are rare species more specialized in their choice of flowers? *Apidologie* **35**(1): 55-63.

Goulson, D., M. E. Hanley, B. Darvill, J. Ellis and M. E. Knight (2005). Causes of rarity in bumblebees. *Biol. Conserv.* **122**(1): 1-8.

Goulson, D., G. C. Lye and B. Darvill (2008). Diet breadth, coexistence and rarity in bumblebees. *Biodivers. Conserv.* **17**(13): 3269-3288.

Goulson, D., E. Nicholls, C. Botías and E. L. Rotheray (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* **347**(6229): 1435.

Goulson, D., S. O'Connor and K. J. Park (2018a). The impacts of predators and parasites on wild bumblebee colonies. *Ecol. Entomol.* **43**(2): 168-181.

Goulson, D., S. O'Connor and K. J. Park (2018b). Causes of colony mortality in bumblebees. *Anim.* **21**(1): 45-53.

Goulson, D., P. Whitehorn and M. Fowley (2012). Influence of urbanisation on the prevalence of protozoan parasites of bumblebees. *Ecol. Entomol.* **37**(1): 83-89.

Goulson, D. and P. Williams (2001). *Bombus hypnorum* (L.)(Hymenoptera: Apidae), a new British bumblebee? *Br. J. Entomol. Nat. Hist* **14**(3): 129-131.

Graystock, P., E. J. Blane, Q. S. McFrederick, D. Goulson and W. O. Hughes (2016). Do managed bees drive parasite spread and emergence in wild bees? *Int. J. Parasitol. Par.* **5**(1): 64-75.

Graystock, P., D. Goulson and W. O. Hughes (2014). The relationship between managed bees and the prevalence of parasites in bumblebees. *PeerJ* **2**: e522.

Graystock, P., D. Goulson and W. O. Hughes (2015). Parasites in bloom: flowers aid dispersal and transmission of pollinator parasites within and between bee species. *Proc. R. Soc. B* **282**(1813): 20151371.

Graystock, P., K. Yates, B. Darvill, D. Goulson and W. O. H. Hughes (2013a). Emerging dangers: Deadly effects of an emergent parasite in a new pollinator host. *J. Invertebr.* **114**(2): 114-119.

Graystock, P., K. Yates, S. E. F. Evison, B. Darvill, D. Goulson and W. O. H. Hughes (2013b). The Trojan hives: pollinator pathogens, imported and distributed in bumblebee colonies. *J. Appl. Ecol.* **50**(5): 1207-1215.

Griffin, R., R. Macfarlane and H. Van Den Ende (1990). *Rearing and domestication of long tongued bumble bees in New Zealand*. VI International Symposium on Pollination 288.

Grixti, J. C., L. T. Wong, S. A. Cameron and C. Favret (2009). Decline of bumble bees (*Bombus*) in the North American Midwest. *Biol. Conserv.* **142**(1): 75-84.

Hall, D. M., G. R. Camilo, R. K. Tonietto, J. Ollerton, K. Ahrné, M. Arduser, J. S. Ascher, K. C. R. Baldock, R. Fowler, G. Frankie, D. Goulson, B. Gunnarsson, M. E. Hanley, J. I. Jackson, G. Langellotto, D. Lowenstein, E. S. Minor, S. M. Philpott, S. G. Potts, M. H. Sirohi, E. M. Spevak, G. N. Stone and C. G. Threlfall (2017). The city as a refuge for insect pollinators. *Conserv. Biol.* **31**(1): 24-29.

Hallmann, C. A., M. Sorg, E. Jongejans, H. Siepel, N. Hofland, H. Schwan, W. Stenmans, A. Müller, H. Sumser and T. Hörren (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS One* **12**(10): e0185809.

Hanife, G. (2006). General principles of insect nutritional ecology. *Trakya Univ J Sci* **7**(1): 53-57.

Hanley, M. E., A. J. Awbi and M. Franco (2014). Going native? Flower use by bumblebees in English urban gardens. *Ann. Bot.* **113**(5): 799-806.

Hanley, M. E., M. Franco, S. Pichon, B. Darvill and D. Goulson (2008). Breeding system, pollinator choice, and variation in pollen quality in British herbaceous plants. *Funct. Ecol.* **22**(4): 592-598.

Hanley, M. E. and D. Goulson (2003). Introduced weeds pollinated by introduced bees: Cause or effect? *Weed Biol. Manag.* **3**: 204-212.

Hansen, R. W. and E. A. Osgood (1983). Insects visiting flowers of wild red raspberry in spruce-fir forested areas of eastern Maine. *Entomol. News* **94**: 147-151.

Hanski, I. (1982). *Communities of bumblebees: testing the core-satellite species hypothesis*. Ann. Zool. Fenn., JSTOR.

Harder, L. and R. Barclay (1994). The functional significance of poricidal anthers and buzz pollination: controlled pollen removal from Dodecatheon. *Funct. Ecol.*: 509-517.

Hasselrot, T. (1952). A new method for starting bumblebee colonies. *Agron. J.* **44**: 218-219.

Heard, M. S., C. Carvell, N. L. Carreck, P. Rothery, O. J. L. and A. F. G. Bourke (2007). Landscape context not patch size determines bumblebee density on flower mixtures sown for agri-environment schemes. *Biol. Lett.* **3**: 638-641.

Heinrich, B. (1974). Pheromone induced brooding behavior in *Bombus vosnesenskii* and *B. edwardsii* (Hymenoptera: Bombidae). *J. Kans. Entomol. Soc.* **47**: 396-404.

Heinrich, B. (1975). Energetics of pollination. *Annu. Rev. Ecol. Evol. Syst.* **6**(1): 139-170.

Heinrich, B. (2004). *Bumblebee economics*, Harvard University Press.

Hendriksma, H. P. and S. Shafir (2016). Honey bee foragers balance colony nutritional deficiencies. *Behav. Ecol. Sociobiol.*: 1-9.

Henson, K. S., P. G. Craze and J. Memmott (2009). The restoration of parasites, parasitoids, and pathogens to heathland communities. *Ecology* **90**(7): 1840-1851.

Herrmann, F., C. Westphal, R. F. Moritz and I. Steffan-Dewenter (2007). Genetic diversity and mass resources promote colony size and forager densities of a social bee (*Bombus pascuorum*) in agricultural landscapes. *Mol. Ecol.* **16**(6): 1167-1178.

Hobbs, G. (1962). Further studies on the food-gathering behaviour of bumble bees (Hymenoptera: Apidae). *The Canadian Entomologist* **94**(5): 538-541.

Hobbs, G. (1964a). Phylogeny of bumble bees based on brood-rearing behaviour. *Can. Entomol.* **96**(1-2): 115-116.

Hobbs, G. (1964b). Ecology of Species of *Bombus* Latr.(Hymenoptera: Apidae) in Southern Alberta. I. Subgenus *Alpinobombus* Skor. *The Canadian Entomologist* **96**(11): 1465-1470.

Hobbs, G., W. Nummi and J. Virostek (1961). Food-gathering behaviour of honey, bumble, and leaf-cutter bees (Hymenoptera: Apoidea) in Alberta. *Can. Entomol.* **93**(6): 409-419.

Hoffmann, W. R., A. Torres and P. Neumann (2004). A scientific note on the nest and colony development of the Neotropical bumble bee *Bombus* (*Robustobombus*) *melaleucus*. *Apidologie* **35**(4): 449-450.

Holloway, B. A. (1976). Pollen-feeding in hover-flies (Diptera: Syrphidae). *N. Z. J. Zool.* **3**: 339-350.

Holm, S. N. (1966). The utilization and management of bumble bees for red clover and alfalfa seed production. *Annu. Rev. Entomol.* **11**(1): 155-182.

Holzschuh, A., I. Steffan-Desenter, D. Kleijn and T. Tscharntke (2007). Diversity of flower-visiting bees in cereal fields: effects of farming system, landscape composition and regional context. *J. Appl. Ecol.* **44**(1): 41-49.

House, H. (1961). Insect nutrition. *Annu. Rev. Entomol.* **6**(1): 13-26.

Howard, D., J. Watkins, R. Clarke, C. Barnett and G. Stark (2003). Estimating the extent and change in broad habitats in Great Britain. *J. Environ. Manage.* **67**(3): 219-227.

Huang, Z. (2012). Pollen nutrition affects honey bee stress resistance. *Terr. Arthropod Rev.* **5**(2): 175-189.

Imhoof, B. and P. Schmid-Hempel (1998). Single-clone and mixed-clone infections versus host environment in *Crithidia bombi* infecting bumblebees. *Parasitology* **117**(04): 331-336.

Imhoof, B. and P. Schmid-Hempel (1999). Colony success of the bumble bee, *Bombus terrestris*, in relation to infections by two protozoan parasites, *Crithidia bombi* and *Nosema bombi*. *Insectes Soc.* **46**(3): 233-238.

Iserbyt, S. and P. Rasmont (2012). The effect of climatic variation on abundance and diversity of bumblebees: a ten years survey in a mountain hotspot. *Ann. Soc. Entomol. Fr.* **48**(3-4): 261-273.

Ito, M., T. Matsumura and S. F. Sakagami (1984). A nest of the Himalayan bumblebee *Bombus* (Festivobombus) *festivus*. *昆蟲* **52**(4): 537-539.

Janse, M., B. Zimmerman, L. Geerlings, C. Brown and L. A. Nagelkerke (2017). Sustainable species management of the elasmobranch populations within

European aquariums: a conservation challenge. *Journal of Zoo and Aquarium Research* **5**(4): 172-181.

Jersáková, J., S. D. Johnson and P. Kindlmann (2006). Mechanisms and evolution of deceptive pollination in orchids. *Biological Reviews* **81**(2): 219-235.

Jervis, M. (1998). Functional and evolutionary aspects of mouthpart structure in parasitoid wasps. *Biol. J. Linn. Soc.* **63**(4): 461-493.

Jie, W., P. Wenjun, A. Jiandong, G. Zhanbao, T. Yueming and L. Jilian (2005). Techniques for year-round rearing of *Bombus terrestris* L.(Hymenoptera, Apoidea) in China. *J Apic Sci* **49**(1): 65-69.

Jones, C. M. and M. J. Brown (2014). Parasites and genetic diversity in an invasive bumblebee. *J. Anim. Ecol.* **83**(6): 1428-1440.

Jones, P. L., M. J. Ryan and L. Chittka (2015). The influence of past experience with flower reward quality on social learning in bumblebees. *Anim.* **101**: 11-18.

Kallioniemi, E., J. Åström, G. M. Rusch, S. Dahle, S. Åström and J. O. Gjershaug (2017). Local resources, linear elements and mass-flowering crops determine bumblebee occurrences in moderately intensified farmlands. *Agr. Ecosyst. Environ.* **239**: 90-100.

Kämper, W., P. K. Werner, A. Hilpert, C. Westphal, N. Blüthgen, T. Eltz and S. D. Leonhardt (2016). How landscape, pollen intake and pollen quality affect colony growth in *Bombus terrestris*. *Landsc. Ecol.* **31**(10): 2245-2258.

Kapahi, P., D. Chen, A. N. Rogers, S. D. Katewa, P. W.-L. Li, E. L. Thomas and L. Kockel (2010). With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. *Cell Metab.* **11**(6): 453-465.

Kato, K. and N. Hijii (1993). Optimal clutch size of the chestnut gall-wasp, *Dryocosmus kuriphilus yasumatsu* (Hymenoptera: Cynipidae). *Popul. Ecol.* **35**(1): 1-14.

Kearns, C. A. (2001). North American dipteran pollinators: assessing their value and conservation status. *Conserv. Ecol.* **5**(1): 5. [online].

Kendle, A. D. and J. E. Rose (2000). The aliens have landed! What are the justifications for ‘native only’ policies in landscape plantings? *Landscape Urban Plan.* **47**(1-2): 19-31.

Kennedy, C. M., E. Lonsdorf, M. C. Neel, N. M. Williams, T. H. Ricketts, R. Winfree, R. Bommarco, C. Brittain, A. L. Burley, D. Cariveau, L. G. Carvalheiro, N. P. Chacoff, S. A. Cunningham, B. N. Danforth, J.-H. Dudenhöffer, E. Elle, H. R.

Gaines, L. A. Garibaldi, C. Gratton, A. Holzschuh, R. Isaacs, S. K. Javorek, S. Jha, A. M. Klein, K. Krewenka, Y. Mandelik, M. M. Mayfield, L. Morandin, L. A. Neame, M. Otieno, M. Park, S. G. Potts, M. Rundlöf, A. Saez, I. Steffan-Dewenter, H. Taki, B. F. Viana, C. Westphal, J. K. Wilson, S. S. Greenleaf and C. Kremen (2013). A global quantitative synthesis of local and landscape effects on wild bee pollinators in agroecosystems. *Ecol.* **16**(5): 584-599.

Kevan, P. G. and H. G. Baker (1983). Insects as flower visitors and pollinators. *Annu. Rev. Entomol.* **28**: 407-453.

Kim, Y. S. and B. H. Smith (2000). Effect of an amino acid on feeding preferences and learning behavior in the honey bee, *Apis mellifera*. *J. Insect Physiol.* **46**(5): 793-801.

Kirchner, W. and J. Röschard (1999). Hissing in bumblebees: an interspecific defence signal. *Insectes Soc.* **46**(3): 239-243.

Klee, J., W. T. Tay and R. J. Paxton (2006). Specific and sensitive detection of *Nosema bombi* (Microsporidia: Nosematidae) in bumble bees (*Bombus* spp.; Hymenoptera: Apidae) by PCR of partial rRNA gene sequences. *J. Invertebr.* **91**(2): 98-104.

Kleijn, D. and I. Raemakers (2008). A retrospective analysis of pollen host plant use by stable and declining bumble bee species. *Ecology* **89**(7): 1811-1823.

Klein, A. M., B. E. Vaissiere, J. H. Cane, I. Steffan-Dewenter, S. A. Cunningham, C. Kremen and T. Tscharntke (2007). Importance of pollinators in changing landscapes for world crops. *Proc. Royal Soc. B* **274**: 303-313.

Klowden, M. J. (2013). *Physiological systems in insects*, Academic press.

Knight, M. E., J. L. Osborne, R. A. Sanderson, R. J. Hale, A. P. Martin and D. Goulson (2009). Bumblebee nest density and the scale of available forage in arable landscapes. *Insect Conserv. Diver.* **2**(2): 116-124.

Knox, D. A., H. Shimanuki and E. Herbert (1971). Diet and the longevity of adult honey bees. *J. Econ. Entomol.* **64**(6): 1415-1416.

Koch, H. and P. Schmid-Hempel (2011). Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *PNAS* **108**(48): 19288-19292.

Koch, J. B. and J. P. Strange (2012). The status of *Bombus occidentalis* and *B. moderatus* in Alaska with special focus on *Nosema bombi* incidence. *Northwest Sci.* **86**(3): 212-220.

Kohler, F., J. Verhulst, R. van Klink and D. Kleijn (2008). At what spatial scale do high-quality habitats enhance the diversity of forbs and pollinators in intensively farmed landscapes? *J. Appl. Ecol.* **45**(3): 753-762.

Konovalova, I. (2007). The first record of the rare oligolectic bumblebee *Bombus gerstaeckeri* Morawitz (Hymenoptera: Apidae: *Bombini*) lrom Ukraine. *Ann. Soc. Entomol. Fr.* **43**(4): 441-443.

Konzmann, S. and K. Lunau (2014). Divergent rules for pollen and nectar foraging bumblebees–a laboratory study with artificial flowers offering diluted nectar substitute and pollen surrogate. *PLoS One* **9**(3): e91900.

Korner, P. and P. Schmid-Hempel (2005). Correlates of parasite load in bumblebees in an Alpine habitat. *Entomol. Sci.* **8**(2): 151-160.

Krenn, H. W. (2010). Feeding mechanisms of adult Lepidoptera: structure, function, and evolution of the mouthparts. *Annu. Rev. Entomol.* **55**: 307-327.

Krenn, H. W., J. D. Plant and N. U. Szucsich (2005). Mouthparts of flower-visiting insects. *Arthropod Struct Dev* **34**(1): 1-40.

Krimmer, E., E. A. Martin, J. Krauss, A. Holzschuh and I. Steffan-Dewenter (2019). Size, age and surrounding semi-natural habitats modulate the effectiveness of flower-rich agri-environment schemes to promote pollinator visitation in crop fields. *Agr. Ecosyst. Environ.* **284**: 106590.

Kristensen, T. N., J. Overgaard, V. Loeschcke and D. Mayntz (2011). Dietary protein content affects evolution for body size, body fat and viability in *Drosophila melanogaster*. *Biol. Lett.* **7**(2): 269-272.

Kunieda, T., T. Fujiyuki, R. Kucharski, S. Foret, S. Ament, A. Toth, K. Ohashi, H. Takeuchi, A. Kamikouchi and E. Kage (2006). Carbohydrate metabolism genes and pathways in insects: insights from the honey bee genome. *Insect Mol. Biol.* **15**(5): 563-576.

Kwon, Y. J., S. Saeed and M. J. Duchateau (2003). Stimulation of colony initiation and colony development in *Bombus terrestris* by adding a male pupa: the influence of age and orientation. *Apidologie* **34**(5): 429-437.

Labandeira, C. C. (1998). How old is the flower and the fly? *Science* **280**(5360): 57-59.

Laroca, S. (1972). Sobre a bionomia de *Bombus brasiliensis* (Hymenoptera, Apoidea). *Acta Biol Parana* **1**.

Laverty, T. M. (1980). The flower-visiting behaviour of bumble bees: floral complexity and learning. *Can. J. Zool.* **58**(7): 1324-1335.

Leonhardt, S. D. and N. Blüthgen (2012). The same, but different: pollen foraging in honeybee and bumblebee colonies. *Apidologie* **43**(4): 449-464.

Lesage, L., M. Crête, J. Huot and J.-P. Ouellet (2001). Evidence for a trade-off between growth and body reserves in northern white-tailed deer. *Oecologia* **126**(1): 30-41.

Lhomme, P., A. Sramkova, K. Kreuter, T. Lecocq, P. Rasmont and M. Ayasse (2013). A method for year-round rearing of cuckoo bumblebees (Hymenoptera: Apoidea: *Bombus* subgenus *Psithyrus*). *Ann. Soc. Entomol. Fr.* **49**(1): 117-125.

Li, X., R. Palmer, J. Trout and R. Fayer (2003). Infectivity of microsporidia spores stored in water at environmental temperatures. *J. Parasitol.* **89**(1): 185-188.

Lichtenberg, E. M., C. M. Kennedy, C. Kremen, P. Batáry, F. Berendse, R. Bommarco, N. A. Bosque-Pérez, L. G. Carvalheiro, W. E. Snyder and N. M. Williams (2017). A global synthesis of the effects of diversified farming systems on arthropod diversity within fields and across agricultural landscapes. *Glob. Change Biol.* **23**(11): 4946-4957.

Lihoreau, M., T. Gómez-Moracho, C. Pasquaretta, J. T. Costa and J. Buhl (2018). Social nutrition: an emerging field in insect science. *Current opinion in insect science* **28**: 73-80.

Lindhard, E. (1912). Humlebien som Husdyr: spredte Traek af nogle danske *Humlebiartes biologi*. *Tidsskr PlAvl* **19**: 319-326.

Lipa, J. T., 0. (1980). *Crithidia bombi* sp. n. a flagellated parasite of a bumble-bee *Bombus terrestris* L.(Hymenoptera, Apidae). *Acta Protozool* **27**: 287-290.

Littlewood, N. A., A. J. Stewart and B. A. Woodcock (2012). Science into practice–how can fundamental science contribute to better management of grasslands for invertebrates? *Insect Conserv. Diver.* **5**(1): 1-8.

LoCascio, G. M., L. Aguirre, R. E. Irwin and L. S. Adler (2019). Pollen from multiple sunflower cultivars and species reduces a common bumblebee gut pathogen. *R. Soc. Open Sci.* **6**(4): 190279.

Logan, A., M. Ruiz-González and M. Brown (2005). The impact of host starvation on parasite development and population dynamics in an intestinal trypanosome parasite of bumble bees. *Parasitology* **130**(06): 637-642.

Loken, A. (1961). Observations on Norwegian bumblebee nests. (Hymenoptera, Apidae, *Bombus*). *Norsk. entom. Tidsskr.* **11**: 255-268.

Loken, A. (1973). Studies on Scandinavian bumblebees (Hymenoptera, Apidae). *Nor. J. Entomol.* **20**: 1-218.

Lopez-Vaamonde, C., J. W. Koning, R. M. Brown, W. C. Jordan and A. F. Bourke (2004). Social parasitism by male-producing reproductive workers in a eusocial insect. *Nature* **430**(6999): 557.

Loram, A., K. Thompson, P. H. Warren and K. J. Gaston (2008). Urban domestic gardens (XII): the richness and composition of the flora in five UK cities. *J. Veg. Science* **19**(3): 321-330.

Lowenstein, D. M., K. C. Matteson and E. S. Minor (2019). Evaluating the dependence of urban pollinators on ornamental, non-native, and 'weedy' floral resources. *Urban Ecosyst.* **22**(2): 293-302.

Lunau, K. (1990). Colour saturation triggers innate reactions to flower signals: Flower dummy experiments with bumblebees. *J. Comp. A* **166**(6): 827-834.

Lunau, K. and E. Maier (1995). Innate colour preferences of flower visitors. *Journal of Comparative Physiology A* **177**(1): 1-19.

Lundgren, J. G. and M. P. Seagraves (2011). Physiological benefits of nectar feeding by a predatory beetle. *Biol. J. Linn. Soc.* **104**(3): 661-669.

MacDonald, M. (2001). The colonization of northern Scotland by *Bombus terrestris* (L.) and *B. lapidarius* (L.)(Hym., Apidae), and the possible role of climate change. *Entomol. Mon. Mag.* **137**: 1-13.

Macfarlane, R. P., J. J. Lipa and H. J. Liu (1995). Bumble bee pathogens and internal enemies. *Bee World* **76**(3): 130-148.

Maddox, J. V. and L. F. Solter (1996). Long-term storage of infective microsporidian spores in liquid nitrogen. *J. Eukaryot. Microbiol.* **43**(3): 221-225.

Majewska, A. A. and S. Altizer (2018). Planting gardens to support insect pollinators. *Conserv. Biol.* **2019**.

Malone, L. A., H. S. Gatehouse and E. L. Tregidga (2001). Effects of time, temperature, and honey on *Nosema apis* (Microsporidia: Nosematidae), a parasite of the honeybee, *Apis mellifera* (Hymenoptera: Apidae). *J. Invertebr.* **77**(4): 258-268.

Mandelik, Y., R. Winfree, T. Neeson and C. Kremen (2012). Complementary habitat use by wild bees in agro-natural landscapes. *Ecol. Appl.* **22**(5): 1535-1546.

Manino, A., F. Marletto, M. Porporato and L. Allais (1994). Research on the rearing of bumblebees in artificial nests. *Ethol. Ecol. Evol.* **3**(Special Issue): 95-99.

Manson, J., M. Otterstatter and J. Thomson (2010). Consumption of a nectar alkaloid reduces pathogen load in bumble bees. *Oecologia* **162**(1): 81-89.

Marden, J. H. (1984). Remote perception of floral nectar by bumblebees. *Oecologia* **64**: 232-240.

Martín-Hernández, R., A. Meana, L. Prieto, A. M. Salvador, E. Garrido-Bailón and M. Higes (2007). Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. *Appl. Environ. Microbiol.* **73**(20): 6331-6338.

Martinet, B., P. Rasmont, B. Cederberg, D. Evrard, F. Ødegaard, J. Paukkunen and T. Lecocq (2015). *Forward to the north: two Euro-Mediterranean bumblebee species now cross the Arctic Circle*. Ann. Soc. Entomol. Fr., Taylor & Francis.

Mayack, C. and D. Naug (2009). Energetic stress in the honeybee *Apis mellifera* from *Nosema ceranae* infection. *J. Invertebr.* **100**(3): 185-188.

McArt, S. H., H. Koch, R. E. Irwin and L. S. Adler (2014). Arranging the bouquet of disease: floral traits and the transmission of plant and animal pathogens. *Ecol.* **17**(5): 624-636.

McAulay, M. and J. Forrest (2019). How do sunflower pollen mixtures affect survival of queenless microcolonies of bumblebees (*Bombus impatiens*)? *Arthropod-Plant Inte.* **13**(3): 517-529.

McCallum, K. P., F. O. McDougall and R. S. Seymour (2013). A review of the energetics of pollination biology. *J. Comp. B* **183**(7): 867-876.

McFrederick, Q. S. and G. LeBuhn (2006). Are urban parks refuges for bumble bees *Bombus* spp. (Hymenoptera: Apidae)? *Biol. Conserv.* **129**(3): 372-382.

McFrederick, Q. S., W. T. Wcislo, D. R. Taylor, H. D. Ishak, S. E. Dowd and U. G. Mueller (2012). Environment or kin: whence do bees obtain acidophilic bacteria? *Molecular ecology* **21**(7): 1754-1768.

McIvor, C. A. and L. A. Malone (1995). *Nosema bombi*, a microsporidian pathogen of the bumble bee *Bombus terrestris* (L.). *New Zeal. J. Zool.* **22**(1): 25-31.

Medzhitov, R., D. S. Schneider and M. P. Soares (2012). Disease tolerance as a defense strategy. *Science* **335**(6071): 936-941.

Meeus, I., D. De Graaf, K. Jans and G. Smagghe (2010). Multiplex PCR detection of slowly-evolving trypanosomatids and neogregarines in bumblebees using broad-range primers. *J. Appl. Microbiol.* **109**(1): 107-115.

METOffice. (2018). from https://www.metoffice.gov.uk/climate/uk/summaries/2018/june.

Michener, C. (2007). The Bees of the World. Johns Hopkins University, Baltimore.

Mitra, B. and D. Banerjee (2007). Fly pollinators: assessing their value in biodiversity conservation and food security in India. *Rec Zool Surv India* **107**(1): 33-48pp.

Moerman, R., N. Roger, R. De Jonghe, D. Michez and M. Vanderplanck (2016). Interspecific variation in bumblebee performance on pollen diet: new insights for mitigation strategies. *PLoS One* **11**(12): e0168462.

Moerman, R., M. Vanderplanck, D. Fournier, A. L. Jacquemart and D. Michez (2017). Pollen nutrients better explain bumblebee colony development than pollen diversity. *Insect Conserv. Diver.* **10**(2): 171-179.

Moerman, R., M. Vanderplanck, N. Roger, S. Decleves, B. Wathelet, P. Rasmont, D. Fournier and D. Michez (2015). Growth rate of bumblebee larvae is related to pollen amino acids. *J. Econ. Entomol.*

Moquet, L., C. Mayer, D. Michez, B. Wathelet and A.-L. Jacquemart (2015). Early spring floral foraging resources for pollinators in wet heathlands in Belgium. *J. Insect Conserv.* **19**(5): 837-848.

Moret, Y. and P. Schmid-Hempel (2000). Survival for immunity: the price of immune system activation for bumblebee workers. *Science* **290**(5494): 1166-1168.

Moret, Y. and P. Schmid-Hempel (2001). Entomology: immune defence in bumble-bee offspring. *Nature* **414**(6863): 506.

Moret, Y. and P. Schmid-Hempel (2009). Immune responses of bumblebee workers as a function of individual and colony age: senescence versus plastic adjustment of the immune function. *Oikos* **118**(3): 371-378.

Murawski, D. and L. E. Gilbert (1986). Pollen flow in *Psiguria warscewiczii*: a comparison of *Heliconius* butterflies and hummingbirds. *Oecologia* **68**(2): 161-167.

Murray, T. E., M. F. Coffey, E. Kehoe and F. G. Horgan (2013). Pathogen prevalence in commercially reared bumble bees and evidence of spillover in conspecific populations. *Biol. Conserv.* **159**: 269-276.

Naug, D. and A. Gibbs (2009). Behavioral changes mediated by hunger in honeybees infected with *Nosema ceranae*. *Apidologie* **40**(6): 595-599.

Naumann, I. (1991). Hymenoptera. The Insects of Australia. *Naumann, ID, Carne, PB, Lawerence, JF et al. Melbourne, Melbourne University Press* **2**: 916-1000.

Neff, J. L. and B. B. Simpson (1993). Bees, pollination systems and plant diversity. *Hymenoptera and biodiversity*. J. Lasalle and I. D. Gauld. Wallingford, UK, C and B International**:** 143-167.

Nepi, M. and M. Stpiczyńska (2007). Nectar resorption and translocation in *Cucurbita pepo* L. and *Platanthera chlorantha* Custer (Rchb.). *Plant Biol.* **9**(1): 93-100.

Nicolson, S. W. and R. W. Thornburg (2007). Nectar chemistry. *Nectar and Nectaries*. S. W. Nicolson, M. Nepi and E. Pacini. Dordrecht, Springer**:** 215-264.

Nieto, A., S. Roberts, J. Kemp, P. Rasmont, M. Kuhlmann, C. M. Garcia, J. Biesmeijer, D. H. BoguschpP, P. De La Rua and T. De Meulemeester (2014). European Red List of bees. Publication Office of the European Union, Luxembourg.

Nilsson, S. G., M. Franzén and L. Pettersson (2013). Land-use changes, farm management and the decline of butterflies associated with semi-natural grasslands in southern Sweden. *Nat Conserv* **6**: 31.

Obenchain, F. D. and J. H. Oliver Jr (1973). A qualitative analysis of the form, function and interrelationships of fat body and associated tissues in adult ticks (*Acari-ixodoidea*). *J Exp Zool* **186**(3): 217-235.

Öckinger, E. and H. G. Smith (2007). Semi-natural grasslands as population sources for pollinating insects in agricultural landscapes. *J. Appl. Ecol.* **44**(1): 50-59.

Ollerton, J. (2017). Pollinator diversity: distribution, ecological function, and conservation. *Annual Review of Ecology, Evolution, and Systematics* **48**: 353-376.

Ollerton, J., H. Erenler, M. Edwards and R. Crockett (2014). Extinctions of aculeate pollinators in Britain and the role of large-scale agricultural changes. *Science* **346**(6215): 1360-1362.

Ollerton, J., R. Winfree and S. Tarrant (2011). How many flowering plants are pollinated by animals? *Oikos* **120**(3): 321-326.

Orford, K. A., I. P. Vaughan and J. Memmott (2015). *The forgotten flies: the importance of non-syrphid Diptera as pollinators*.

Osborne, J. L., A. P. Martin, C. R. Shortall, A. D. Todd, D. Goulson, M. E. Knight, R. J. Hale and R. A. Sanderson (2008). Quantifying and comparing bumblebee nest densities in gardens and countryside habitats. *J. Appl. Ecol.* **45**(3): 784-792.

Otterstatter, M. C., R. J. Gegear, S. R. Colla and J. D. Thomson (2005). Effects of parasitic mites and protozoa on the flower constancy and foraging rate of bumble bees. *Behav. Ecol. Sociobiol.* **58**(4): 383-389.

Otterstatter, M. C. and J. D. Thomson (2007). Contact networks and transmission of an intestinal pathogen in bumble bee (*Bombus impatiens*) colonies. *Oecologia* **154**(2): 411-421.

Otterstatter, M. C. and J. D. Thomson (2008). Does pathogen spillover from commercially reared bumble bees threaten wild pollinators? *PLoS One* **3**(7): e2771.

Otti, O. and P. Schmid-Hempel (2007). *Nosema bombi:* a pollinator parasite with detrimental fitness effects. *J. Invertebr.* **96**(2): 118-124.

Palmer-Young, E. C. (2017). Pollen extracts increase growth of a trypanosome parasite of bumble bees. *PeerJ Preprints* **5**: e2730v2731.

Palmer-Young, E. C., B. M. Sadd, P. C. Stevenson, R. E. Irwin and L. S. Adler (2016). Bumble bee parasite strains vary in resistance to phytochemicals. *Sci. Rep.* **6**.

Parmigiani, S. and F. S. Vom Saal (1994). *Infanticide and parental care*, Taylor & Francis.

Patel, N. G. (1971). Protein synthesis during insect development. *Insect Biochemistry* **1**(4): 391-427.

Paull, S. H., S. Song, K. M. McClure, L. C. Sackett, A. M. Kilpatrick and P. T. Johnson (2012). From superspreaders to disease hotspots: linking transmission across hosts and space. *Front. Ecol. Environ.* **10**(2): 75-82.

Pauw, A. (2007). Collapse of a pollination web in small conservation areas. *Ecology* **88**(7): 1759-1769.

Peakall, R., A. Beattie and S. James (1987). Pseudocopulation of an orchid by male ants: a test of two hypotheses accounting for the rarity of ant pollination. *Oecologia* **73**(4): 522-524.

Peat, J. and D. Goulson (2005). Effects of experience and weather on foraging efficiency and pollen versus nectar collection in the bumblebee, *Bombus terrestris*. *Behav. Ecol. Sociobiol.* **58**: 152-156.

Peeters, T. M. (2012). Bijen: *Bombus hommels*. *Natuur van Nederland* **11**(1): 250-276.

Pereboom, J. (2000). The composition of larval food and the significance of exocrine secretions in the bumblebee *Bombus terrestris*. *Insectes Soc.* **47**(1): 11-20.

Pereboom, J., H. Velthuis and M. Duchateau (2003). The organisation of larval feeding in bumblebees (Hymenoptera, Apidae) and its significance to caste differentiation. *Insectes Soc.* **50**(2): 127-133.

Phillips, B. B., R. F. Shaw, M. J. Holland, E. L. Fry, R. D. Bardgett, J. M. Bullock and J. L. Osborne (2018). Drought reduces floral resources for pollinators. *Glob. Change Biol.* **24**(7): 3226-3235.

Pinto da Silva, C., A. Silva, G. Duran Cordeiro and I. Alves dos Santos (2015). The body size of the oil-collecting bee *Tetrapedia diversipes* (Apidae).

Piot, N., I. Meeus, D. Kleijn, J. Scheper, T. Linders and G. Smagghe (2019). Establishment of wildflower fields in poor quality landscapes enhances micro-parasite prevalence in wild bumble bees. *Oecologia* **189**(1): 149-158.

Plath, O. (1927). The natural grouping of the Bremidæ (Bombidæ) with special reference to biological characters. *The Biological Bulletin* **52**(5): 394-410.

Plowright, R. and S. Jay (1977). On the size determination of bumble bee castes (Hymenoptera: Apidae). *Can. J. Zool.* **55**(7): 1133-1138.

Plowright, R. C. and S. C. Jay (1966). Rearing bumble bee colonies in captivity. *J. Apic. Res.* **5**(3): 155-165.

Pomeroy, N. and R. Plowright (1979). Larval ejection following narcosis of bumble bees (Hymenoptera: Apidae). *J. Kans. Entomol. Soc.* **52**: 215-217.

Pomeroy, N. and R. Plowright (1980). Maintenance of bumble bee colonies in observation hives (Hymenoptera: Apidae). *Can. Entomol.* **112**(3): 321-326.

Ponton, F., K. Wilson, S. C. Cotter, D. Raubenheimer and S. J. Simpson (2011). Nutritional immunology: a multi-dimensional approach. *PLoS Pathogens* **7**(12): e1002223.

Ponton, F., K. Wilson, A. J. Holmes, S. C. Cotter, D. Raubenheimer and S. J. Simpson (2013). Integrating nutrition and immunology: a new frontier. *J. Insect Physiol.* **59**(2): 130-137.

Popp, M. and H. M. G. Lattorff (2011). A quantitative in vitro cultivation technique to determine cell number and growth rates in strains of *Crithidia bombi* (Trypanosomatidae), a parasite of bumblebees. *J. Eukaryot. Microbiol.* **58**(1): 7-10.

Porrini, M. P., E. G. Sarlo, S. K. Medici, P. M. Garrido, D. P. Porrini, N. Damiani and M. J. Eguaras (2011). *Nosema ceranae* development in *Apis mellifera*: influence of diet and infective inoculum. *J. Apic. Res.* **50**(1): 35-41.

Potts, S. G., J. C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger and W. E. Kunin (2010). Global pollinator declines: trends, impacts and drivers. *Trends Ecol. Evol.* **25**(6): 345-353.

Potts, S. G., B. Vulliamy, A. Dafni, G. Ne'eman and P. Willmer (2003). Linking bees and flowers: how do floral communities structure pollinator communities? *Ecology* **84**(10): 2628-2642.

Pouvreau, A. (2004). *Les Insectes Pollinisateurs*. Lausanne, Delachaux et Niestle.

Power, A. and A. Flecker (2008). *The role of vector diversity in disease dynamics*, Princeton, NJ: Princeton University Press.

Pradervand, J., L. Pellissier, L. Rossier, A. Dubuis, A. Guisan and D. Cherix (2011). Diversity of bumblebees (*Bombus Latreille*, Apidae) in the Alps of the canton Vaud (Switzerland). *Mitt. Schweiz. Entomol. Ges.* **84**(1/2): 45-66.

Praz, C., J., A. Müller and S. Dorn (2008). Host recognition in a pollen-specialist bee: evidence for a genetic basis. *Apidologie* **39**(5): 547-557.

Proctor, M. and P. Yeo (1973). *The pollination of flowers*. New York, NY, Taplinger Publishing Company.

Prŷs-Jones, P. E. and S. A. Corbet (1991). *Bumblebees*. Slough, Richmond Publishing.

Ptacek, V. (1983). Stimulation of brood rearing of bumblebee queens (Hymenoptera, Bornbidae by contacting honey bees (*Apis melliffera* L.) (preliminary report). *Sb. Ved. Pr. Vyzk. Slechtitelskeho Ustavu Picninorskeho, Troubsko Brna* **8**: 157-161.

Ptacek, V. (1985). Testing three methods of rearing bumble bees (Hymenoptera, Bombinae). *Sb. Ved. Pr. VSUP Troubsko* **9**: 56-57.

Ptacek, V. (2001). *Some biological aspects of bumble bee (Bombus, Hymenoptera) management.* Proceedings of the Eighth International Pollination Symposium: pollination: integrator of crops and native plant systems, ISHS.

Ptáček, V. (2008). *Chov čmeláků v laboratoři*, TRIBUN EU.

Ptácek, V., R. Borovec and E. Pernova (2000). The two-queen cascade method as an alternative technique for starting bumble bee [*Bombus*, Hymenoptera, Apidae] colonies in laboratory conditions: a preliminary study. *Pszczelnicze Zesz. Nauk.* **44**(2): 305-309.

Ptáček, V., A. Votavová and O. Komzáková (2015). Experience in rearing common carder bees (*Bombus pascuorum* Scop.), with some notes on three similar species: shrill carder bee (*B. sylvarum* L.), red-shanked carder bee (*B. ruderarius* Müll.), and brown-banded carder bee (*B. humilis* Ill.)(Hymenoptera: Apidae). *Acta Univ. Agric. et Silvic. Mendel. Brun.* **63**(5): 1535-1542.

Pyle, R., M. Bentzien and P. Opler (1981). Insect conservation. *Annu. Rev. Entomol.* **26**(1): 233-258.

Pywell, R., W. Meek, L. Hulmes, S. Hulmes, K. James, M. Nowakowski and C. Carvell (2011). Management to enhance pollen and nectar resources for bumblebees and butterflies within intensively farmed landscapes. *J. Insect Conserv.* **15**(6): 853-864.

Pywell, R. F., E. A. Warman, L. Hulmes, S. Hulmes, P. Nuttall, T. H. Sparks, C. N. R. Critchley and A. Sherwood (2006). Effectiveness of new agri-environment schemes in providing foraging resources for bumblebees in intensively farmed landscapes. *Biol. Conserv.* **129**(2): 192-206.

Quezada-Euán, J., A. López-Velasco, J. Pérez-Balam, H. Moo-Valle, A. Velazquez-Madrazo and R. Paxton (2011). Body size differs in workers produced across time and is associated with variation in the quantity and composition of larval food in *Nannotrigona perilampoides* (Hymenoptera, Meliponini). *Insectes Soc.* **58**(1): 31-38.

Raguso, R. A. (2008). Wake up and smell the roses: The ecology and evolution of floral scent. *Annual Review of Ecology, Evolution, and Systematics* **39**(1): 549-569.

Rasmont, P. and P. Mersch (1988). Première estimation de la dérive faunique chez les bourdons de la Belgique (Hymenoptera: Apidae). *Ann. Soc. R. Zool. Belg.* **118**(2): 141-147.

Rau, P. (1941). The nesting habits of *Bombus medius* Cresson, the Mexican bumblebee. *Psyche: A Journal of Entomology* **48**(4): 166-168.

Reisenman, C. E. and M. Giurfa (2008). Chromatic and achromatic stimulus discrimination of long wavelength (red) visual stimuli by the honeybee *Apis mellifera*. *Arthropod-Plant Interactions* **2**(3): 137.

Richardson, L. L., L. S. Adler, A. S. Leonard, J. Andicoechea, K. H. Regan, W. E. Anthony, J. S. Manson and R. E. Irwin (2015). Secondary metabolites in floral nectar reduce parasite infections in bumblebees. *Proc. R. Soc. B* **282**(1803).

Riddell, C. E. and E. B. Mallon (2006). Insect psychoneuroimmunology: immune response reduces learning in protein starved bumblebees (*Bombus terrestris*). *Brain Behav. Immun* **20**(2): 135-138.

Ring, R. A. and D. Tesar (1981). Adaptations to cold in Canadian arctic insects. *Cryobiology* **18**(2): 199-211.

Roger, N., D. Michez, R. Wattiez, C. Sheridan and M. Vanderplanck (2017b). Diet effects on bumblebee health. *J. Insect Physiol.* **96**: 128-133.

Roger, N., R. Moerman, L. G. Carvalheiro, J. Aguirre-Guitiérrez, A. L. Jacquemart, D. Kleijn, G. Lognay, L. Moquet, M. Quinet and P. Rasmont (2017a). Impact of pollen resources drift on common bumblebees in NW Europe. *Glob. Change Biol.* **23**(1): 68-76.

Röseler, P.-F. (1985). A technique for year-round rearing of *Bombus terrestris* (Apidae, *Bombini*) colonies in captivity. *Apidologie* **16**(2): 165-170.

Roulston, T. a. H., J. H. Cane and S. L. Buchmann (2000). What governs protein content of pollen: pollinator preferences, pollen-pistil interactions, or phylogeny? *Ecol.* **70**(4): 617-644.

Roulston, T. a. H. and K. Goodell (2011). The role of resources and risks in regulating wild bee populations. *Annu. Rev. Entomol.* **56**(1): 293-312.

Roulston, T. H. and J. H. Cane (2000). Pollen nutritional content and digestibility for animals. *Plant Syst. Evol.* **222**(1-4): 187-209.

Ruedenauer, F. A., J. Spaethe and S. D. Leonhardt (2015). How to know which food is good for you: bumblebees use taste to discriminate between different concentrations of food differing in nutrient content. *J. Exp. Biol.* **218**(14): 2233-2240.

Ruedenauer, F. A., J. Spaethe and S. D. Leonhardt (2016). Hungry for quality—individual bumblebees forage flexibly to collect high-quality pollen. *Behav. Ecol. Sociobiol.* **70**(8): 1209-1217.

Ruiz-González, M. X., J. Bryden, Y. Moret, C. Reber-Funk, P. Schmid-Hempel and M. J. F. Brown (2012). Dynamic transmission, host quality, and population structure in a multihost parasite of bumblebees. *Evolution* **66**(10): 3053-3066.

Russell, A. L., S. J. Morrison, E. H. Moschonas and D. R. Papaj (2017). Patterns of pollen and nectar foraging specialization by bumblebees over multiple timescales using RFID. *Sci. Rep.* **7**: 42448.

Rutrecht, S., J. Klee and M. Brown (2007). Horizontal transmission success of *Nosema bombi* to its adult bumble bee hosts: effects of dosage, spore source and host age. *Parasitology* **134**(12): 1719-1726.

Rutrecht, S. T. and M. J. Brown (2008). Within colony dynamics of *Nosema bombi* infections: disease establishment, epidemiology and potential vertical transmission. *Apidologie* **39**(5): 504-514.

Sakagami, S. F. (1976). Specific differences in the bionomic characters of bumblebees.: A comparative review *北海道大學理學部紀要* **20**(3): 390-447.

Sakagami, S. F., Y. Akahira and R. Zucchi (1967). Nest architecture and brood development in a neotropical bumblebee, *Bombus atratus*. *Insectes Soc.* **14**(4): 389-413.

Salathé, R. M. and P. Schmid-Hempel (2011). The genotypic structure of a multi-host bumblebee parasite suggests a role for ecological niche overlap. *PLoS One* **6**(8): e22054.

Salisbury, A., J. Armitage, H. Bostock, J. Perry, M. Tatchell and K. Thompson (2015). Enhancing gardens as habitats for flower-visiting aerial insects (pollinators): should we plant native or exotic species? *J. Appl. Ecol.* **52**(5): 1156-1164.

Salvarrey, S., N. Arbulo, E. Santos and C. Invernizzi (2013). Cría artificial de abejorros nativos *Bombus atratus* y *Bombus bellicosus* (Hymenoptera, Apidae). *Agrociencia Uruguay* **17**(2): 75-82.

Samnegård, U., A. S. Persson and H. G. Smith (2011). Gardens benefit bees and enhance pollination in intensively managed farmland. *Biol. Conserv.* **144**(11): 2602-2606.

Sanger, T. J., J. B. Losos and J. J. Gibson-Brown (2008). A developmental staging series for the lizard genus Anolis: a new system for the integration of evolution, development, and ecology. *J. Morphol.* **269**(2): 129-137.

Saunders, M. E., J. K. Janes and J. C. O'Hanlon (2020). Moving on from the insect apocalypse narrative: engaging with evidence-based insect conservation. *Bioscience* **70**(1): 80-89.

Schaefer, H. M. and G. D. Ruxton (2009). Deception in plants: mimicry or perceptual exploitation? *Trends Ecol. Evol.* **24**(12): 676-685.

Schaub, G. (1994). Pathogenicity of trypanosomatids on insects. *Parasitol. Today* **10**(12): 463-468.

Scheper, J., R. Bommarco, A. Holzschuh, S. G. Potts, V. Riedinger, S. P. M. Roberts, M. Rundlöf, H. G. Smith, I. Steffan-Dewenter, J. B. Wickens, V. J. Wickens and D. Kleijn (2015). Local and landscape-level floral resources explain effects of wildflower strips on wild bees across four European countries. *J. Appl. Ecol.* **52**(5): 1165-1175.

Scheper, J., A. Holzschuh, M. Kuussaari, S. G. Potts, M. Rundlöf, H. G. Smith and D. Kleijn (2013). Environmental factors driving the effectiveness of European agri-environmental measures in mitigating pollinator loss – a meta-analysis. *Ecol.* **16**(7): 912-920.

Schiestl, F. P., M. Ayasse, H. F. Paulus, C. Löfstedt, B. S. Hansson, F. Ibarra and W. Francke (1999). Orchid pollination by sexual swindle. *Nature* **399**(6735): 421-421.

Schiestl, F. P. and S. D. Johnson (2013). Pollinator-mediated evolution of floral signals. *Trends Ecol. Evol.* **28**(5): 307-315.

Schmid-Hempel, P. (1998). *Parasites in social insects*, Princeton University Press.

Schmid-Hempel, P. (2001). On the evolutionary ecology of host-parasite interactions: addressing the question with regard to bumblebees and their parasites. *Naturwissenschaften* **88**(4): 147-158.

Schmid-Hempel, P. and R. Loosli (1998). A contribution to the knowledge of *Nosema* infections in bumble bees, *Bombus spp*. *Apidologie* **29**: 525-536.

Schmid-Hempel, P., K. Puhr, N. Kruger, C. Reber and R. Schmid-Hempel (1999). Dynamic and genetic consequences of variation in horizontal transmission for a microparasitic infection. *Evolution* **53**: 426-434.

Schowalter, T. D., M. R. Willig, S. J. Presley and M. Pandey (2019). Warnings of an "insect apocalypse" are premature. *Frontiers in Ecology and the Environment* **17**(10): 547-547.

Senapathi, D., L. G. Carvalheiro, J. C. Biesmeijer, C.-A. Dodson, R. L. Evans, M. McKerchar, R. D. Morton, E. D. Moss, S. P. M. Roberts, W. E. Kunin and S. G. Potts (2015). The impact of over 80 years of land cover changes on bee and wasp pollinator communities in England. *Proc. R. Soc. B* **282**(1806).

Shreeve, T. (1981). Flight patterns of butterfly species in woodlands. *Oecologia* **51**(2): 289-293.

Shykoff, J. and P. Schmid-Hempel (1991). Incidence and effects of four parasites in natural populations of bumble bees in Switzerland. *Apidologie* **22**(2): 117-125.

Simmons, B. I., A. Balmford, A. J. Bladon, A. P. Christie, A. De Palma, L. V. Dicks, J. Gallego-Zamorano, A. Johnston, P. A. Martin and A. Purvis (2019). Worldwide insect declines: An important message, but interpret with caution. *Ecol. Evol.* **9**(7): 3678-3680.

Simone-Finstrom, M., H. Li-Byarlay, M. H. Huang, M. K. Strand, O. Rueppell and D. R. Tarpy (2016). Migratory management and environmental conditions affect lifespan and oxidative stress in honey bees. *Scientific Reports* **6**: 32023.

Simpson, S. and D. Raubenheimer (1995). The geometric analysis of feeding and nutrition: a user's guide. *Journal of Insect Physiology* **41**(7): 545-553.

Simpson, S. J. and D. Raubenheimer (2009). Macronutrient balance and lifespan. *Aging* **1**(10): 875.

Simpson, S. J. and D. Raubenheimer (2012). *The nature of nutrition: a unifying framework from animal adaptation to human obesity*, Princeton university press.

Singh, R., A. L. Levitt, E. G. Rajotte, E. C. Holmes, N. Ostiguy, D. vanEngelsdorp, W. I. Lipkin, C. W. dePamphilis, A. L. Toth and D. L. Cox-Foster (2010). RNA viruses in Hymenopteran pollinators: Evidence of inter-taxa virus transmission via pollen and potential impact on non-*Apis* Hymenopteran species. *PLoS One* **5**(12): e14357.

Sladen, F. (1912). *The humble-bee, its life-history and how to domesticate it*. London, Macmillan & Co.

Slansky, F. and J. G. Rodriguez (1987). *Nutritional ecology of insects, mites, spiders, and related invertebrates*, Wiley.

Smith, R. (1985). Oviposition, ovicide and larval competition in granivorous insects. *Behav. Ecol.* **25**: 423-488.

Smith, R. M., K. Thompson, J. G. Hodgson, P. H. Warren and K. J. Gaston (2006b). Urban domestic gardens (IX): composition and richness of the vascular plant flora, and implications for native biodiversity. *Biol. Conserv.* **129**(3): 312-322.

Smith, R. M., P. H. Warren, K. Thompson and K. J. Gaston (2006a). Urban domestic gardens (VI): environmental correlates of invertebrate species richness. *Biodivers. Conserv.* **15**(8): 2415-2438.

Smith, V. (2007). Host resource supplies influence the dynamics and outcome of infectious disease. *Integr. Comp. Biol.* **47**(2): 310-316.

Somerville, D. and D. Collins (2007). *Field trials to test supplementary feeding strategies for commercial honeybees*. Kingston, Rural Industries Research and Development Corporation.

Somme, L., M. Vanderplanck, D. Michez, I. Lombaerde, R. Moerman, B. Wathelet, R. Wattiez, G. Lognay and A.-L. Jacquemart (2015). Pollen and nectar quality

drive the major and minor floral choices of bumble bees. *Apidologie* **46**(1): 92-106.

Stabler, D., P. P. Paoli, S. W. Nicolson and G. A. Wright (2015). Nutrient balancing of the adult worker bumblebee (*Bombus terrestris*) depends on the dietary source of essential amino acids. *J. Exp. Biol.* **218**(5): 793-802.

Stange, E., G. Zulian, G. Rusch, D. Barton and M. Nowell (2017). Ecosystem services mapping for municipal policy: ESTIMAP and zoning for urban beekeeping. *One Ecosystem* **2**: e14014.

Stone, E. C. (2018). *The effect of bumblebee caste on individuals' response to medicinal pollen*. Honors Project, Smith College, Northampton.

Sutcliffe, G. and R. Plowright (1990). The effects of pollen availability on development time in the bumble bee *Bombus terricola* K.(Hymenoptera: Apidae). *Can. J. Zool.* **68**(6): 1120-1123.

Tanaka, C. S., M. Ikemoto, A. Nikkeshi, Y. Kanbe, M. Mitsuhata and T. Yokoi (2019). Ovarian development related to pollen feeding in workers of the bumblebee *Bombus ignitus* (Hymenoptera: Apidae). *Appl. Enotomol. Zool.* **54**(1): 85-89.

Tasei, J.-N. and P. Aupinel (2008). Nutritive value of 15 single pollens and pollen mixes tested on larvae produced by bumblebee workers (*Bombus terrestris*, Hymenoptera: Apidae). *Apidologie* **39**(4): 397-409.

Tasei, J. (1994). Effect of different narcosis procedures on initiating oviposition of prediapausing *Bombus terrestris* queens. *Entomol. Exp. Appl.* **72**(3): 273-279.

Tay, W. T., E. M. O'Mahony and R. J. Paxton (2005). Complete rRNA gene sequences reveal that the microsporidium *Nosema bombi* infects diverse bumblebee (*Bombus* spp.) hosts and contains multiple polymorphic sites. *J. Eukaryot. Microbiol.* **52**(6): 505-513.

Theodorou, P., R. Radzevičiūtė, J. Settele, O. Schweiger, T. E. Murray and R. J. Paxton (2016). Pollination services enhanced with urbanization despite increasing pollinator parasitism. *Proc. Royal Soc. B* **283**(1833).

Thomas, J. A., M. Telfer, D. B. Roy, C. D. Preston, J. Greenwood, J. Asher, R. Fox, R. Clarke and J. Lawton (2004). Comparative losses of British butterflies, birds, and plants and the global extinction crisis. *Science* **303**(5665): 1879-1881.

Thompson, K., J. G. Hodgson, R. M. Smith, P. H. Warren and K. J. Gaston (2004). Urban domestic gardens (III): composition and diversity of lawn floras. *J. Veg. Science* **15**(3): 373-378.

Thorp, R. W. (1979). Structural, behavioral and physiological adaptations of bees (Apoidea) for collecting pollen. *Ann. Missouri Bot. Gard.* **66**: 788-812.

Thorp, R. W., D. S. Horning and L. L. Dunning (1983). *Bumble bees and cuckoo bumble bees of California (Hymenoptera, Apidae)*, University of California Press.

Timberlake, T. P., I. P. Vaughan and J. Memmott (2019). Phenology of farmland floral resources reveals seasonal gaps in nectar availability for bumblebees. *J. Appl. Ecol.* **56**(7): 1585-1596.

Tiusanen, M., P. D. Hebert, N. M. Schmidt and T. Roslin (2016). One fly to rule them all—muscid flies are the key pollinators in the Arctic. *Proceedings of the Royal Society B: Biological Sciences* **283**(1839): 20161271.

Tschapka, M. and S. Dressler (2002). Chiropterophily: On bat-flowers and flower-bats. *Curtis's Bot. Mag.* **19**(2): 114-125.

Turo, K. J. and M. M. Gardiner (2019). From potential to practical: conserving bees in urban public green spaces. *Front. Ecol. Environ.* **17**(3): 167-175.

Tyler, E. R., S. Adams and E. B. Mallon (2006). An immune response in the bumblebee, *Bombus terrestris* leads to increased food consumption. *BMC Physiol.* **6**(1): 6.

Van den Eijnde, J., A. De Ruijter and J. Van der Steen (1990). *Method for rearing Bombus terrestris continuously and the production of bumblebee colonies for pollination purposes*. VI International Symposium on Pollination 288.

van den Eijnde, J. and N. Vette (1993). *Nosema infection in honey bees (Apis mellifera L.) and bumble bees (Bombus terrestris L.)*. Proc. Exp. Appl. Entomol. NEV Amsterdam.

van Der Steen, J. J. (2008). Infection and transmission of *Nosema bombi* in *Bombus terrestris* colonies and its effect on hibernation, mating and colony founding. *Apidologie* **39**(2): 273-282.

Van Noordwijk, A. J. and G. de Jong (1986). Acquisition and allocation of resources: their influence on variation in life history tactics. *The American Naturalist* **128**(1): 137-142.

Vanbergen, A. J. and I. P. Initiative (2013). Threats to an ecosystem service: pressures on pollinators. *Front. Ecol. Environ.* **11**(5): 251-259.

Vanderplanck, M., R. Moerman, P. Rasmont, G. Lognay, B. Wathelet, R. Wattiez and D. Michez (2014). How does pollen chemistry impact development and feeding behaviour of polylectic bees? *PLoS One* **9**(1): e86209.

Vásquez, A., E. Forsgren, I. Fries, R. J. Paxton, E. Flaberg, L. Szekely and T. C. Olofsson (2012). Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. *PloS one* **7**(3).

Vaudo, A. D., L. M. Farrell, H. M. Patch, C. M. Grozinger and J. F. Tooker (2018). Consistent pollen nutritional intake drives bumble bee (*Bombus impatiens*) colony growth and reproduction across different habitats. *Ecology and evolution* **8**(11): 5765-5776.

Vaudo, A. D., H. M. Patch, D. A. Mortensen, J. F. Tooker and C. M. Grozinger (2016). Macronutrient ratios in pollen shape bumble bee (*Bombus impatiens*) foraging strategies and floral preferences. *Proc. Natl. Acad. Sci. U.S.A.*: 201606101.

Vaudo, A. D., J. F. Tooker, C. M. Grozinger and H. M. Patch (2015). Bee nutrition and floral resource restoration. *Curr. Opin. Insect Sci.* **10**: 133-141.

Velthuis, H. H. and A. Van Doorn (2006). A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie* **37**(4): 421-451.

Velthuis, H. H. W. (2002). The historical background of the domestication of the bumblebee, *Bombus terrestris*, and its introduction in agriculture. *Pollinating Bees-The Conservation Link Between Agriculture And Nature*. P. Kevan and V. L. I. Fonseca. Brazil, Ministry of Environment**:** 177-184.

Venales, B. and E. M. Barrows (1985). Skippers: Pollinators or nectar thieves? *J. Lepid. Soc.* **39**(4): 299-312.

Vogt, F. D., B. Heinrich and C. Plowright (1998). Ovary development in bumble bee queens: the influence of abdominal temperature and food availability. *Can. J. Zool.* **76**(11): 2026-2030.

Wacht, S., K. Lunau and K. Hansen (2000). Chemosensory control of pollen ingestion in the hoverfly *Eristalis tenax* by labellar taste hairs. *J. Comp. A* **186**(2): 193-203.

Walther-Hellwig, K. and R. Frankl (2000). Foraging habitats and foraging distances of bumblebees, *Bombus* spp. (Hym., apidae), in an agricultural landscape. *J. Appl. Enotomol.* **124**(7-8): 299-306.

Wardhaugh, C. W. (2015). How many species of arthropods visit flowers? *Arthropod-Plant Interactions* **9**(6): 547-565.

Wardhaugh, C. W., W. Edwards and N. E. Stork (2015). The specialization and structure of antagonistic and mutualistic networks of beetles on rainforest canopy trees. *Biol. J. Linn. Soc.* **114**(2): 287-295.

Warren, M., J. Hill, J. Thomas, J. Asher, R. Fox, B. Huntley, D. Roy, M. Telfer, S. Jeffcoate and P. Harding (2001). Rapid responses of British butterflies to opposing forces of climate and habitat change. *Nature* **414**(6859): 65-69.

Watts, S. A., M. Powell and L. R. D'Abramo (2012). Fundamental approaches to the study of zebrafish nutrition. *ILAR J* **53**(2): 144-160.

Weidenmüller, A., C. Kleineidam and J. Tautz (2002). Collective control of nest climate parameters in bumblebee colonies. *Animal Behaviour* **63**(6): 1065-1071.

Wepprich, T., J. R. Adrion, L. Ries, J. Wiedmann and N. M. Haddad (2019). Butterfly abundance declines over 20 years of systematic monitoring in Ohio, USA. *PloS one* **14**(7).

Westerkamp, C. (1996). Pollen in bee-flower relations - Some considerations on melittophily. *Bot Acta* **109**(4): 325-332.

Westphal, C., I. Steffan-Dewenter and T. Tscharntke (2009). Mass flowering oilseed rape improves early colony growth but not sexual reproduction of bumblebees. *J. Appl. Ecol.* **46**(1): 187-193.

Weyrauch, W. (1934). Über einige baupläne der wabenmasse in hummelnestern. *Zeitschrift für Morphologie und Ökologie der Tiere*: 497-552.

Whitehorn, P. R., M. C. Tinsley, M. J. F. Brown, B. Darvill and D. Goulson (2010). Genetic diversity, parasite prevalence and immunity in wild bumblebees. *Proc. Royal Soc. B.*

Whittington, R., M. L. Winston, C. Tucker and A. L. Parachnowitsch (2004). Plant-species identity of pollen collected by bumblebees placed in greenhouses for tomato pollination. *Can J Plant Sci* **84**(2): 599-602.

Whitton, P., R. Strang and R. Nicholson (1987). The distribution of taurine in the tissues of some species of insects. *Insect Biochem.* **17**(4): 573-577.

Wiermann, R. and S. Gubatz (1992). Pollen wall and sporopollenin. *Int. Rev. Cytol.*, Elsevier. **140:** 35-72.

Wiklund, C., T. Eriksson and H. Lundberg (1979). The wood white butterfly *Leptidea sinapis* and its nectar plants: a case of mutualism or parasitism? *Oikos*: 358-362.

Williams, I. H., S. A. Corbet and J. L. Osborne (1991). Beekeeping, wild bees and pollination in the European Community. *Bee World* **72**(4): 170-180.

Williams, N. M., D. Cariveau, R. Winfree and C. Kremen (2011). Bees in disturbed habitats use, but do not prefer, alien plants. *Basic Appl. Ecol.* **12**(4): 332-341.

Williams, N. M. and J. D. Thomson (1998). Trapline foraging by bumble bees: III. Temporal patterns of visitation and foraging success at single plants. *Behav. Ecol.* **9**(6): 612-621.

Williams, P. (2005). Does specialization explain rarity and decline among British bumblebees? A response to Goulson et al. *Biol. Conserv.* **122**(1): 33-43.

Williams, P., S. Colla and Z. Xie (2009a). Bumblebee vulnerability: Common correlates of winners and losers across three continents. *Conserv. Biol.* **23**(4): 931-940.

Williams, P., Y. Tang, J. Yao and S. Cameron (2009b). The bumblebees of Sichuan (Hymenoptera: Apidae, Bombini). *Syst. Biodivers.* **7**: 101-189.

Williams, P. H. (1982). The distribution and decline of British bumble bees (*Bombus* Latr.). *J. Apic. Res.* **21**(4): 236-245.

Williams, P. H. (1994). Phylogenetic relationships among bumble bees (Bombus Latr.): a reappraisal of morphological evidence. *Syst. Entomol.* **19**(4): 327-344.

Williams, P. H. and J. L. Osborne (2009). Bumblebee vulnerability and conservation world-wide. *Apidologie* **40**(3): 367-387.

Willmer, P. (2011). *Pollination and floral ecology*. Princeton, New Jersey, Princeton University Press.

Winfree, R., R. Aguilar, D. P. Vázquez, G. LeBuhn and M. A. Aizen (2009). A meta-analysis of bees' responses to anthropogenic disturbance. *Ecology* **90**(8): 2068-2076.

Winfree, R., I. Bartomeus and D. P. Cariveau (2011). Native pollinators in anthropogenic habitats. *Annu. Rev. Ecol. Evol. Syst.* **42**(1): 1-22.

Wood, T. J., J. M. Holland and D. Goulson (2017). Providing foraging resources for solitary bees on farmland: current schemes for pollinators benefit a limited suite of species. *J. Appl. Ecol.* **54**(1): 323-333.

Wood, T. J., J. M. Holland, W. O. Hughes and D. Goulson (2015). Targeted agri-environment schemes significantly improve the population size of common farmland bumblebee species. *Mol. Ecol.* **24**(8): 1668-1680.

Wood, T. J., I. Kaplan and Z. Szendrei (2018). Wild bee pollen diets reveal patterns of seasonal foraging resources for honey bees. *Front. Ecol. Evol.* **6**: 210.

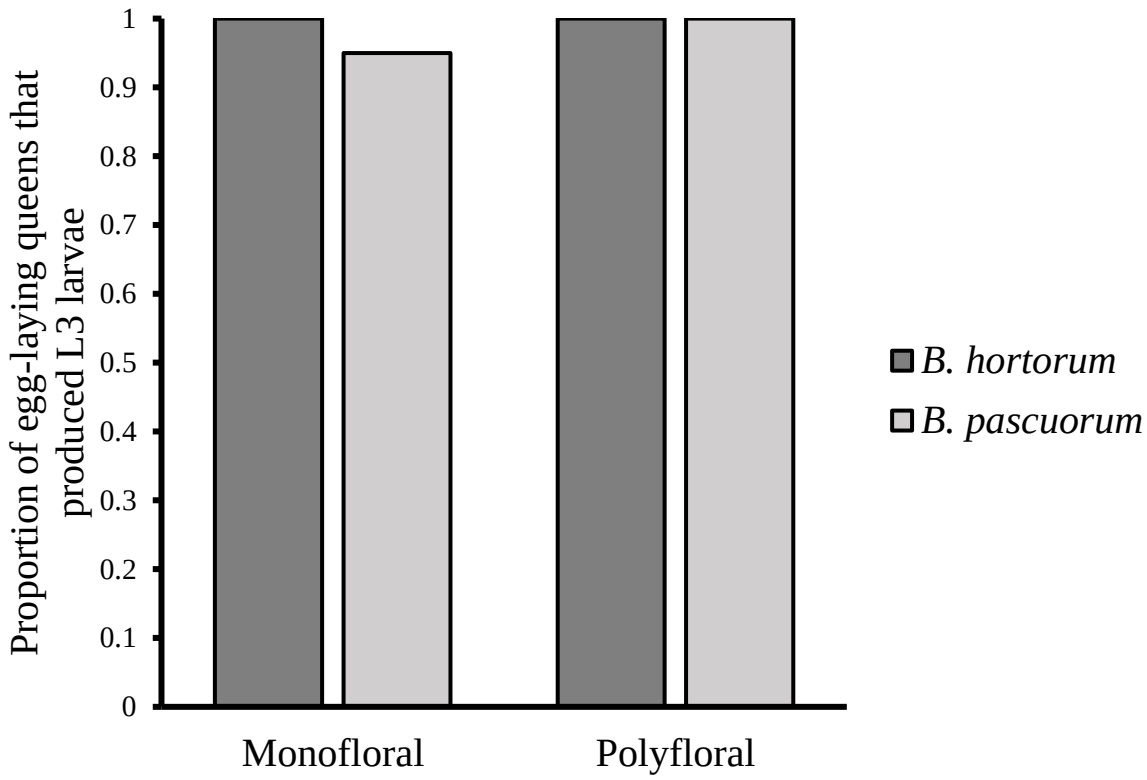
Woodard, S. H. and S. Jha (2017). Wild bee nutritional ecology: predicting pollinator population dynamics, movement, and services from floral resources. *Curr. Opin. Insect Sci.* **21**: 83-90.

Yoneda, M. (2008). Induction of colony initiation by Japanese native bumble bees using cocoons of the exotic bumblebee *Bombus terrestris*. *Entomol. Sci.* **11**(1): 123-126.

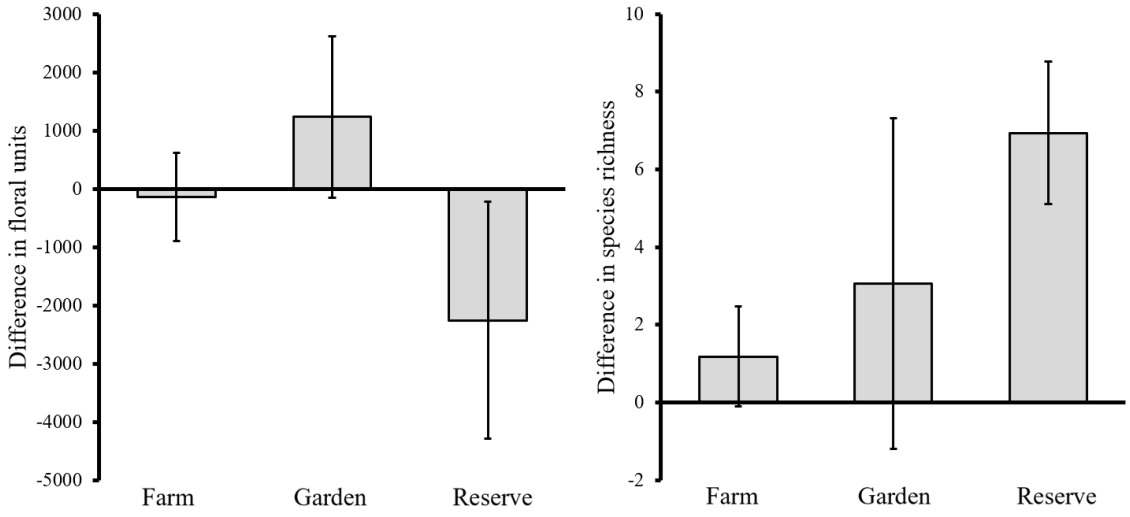
Yoon, H. J., S. E. Kim and Y. S. Kim (2002). Temperature and humidity favorable for colony development of the indoor-reared bumblebee, *Bombus ignitus*. *Appl. Enotomol. Zool.* **37**(3): 419-423.

Supplementary Figures

Supplementary Figure 3.1 Incipient colony success of wild-caught *B. hortorum* (n = 20; dark grey) and *B. pascuorum* (n = 61; light grey) bumblebee queens measured by the proportion of egg-laying queens (n = 52) that produced L3 larvae (n = 51). Queens were randomly assigned a diet treatment of either monofloral hawthorn pollen or a polyfloral wildflower pollen mix.



Supplementary Figure 5.1 Mean (± s.e.) difference in floral unit abundance and species richness between two surveying methods: flower-rich plots and transect, calculated by transect subtracted from plot. Data were collected across farmland (n = 4), gardens (n = 4) and nature reserves (n = 4) between May and August 2016. Each site was surveyed once a month using both survey methods on the same day. Flower-rich plots were selected each month based on visual assessments of flower availability. One transect was developed for each site that was considered representative of its smaller habitats and remained consistent throughout the sampling period.



Supplementary Tables

Supplementary Table 2.1 Survival, pairing and reproductive success of each wild-caught *B. pascuorum* queen (N = 57) reared in captivity that produced workers. All queens were initially paired to encourage one female to establish dominance. * indicates when a queen died (where [queen name]* = died in initial pairing and 1* = died after being given own nest box). Based on their behaviour, reproductive success and survival, queens were either left as a pair or separated and given their own nest box. Column 1 specifies the queen and number of days she survived. Columns 2-6 show the response of queens to pairing and their reproductive success during and (if applicable) after: the queen she was paired with and the corresponding Supplementary Table that queen is found; the number of days the queens were paired; the reproductive success (RS) of the queen in her pair (where ● = eggs produced, ■ = workers produced, - = no progress and [2] = eggs or workers were produced by the queen or her partner and which one could not be ascertained); the corresponding action that was taken (where 1 = separated and given own nest box and - = no action taken) and the justification for that action (where CO = both queens were settled and contributed to brood care, NE = no engagement by submissive queen); and any further progress made by queens that were given their own nest box. Columns 7-9 specify the day the queen laid her first egg/s, the day stimuli (a *B. terrestris* cocoon) was added to the nest box, and the day the first long-tongued worker eclosed. Columns 10-11 show whether the queen was paired (P) or single (S) when she was given the stimuli (and if she was paired, which queen she was paired with), and when she died.

Queen (days survived)	Events and progress in first pairing				Further progress	Day first			State	
	(S. Table) Queen	Days paired	RS	Action & justification		Egg was laid	Stimulus was added	Worker eclosed	When stimulus was added	At death
BpAD (123)	(2.1) BpAZ*	61	●	-; CO	■	13	53	64	(P) BpAZ	(P)
BpAL* (65)	(2.1) BpAM*	65	■[2]	-; CO		16	51	64	(P) BpAM	(P)
BpAM* (65)	(2.1) BpAL*	65	■[2]	-; CO		16	51	64	(P) BpAL	(P)
BpAQ (136)	(2.2) BpH	7	●	1*; NE	■	16	59	25	(S)	(S)
BpAZ* (61)	(2.1) BpAD	61	●	-; CO		13	53	64	(P) BpAD	(P)
BpB (114)	(2.3) BpAV	3		1*; NE	■	23	69	84	(S)	(S)
BpBC* (123)	(2.1) BpK*	123	■[2]	-; CO		9	54	95	(P) BpK	(P)
BpK* (124)	(2.1) BpBC*	123	■[2]	-; CO		9	54	95	(P) BpBC	(P)

Supplementary Table 2.2 Survival, pairing and reproductive success of each wild-caught *B. pascuorum* queen (N = 57) reared in captivity that produced only eggs. All queens were initially paired to encourage one female to establish dominance. * indicates when a queen died (where [queen name]* = died in initial pairing, 1* = died after being given own nest box and 2* = died in subsequent pairing). Based on their behaviour, reproductive success and survival, queens were either left as a pair or separated and given their own nest box. Column 1 specifies the queen and number of days she survived. Columns 2-14 show the response of queens to pairing and their reproductive success during and (if applicable) after: the queen she was paired with and the corresponding Supplementary Table that queen is found; the number of days the queens were paired; the reproductive success (RS) of the queen in her pair (where ● = eggs produced, - = no progress, [2] = eggs were produced by the queen or her partner and which one could not be ascertained, (●) = eggs laid by partner queen); the corresponding action that was taken (where 1 = separated and given own nest box, 2 = re-paired with a new queen, and - = no action taken) and the justification for that action (where CO = both queens were settled and contributed to brood care, SE = queens were undisturbed by each other's presence but only exhibited brood care behaviours; NE = no engagement by submissive queen, AG = aggression between queens); further progress made by queens that were given their own nest box. Columns 15-16 specify the day the queen laid her first egg/s, the day stimuli (a *B. terrestris* cocoon) was added to the nest box, and the day the first long-tongued worker eclosed. Columns 17-18 show whether the queen was paired (P) or single (S) when she was given the stimuli (and if she was paired, which queen she was paired with), and when she died.

Queen (days survived)	Events and progress in first pairing				Events and progress in second pairing				Events and progress in third pairing				Additional progress	Day of first		State (paired or single)	
	(S. Table) Queen	Days paired	RS	Action & justification	(S. Table) Queen	Days paired	RS	Action	(S. Table) Queen	Days paired	RS	Action		Egg was laid	Stimulus was added	When stimulus was added	At death
BpAR* (129)	(2.2) BpAP	129	●[2]	-; CO										19	55	(P) BpAR	(P)
BpAT* (23)	(2.2) BpL	23	●	-; SE										20	48	(S)	(S)
BpAX* (47)	(2.2) BpH	47	●[2]	-; CO										9	-	-	(P)
BpAY* (60)	(2.2) BpN	60	●[2]	-; CO										46	41	(P) BpN	(P)
BpBF* (50)	(2.2) BpBE	50	●[2]	-; CO										14	48	(P) BpBE	(P)
BpM* (77)	(2.2) BpW	77	●[2]	-; CO										36	34	(P) BpW	(P)
BpAE (76)	(2.2) BpJ	74	●[2]	1*; CO			●	-						13	59	(P) BpJ	(S)
BpAF (140)	(2.2) BpAG	47	(●)	1*; SE			●	-						13	48	(S)	(S)
BpBA (132)	(2.2) BpBB*	58	(●)	-; SE			●	-						20	56	(P) BpBB	(S)
BpBE (52)	(2.2) BpBF*	50	●[2]	-; CO			-	-						14	48	(P) BpBF	(S)
BpHI (136)	(2.2) BpAS*	40	●	-; SE			●	-						30	50	(S)	(S)
BpN (82)	(2.2) BpAY*	60	●[2]	-; CO			●	-						46	41	(P) BpAY	(S)
BpP (132)	(2.3) BpG	6	●	1*; US			●	-						5	-	-	(S)
BpAG (69)	(2.2) BpAF	47	●	1*; SE			-	-						13	48	(S)	(S)
BpBB (62)	(2.2) BpBA	58	●	1*; SE			-	-						20	56	(P) BpBA	(S)
BpAP (149)	(2.2) BpAR*	129	●[2]	-; CO			●	-						19	55	(P) BpAR	(S)
BpW (92)	(2.2) BpM*	77	●[2]	-; CO			●	-						36	34	(P) BpM	(S)
BpH (132)	(2.1) BpAQ	7	-	2; US	(2.2) BpO	11	●	2; US	(2.2) BpAX	47	●[2]	-; CO		4	58	(P) BpAX	(S)
BpAC (80)	(2.2) BpZ	22	-	1*; US			●	-						17	59	(S)	(S)
BpAH (153)	(2.3) BpAU	19	-	1*; NE			●	-						28	50	(S)	(S)
BpV (119)	(2.3) BpAB	2	-	1*; US			●	-						28	45	(S)	(S)
BpAO (117)	(2.3) BpAU*	9	-	-; NE			●	-						12	35	(S)	(S)
BpAS (71)	(2.3) BpAJ	2	●	2*; US	(2.2) BpHI	67	●	-; SE						30	50	(P) BpHI	(P)
BpAU (28)	(2.2) BpAH	19	-	2*; NE	(2.2) BpAO	9	●	-; SE						21	-	-	(P)
BpJ (133)	(2.2) BpI	2	-	2; US	(2.2) BpAE*	74	●[2]	1*; CO			●	-		13	59	(P) BpAE	(S)
BpL (130)	(2.3) BpAK	11	-	2; NE	(2.2) BpAT*	23	(●)	-; NE			●	-		20	48	(S)	(S)
BpO (126)	(2.3) BpS*	12	-	2; US	(2.2) BpH	11	(●)	1*; US			●	-		16	49	(S)	(S)
BpY (142)	(2.3) BpT*	9	-	-; NE			●	-						21	35	(S)	(S)
BpF (12)	(2.3) BpD*	5	-	2; NE	(2.2) BpI	6	-	1*; US*			●	-		11	-	-	(S)
BpI (141)	(2.2) BpJ	2	-	2; NE	(2.2) BpX	10	-	2; NE	(2.3) BpF	6	-	1*; US	●	38	58	(S)	(S)
BpX (103)	(2.2) BpI	10	-	2; NE	(2.3) BpAB*	1	-	-; US			●	-		22	57	(S)	(S)
BpZ (160)	(2.2) BpAC	22	-	2; NE	(2.3) BpG*	1	-	1*; AG			●	-		55	62	(S)	(S)

Supplementary Table 2.3 Survival, pairing and reproductive success of each wild-caught *B. pascuorum* queen (N = 57) reared in captivity that did not produce eggs. All queens were initially paired to encourage one female to establish dominance. * indicates when a queen died (where [queen name]* = died in initial pairing, 1* = died after being given own nest box and 2* = died in a subsequent pairing). Based on their behaviour, reproductive success and survival, queens were either left as a pair or separated and given their own nest box. Column 1 specifies the queen and number of days she survived. Columns 2-13 show the response of queens to pairing and their reproductive success during and (if applicable) after: the queen she was paired with and the corresponding Supplementary Table that queen is found; the number of days the queens were paired; the reproductive success (RS) of the queen in her pair (where - = no progress and (●) = eggs laid by partner queen); the corresponding action that was taken (where 1 = separated and given own nest box, 2 = re-paired with a new queen, and - = no action taken) and the justification for that action (where SE = queens were undisturbed by each other's presence but only exhibited brood care behaviours; NE = no engagement by submissive queen); further progress made by queens that were given their own nest box. Column 14 shows whether the queen was paired (P) or single (S) when she died.

Queen (days survived)	Events and progress in first pairing				Events and progress in second pairing				Events and progress in third pairing				State at death
	(S. Table) Queen	Days paired	RS	Action & justification	(S. Table) Queen	Days paired	RS	Action & justification	(S. Table) Queen	Days paired	RS	Action & justification	
BpC* (3)	(2.3) BpAW	3	-	-; NE									(P)
BpAA* (22)	(2.3) BpU	22	-	-; SE									(P)
BpT* (9)	(2.2) BpY	9	-	-; NE									(P)
BpD* (5)	(2.3) BpF	5	-	-; NE									(P)
BpBD* (8)	(2.3) BpE*	8	-	-; SE									(P)
BpE* (8)	(2.3) BpBD*	8	-	-; SE									(P)
BpQ (17)	(2.3) BpAJ	16	-	1*; NE			-	-					(S)
BpAK (25)	(2.2) BpL	11	-	1*; NE			-	-					(S)
BpAW (6)	(2.3) BpC*	3	-	2; US	(2.3) BpR*	2	-	1*; US; 1*			-	-	(P)
BpS (16)	(2.2) BpO	12	-	2; US	(2.3) BpAJ*	2	-	1*; US; 1*			-	-	(S)
BpAV (22)	(2.1) BpB	3	(●)	2; NE	(2.3) BpAN	16	-	1*; NE; 1*			-	-	(S)
BpAN (24)	(2.3) BpU	1	-	2; NE	(2.3) BpR*	5	-	2; US; 2	(2.3) BpAV	16	1*	-; NE	(S)
BpAJ (20)	(2.2) BpAS	2	(●)	2; NE	(2.3) BpQ*	16	-	2*; NE; 2*	(2.3) BpS*	2	-	-; NE	(P)
BpR (7)	(2.3) BpAW*	2	-	2*; US	(2.3) BpAN	5	-						(S)
BpU (23)	(2.3) BpAN	1	-	2*; NE	(2.3) BpAA*	22	-						(P)
BpG (7)	(2.2) BpP	6	(●)	2*; NE	(2.2) BpZ	1	-						(P)
BpAB (3)	(2.2) BpV	2	-	2*; NE	(2.2) BpX	1	-						(P)

Supplementary Table 3.1 Parameters and output of the generalised linear models used to test the effect of species, diet and their interaction on five measures of queen success. All queens (n = 81) were included in the first two models on early-stage colony development and thereafter models only included either queens that produced L3 larvae (n = 51), or queens that produced workers (n = 21). Link functions were selected based on the data distribution and lowest AIC values.

Measure of queen success	No. queens included in model	Model parameters	Sig.
Proportion of queens that produced eggs	81	Binomial, logit	Species p = 0.862 Diet p = 0.019 Species x diet p = 0.045
Proportion of queens that produced L3 larvae	81		Species p = 0.77 Diet p = 0.026 Species x diet p = 0.032
Proportion of queens that produced workers	51		Species p = 0.006 Diet p = 0.073 Species x diet p = 0.049
No. weeks until L3 larvae were produced	51	Poisson, log	Species p = 0.643 Diet p = 0.080 Species x diet p = 0.035
Mean no. workers produced	21		Species p = 0.698 Diet p = 0.914 Species x diet p = 0.539

Supplementary Table 4.1 Study sites in East and West Sussex.

Habitat Type	Grid Reference
Farm	TQ 40805 13200
	TQ 48928 08008
	TQ 28925 10207
	TQ 23768 18640
	TQ 37217 06414
	TQ 44482 08920
	SU 27551 23361
	TQ 49351 17804
	TQ 17631 29522
	TQ 19068 24678
	TQ 65138 14389
	TQ 63756 11558
Garden	TQ 32222 26466
	TQ 27691 30729
	TQ 55906 09395
	TQ 06045 14440
	TQ 79202 37741
	TQ 68736 35377
	TQ 533 37700
	TQ 4128823969
	TQ 1324 2800
	TQ 23097 18089
	TQ 47830 45188
	TQ 82137 25122
	TQ 18925 10598
	TQ 39013 35677
	TQ 67083 23799

Supplementary Table 4.2 Primer sets and cycling conditions used for the molecular detection of bumblebee pathogens. Assay mix/ sample: µl/sample: 4.8 µl H_2O, 2µl buffer, 1.25µl $MgCl_2$, 0.5µl dNTPs, 0.2µl primer (forward), 0.2µl primer (reverse), 0.05µl Taq.

Target	Primer	Sample dilution	Cycling conditions		Size (bp)
			Cycles	Time: Temp °C	
Apidae host control (Meeus *et al.* 2010)	ApidaeF: AGATGGGGGCATTCGTATTG ApidaeR: ATCTGATCGCCTTCGAACCT	1/100	Denaturing	2m:95	130
			Replication	35x 30s:95 30s:56 60s:72	
			Elongation	7m:72	
C. bombi (Meeus *et al.* 2010)	SEF: CTTTTGGTCGGTGGAGTGAT SER: GGACGTAATCGGCACAGTTT	1/100	Denaturing	2m:94	420
			Replication	35x 30s:95 30s:57 45s:72	
			Elongation	3m:72	
N. bombi (Klee *et al.* 2006)	NBF: CCATGCATGTTTTTGAAGATTATTAT NBR: CATATATTTTTAAAATATGAAACAATAA	1/10	Denaturing	4m:95	323
			Replication	45x 60s:95 60s:50 60s:72	
			Elongation	4m:72	
N. ceranae (Martín-Hernández *et al.* 2007)	MITOCF: CGGCGACGATGTGATATGAAAATATTAA MITOCR: CCCGGTCATTCTCAAACAAAAAACCG	1/10	Denaturing	5m:98	218
			Replication	35x 15s:95 30s:58 45s:72	
			Elongation	7m:72	

Supplementary Table 5.1 Study sites in East and West Sussex.

Habitat Type	Site	Grid Reference
Farm	F1	TQ 40805 13200
	F2	TQ 48928 08008
	F3	TQ 28925 10207
	F4	TQ 23768 18640
Garden	G1	TQ 32222 26466
	G2	TQ 27691 30729
	G3	TQ 55906 09395
	G4	TQ 06045 14440
Reserve	R1	TQ 37217 06414
	R2	SU 98565 09126
	R3	TQ 54468 01849
	R4	TQ 44482 08920

Supplementary Table 5.2 Primer sets and cycling conditions used for the molecular detection of bumblebee pathogens. Assay mix/ sample: µl/sample: 4.8 µl H_2O, 2µl buffer, 1.25µl $MgCl_2$, 0.5µl dNTPs, 0.2µl primer (forward), 0.2µl primer (reverse), 0.05µl Taq.

Target	Primer	Sample dilution	Cycling conditions		Size (bp)
			Cycles	Time: Temp °C	
Apidae host control (Meeus *et al.* 2010)	ApidaeF: AGATGGGGGCATTCGTATTG ApidaeR: ATCTGATCGCCTTCGAACCT	1/100	Denaturing	2m:95	130
			Replication	35x 30s:95 30s:56 60s:72	
			Elongation	7m:72	
C. bombi (Meeus *et al.* 2010)	SEF: CTTTTGGTCGGTGGAGTGAT SER: GGACGTAATCGGCACAGTTT	1/100	Denaturing	2m:94	420
			Replication	35x 30s:95 30s:57 45s:72	
			Elongation	3m:72	
N. bombi (Klee *et al.* 2006)	NBF: CCATGCATGTTTTTGAAGATTATTAT NBR: CATATATTTTTAAAATATGAAACAATAA	1/10	Denaturing	4m:95	323
			Replication	45x 60s:95 60s:50 60s:72	
			Elongation	4m:72	
N. ceranae (Martín-Hernández *et al.* 2007)	MITOCF: CGGCGACGATGTGATATGAAAATATTAA MITOCR: CCCGGTCATTCTCAAACAAAAAACCG	1/10	Denaturing	5m:98	218
			Replication	35x 15s:95 30s:58 45s:72	
			Elongation	7m:72	

Supplementary Table 5.3 Taxonomic composition of clusters 1-3, identified in the hierarchical cluster analysis, based on species richness data collected from 48 site visits in farmland, gardens and nature reserves between May and August 2016. Frequency is the number of times a species of that genera was recorded. For clusters 1-2, only genera in which a species was recorded more than once are included.

	Cluster 1	Cluster 2	Cluster 3
No. site visits	6	2	1
GENUS	**Frequency**		
Achillea	4	3	1
Agapanthus	2	2	-
Alchemilla	3	-	-
Allium	3	2	-
Alstroemeria	3	-	1
Antirrhinum	-	-	1
Aquilegia	2	-	-
Aster	-	-	1
Astilbe	-	2	-
Bellis	-	2	1
Bessera	-	-	1
Bistorta	2	2	-
Brachysome	-	-	1
Buddleja	2	-	-
Campanula	-	-	1
Centaurea	2	-	1
Carduus	2	-	-
Chamaenerion	4	-	-
Cistus	-	-	1
Clematis	-	3	-
Convolvulus	2	-	-
Coreopsis	-	-	1
Corydalis	2	-	-
Cosmos	-	-	1
Dahlia	2	-	1
Diascia	-	-	1
Digitalis	3	2	1
Erigeron	-	2	1
Eschscholzia	4	2	1
Eupatoria	-	2	-
Eupatorium	2	-	-
Fuchsia	-	-	1
Galium	2	-	1
Geranium	4	3	1
Geum	-	2	-
Helianthemum	-	-	1
Helianthus	2	-	-
Helichrysum	-	-	1
Heuchera	-	-	1
Hippocrepis	-	-	1
Hydrangea	-	3	-
Hypercium	3	-	1
Iris	2	-	-
Knautia	3	-	1
Kniphofia	-	-	1
Lamium	2	2	1
Lapsana	-	-	1
Lathyrus	3	-	1
Lavandula	-	-	1

Supplementary Table 5.3 cont.

	Cluster 1	Cluster 2	Cluster 3
GENUS	**Frequency**		
Leucanthemum	-	-	1
Leycesteria	-	-	1
Linaria	-	-	1
Lobelia	-	-	1
Lotus	-	2	-
Lychnis	2	2	-
Malva	2	-	-
Monarda	-	2	-
Myosotis	-	2	-
Nepeta	4	2	1
Nigella	-	2	-
Oenothera	-	-	1
Papaver	2	2	-
Pentaglottis	2	-	-
Persicaria	4	3	-
Phlomis	3	-	-
Phlox	4	3	1
Phuopsis	-	-	1
Polemonium	-	2	-
Primula	2	-	-
Prunella	-	-	1
Pulicaria	3	-	-
Ranunculus	3	2	1
Rhododendron	-	-	1
Rosa	3	2	1
Rudbeckia	3	2	1
Salvia	4	3	1
Sambucus	-	2	-
Sanguisorba	2	-	1
Scabiosa	3	-	-
Sedum	2	-	-
Silene	2	2	-
Sisyrinchium	2	3	1
Solanum	-	-	1
Solidago	2	-	-
Spirea	-	-	1
Stachys	2	-	-
Symphytum	2	-	1
Tanacetum	-	2	-
Taraxacum	2	-	-
Tithonia	2	-	-
Trifolium	2	2	1
Verbascum	-	2	-
Verbena	-	3	1
Veronica	3	-	-
Vicia	-	-	1
Viola	-	-	1
Wisteria	2	-	-

Supplementary Table 5.4 Taxonomic composition of clusters 4-6, identified in the hierarchical cluster analysis, based on species richness data collected from 48 site visits in farmland, gardens and nature reserves between May and August 2016. Frequency is the number of times a species of that genera was recorded. Only genera in which a species was recorded more than once are included.

	Cluster 4	Cluster 5	Cluster 6
No. site visits	24	7	7
GENUS	**Frequency**		
Achillea	-	2	2
Agrimonia	-	-	2
Allium	-	2	-
Anacamptis	-	-	3
Bellis	5	3	3
Buddleja	2	-	-
Calystegia	2	-	-
Carduus	3	-	-
Centaurea	5	4	2
Centaurium	-	-	2
Cerastium	2	-	-
Chamaenerion	2	-	3
Cirsium	8	2	2
Clinopodium	-	-	2
Convolvulus	5	-	2
Crataegus	5	-	-
Crepis	-	-	4
Dactylorhiza	-	-	3
Digitalis	-	-	2
Echium	-	-	3
Epilobium	3	-	-
Filipendula	-	-	2
Galium	2	-	4
Geranium	2	2	4
Glechoma	5	-	-
Hippocrepis	3	2	3
Hyacinthoides	-	2	-
Hypercium	-	2	-
Hypochaeris	-	3	4
Knautia	4	-	3
Lamium	2	2	-
Lathyrus	4	2	2
Leontodon	5	4	4
Leucanthemum	4	2	3
Linum	2	-	-
Lotus	6	3	6
Magnolia	-	2	-
Medicago	3	-	6
Myosotis	3	-	-
Picris	2	-	-

Supplementary Table 5.4 cont.

Cluster 4	Cluster 5	Cluster 6	Cluster 4
GENUS	**Frequency**		
Odonites	-	-	2
Origanum	-	2	-
Polygala	-	-	3
Potentilla	2	3	2
Primula	3	-	-
Prunella	5	3	3
Pulicaria	2	-	-
Ranunculus	12	4	6
Rhinanthus	-	-	2
Rhododendron	-	2	-
Rubus	6	4	5
Rumex	4	-	-
Sambucus	-	-	2
Scorzoneroides	2	-	-
Senecio	5	2	-
Silene	7	-	3
Sonchus	2	-	-
Stachys	5	-	-
Stellaria	8	-	3
Taraxacum	5	4	4
Thymus	2	-	2
Tragopogon	2	-	-
Trifolium	15	2	6
Veronica	3	-	-
Vicia	3	-	4

Appendices

The following details additional work conducted during this PhD, which was either performed for exploratory purposes or is intended for publication but incomplete at the point of submission.

1. Exploratory work: bumblebee mating and hibernating
2. Exploratory work: bumblebee larval rearing
3. Development of dissection protocol
4. Additional photos of long-tongued bee rearing
5. Disclaimer and additional work for Chapter 4: Habitat type affects the prevalence of bumblebee pathogens in gardens and farmland, but not rates of transmission
6. Additional work for Chapter 5: Floral resource availability, bumblebee health and pathogen prevalence across habitats
7. Additional project: The prevalence of bumblebee pathogens on flowers
8. Additional project: Gut bacterial diversity in pollinators and flowers

Appendix 1: Exploratory work: bumblebee mating and hibernating

Mating queens

Fourteen new queens from *Bombus terrestris* colonies (Biobest) were removed and mated with males from other colonies. Each queen was placed in a plastic nest box and given pollen and sugar water *ad libitum* for 1 hr (food and nest boxes as used in Chapters 2 and 3), before males were added. Bees were kept in daylight. Males and females mated readily and some females mated up to three times. Most only mated once and were aggressive towards males thereafter.

Males showed little aggression towards each other and one male tried to separate a mating male and female.

Figure A1.1 Mating of *B. terrestris* males and young queens. Mating occurred in daylight at 22^{o}C, 55% rh.

Figure A1.2 Male and female *B. terrestris* mating.

Hibernating mated queens

Mated queens were placed in a 50 ml falcon tube sealed with wet cotton wool and hibernated in a SANYO incubator (MIR-154) for 4 months at 4^{o}C and 70% R.H. Seven queens survived this period and produced colonies.

Appendix 2: Exploratory work: bumblebee larval rearing

Work carried out with Danielle Beckett and Rosaline A. Hulse.

Dissection of colonies

Queens and workers were removed from four Biobest *B. terrestris audax* colonies and each life stage separated.

Figure A2.1 *B. terrestris* pupae in casing.

Figure A2.2 *B. terrestris* larvae (left) and pupae (right).

Figure A2.3 *B. terrestris* life stages from larvae (left) to developed pupae (right).

In vitro rearing of *B. terrestris* larvae

Thirty larvae were removed from Biobest *B. terrestris audax* colonies. It is necessary to note that the colonies were quite old and better results would be achieved with young colonies. Larvae were reared in a SANYO incubator (MIR-154) at 33ºC in culture plates (Figure A.2). Without the use of K_2SO_4, pots of water were used to maintain relative humidity. Larvae were fed a 50% sugar solution (250 g sucrose and 500 g water) mixed with 2.5 g ground pollen (35% v/v), 2 µl three times a day, 5 days a week. The food was pipetted into the centre of the well in front of the larvae. Larvae were observed to feed under a dissection microscope. Nearly 20% of larvae pupated and it was theorised that 3 feedings a day was sufficient, but more than 2 µl was required at each feeding.

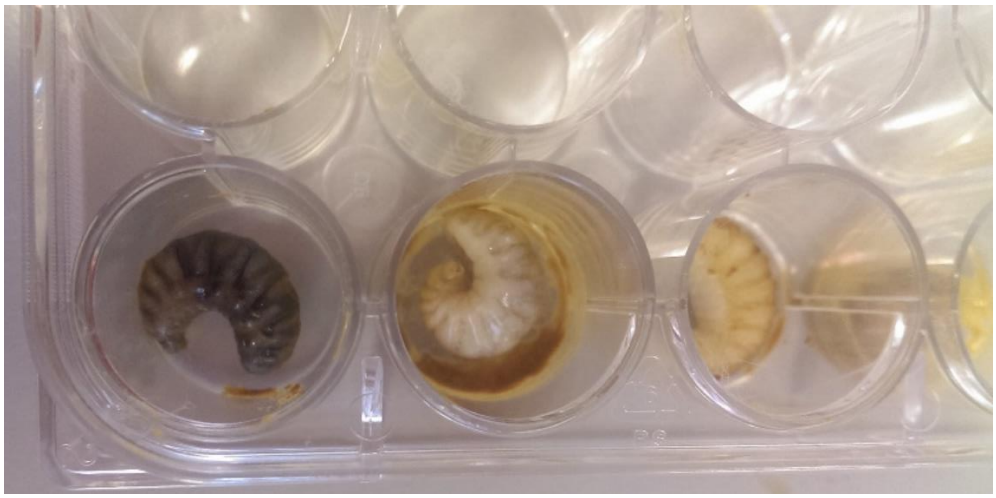


Figure A2.4 *B. terrestris* larvae extracted from Biobest colonies and kept in culture plates. Living larvae are white. Pollen was pipetted in the centre of the well, on the interior side of the larva's abdomen.

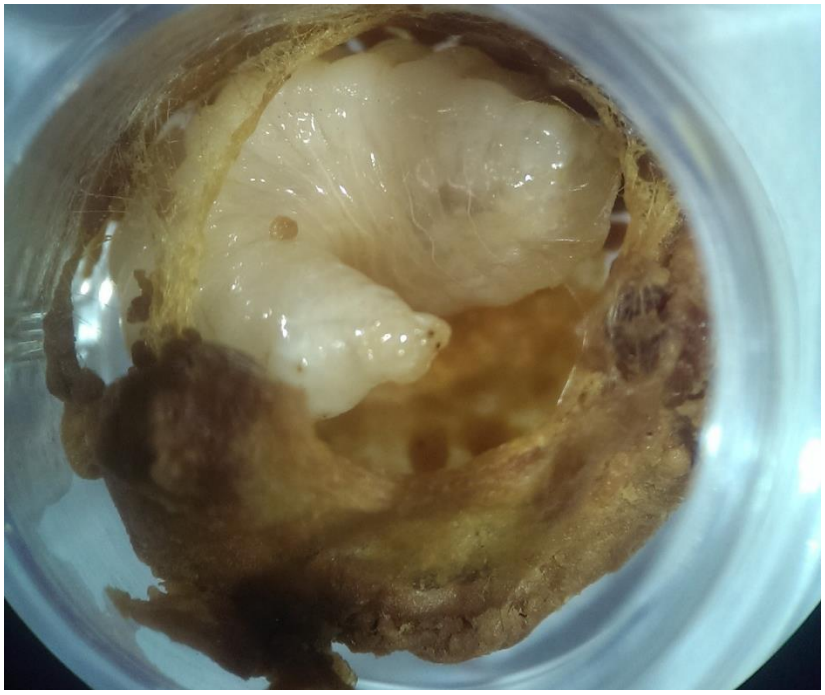


Figure A2.5 *B. terrestris* larvae.

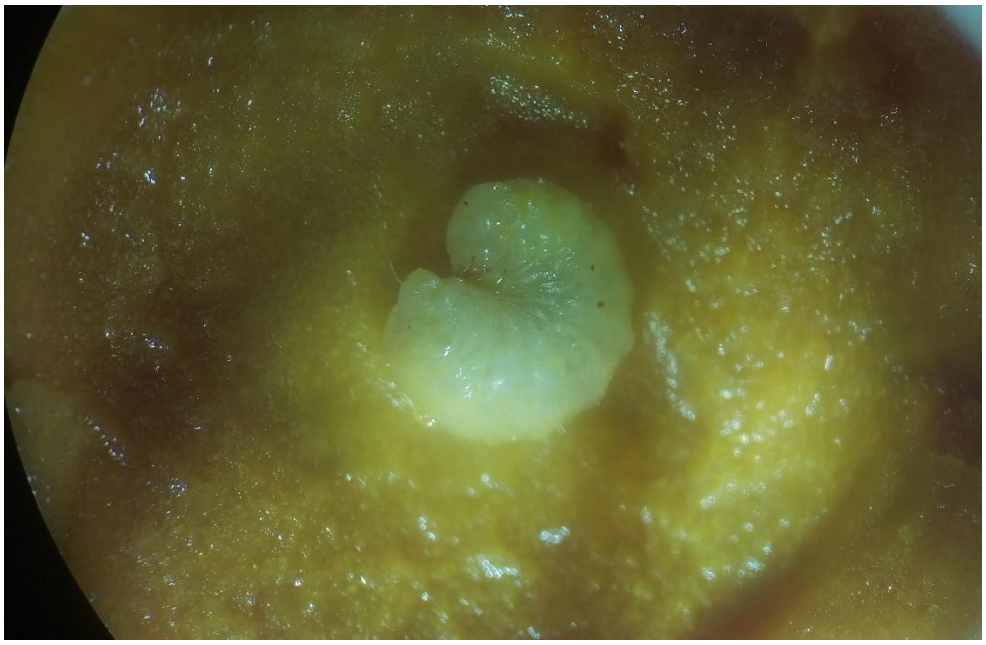


Figure A2.6 A *B. pascuorum* larva that was ejected by its queen was maintained under the same conditions described above, but on a ground surface of pollen (as prepared in Chapters 2 and 3). The larva had died by the following day and I found no evidence it had consumed the pollen.

Appendix 3: Development of dissection protocol

Dissection

My early method of dissection required the use of a scalpel to separate off the abdomen of an ethanol-stored bee, and scissors to cut a straight line down the underside. I found later it was quicker and more economical to dry-freeze the bee and dispense with the scalpel and scissors completely, using only two forceps to tear open the abdomen and remove the desired parts of the gut (Figure A3.1) To screen for all bumblebee pathogens, it was necessary to take samples of the midgut, hindgut, fat body and Malpighian tubules. Bursting the crop was avoided as the pollen and sugar adheres to forceps and the rest of the gut. The gut samples were placed immediately in the extraction buffer contained in 96-well plates and frozen until later use. The remaining carcass of the bee was stored in ethanol.

Extraction

The Proteinase-Chelex extraction (see Chapters 4 and 5), was particularly effective for gut samples taken from bees that contained contents of the bee's digestive tract, which had rotted, or where whole guts were used, but became used as standard for all molecular screening of bumblebees in our lab.

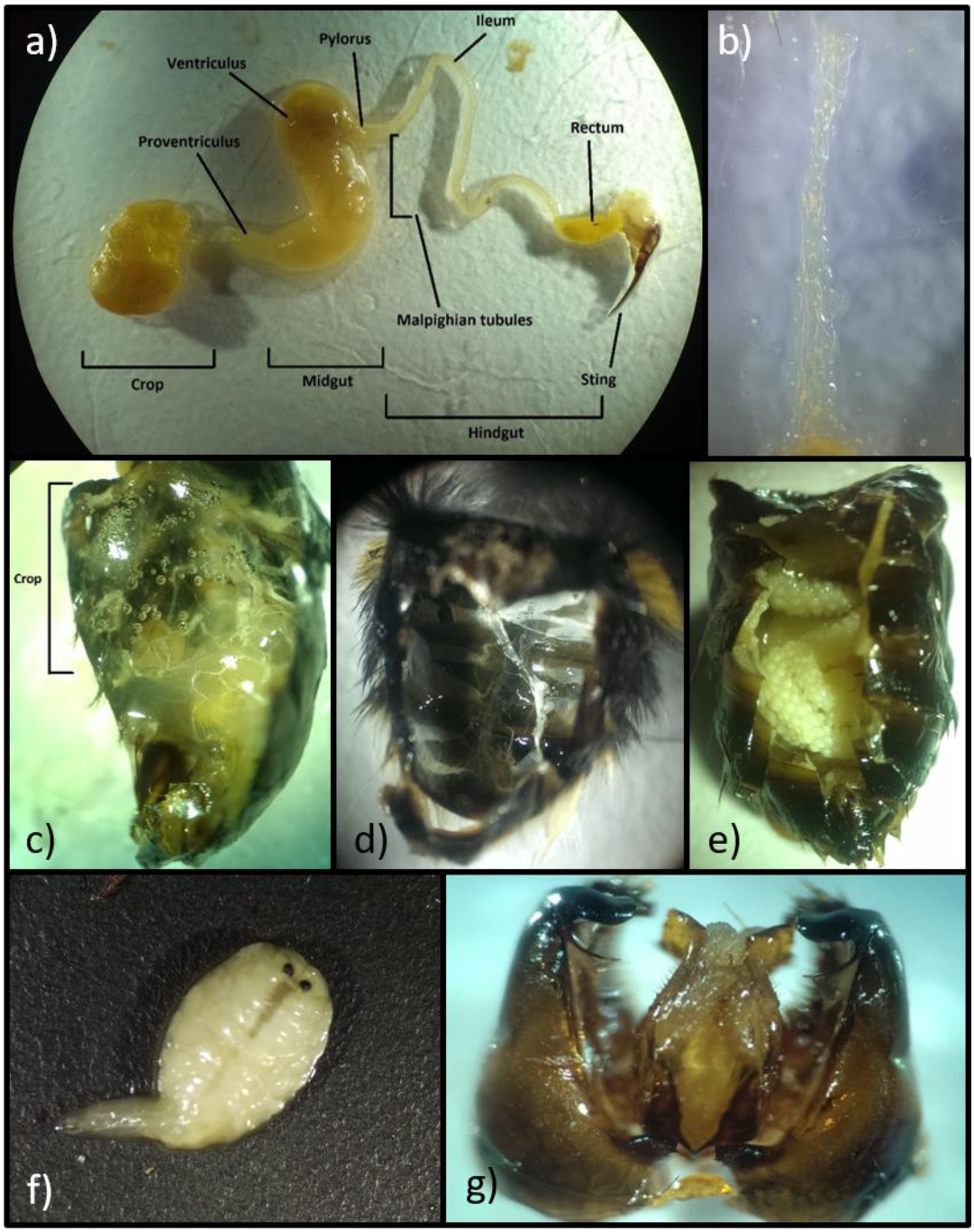


Figure A3.1 a) The organs of the bumblebee gut (from a *B. terrestris* worker). Note the yellow of the pollen throughout the digestive tract; b) the string-like Malpighian tubules; c) a nectar or water-filled crop is clear; d) devoid of gut, the fat body and muscle are visible as white elastic tissue; e) *Sphaerularia bombi*, a parasitic nematode of bumblebees; f) a conopid (Conopidae) fly larvae removed from a *B. lapidarius* worker; g) *B. terrestris* male genetalia.

Appendix 4: Additional photos of long-tongued bee rearing

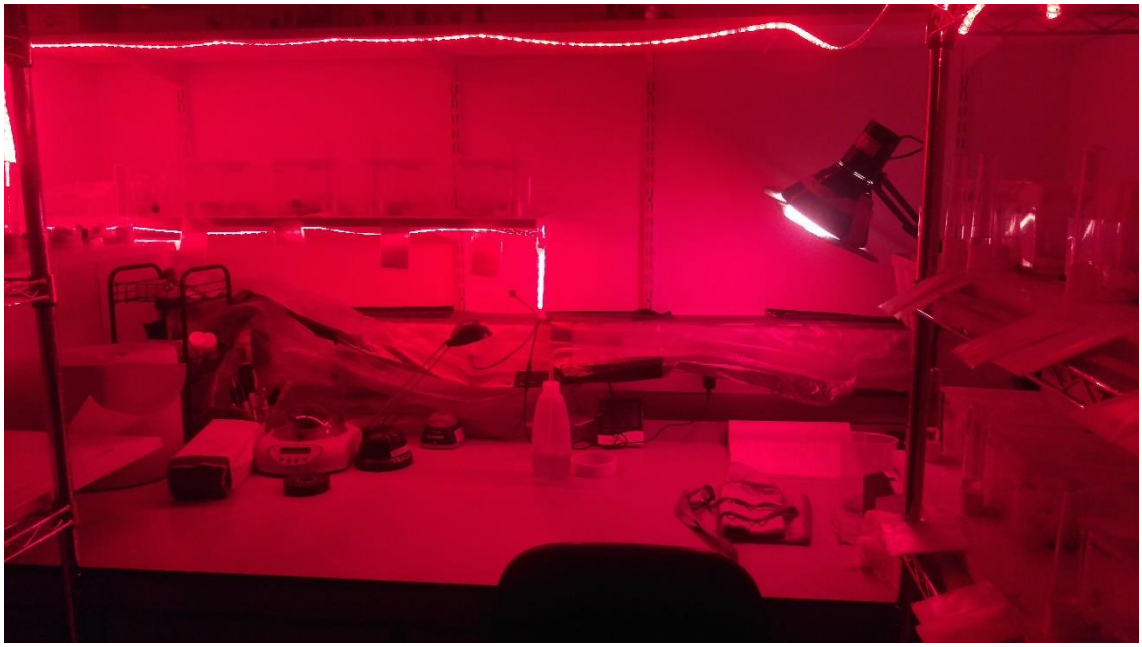


Figure A4.1 Rearing room under red light.

Figure A4.2 Long-tongued bee colonies, including a large *B. hortorum* colony, under red light.

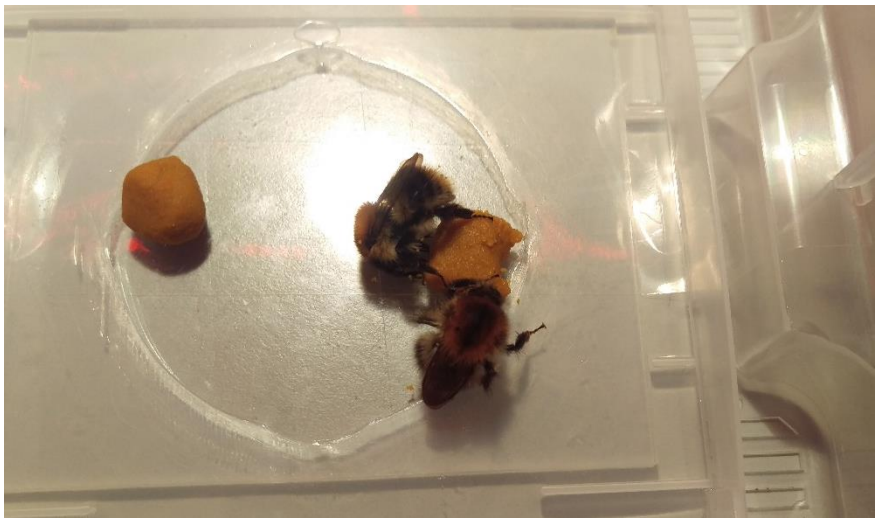


Figure A4.3 Two *B. pascuorum* queens on the day of their capture. Many queens fed immediately on the pollen and showed little or no reaction to the other queen.

Figure A4.4 Two *B. pascuorum* queens on their fourth day paired. A nectar cup made from cotton wool can be seen on the left, and bite marks on the pollen.

Figure A4.5 Two *B. pascuorum* queens both incubating brood cells beside a nectar pot, constructed from pollen and cotton wool.

Figure A4.6 A *B. pascuorum* queen incubating her remaining brood. Openings in the brood casing show where she has ejected eggs and larvae. Cotton wool was used widely by queens as nesting material. This originally provided as cut solid piece – the queens pulled it apart.

Figure A4.7 A *B. pascuorum* queens and worker, with a new pollen pocket to the right of the worker. All workers of this species were very small.

Figure A4.8 A *B. hortorum* queen and her *B. terrestris* worker feeding on sugar water stored in cups made from cotton wool.

Figure A4.9 A *B. pascuorum* queen incubating brood cells. Ejected larval can be seen above her to the right.

Figure A4.10 A *B. pascuorum* nest with three *B. pascuorum* workers and one *B. terrestris* from a donor cocoon.

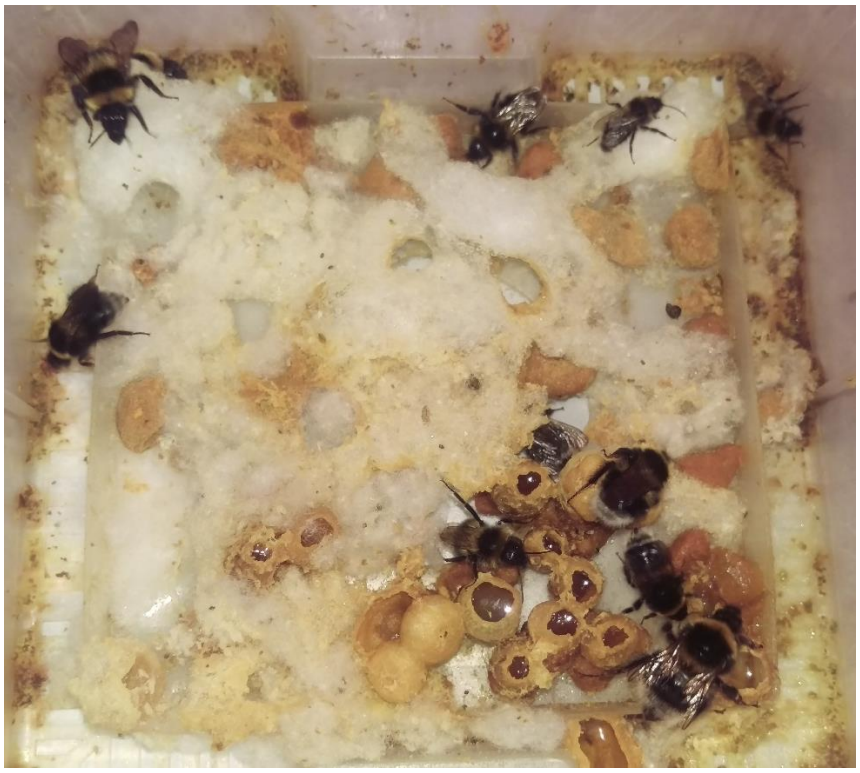


Figure A4.11 *B. hortorum* nest with multiple workers, large and small.

Figure A4.12 *B. hortorum* nest with both young brood pocket visible (top right) and late-stage (L4) larvae (lower left).

Figure A4.13 The largest *B. hortorum* colony with multiple cocoons present.

Appendix 5: Disclaimer and additional work for Chapter 4: Habitat type affects the prevalence of bumblebee pathogens in gardens and farmland

Disclaimer

Data for this study were initially collected based on the assumption that approximately ten bees could be collected from approximately 40 sites. This was based on previous years' experience in the field and knowledge of the sites used. However, it was not possible to visit as many sites or collect as many bees as planned and the chapter was revised accordingly.

Additional work

In addition to molecular screening, each bee would also undergo a PO/proPO analysis. Melanisation, an immune response in bumblebees, involves the enzyme phenoloxidase (PO). Its inactive form, prophenoloxidase (proPO), is always present in the haemolymph. Levels of the two are to be compared in each bee. In tangent with the molecular screening for pathogens, we hope to offer evidence to distinguish between presence and infection.

Materials and Methods

Workers were freeze-killed in dry ice and stored -80^{o}C until further use. After the gut was removed for molecular screening, the remaining thoraces were placed in 96-well plates and each was mechanically homogenised in a 700µl phosphate-buffered saline (8.74g NaCl; 1.78g $Na_2HPO_4{\cdot}H_2O$; 1000ml distilled water (pH= 6.5). The samples were vortexed and centrifuged at 4000G (4^{o}C for 10 minutes) and the supernatant recovered. The reaction mixture for measuring active PO was prepared on ice (140µl MQ water; 20µl PBS; 20µl supernatant; 20µl L-DOPA solution: $4mg^{-1}$ in MQ water) and pipetted into a new 96-well plate. Total PO and proPO was prepared on ice (120µl distilled water; 20µl PBS; 20µl supernatant; 20µl bovine α-chymotrypsin solution: $2mgml^{-1}$ MQ water) and incubated at room temperature for 5 m before 20µl of L-DOPA was added to the reaction mixture and pipetted into a new 96-well plate. 20µl of diluted thorax sample was pipetted into each plate. For both PO and total PO/proPO measures, blank and control mixes were used. Plates were placed in a preheated plate reader (30^{o}C) and measure absorbance every 20s for 50 minutes at 480nm. Enzyme activity was measured as the slope of the reaction curve during the linear phase of the reaction. PO measures were

normalised to the protein concentration of haemolymph per sample (determined by the Bradford assay with a bovine serum albumin standard).

Appendix 6: Additional work for Chapter 5: Floral resource availability, bumblebee health and pathogen prevalence across habitats

Bee size analysis

Measurements of head width, head length, intertegular span, hind tibia length and marginal cell length were taken as proximates for bee size.

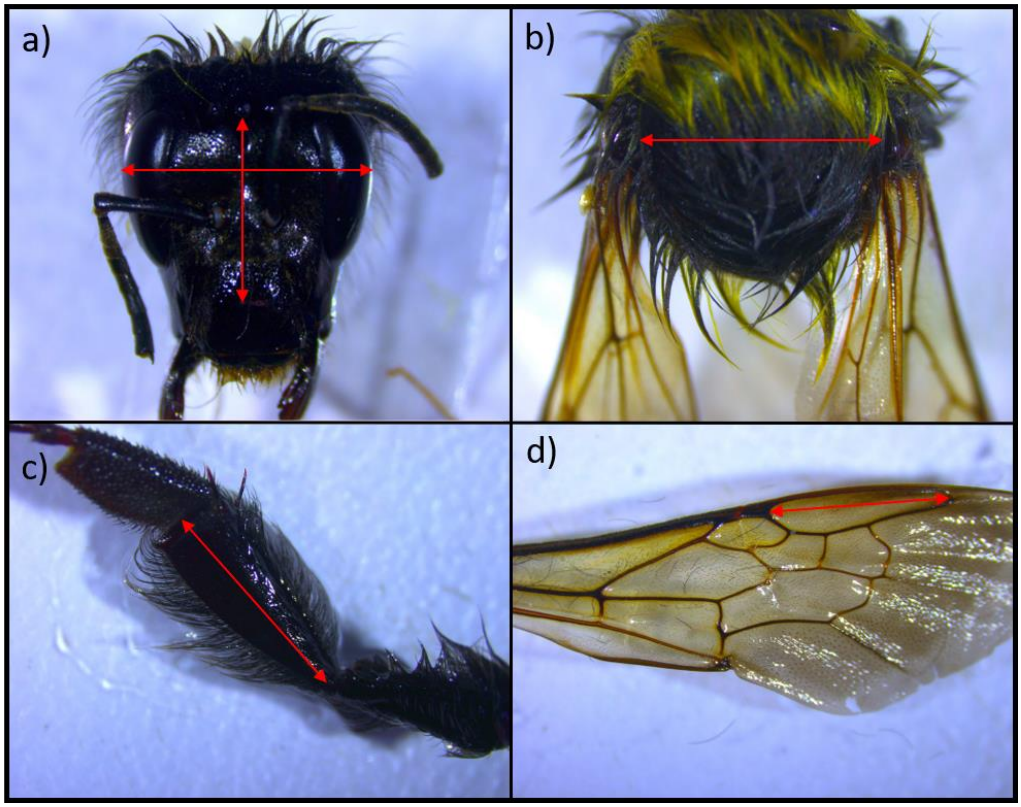


Figure A6.1 Body size was originally based on linear measures of a) head width (maximum distance between external lateral margins of eye), head length (from central ocellus to the fissure of the labrum), b) intertegular span (the shortest distance between wing tegulae across the thoracic dorsum), c) hind tibia length, and d) marginal cell length of the forewing (Cane 1987, Pinto da Silva *et al.* 2015). Head and thorax measurements were taken under 1.6 x 40 magnification and tibia and wing measures were taken under 1.25 x 40 magnification.

Pollen analysis

Pollen analysis was considered for this project to evidence the plant species on which bumblebees were foraging on and scopal pollen loads were harvested from all bumblebees if present. It was later decided there was not sufficient time to carry out the analysis.

Slide preparation

Pollen grains removed from the scopae of wild bumblebees were stored in 100% EthOH before they were transferred to a microscope slide and the EthOH allowed to evaporate. A drop of water containing glycerine jelly stained with fuchsin was added and the slide sealed with a coverslip.

Appendix 7: Additional project: The prevalence of bumblebee pathogens on flowers

Rationale

Flowers are important ecological intermediaries for parasite transmission. All microbial pathogens known to infect bumblebees are horizontally transmitted in this way. Previous research has established how prevalent each of these pathogens are in bumblebees, however no attempt has been made to establish their prevalence in flowers, which may not be the same. In this environment, the pathogens are exposed to diverse biochemical conditions associated with pollen and nectar secretions as well as UV light and fluctuating temperatures. However, some flowers may constitute harsher environments than others, which may have implications for transmission efficiency and pathogen virulence.

Materials and methods

This study was carried out in Sussex (southern England) using four sites (three non-domestic gardens and the university campus). Prior to field sampling, six plant species known to be visited by bumblebees and which were flowering in the sites during the survey period were selected (*Trifolium repens, Geranium pratense, Hippocrepis comosa, Lotus corniculatus, Lathyrus pratensis and Prunella vulgaris*). These functioned as baseline species, to which all others could be compared.

Bee and floristic surveys

Site visits were carried out at the end of June 2017. Each site was visited once, in which 20 short-tongued bumblebee workers (including *B. terrestris/ B. lucorum*) and 20 long-tongued workers (including *B. pascuorum* and *B. hortorum*) were collected. In addition to the six plants selected beforehand, up to ten other plants visited by bumblebees were chosen at each site. Flower samples were taken from all selected plants, each containing three single flowers or three inflorescences. Up to 20 samples were collected for each species (from 1-10 plants) and stored immediately in 100% ethanol. In addition, all species were observed for 4 minutes to quantify insect visitation. For each plant species we recorded the estimated floral units in the observatory area and the number of (i) *B. terrestris/ B. lucorum,* (ii) *B. lapidarius,* (iii) *B. hortorum,* (iv) *B. pascuorum,* (v) *B.*

pratorum, (vi) *B. hypnorum,* (vii) other *Bombus,* (viii) *Apis mellifera,* (ix) Lepidoptera, (x) Diptera, and (xi) other.

Bumblebee dissection and pathogen screening

For pathogen screening a sample of the Malpighian tubules, fat body, midgut and hindgut from each bee was transferred to a digestive solution for 20 min, containing STE buffer (100 mM NaCl, 10 mM Tris pH8, 25 mM EDTA and 0.5% SDS), proteinase K (0.1 µg/µl) and 50% Chelex. The samples were then homogenised using sterile toothpicks and incubated for 6 h at 55^{o}C and 15 min at 95^{o}C. Two elution steps were carried out, the first using 1:1 isopropanol and the second using 70% EthOH. Between each elution the samples were centrifuged for 1 h and the supernatant discarded. The remaining DNA pellets were resuspended in molecular grade water and stored at -4^{o}C until screening (Supplementary Table 5.2). Negative controls were included in each extraction plate and positive controls in each PCR.

Flower processing and pathogen screening

Flower samples will be vortexed before the flowers are removed and the remaining ethanol solution containing cells and spores, centrifuged. Following a Chelex extraction, the DNA pellets will be suspended in molecular grade water and screened for bumblebee pathogens (Supplementary Table 5.2). Negative controls will be included in each extraction plate and positive controls in each PCR.

Appendix 8: Additional project: Gut bacterial diversity in wild bees and their flowers

Introduction

Sociality is known to play an important role in the transmission of gut bacteria in bees. Transmission occurs via the oral-faecal route and the bacterial communities in bumblebees are recognised as simple, species-poor and highly distinctive. Upon emergence, callow workers would be immediately exposed to the dominant bacterial groups abundant within their colony and by the time they leave to begin foraging, their gut microbiome may be fully formed. Research thus far has shown little to no variation in the bacterial communities of bees living in different habitats or even in different continents, suggesting that host species have distinct gut communities independent of geography. Host-specific strains of bacteria support the hypothesis of coevolved, vertically transmitted bacteria rather than a free exchange amongst bee species. Where habitat-specific differences are seen, they are amongst the non-core (rare) bacterial groups. We suggest this could be explained by the regular (but sparse) influx of new bacteria that are transmitted via flowers, which are unlikely to get a foothold in the gut of the worker or its colony.

Solitary bees, for lack of in-colony transmission, acquire all of their gut floral through shared flowers. Given this difference, we expect to see a difference in the degree of similarity between bumblebees and their flowers, and solitaries and theirs. Flowers are colonised by various transient microbial organisms using them as vectors, as well as their own microbiota. As such, the solitary bee gut flora should sit within the range of bacteria found on flowers. For bumblebees and their flowers, there should be fewer similarities, but also less structural overlap, due to their second (dominant) source of bacteria. In some cases, the bacterial community in bumblebees may exist almost entirely independently of floral resources.

Given this difference between solitary and social, we would expect to see a difference in the degree of similarity between bumblebees and their flowers, and solitaries and theirs. If flowers carry bee-related microbial organisms as well as their own microbiota plus other things looking to colonise non-bee taxa, the solitary bee gut floral should sit within

the range of the bacteria found on flowers. For bumblebees and their flowers, there should be fewer similarities, but also less overlap, because they have a second (dominant) source of bacteria from elsewhere (the colony). In some cases, the bacterial community may exist almost independently of floral resources. This study aims to determine how the similarity of the gut flora between bees and their preferred foodplants is affected by sociality.

Materials and Methods

Between April and July 2018, pollinators actively foraging on flowers were collected through Sussex, southeast England. Insects were immediately freeze-killed in dry ice. A sample of 20 flowers (or 20 inflorescences) were taken from each visited flower. All insects and flowers were barcoded to confirm a species identification. Samples were pooled by each plant-pollinator interaction and deep-level sequencing will be carried out.