The emergence and spillover of bumblebee parasites

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The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Author contributions are as follows: PG, BD, DG & WOHH designed the study. PG, performed experimental work with assistance by KY and SEFE. PG performed molecular work, data analysis and manuscript drafting. WOHH supervised the work and assisted with data analysis and manuscript drafting. BD & DG assisted with manuscript drafts.

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“I'm a success today because I had a family who believed in me and I didn't have the heart to let them down.”

- *Anonymous*

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Abstract

Pollinators and in particular, bumblebees are currently experiencing significant declines. In Britain, bumblebee populations have been declining since the industrial revolution. Modern farming now requires large, monoculture fields to be effectively pollinated, yet the very nature of such large, homogeneous environments prevents many wild pollinators to thrive there. Since the mid-1980s bumblebees have been reared and imported on an industrial scale to aid the pollination of many valuable crops such as tomatoes and raspberry. There is great concern that the intensive rearing and importation of these bumblebees may permit the introduction of exotic parasites to native bumblebees. These concerns follow suggestions that parasite spillover from commercially reared bumblebees may be occurring; with declines of wild bumblebees in North and South America correlated with commercial bumblebee use. It's believed that around 50, 000 bumblebee hives are imported into the UK every year and whilst they are purported to be disease free, no independent testing is carried out. Here, I assess what risk the use of commercial bumblebees has on native bees. By screening commercially reared and imported bumblebee colonies for a range of bumblebee and honey bee parasites, I identified the majority have infections. The parasites detected include the emerging diseases *Apicystis bombi* and *Nosema ceranae* which are found to be lethal to infected bumblebees. Shared flowers between bumblebees and honey bees are shown to be platforms for the dispersal of many of these parasites. The frequent mixing between domesticated and wild bumblebees allows potential transmission of these parasites. The deployment of commercial bumblebees was shown to increase parasite prevalence within local populations of wild bumblebees and when bumblebees have increased competition in the form of domesticated honey bees, they once again have higher parasite prevalence. Here I show that not only are current import regulation inadequate to avoid introducing infected bumblebees into England, but that there are clear opportunities and evidence that transmission is occurring.

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Chapter 1: General Introduction

1.1 Pollinators and their role in the environment

Plants are essential for life on earth, as primary food producers, and responsible for the majority of the world's atmospheric oxygen. The dominant land plants are angiosperms (flowering plants) with an estimated *ca* 352,000 species, all of which rely on pollination for their successful reproduction (Paton *et al.* 2008). Pollination is the process by which pollen is transferred from the anther to the stigma of a flower, achieving fertilization, thus enabling the plant to reproduce (Raven *et al.* 1998). Whilst some angiosperms can be pollinated abiotically, more than two thirds rely on the assistance of animal pollinators, without which these species would be unable to reproduce and therefore face extinction (Kearns & Inouye 1997; Ashman *et al.* 2004; Fontaine *et al.* 2006; Biesmeijer *et al.* 2006; Klein *et al.* 2007; Ollerton *et al.* 2011). This reliance is the result of 65-135 million years of co-evolution between flowers and pollinators (Poinar & Danforth 2006; Friis *et al.* 2006). Essential for the lifecycles of flowering plants, pollinators thus help sustain ecosystem biodiversity and productivity (Buchmann & Nabhan 1996; Kearns *et al.* 1998; Ashman *et al.* 2004). A huge diversity of animals act as pollinators, including birds, bats and monkeys (Gautier-Hion & Maisels 1994; Cronk & Ojeda 2008; Fleming *et al.* 2009). However, the oldest and most diverse group of animal pollinators are the insects (Pellmyr 1992). Several orders of insect can act as pollinators. The following four are frequently found on flowers;

1. Coleoptera: Widely considered the most primitive pollinators, many beetles have a solely floral diet as adults (Faegri & Pijl 1966). Pollination by beetles is often cumbersome and primarily requires flowering plants to entice the beetles with easy access and heavy scent to their flowers (Kevan & Baker 1983; Endress 1994). The mutualism between beetles and some flowers is believed to be important for the maintenance of flowers in several semi-arid ecosystems such as Southern Africa and the North American state of California (Jones & Jones 2001).

2. Diptera: Pollinating Dipterans are also considered to be primitive pollinators, generally displaying only slight adaptations for pollination such as lapping mouth parts, requiring flowers to provide easily accessible nectar (Thien 1980). Dipterans are

known to visit in excess of 555 flowering plant species with several of these being important crops such as cashew, mango and onion (Larson *et al.* 2001; Ssymank *et al.* 2008). As a result, Some Dipterans such as *Lucilia caesar* are commercially reared for crop/seed production, though producers encourage their use to be supplementary to bumblebee pollination (Koppert; Currah & Ockendon 1984).

3. Lepidoptera: Most Lepidopterans have mouthparts adapted to feed extensively on floral nectar. The proboscis length is variable across the order, with the moth *Xanthopan morgani* having the longest at up to 30 cm long whilst moths in the family's Micropterigidae, Agathiphagidae, and Heterobathmiidaesome have no proboscis (Nilsson 1998; Krenn 2010). Though some mutualisms exist, such as between the orchid *Angraecum sesquipedale* and *X. morgana*, Lepidopterans are generally not considered to be major pollinators due to their non-pollen diet (Kevan & Baker 1983; Jay 1986; Tangmitcharoen & Owens 1997; Anderson & Johnson 2008; Krenn 2010) .

4. Hymenoptera: The diversity and efficiency of Hymenopteran pollinators make this order the most important for angiosperms in general. Ants represent the least adapted pollinator family in this order, with some ants secreting chemicals that inadvertently reduce pollen viability (Dutton & Frederickson 2012). Despite this, some ant pollinators do exist (Rico-Gray & Oliveira 2007). Most wasps are carnivorous, playing little part in pollination however some social wasps such as *Polistes versicolor*, are able to pollinate (Sühs *et al.* 2009). Sawflies, again, do not have mouthparts adapted for pollination yet nectar forms a large part of their diet and during consumption pollen will become attached to the sawfly's body (Kevan & Baker 1983). Fig wasps engage in an intimate mutualism with figs; over 750 species of fig depend on fig wasps for their pollination, during which fig wasps are able to complete their lifecycle (Cook & Rasplus 2003). Unlike sawflies, wasps and ants, bees have mouthparts adapted for pollination and large hairy bodies that also aid pollen transfer. Bees are not only the main hymenopteran pollinators, but are considered the main animal pollinator in most ecosystems (Kevan & Baker 1983; Neff & Simpson 1993; Williams 1998a),

1.2 Success and domestication of pollinators

Pollinators play an important role in the lifecycle and seed/fruit formation of plants; they are inherently critical not just to the ecosystems they inhabit but also for the productivity of farmland crops (Buchmann & Nabhan 1996). Animal pollination is directly responsible for 22.6% and 14.7% of agricultural production in the developed and developing world, respectively (Aizen *et al.* 2008). With the addition of foods that rely indirectly on animal pollination, 35% of human diet benefits from the role of pollinators (Klein *et al.* 2007). Worldwide the valuation of 'animal pollination services' is estimated at around €153 billion pa (Richards 1992; Williams 1994; Gallai *et al.* 2009). Their direct value to agriculture is estimated at €14.2 billion for Europe and €14.4 billion for North America (excluding Mexico) (Gallai *et al.* 2009). In Europe, the yields of 84% of more than 150 crop species rely on, or are improved by insect pollination (Klein *et al.* 2007). Modern farming is increasingly intensive, creating large areas with limited proximity to pollinator rich areas, such as wild meadows or even hedgerows. When flowering crops are used in such fields, they suffer from suboptimal crop production due to the field centres providing few-to-no habitable areas for pollinators such as bumblebees to nest (Kremen *et al.* 2002; Free & Williams 2009). Additionally, areas can be planted in such densities that natural pollinator populations are unable to service all crops. As such, to increase crop yields, it is often beneficial to boost the numbers of pollinators on-site by utilizing domesticated bees (Batra 1995; VanEngelsdorp & Meixner 2010; Lye *et al.* 2011).

Honey bees (genus: *Apis*) and bumblebees (genus: *Bombus*) are considered to be the two main groups of pollinating bee and have been successfully domesticated to further utilised their pollination services (Velthuis 2002; VanEngelsdorp & Meixner 2010). Whilst the life histories of *Apis* and *Bombus* are not the same, much of their success and domestication has come from their eusocial lifestyle. Eusocial insects include social bees, ants and wasps and despite only comprising 2% of known insect species, form the majority of the planet's insect biomass (Wilson 1990). Eusociallity is typified by a division of labour with reproductive and more or less sterile individuals (often split into 'castes'), occurring in overlapping generations and employing cooperative brood care (Wilson 1971). Eusocial bees are typified by having a large number of workers (pollinators) all foraging to increase colony growth and reproductive success of their reproductive caste. As a result, a single, successful

colony can be home to hundreds (in the case of bumblebees) or thousands (in the case of honey bees) of pollinators.

1.2.1 Honey bee domestication

Despite often being thought of as the most common bee, there are only nine species of honey bee worldwide (Koeniger & Koeniger 2000). Eight of these are naturally distributed within Asia, whilst the ninth, *Apis mellifera*, has a natural range from central Asia into Europe and Africa (Seeley 1985; Ruttner 1988; Sheppard & Meixner 2003). Honey bees do not hibernate in the true sense, and instead, owe their survival through months of scarce floral resources to the stockpiling of honey, allowing them to 'over-winter'. Stocks of honey are produced by worker bees following nectar collection during the summer and stored in the hives. Colonies stockpile enough honey to sustain the colony until the following spring when foraging can begin again. A strong colony has the capacity to stockpile more than enough honey, allowing it to be farmed by apiarists (Figure 1.2.1.1).

Honey bees have been managed since 2600 BCE in Ancient Egypt for their honey (Ransome 2004). Domesticated honey bees are a source of beeswax, pollen and honey plus their ability to pollinate has ensured their translocation along with every large scale human migration (Crane 1975, 1999; VanEngelsdorp & Meixner 2010). This movement across the world has caused some conservation concerns, with changes to natural floral sets and local pollinators suffering reduced fitness following the introduction of managed honey bees (Huryn 1997; Goulson 2003b; Goulson & Sparrow 2008). Despite these concerns, *Apis mellifera* is now the most commonly managed pollinator and found in nearly all habitable regions (Engel 1999; Ransome 2004; VanEngelsdorp & Meixner 2010). The generalist pollinating services of the honey bee allow this widespread pollinator to be regarded as the most economically valuable pollinator of monoculture crops (McGregor 1976; Watanabe 1994). Globally, around 1.4 million tonnes of honey are taken to market a year with an estimated total valued in excess of €1 billion (VanEngelsdorp & Meixner 2010). The European Union consumes approximately 310, 000 tonnes of honey a year, 20% of worldwide production, with China and the USA accounting for 15% and 10% of its consumption respectively (Eurostat 2009). In these top 3 consumers; China is the only country to produce enough honey to satisfy its own demands (FAO 2009). The EU is also the

largest consumer of beeswax, accounting for a third of global imports in 2006. An estimated 10, 000 tonnes of beeswax is purchased annually in the EU, though this is expected to be lower than the actual figure due to incomplete data reports. Spain is the largest European producer of beeswax, accounting for 55% of European production; total production was calculated as 4169 tonnes in 2005 (CBI 2009). Spain is also believed to be the only country in the EU to produce significant volumes of other bee products such as propolis, royal jelly and bee pollen.

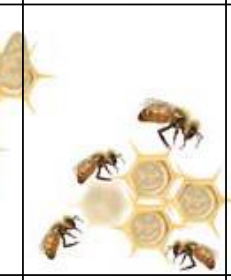
	Spring	Early Summer	Late Summer	Autumn	
Outside					During Summer, additional hive segments (Supas) are added by an apiarist to encourage additional honey production. By autumn these are harvested .
Inside					A honey bee queen can last several years and every year, more queens will be produced
	Spring flowers generate increased foraging from overwintering workers. Queen will lay between 1500-2000 eggs a day.	Peak activity: high nectar returns converted to honey and stored in the nest comb. High pollen returns ensure a high larvae/worker production. Triggered by high worker density, special brood cells for new queens are constructed. Upon emergence new queens will leave to establish their own nest with a cohort of maternal workers.		Diminishing resources trigger reduced activity within the hive. The colony will exist on stored food throughout the winter with occasional scouts for water.	

Figure 1.2.1.1 The annual cycle of a honey bee hive. Highlighting honey harvesting by apiarists (outside row) and natural queen and worker production (inside row).

The most important and valuable service performed by honey bees though is their pollination provision. Fifty-two of the top 115 global food commodities depend on honey bee pollination for either fruit or seed set (Klein *et al.* 2007). Capable of increasing the yields in 96% of animal pollinated crops, honey bees are the most important pollinator for most crops worldwide (McGregor 1976; Delaplane & Mayer 2000; Klein *et al.* 2007). Recently it's been estimated that projected honey bee

populations will not be able to satisfy agricultural pollination demands in the future (Aizen & Harder 2009).

1.2.2 Bumblebee domestication

Bumblebees are much more diverse than honey bees, with over 250 bumblebee species worldwide, confined mostly to temperate-alpine areas of the northern hemisphere and South America (Williams 1998b). With the diversity of species, comes a diversity of adaptations, the variation that exists between species enable the *Bombus* genus to pollinate a large range of flower types, some of which are solely reliant on pollination by bumblebees and therefore their declines have been inextricably linked with declines in coevolved flowers (Biesmeijer *et al.* 2006; Goulson 2010). *Bombus terrestris* is the most widespread species of bumblebee with a native range covering Europe and coastal North Africa. For many important plants and valuable crops such as raspberry and tomatoes, honey bees are inefficient pollinators. (Batra 1995; Cane 2005; Velthuis & Van Doorn 2006; Greenleaf & Kremen 2006). Several adaptations make bumblebees ideal pollinators for many such plant/crop species (Velthuis & Van Doorn 2006). Bumblebees can perform buzz pollination (sonication), essential for the pollination of tomatoes, whereby high frequency vibrations from the bee stimulates the flower to release pollen (Goulson 2010). Their large bodies have a thick coat of hairs that serves to transfer pollen from one flower to the next; this coat provides insulation allowing them to forage in cooler climates and to begin foraging earlier and finish later in the day than honey bees (Heinrich 1993). Some bumblebee species have also evolved long tongues as part of a mutualism with some plant species that have coevolved long corollas often preventing other pollinators accessing their nectar (Nilsson 1988). It is the combination of these pollinator features that allows for a wide range of plants to be serviced by bumblebees and highlights the value of bumblebee pollination to the environment and our economy (Goulson 2010). Unlike honey bees, bumblebees have an annual lifestyle so no effort is spent stockpiling resources or overwintering after reproductive individuals have been produced. Generally speaking, bumblebee queens will emerge from hibernation in the spring and will start searching for a suitable nest site. Differences in emergence timings do exist between bumblebee species though, with some species timing emergence with resource availability such as *B. frigidus* timing its emergence within 24 hours of the first appearance of willow

catkins (Vogt *et al.* 1994). Following emergence, a bumblebee queen will find a suitable nest before rearing a batch of workers, and then switching to queen and male production (resource dependent). Newly produced queens are the only individuals that will survive to the following spring (Goulson 2010; Figure 1.2.2.1).

Colony founding	Establishment	Mating	Hibernating
			
A queen emerges from hibernation and finds a nest site, such as an abandoned rodent burrow. She creates wax pots to hold nectar and pollen, on which she lays and incubates eggs.	When her daughters emerge as adults, they take over foraging and other duties. The colony will grow to between 50-300 individuals depending on species	In autumn the colony produces new queens and/or male bees, who leave to find mates.	Newly mated queens hibernate and the rest of the bees die.

Figure 1.2.2.1 The annual life cycle of bumblebees. After hibernation, bumblebee queens will found a colony, raise workers and reproductives, and then die. Only the newly produced queens will survive until the following year.

Unlike honey bees, bumblebees do not produce honey or hive material fit for mass market. Their commercial use is purely based on demand for their pollination services. For many crops, honey bees are not the most efficient pollinator (VanEngelsdorp & Meixner 2010). Up to 8% of all flowering plants including many important crops such as tomatoes and potatoes require pollination via sonication, plus flowers with long corollas are not successfully pollinated by honey bees due to being unable to sonicate and having an insufficient proboscis length (Hobbs 1961; Buchmann 1983). In such instances, bumblebees are often an efficient pollinator and more suitable than honey bees (Nilsson 1988; Velthuis & Van Doorn 2006; Goulson 2010). Prior to bumblebee domestication, crops requiring sonication such as tomatoes were mechanically vibrated to achieve pollination. The cost of mechanically vibrating the plants was over €10, 000 per ha per year in 1988 (Velthuis & Van Doorn 2006). Like honey bees, bumblebees have, more recently, been translocated by humans to aid crop production. Between 1885 and 1906, hundreds of bumblebee queens were translocated from the UK to New Zealand to establish colonies and improve the seed set of red clover. Four native UK

species; *B. hortorum*, *B. ruderatus*, *B. subterraneus* and *B. terrestris* all became naturalised in New Zealand as a result (Hopkins 1914). Following this agricultural success in New Zealand, *Bombus ruderatus* queens from the newly naturalised stock were translocated to Chile for their red clover pollination in 1981 and 1982 (Arretz & Macfarlane 1986). By the 1960's there was a clear demand to utilise bumblebees as pollinators on an intensive scale, requiring their domestication (Holm 1966; Free 1970; Velthuis 2002; Velthuis & Van Doorn 2006). Unlike honey bees, bumblebees require diapause initiation after mating and then need to be stimulated to lay eggs following emergence (Goulson 2010). Various protocols were developed, to achieve this, such as the inclusion of honey bee workers to stimulate egg laying from bumblebee queens (Ptacek 1985; Heemert *et al.* 1990; Van der Eijnde 1990). By the late 1970's enough progress had been made to fully domesticate the most common bumblebee species, *B. terrestris* (Röseler 1977; Velthuis & Van Doorn 2006). With the joining of both demand and technology, commercial production of bumblebee colonies began in 1987 and by 1989 three Dutch companies had begun production (Biobest, Koppert and Bunting Brinkman Bees [Later purchased by Syngenta]) (Velthuis & Van Doorn 2006). Today the industry estimated to be worth in excess of €55 million pa and is served by over 30 factories (Velthuis & Van Doorn 2006). Globally, over a million bumblebee colonies are imported with 40,000 – 50,000 being imported under licence specifically to the UK (Velthuis & Van Doorn 2006; Natural England 2012). Most commercially reared bumblebees are *B. terrestris*, though differing subspecies of *B. terrestris* are used with non-native species and subspecies often deployed. In Japan and Argentina *B. terrestris* is used despite originating from different continents (Velthuis & Van Doorn 2006). Similarly, until recently, only the non-native subspecies *B. terrestris dalmatinus* was deployed in the UK with colonies costing approximately £44 each. Since 2010 *B. t. audax* has also been offered, which is the native subspecies in the UK and costs just £7 more per hive than *B. t. dalmatinus*. In order to maintain worldwide demand for commercially reared bumblebees, its estimated around 500 tonnes of pollen is required to feed the colonies in the rearing facilities (Goulson 2013). This pollen is harvested by honey bees and removed from their pollen baskets by 'pollen traps'. The geographical origin of the pollen is not disclosed by the commercial companies though Spain and China are believed to have the best set-up for pollen harvesting (Centre for the Promotion of Imports from developing countries 2009).

1.3 Pollinator declines

Due to the clear value pollinators have both in agriculture and in the diverse ecosystems they naturally service, populations are frequently monitored. The productivity of diverse ecosystems are often seen as being resilient against species declines as alternatively available organisms may provide similar roles in the community (McCann *et al.* 1998; Dunne *et al.* 2002). However, ecosystem collapse is predicted to occur if key species such as pollinators are removed (Heywood 1995). Currently both managed and wild pollinators and are suffering alarming declines in many parts of the world (Potts *et al.* 2010b). Managed honey bees in North America and Europe are the worst hit currently. In the USA from 1947 to 2005 the number of honey bee hives had dropped 59% and in Europe they have dropped 25% between 1985 to 2005 (Natural Research Council 2007; VanEngelsdorp *et al.* 2008; Potts *et al.* 2010a). Worldwide, the numbers of managed honey bee colonies is on the increase, however with large regional declines in the USA and Europe, agricultural demand is increasing 3 times greater than net honey bee population (Aizen & Harder 2009). Wild and feral honey bees populations in the USA and Europe are now significantly reduced to near absence (Kraus & Page 1995; Moritz *et al.* 2007). Over 70% of British butterflies have also suffered from reducing ranges in the past 30 years (Warren *et al.* 2001). The strongest declines are found in pollinating species with long proboscis such as bumblebees. The uniformity of this pattern has prevented other pollinators to compensate for the lack of pollination to long corolla flowers (Rasmont *et al.* 1993, 2005). Over 25% of wild bees in Belgium are suffering declines whilst In Hungary 47% of native bumblebee species have exhibited recent declines (Sárospataki *et al.* 2005). Similar declines in wild pollinators are found in Britain, Ireland, Spain, France, Morocco, Corsica, Tibet, Brazil and Madagascar (Williams 1982; Rasmont *et al.* 2005; Fitzpatrick *et al.* 2007; Kosior *et al.* 2007; Xie *et al.* 2008; Martins & Melo 2010). In the North American mid-west, half of the native bumblebee species have declined during the mid-twentieth century, coinciding with increased agricultural land change (Grixti *et al.* 2009). Whilst in Eastern North America between 1971 and 2006, bumblebees have declined in diversity, evenness and abundance (Colla & Packer 2008). Today 11% of all bumblebee species worldwide are listed in a threat category in the IUCN Red list (Williams & Osborne 2009).

1.3.1 Causes of pollinator decline

The cause of this wide scale pollinator decline is believed to be multicomponent (Natural Research Council 2007; Didham *et al.* 2007; Potts *et al.* 2010b). The main drivers are likely to be climate change, land use change, pesticides, and introduced species and parasites (Potts *et al.* 2010b; Vanbergen & Initiative 2013);

1.3.1.1 Climate change

A changing global climate is forcing migratory movements in many species living on the edge of their climatic range. In general this is seeing poleward movements by insects such as butterflies seeking to avoid rising temperatures (Hickling *et al.* 2006). In instances where species are unable to move with the shifting climatic range, population declines or extinction may be expected (Schweiger *et al.* 2008; Williams & Osborne 2009). Climatic changes could cause phenological mismatches between pollinator and flowers. Emergence of hibernating pollinators is often timed with floral abundance. *Bombus frigidus* for example emerges within 24 hours of the first appearance of willow catkins (Vogt *et al.* 1994). Mismatching pollinator activity with floral availability would cause significant declines in pollinator (and plant) fitness (Memmott *et al.* 2007; Pauw & Hawkins 2011).

1.3.1.2 Land use change

Increased urbanisation and agricultural intensification has dramatically reduced natural and semi-natural land availability to pollinators (Goulson *et al.* 2005, 2006; Biesmeijer *et al.* 2006; Carvell *et al.* 2006; Ricketts *et al.* 2008; Goulson 2010). Since the 1940s, Britain has lost 70% of its semi-natural habitat (Asher *et al.* 2001). Such land-use change and fragmentation can disturb nesting and foraging sites of pollinators (Goulson *et al.* 2008; Kleijn & Raemakers 2008; Garibaldi *et al.* 2011). The most specialised pollinators tend to be the ones most vulnerable to habitat change (Biesmeijer *et al.* 2006; Williams & Osborne 2009). Bees particularly have suffered local extinctions following land use changes (Williams & Osborne 2009; Burkle *et al.* 2013). However when land is reverted back to semi-natural, local pollinator declines are reduced (Carvalheiro *et al.* 2013).

1.3.1.3 Pesticides

Species richness is found to be low in areas found to have high levels of pesticides (Brittain *et al.* 2010). In the developed world, crops are routinely treated with quantities of pesticides deemed non-lethal to pollinators. Increasingly though, evidence is showing that non-lethal levels of pesticide are enough to reduce the fitness of exposed pollinators. Individually, reduced brain function and learning has been shown by honey bees exposed to sub-lethal levels of pesticide (Henry *et al.* 2012; Palmer *et al.* 2013). Exposed bumblebees have significantly retarded colony growth with only a few reproductives produced (Whitehorn *et al.* 2012). Additionally, within a mosaic of fields, pollinators are likely to encounter a cocktail of different pesticides. Pesticides have been shown to have synergistic effects with each other and additional stressors such as parasites (Vidau *et al.* 2011; Gill *et al.* 2012).

1.3.1.4 Introduced species

Non-native pollinators can have negative impacts on native species due to a number of factors. Many non-native pollinator introductions occur as a result of translocation by man and are thus, commonly the agriculturally used generalist pollinators *A. mellifera* or *B. terrestris*. These generalist pollinators will compete with native pollinators for floral resources. In the USA, the range of plant species foraged upon by managed honey bees, overlaps with those of native bumblebees by up to 90% (Thomson 2006). Following the escape and naturalisation of the European bumblebee *B. terrestris* from greenhouses in Japan, it was discovered they have a 70% resource overlap with these native bumblebees in Japan (Matsumura *et al.* 2004). When investigated, competing wild bees have been found to suffer from retarded body growth and reproductive success, though not in all cases (Elbagrmi *et al.*; Steffan-Dewenter & Tscharntke 2000; Roubik & Wolda 2001; Thomson 2006; Goulson & Sparrow 2008). This suggests the intensity of competition and availability of alternative resources may play a role. There is also evidence of genetic dilution when native species hybridise with exotic species and a disturbance to the pollinator-flora mutualisms (Franck *et al.* 1998; Winter *et al.* 2006; Dafni *et al.* 2010).

1.3.1.5 Introduced parasites

One of the greatest concerns about introduced species, is the introduction of parasites to native, naïve populations (Goulson 2003b). Parasites are believed to be a contributor to the large-scale losses in honey bees. The introduction of the parasitic mite *Varroa destructor* from Asia is believed to be responsible for significant losses to managed, feral and wild honey bees (Kraus & Page 1995; Sammataro *et al.* 2000; Moritz *et al.* 2007). Whilst the cause of the enigmatic syndrome of Colony Collapse Disorder (CCD) is still unclear, parasites are believed to be a likely driver (Natural Research Council 2007; Cox-Foster *et al.* 2007; VanEngelsdorp *et al.* 2009; Dainat *et al.* 2012).

As a group of specialised pollinators, bumblebees are believed to be vulnerable to the same threats facing pollinators, and indeed account for much of the evidence available of reduced pollinator numbers. Of the 25 native UK bumblebee species for example, two have recently gone extinct with a further 8 having decreased in numbers substantially since the 1940s (Goulson *et al.* 2008). Many bumblebee populations are now much reduced and fragmented, which combined with their inherently low genetic diversity as a result of being eusocial, are now facing increased vulnerability to infectious diseases (Daszak *et al.* 2001; Goulson *et al.* 2008; Whitehorn *et al.* 2011). Unlike honey bees, very few bumblebee parasites are known, less so their pathology. This is likely due to publication bias with comparatively few studies looking at bumblebee parasites (Schmid-Hempel 1998). As a result, the role of parasites in bumblebee declines in relatively unknown despite parasites being a major component of animal ecology (Hatcher & Dunn 2011). This is a particular concern following the recent domestication and subsequent translocation of bumblebees globally.

1.4 Parasites and their role in the environment

Symbiosis is a ubiquitous feature of life on earth. The pioneering plant pathologist Anton de Bary, working primarily on fungal and oomycete diseases, emphasized that symbiosis was the “living together” of two distinct organisms (Sapp 1999). The spectrum of symbiosis is wide and ranges from mutualistic associations to parasitism. A parasitic relationship is always asymmetrical, with one party being exploited by the other. The co-evolutionary relationship that exists between a parasite and its natural host can therefore be described as an evolutionary arms race (Van Valen 1973),

between the negative effects caused by parasite infection, and the host's resistance to such effects. The degree to which a parasite negatively affects the fitness of its host is regarded as the parasite's virulence. Over time, host populations can adapt resistance mechanisms to combat this and reduce the parasite's virulence. Often parasites have a larger population size and shorter generation time than their hosts, allowing them to adapt to host defences faster than the host can evolve counter adaptions. This has been demonstrated in transplant experiments for a range of host-parasite assemblages (Parker 1985; Lively 1989; Ebert 1994; Lively *et al.* 2000; see Kaltz *et al.* 1998 for a review). Any virulent effects on a host, no matter how small, will act as an additional stressor on the host population. Such things may alter the equilibrium between the naïve host and their environment. A parasite-induced shift in this equilibrium may leave the new hosts vulnerable to indirect impacts of infection such as increased vulnerability to predators, reduced ability to obtain prey/resources or a reduction in competitiveness (Prenter *et al.* 2004; Hatcher & Dunn 2011). In addition to this, some parasites have been found to be equally, if not more virulent in sympatric hosts (Morand *et al.* 1996; Dufva 1996; Imhoof & Schmid-Hempel 1998; Oppliger *et al.* 1999; Mutikainen *et al.* 2000). In such instances, parasites may also pave the way for invading host species to become naturalised, as seen when the invasive grey squirrel introduced the pox virus into British native red squirrels. In this example, the native populations were weakened by the exotic virus allowing the invasive grey squirrels to outcompete them (Tompkins *et al.* 2003). Such parasite effects can place the naïve, susceptible species at a particular disadvantage due to contending with both an increased parasite and competitive threat.

1.4.1 Emergent infectious diseases

Emergent infectious diseases are ranked as one of the top five causes of species extinction worldwide (Daszak *et al.* 2000). They are defined broadly as diseases that have recently increased in either incidence, demographic or host range, or that have recently evolved or been discovered (Lederberg *et al.* 1992; Morse 1993; Daszak *et al.* 2000, 2001). The effects of emergent diseases can vary, but are responsible for significant costs to society, the economy, and conservation of species or resources. There are many well documented examples of emergent diseases. These included the H1N1 swine flu pandemic, human immunodeficiency virus (HIV), *Chytridiomycosis*

decimating amphibian populations in the Americas, and the widespread famine caused by the emergence of Late Potato Blight in cultivated crops from wild populations (Berger *et al.* 1998; Anderson *et al.* 2004; Neumann *et al.* 2009). Many instances of EIDs in wild animals are the result of interactions with domesticated species (see figure 1.4.1.1). The main conservation concern regarding the emerging industry of bumblebee production and importation, is that of exotic parasite introduction and spillover/spillback to wild populations. By allowing domesticated, imported bumblebees to mix with wild bumblebees, there is the potential of disease emergence in wild bumblebees (Daszak *et al.* 2000, 2001; Jones *et al.* 2008).

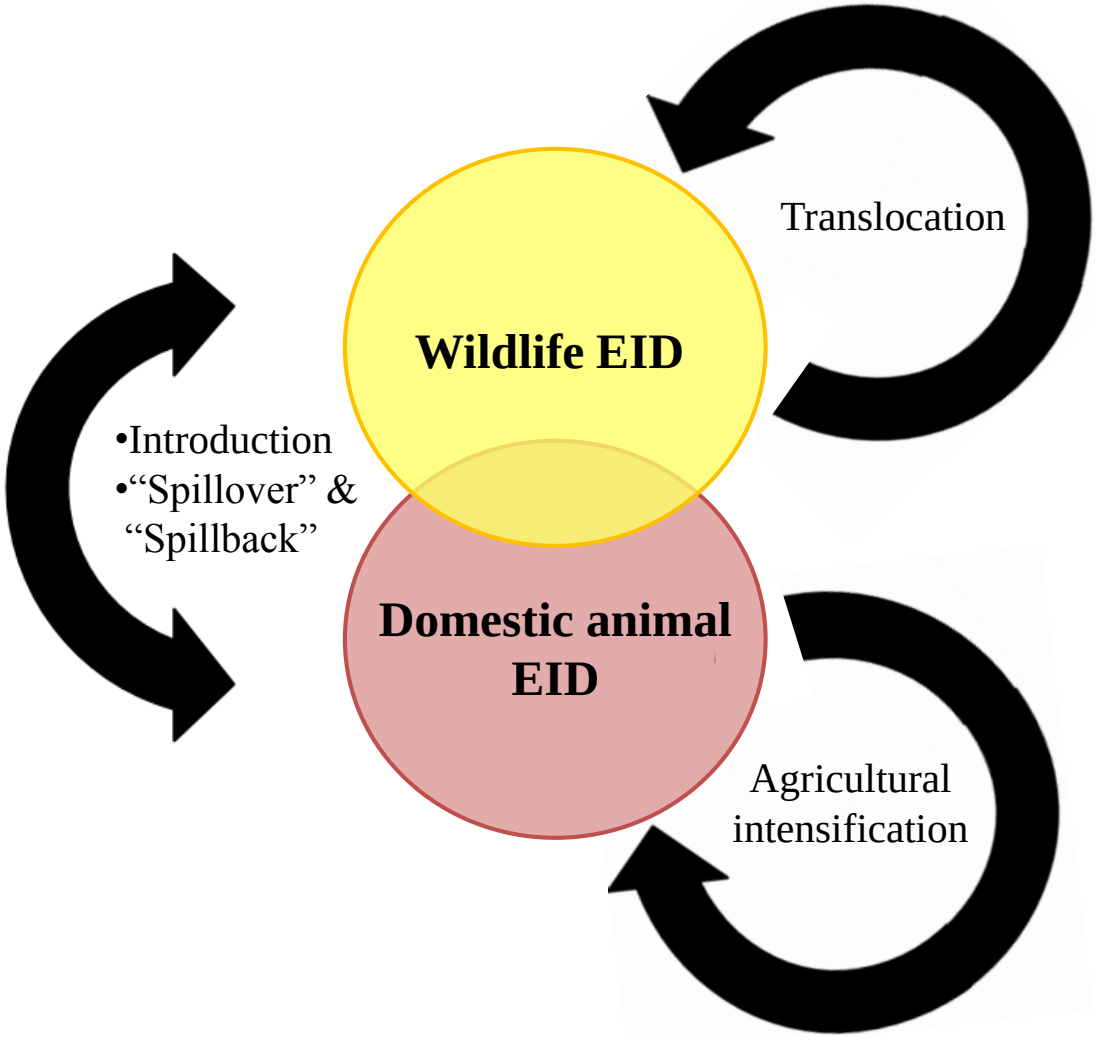


Figure 1.4.1.1 The key factors that drive disease emergence within and between populations of domestic and wild animals. Adapted from Daszak *et al.* (2000).

1.4.2 Parasite spillover

A common mechanism that can lead to disease emergence occurs following interactions between uninfected 'susceptible' populations with an infected 'reservoir' population. This is known as 'spillover' and can occur by direct transmission from infected individuals, or may involve vectors or environmental contamination. In addition, if transmission rates are low, infection of susceptible individuals may occur in many instances before the parasite emerges at a population level, or its emergence may require continued mixing with the source/reservoir population (Hatcher & Dunn 2011). The likelihood of successful pathogen spillover to a new host population will however vary depending on the compatibility of the host and pathogen. Closely related, sympatric hosts have a greater potential to transmit pathogens between them (Perlman & Jaenike 2003). As a result, strict controls are often put in place to avoid mixing between domestic and wild animals that could transmit or become infected with parasites from one another (Daszak *et al.* 2000; Foufopoulos *et al.* 2002). Examples of this include the quarantining of dogs with suspected rabies to prevent transmission to wild animals (Brown *et al.* 2011).

Parasite spillover does not only occur from domesticated to wild species. In some instances, wild animals can act as the parasite reservoir. The microsporidian parasite *Nosema ceranae* serves as a good example of a parasite spilling over from wild to domestic animals. *Nosema ceranae* spilt over from its natural wild host *Apis ceranae* to the domesticated European honey bee *A. mellifera.* Transmittable horizontally in honey bee hosts via the faecal-oral route, *N. ceranae* spores are persistent and found not only within host bees, but also in their hive materials and pollen, where spores can stay viable for long periods (Higes *et al.* 2010). *Nosema ceranae* originates from the Asian honey bee, *Apis ceranae* (Botías *et al.* 2012). Over the past decade the translocation of *Apis mellifera* honey bee hives have brought *N. ceranae* into contact with this novel host species. Following this, *N. ceranae* has been able to successfully spillover to multiple sympatric *Apis* species worldwide. Infected honey bees suffer from immune assaults and reduced lifespan with *N. ceranae* now considered to be a contributing factor to the collapse of some managed honey bee hives (Fries *et al.* 1996; Higes *et al.* 2006, 2008b; Paxton *et al.* 2007; Klee *et al.* 2007; Chen *et al.* 2008; Antúnez *et al.* 2009; Chaimanee *et al.* 2010; Botías *et al.* 2012).

1.5 Bumblebee parasites

Knowledge of parasite prevalence in bumblebees is largely limited to the visual detection of three protozoa; the trypanosome *Crithidia bombi* (Lipa & Triggiani 1988), the microsporidian *Nosema bombi* (Fantham & Porter 1914) and the neogregarine *Apicystis bombi* (previously referred to as *Mattesia bombi*) (Liu *et al.* 1974; Lipa & Triggiani 1996). Most bumblebee parasite studies are based on *C. bombi* and *N. bombi* infections with the effects and mechanisms of *A. bombi* infection being mostly based on non-quantitative reports (Macfarlane *et al.* 1995; Schmid-Hempel 1998; Imhoof & Schmid-Hempel 1999; Rutrecht & Brown 2008):

1.5.1 *Crithidia bombi*

Crithidia bombi is a single-celled flagellate parasite that resides in the intestinal tract of infected bumblebees (Lipa & Triggiani 1988). This parasite is the best studied of all the bumblebee parasites. Recent molecular work has shown that there are two distinct species of *Crithidia* that have both previously been referred to as *Crithidia bombi* (Schmid-Hempel & Tognazzo 2010). As yet, there have been no attempts to describe differences in the pathology of the newly named *Crithidia expoeki* compared to the species designated *Crithidia bombi*. As a result, I will refer to both species as '*Crithidia bombi*'. The cells of the parasite attach to the walls of the mid and hindgut of infected bumblebees and multiply rapidly. New parasite cells are then released from two to five days after the initial infection and pass out in the faeces, increasing in numbers for 8-13 days, after which a consistently high faecal pathogen load remains (Schmid-Hempel & Schmid-Hempel 1993; Logan *et al.* 2005). The parasite is horizontally transmitted between bumblebees via the faecal-oral route following contact with infective parasite cells in the nest or whilst foraging (Durrer & Schmid-Hempel 1994; Otterstatter & Thomson 2007). The prevalence of *C. bombi* among bumblebees can vary among host species but is typically high with between 30-50% of bees infected, depending on locality and time of year (Gillespie 2010; Whitehorn *et al.* 2011). Within a single host, multiple strains of *C. bombi* are commonly detected, with frequent transmission and mixing within host populations (Salathé & Schmid-Hempel 2011; Ruiz-González *et al.* 2012). The virulence of *C. bombi* is generally considered to be low (Imhoof & Schmid-Hempel 1999). The negative effects on infected colonies are greater when non-native strains infect individuals (Imhoof & Schmid-Hempel

1998), or when individuals are under additional stresses such as starvation (Logan *et al.* 2005). In such cases, fatalities of up to 50% of workers, retarded colony development and reductions in ovary size in queens and workers is experienced (Shykoff & Schmid-Hempel 1991a; Imhoof & Schmid-Hempel 1998; Brown *et al.* 2000; Logan *et al.* 2005). Additionally, *C. bombi* infected workers have been found to display sub-lethal 'trait effects' such as impaired flower choice and handling, and overall foraging performance is negatively correlated with infection intensity (Scheiner *et al.* 2001; Gegear *et al.* 2005, 2006; Otterstatter & Thomson 2006). Such a reduction in foraging efficiency can have substantial knock-on effects at a colony level, severely reducing the reproductive output (Schmid-Hempel 1998; Schmid-Hempel & Schmid-Hempel 1998).

1.5.2 *Nosema bombi*

Nosema bombi is a microsporidian and single-celled intestinal parasite of bumblebees (Microsporidia, Nosematidae, Fantham & Porter, 1914). Following ingestion, *N. bombi* spores germinate in the gut lumen of infected bumblebees and subsequently display tissue tropism, invading mid-gut cells, malpighian tubules, fat body, nerve tissue, tracheae and reproductive organs (Schmid-Hempel 1998; Larsson 2007). Infected host cells rupture, releasing mature spores back to the gut lumen where they are passed out in the bumblebees faeces. This process can take up to three weeks following infection (McIvor & Malone 1995). The infectivity of *N. bombi* is age specific, with larvae and newly emerged bumblebees being more susceptible than mature workers (Schmid-Hempel & Loosli 1998; Rutrecht *et al.* 2007). The virulence of *N. bombi* is low with otherwise healthy colonies showing no apparent changes in productivity (Fisher & Pomeroy 1989; Imhoof & Schmid-Hempel 1999; Whittington & Winston 2003). Experimental infection however, increases worker mortality by 500%, reduces sperm viability and infected gynes can have distended abdomens with crippled wings, together significantly reducing reproductive success (Macfarlane *et al.* 1995). Field studies have also found that colonies headed by infected queens are significantly smaller and yield no reproductive offspring (Otti & Schmid-Hempel 2008). As with *C. bombi*, the prevalence of *N. bombi* varies spatially, temporally and across species (Cordes *et al.* 2012).

1.5.3 *Apicystis bombi*

Apicystis bombi was originally described as *Mattesia bombi*, until further analysis determined it was not from the *Mattesia* Genus. *Apicystis bombi* is a cosmopolitan Neogregarine that can be found infecting both bumblebees and honey bees (Liu *et al.* 1974; Lipa & Triggiani 1996). However, very little is known about the pathology of this parasite. Horizontal transmission is believed to occur via the oral-faecal route. Following ingestion, spores likely penetrate the gut wall and can be found infecting the fat body. Spores produced in the fat body are then excreted in the faeces (Macfarlane *et al.* 1995). The infection of the fat body is believed to be the primary reason that infected gynes have reduced fat body though this has never been quantified (Durrer & Schmid-Hempel 1995; Macfarlane *et al.* 1995). A degradation of fat would have major consequences whilst overwintering and would explain the death of all *A. bombi* infected queens in survey studies (Schmid-Hempel 2001; Rutrecht & Brown 2008). Prevalence in wild bumblebees seem to be low following visual screening for spores with studies tending to have < 10% prevalence (Colla *et al.* 2006; Kissinger *et al.* 2011). *Apicystis bombi* has been labelled an emerging parasite of bumblebees in some areas (namely South America) due to its apparent rapid appearance in local fauna (Plischuk *et al.* 2011; Arbetman *et al.* 2012).

1.5.4 Other parasites of bumblebees:

Two reviews compile limited reports of other parasites including the bacteria *Spiroplasma* sp., *Aerobacter cloaca*; a queen infecting nematode, *Sphaerularia bombi*; an entomopox-like virus and several fungus groups; *Beauveria, Acrostalagmus, Aspergillus, Candida, Cephalosporium, Hirsutella, Metarhizium, and Paecilomyces* all being found in bumblebees (Schmid-Hempel 1998; Boomsma *et al.* 2005). Many more pathogens are known to infect honey bees including several viruses although this is possibly due to research bias (Schmid-Hempel 1998; Goulson 2010). Given the similarity between the lifestyles of these two genera, it is not surprising that some honey bee parasites have been found in bumblebees. The honey bee virus, deformed wing virus (DWV), highly prevalent in honey bee colonies (Baker & Schroeder 2008), has recently been found infecting bumblebees, in some cases causing symptomatic wing deformities (Genersch *et al.* 2006; Evison *et al.* 2012). The prevalence and full pathology of this virus in bumblebees is still unknown but in honey bees it has been

implicated in colony losses (Highfield *et al.* 2009). Additionally, acute bee paralysis virus (ABPV) (Bailey & Gibbs 1964), and *Nosema ceranae* (Plischuk *et al.* 2009) which additionally has been found to be more lethal to bees that are also exposed to pesticides (Alaux *et al.* 2010; Vidau *et al.* 2011; Pettis *et al.* 2012), have been identified in bumblebees. Unfortunately the prevalence and effects of these honey bee pathogens in bumblebees is also unknown.

1.6 Pathogen spillover to bumblebees

The incidence of bee pathogens in the environment will innately fluctuate depending on natural phenomena such as climatic changes and the lifecycles of the hosts and parasites. Additionally, bumblebee parasites have been found to be more prevalent around sites using commercial bumblebees (Colla *et al.* 2006; Otterstatter & Thomson 2008; Murray *et al.* 2013). Though, this is limited, correlative evidence of potential pathogen spillover from commercially reared bumblebees (the reservoir population) to native bees (susceptible population). This is also based on just visual detection of parasites and does not take into account the densities of hosts around the sites using commercial bumblebees, which will be unnaturally high. The process of spillback of parasites originating in native bumblebees could then also be the cause of this correlative evidence (Kelly *et al.* 2009). In spillback, parasites from native bumblebees could infect commercial bumblebees, a heightened prevalence then occurs in this population of dense, domestic hosts, and infection spills back to the native population. For either spillover or spillback to be taking place, transmission between wild and commercially reared bumblebees would have to occur. It is impossible to know if the increased parasite incidence around greenhouses is the result of spillover or spillback without at least knowing if commercial bumblebees are infected upon purchase.

During the initial years of commercial bumblebee use, there was a lack of regulations for the production and use of bumblebees and there were multiple instances of both commercial bumblebees and their parasites colonising the surrounding environment (Goka *et al.* 2001; Matsumura *et al.* 2004; Plischuk & Lange 2009). Following this, farmers are now encouraged to keep their greenhouses closed to prevent mixing between native and commercial bumblebees and production facilities declare all their colonies to be disease free. Recently, studies have identified native bees foraging inside commercial greenhouses (Kraus *et al.* 2010; Lye *et al.* 2011), with commercial bumblebees foraging beyond their greenhouses (Morandin *et al.*

2001; Murray *et al.* 2013). This population mixing increases the amount of shared foraging by commercial and native bees, with such shared flowers being possible sites of parasite transmission (Durrer & Schmid-Hempel 1994). This highlights the need to identify if parasite transmission is occurring between populations. Another route for pathogen spillover is via the commercial facilities that breed bumblebees. Bumblebees are reared on honey bee harvested pollen, though this food source is not screened for disease (Goulson 2013). Many known bee pathogens are transmittable via ingestion (Schmid-Hempel 1998). Parasites including DWV, Black Queen cell virus, Sac-brood virus, *Ascosphaera* and *Nosema ceranae* have all been found in honey bee pollen with some still being infective following ingestion (Flores *et al.* 2005; Chen *et al.* 2006; Higes *et al.* 2008a; Singh *et al.* 2010).

1.6.1 Impacts of pathogen spillover on wild populations

The impacts of parasite emergence and spillover can vary, with some parasites showing little effect on host populations and others being responsible for population declines and extinctions (Cunningham & Daszak 1998; Daszak & Cunningham 1999; Smith *et al.* 2009). The early introduction of commercially-produced bumblebees in Japan, North America and Argentina has been correlated with declines in native bumblebee species, increases in the prevalence of parasites in them, or the introduction of foreign strains or species of parasite (Goka *et al.* 2001; Colla *et al.* 2006; Otterstatter & Thomson 2008; Meeus *et al.* 2011; Arbetman *et al.* 2012).

1.6.1.1 Japan:

The first reported instance of pathogen spillover from commercially reared bumblebees was in Japan. Here, commercial bumblebees have been imported primarily for tomato production since 1991 (Goka *et al.* 2001). Within a decade, over 40,000 colonies of non-native *B. terrestris* were being imported to Japan from Europe annually. On average, 20% of the commercial colonies searched, were found to be infected with the endoparasitic mite *Locustacarus buchneri* (Goka *et al.* 2000, 2001). The mite feeds, reproduces and lives inside the abdominal airsacks of female bumblebees (Yoneda *et al.* 2008a). Bumblebees heavily infected with *L. buchneri* suffer from diarrhoea, lethargy, stop foraging, and have a reduced lifespan thus retarding colony growth (Husband & Sinha 1970; Otterstatter & Whidden 2004). Mites

on bumblebee species native to Japan such as *Bombus hypocrite* were found to be of European decent via commercially reared bumblebees (Goka *et al.* 2001, 2006; Yoneda *et al.* 2008a; b). Since this spillover, commercial breeding facilities are believed to have put into place stricter controls for the mite. A recent report that screened 37 different commercially produced colonies found no evidence of the mites prior to deployment, but found that commercial colonies do become infected by native mites once bumblebees are permitted to forage (Rożej *et al.* 2012).

1.6.1.2 North America:

The declines of several bumblebee species in North America coincided with the local introduction of commercially-reared bumblebees, which are thought to have had high incidences of the microsporidian parasite *Nosema bombi* (Thorp 2005; Thorp & Shepherd 2005; Winter *et al.* 2006; Cameron *et al.* 2011). Whilst *N. bombi* is known to have detrimental effects on bumblebee colonies (Otti & Schmid-Hempel 2007, 2008), it has not yet been proven that *N. bombi* was the cause of the declines in native populations (Brown 2011). However, the recent declines of bumblebees in North America did coincide with relatively high levels of *N. bombi* infections (Cameron *et al.* 2011), suggesting this parasite maybe an emergent infectious disease in the North American bumblebees (Meeus *et al.* 2011). In addition to this, studies have found that the two parasites *N. bombi* and *C. bombi* are more prevalent in bees adjacent to fields using commercial bumblebees than those several km (Colla *et al.* 2006; Otterstatter & Thomson 2008).

1.6.1.3 Argentina:

Commercially reared *B. terrestris* which is not native to South America started being imported to Chile for crop pollination in 1998, by 2006 it had naturalised and was discovered invading Argentina (Torretta *et al.* 2006). When screened, *B. terrestris* was found to be parasitized by *C. bombi* and *A. bombi*, yet these parasites were not present in native bumblebee species (Plischuk & Lange 2009). In a further study using sensitive, molecular screening of fresh and historical bumblebee samples, *A. bombi* was found in samples of native *B. ruderatus* and *B. dahlbomii* collected after the introduction of commercial bumblebees, yet was undetected in samples collected prior to this point (Arbetman *et al.* 2012). Historical records of parasite prevalence in this

area is however weak and cannot guarantee these parasites were absent before the introduction of commercial bumblebees. This perceived 'introduction' of *A. bombi* in native bumblebees also coincided with the collapse and geographical retraction *of B. dahlbomii* (Arbetman *et al.* 2012).

When native populations suffer more than non-native hosts, the spillover of pathogens may additionally lead to disease mediated invasion by the non-native species (Strauss *et al.* 2012). The most commonly used commercial bumblebee, *B. terrestris*, is not native in the majority of the countries it is deployed in (Velthuis & Van Doorn 2006). Additionally, this bumblebee species is considered invasive in many areas and in several instances was introduced following escapes from commercially reared stocks (Dafni & Shmida 1996; Goulson 2003b; Dafni *et al.* 2010).

Faced with criticism and continued suggestions of parasite spillover from their bumblebees, suppliers began to employ basic parasite screening of their bees and where possible, supply native species. Now, supplied bumblebees are commonly cited by their supplier as being disease free and indeed, this is a stipulation of their deployment in most countries (European Commission 1992; Koppert 2005; HM Government 2006; Velthuis & Van Doorn 2006; Winter *et al.* 2006; Syngenta 2012).

1.7 Aims

Whilst it is claimed that commercially reared bumblebees are disease free and pose no risk to native bumblebees, all parasite screening is done in-house by the suppliers. Clearly a potential threat to native bumblebees of pathogen spillover from commercially reared bumblebees exists. In order to effectively conserve these vital pollinators and the ecosystems they service, the parasite risks bumblebees are under and what effect the transportation of commercial colonies has upon this must be identified. Once this is known, relevant controls and tests can be implemented in order to help prevent or reduce further losses to these keystone species. To assess the risks native bumblebees currently face as a result of the commercial deployment of reared bumblebees, a thorough investigation is required. The stages of a parasite invasion are 1) Introduction 2) Establishment 3) Invasion. In my second chapter I will identify if infective parasites are being introduced into England via commercial bumblebees. My

third chapter will look at possible transmission routes for parasites to spillover between domestic and native bee populations. In my fourth chapter I will identify if there is any evidence of parasite establishment and invasion in native bumblebees as a result of managed bees. The fifth and sixth chapters will identify the lethal risks and trait effects some of the previously identified parasites pose to bumblebees. Finally I will synthesise these results, the impacts these findings have and how that fits with current regulations imposed, the practices of commercial bumblebee breeders and conservation needs.

Chapter 2: The Trojan hives: pollinator pathogens, imported and distributed in bumblebee colonies

Abstract

Over a million commercially produced bumblebee colonies are imported annually on a global scale for the pollination of greenhouse crops. After importation, they interact with other pollinators, with an associated risk of any parasites they carry, infecting and harming native bees. National and supranational regulations are designed to prevent this and commercially produced bumblebee colonies are accordingly now often sold and imported as being parasite-free. Here I used molecular methods to examine the occurrence of parasites in bumblebee colonies that were commercially produced in 2011 and 2012 by three producers. I then used controlled experiments to determine whether any parasites present were infectious. I found that 77% of the commercially produced bumblebee colonies from the three producers, which were imported on the basis of being free of parasites, in fact carried microbial parasites, with five different parasites being detected across the total sample of bumblebees and a further three in the pollen supplied with the colonies as food. My controlled experiments demonstrated that at least three of these parasites were infectious to bumblebees with significant negative effects on their health. Furthermore, I also found that at least four of the parasites carried by commercially produced bumblebees were infectious to honey bees, indicating that they pose a risk to other pollinators as well. The results demonstrate that commercially produced bumblebee colonies carry multiple, infectious parasites which pose a significant risk to other native and managed pollinators. More effective disease detection and management strategies are urgently needed to reduce the pathogen spillover threat from commercially produced bumblebees.

2.1 Introduction

Bumblebees are amongst the most ecologically and economically important groups of pollinators in temperate regions, but many bumblebee species and other pollinators are suffering declines worldwide (Potts *et al.* 2010b). Out of 25 bumblebee species in the UK, for example, 2 have gone extinct and 8 decreased substantially in abundance since

1940, while 13 species have gone extinct in at least one European country and four across the entire region (Goulson *et al.* 2008). Other species have undergone similar declines in North America (Cameron *et al.* 2011; Szabo *et al.* 2012), and 11% of all bumblebee species worldwide are listed in a threat category on the IUCN Red list (Williams & Osborne 2009).

The importance of bumblebees for the pollination of many high-value crops has led to the commercial production and importation of over a million colonies per year in Europe, North America, South America, and Asia (Velthuis & Van Doorn 2006). Emergent parasites represent one of the major threats to biodiversity and spillover from introduced organisms to native species can be particularly damaging, either by introducing novel species or strains of parasite, or by increasing the density of infected hosts (Daszak *et al.* 2000). Commercially produced bumblebees interact with native bumblebees and other pollinators after importation during shared flower use (Whittington *et al.* 2004; Murray *et al.* 2013), which can cause bee parasites to be transmitted (Durrer & Schmid-Hempel 1994). The introduction of commercially produced bumblebees in North America, South America and Japan has been correlated with declines in native bumblebee species, increases in the prevalence of parasites, or the introduction of foreign strains or species of parasite (Goka *et al.* 2001; Colla *et al.* 2006; Otterstatter & Thomson 2008; Meeus *et al.* 2011; Szabo *et al.* 2012; Arbetman *et al.* 2012). In addition, there have been a number of reports of bumblebees commercially produced up to 2008 having parasites (Whittington & Winston 2003; Gegear *et al.* 2005; Colla *et al.* 2006; Otterstatter & Thomson 2007; Manson *et al.* 2010; Singh *et al.* 2010; Meeus *et al.* 2011; Murray *et al.* 2013). As a result, the regulatory requirements for bumblebee importation have been tightened in some countries in recent years to stipulate mandatory disease screening and the producers of bumblebee colonies now often claim that their colonies are free of parasites (European Commission 1992; Koppert 2005; HM Government 2006; Velthuis & Van Doorn 2006; Winter *et al.* 2006; The Food and Environment Research Agency 2011; Syngenta 2012). In England for example, the importation licences for the non-native subspecies most commonly imported are specifically limited to parasite-free colonies, and 40–50 thousand colonies are imported annually to the UK on this basis (Natural England 2009, 2012). However, concern remains about whether bumblebee colonies being produced currently may nevertheless carry parasites, and it is also unclear

whether any parasites that may be present in the hives are infectious, making the pathogen spillover risk posed currently uncertain.

Here I examined bumblebee colonies that were commercially produced in 2011 and 2012 for the presence of three bumblebee parasites and six honey bee parasites. I then tested experimentally whether any of the parasites found were infectious to bumblebees or to honey bees. Our results show that parasites are present and infectious in bumblebee colonies that are currently being commercially produced, substantiating concerns about significant risks of pathogen spillover.

2.2 Materials and Methods

2.2.0 Colony screening

Forty-eight commercially produced *Bombus terrestris* (Linnaeus 1758) colonies were purchased in 2011 and 2012 from three of the main producers in Europe, with 15 (five from each producer) being of the non-native *B. terrestris dalmatinus* or *B. t. terrestris*, and the remainder *B. t. audax*. The colonies were imported into the UK by the producers under Natural England licences which are limited to disease-free colonies (Natural England 2009). Immediately upon arrival, 15 workers were removed from each colony, as well as 25 samples of the pollen (originally sourced from honey bees) that were supplied with the colonies as food (5 samples from separate bags or bottles for each of the three producers in 2011, and from Producers A and B in 2012). A ca. 0.2-cm^3 sample of the hind gut, fat body and malpighian tubules was dissected out from each of the workers and homogenised with a micropestle. Pollen samples (0.6 g) were homogenised for 2 min with 0.1 mm zirconia/silica beads in a Qiagen TissueLyser. DNA and RNA was extracted by boiling either the entire homogenate (in the case of worker samples) or 5 µl of the supernatant (in the case of the pollen samples) in 145 µl of 10% Chelex solution, which is effective at isolating viral RNA, as well as the DNA of the other parasites (Rekand *et al.* 2003; Rudenko *et al.* 2004; Evison *et al.* 2012). For 34 of the colonies, I pooled DNA/RNA extracts from the 15 bees such that there was a single pooled sample of bee DNA/RNA per colony, while for the other 14 colonies I ran the 15 bees separately. I screened the bees for the three main bumblebee parasites (the trypanosome *Crithidia bombi*, the microsporidian *Nosema bombi* and the neogregarine *Apicystis bombi*, all of which are faecal–orally

transmitted parasites of adult bees; (Schmid-Hempel 1998), four widespread honey bee parasites (the faecal–orally transmitted microsporidian parasites of adult bees *Nosema apis* and *N. ceranae*, and the orally infecting foulbrood bacteria *Melissococcus plutonius* and *Paenibacillus larvae* of bee larvae; (Morse & Flottum 1997), deformed wing virus (DWV) which is a common parasite in honey bees and bumblebees (Evison *et al.* 2012), and the orally infecting fungal parasite *Ascosphaera* of bee larvae (Aronstein & Murray 2010). I screened the samples for parasites using conventional, nested or hemi-nested PCR, or Taqman RT-PCR for DWV, using parasite-specific primers (see Table A2.2.1.1). Amplification at the host *18S Apidae* gene was used to check for quality of the DNA extractions, and positive and negative controls were included in all sets of samples.

2.2.1 Experiment 1: Infection risk to adult bumblebees

A total of 150 adult *Bombus terrestris audax* workers were collected from three commercially produced colonies that I had found to be free of parasites by PCR screening 15 adult bees per colony (our data on parasite prevalence for a subset of 14 colonies indicated that parasites, when present in a colony, infected >10% of bees within that colony; Table A2.2.2.1). The uninfected status of these colonies was then confirmed by the fact that all control bees used in the experiment were found subsequently to be uninfected (see 2.3 Results). Each bumblebee was placed in a holding harness and individually fed 5 µl of 40% sucrose solution containing either pollen (0.6 g ml^{-1}) or bumblebee faeces (diluted 3:1) from commercially produced bumblebee colonies that had been found by PCR to contain parasites, or sterile sucrose solution control. The pollen used was that supplied with colonies by the commercial producers, with pollen samples from the three producers being mixed in equal measure to produce a single homogenous solution. Faeces was obtained by placing bees in holding pots until they defecated, with faeces then collected from the pot with a syringe and combined to produce a single solution. The pollen and faeces used were confirmed by PCR and RT-PCR to contain *N. bombi, N. ceranae, C. bombi, A. bombi*, and DWV (as well as the *Ascosphaera* parasite of bee larvae), with the pollen also having *N. apis*. The pollen solution contained 8.4 x 10^4 *Nosema* spores and 24 *Apicystis* spores per µl (1.4 x 10^5 *Nosema* spores and 40 *Apicystis* spores per mg of pollen), while the faeces solution contained 6.1 x 10^2 *Nosema* spores and 5 *Apicystis*

spores per µl (2.4 x 10^3 *Nosema* spores and 20 *Apicystis* spores per µl of bumblebee faeces), based on counts under a phase contrast microscope with a haemocytometer. No *Crithidia* were observed in these counts and levels of DWV were not quantified. The bees were then placed in 10 x 6 x 6 cm plastic boxes, with each box containing 10 bees that were from the same colony and had received the same treatment. The boxes of bees were kept at 28°C and 60% RH with 40% sucrose solution provided *ad libitum* for 15 days with mortality checked daily. The proboscis extension response was used to assess the sucrose sensitivity of the bees every 5 days, by placing bees in individual holding harnesses and presenting them with a series of sucrose solutions increasing from 10–80% in increments of 10%. Harnessed bees were initially hand-fed to satiation with 30% sucrose solution and then starved for 5 h, before testing them by touching the test sucrose solutions on to an antenna, with distilled water applied to the antenna for a 60 s period in between each test to prevent conditioning. The concentration at which the bee extended its proboscis to drink was then recorded, with bees scoring from 0 to 8 for low to high sensitivity, with 8 indicating that the bee extended its proboscis in response to all concentrations of sucrose and 1 indicating it only extended it in response to the 80% sucrose solution. After the 15 day experimental period, all surviving bees, as well as all bees that died during the experiment, were screened for parasites of adult bees (*A. bombi*, *C. bombi*, *N. bombi*, *N. ceranae*, *N. apis*, DWV) by PCR and RT-PCR as above, and the numbers visible in the tissue samples counted with light microscopy.

2.2.2 Experiment 2: Infection risk to adult honey bees

Capped brood frames were taken from three *Apis mellifera* honey bee colonies that had been confirmed previously by PCR to be free of parasites, with the exception of the ubiquitous *Varroa* mite and asymptomatic DWV, and placed in an incubator at 34°C and 60% RH for eclosion. A total of 180 freshly eclosed workers were collected from these brood frames and transferred to sterile boxes with *ad libitum* sucrose solution until 2 days of age. As in Experiment 1, the honey bees were then placed in a holding harness and individually fed 5 µl of the 40% sucrose solutions containing either pollen or bumblebee faeces from parasite-infected, commercially produced bumblebee colonies, or sterile sucrose solution control. The pollen solution used in this experiment contained 7.1 x 10^4 *Nosema* spores and 40 *Apicystis* spores per µl (1.2 x

10^5 *Nosema* spores and 67 *Apicystis* spores per mg of pollen), while the faeces solution contained 6.6 x 10^2 *Nosema* spores and 8 *Apicystis* spores per µl (2.6 x 10^3 *Nosema* spores and 32 *Apicystis* spores per µl of bumblebee faeces). The honey bees were then placed in cohorts of 20 like-treated nestmates in a 10 x 6 x 6 cm plastic box, and kept at 34°C and 60% RH with 40% sucrose solution provided *ad libitum* for 14 days with mortality checked daily. After this period, all surviving bees as well as those which had died during the period were screened by PCR as before for parasites of adult bees (*A. bombi*, *C. bombi*, *N. bombi*, *N. ceranae*, *N. apis*; DWV was excluded).

2.2.3 Experiment 3: Infection risk to honey bee larvae

A total of 144 one-day-old larvae were collected from the three honey bee colonies that had been confirmed to be free of disease as above, and placed in 48-well tissue culture plates on 60-µl drops of diet (50% royal jelly, 6% D-glucose, 6% D-fructose, and sterile deionised water), with the plates then placed in sealed boxes containing a pool of 0.04% K_2SO_4 to ensure high RH, at 34°C. Two days later, the larvae were fed 20 µl of a mixture consisting of 4 parts diet to 1 part of either a solution in distilled water of the same parasite-contaminated pollen supplied with commercially produced bumblebee colonies as was used in Experiment 2, a solution of the same pollen but after it had been frozen at -20°C for 24 h and then microwaved at 600 W for 5 s to reduce the viability of any parasites, or sterile distilled water control. In addition to the adult parasites mentioned above, the pollen solution also contained 1.7 x 10^6 spores per µl of the *Ascosphaera* fungal parasite. The larvae were fed the same diets on each subsequent day, increasing by 10 µl per day, until the larvae defecated (indicating the end of larval growth) on about day 6, when the faeces was cleaned from their wells and the larvae were not fed any further. The survival of larvae was checked daily with a dissecting microscope for the 6-day feeding period and a further 4-day period. All larvae which survived to the end of the 10-day experimental period, as well as those which died during the period, were rinsed in TRIS buffer and screened for parasites of bee larvae (*Ascosphaera*, *Paenibacillus larvae* and *Melisococcus plutonius*) as above.

2.2.4 Statistical analysis

All analyses were carried out in IBM SPSS 19. The parasite species richness of the commercially produced bumblebee colonies was compared between the two subspecies, three producers and two years using generalized linear models (GLM) with gamma distribution and log link function on $x + 1$ data, while the numbers of colonies in which each parasite was detected were analysed using GLM with a binomial distribution and log link function. The likelihood ratio χ^2 statistic was used to test for significance and to check for model fit compared to the intercept-only model. The deviance/d.f. ratio was used to check for over- or underdispersion, with cases of overdispersion being dealt with by using the inverse of the deviance/d.f. value as a scale parameter to fit an overdispersed model. When there was a quasi-complete separation of the data, Fisher's exact tests were used to explore the data instead. I also carried out analyses using subsets of the data for which I had information from all three producers (colonies of *dalmatinus/terrestris* or pollen from 2011 only), both subspecies of bumblebees (colonies from Producers A and B in 2011 only), or both years (*B. t. audax* colonies or pollen from Producers A and B only), but these did not materially change the results (see Table A2.2.5.1 for the one exception). The effect of treatment on the survival of bumblebees and honey bees in the three experiments was examined using Cox proportional-hazards regression models, with Kaplan-Meier tests using the Breslow statistic for pairwise comparisons, which accounted for the censored nature of the survival data. The effects of treatment on the sucrose sensitivity of bumblebees in Experiment 1 were examined using a GLM with a gamma distribution and log link function on $x + 1$ data, followed by pairwise comparisons of treatments on each day in which the *P*-value was adjusted by the sequential Bonferroni method. The numbers of individuals in the three experiments in which each parasite was detected were compared between the treatments using GLM with binomial distribution and logit link function, with day of death included as a covariate. Colony of origin was included in both the Cox and GLM models of data from the three experiments. Non-significant terms were removed stepwise in all cases to obtain the minimum adequate models.

2.3 Results

2.3.0 Colony screening

Five of the nine parasites I screened for were present in 13–53% (depending on the parasite) of the commercially produced bumblebee colonies, with a further three parasites being present in the pollen supplied with the colonies as food (Figure 2.3.0.1; Tables A2.2.2.1 and A2.3.1.1). In only 11 of the 48 colonies were the bees screened negative for all of the parasites. Of the 25 pollen samples, only a single sample was free of every parasite. The prevalence of the three bumblebee parasites *C. bombi*, *N. bombi* and *A. bombi* were found in 45%, 48% and 15% of colonies respectively (Figure 2.3.0.1). The commercial bumblebees had a somewhat lower prevalence of the honey bee parasites than of the bumblebee parasites, but the *Ascospaera* fungal parasite was present in 60% of the pollen samples and only *M. plutonius* was completely absent from the samples examined. When examined by microscopy, I did not observe *Crithidia* in the hind guts of the bumblebees, but both *Nosema* and *Apicystis* spores were visible. The richness of parasite communities found in the bumblebees did not differ significantly between the two bumblebee subspecies supplied, the three producers, or years (χ^2 = 0.232, d.f. = 1, P = 0.63, χ^2 = 5.04, d.f. = 2, P = 0.081, and χ^2 = 0.042, d.f. = 1, P = 0.838, respectively), and there was also no difference between the producers or years in the richness of parasites in their pollen (χ^2 = 5.04, d.f. = 2, P = 0.081, and χ^2 = 0.581, d.f. = 1, P = 0.446, respectively). However, DWV was more common in bumblebee colonies from 2011 compared with 2012 (7/30 vs. 0/18 colonies; P = 0.036), while the reverse was true for *C. bombi* (9/30 vs. 11/18 colonies; χ^2 = 4.32, d.f. = 1, P = 0.038). *N. bombi* was found in bumblebees from 5/5 colonies from Producer C, compared with 5/22 from Producer A and 3/13 from Producer B (P = 0.002), while *N. apis* was found only in pollen samples from Producer B (4/10 samples vs. 0/15 from the other two suppliers; P = 0.048). There were no other significant differences between bumblebee subspecies, producers or years in the number of colonies or pollen samples which carried the different parasites (P > 0.05 in all other cases; Table A2.2.5.1, Table A2.3.1.2).

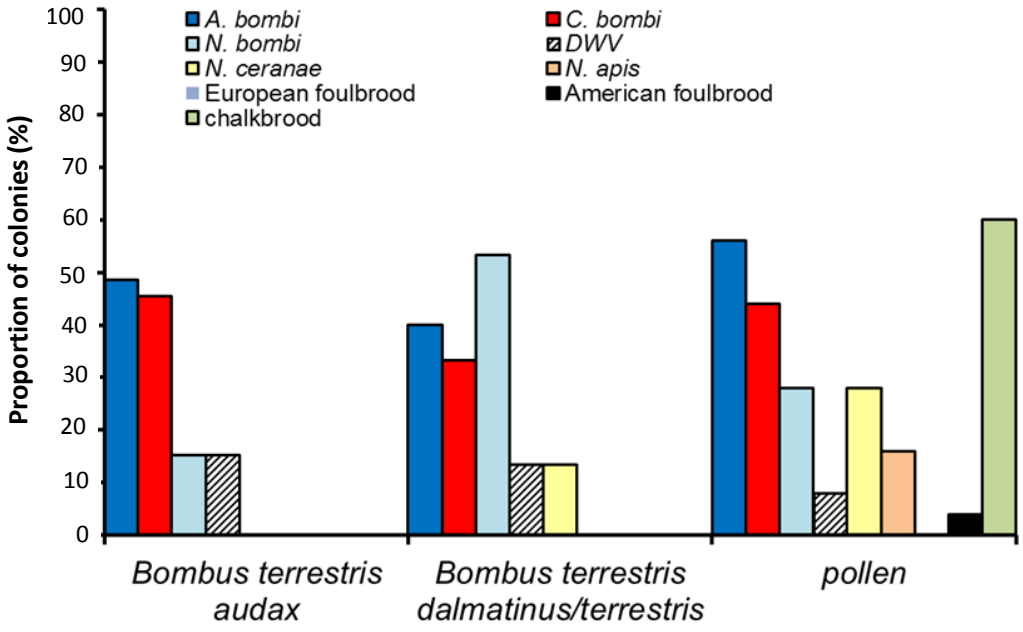


Figure 2.3.0.1 Commercially produced bumblebee colonies contain a diversity of parasites. Prevalence within 48 commercially produced bumblebee colonies of three species of bumblebee parasites and six species of honeybee parasites. Data are for 33 colonies of the bumblebee subspecies native to the UK (*Bombus terrestris audax*), 15 colonies of the most common subspecies produced commercially (*B. t. dalmatinus* and *B. t. terrestris*) and 25 samples of the pollen supplied with the colonies as food. Fifteen adult bumblebee workers were screened per colony. In 34 colonies, the 15 workers were pooled to give a single presence/absence for each colony, while in the other 14 colonies the 15 workers were screened individually (see Table A2.2.2.1).

2.3.1 Experiment 1: Infection risk to adult bumblebees

Bumblebee survival was reduced significantly by ingestion of faeces or pollen from commercially produced colonies (*Wald* = 6.11, d.f. = 2, *P* = 0.047), with the negative effect being very similar for the two treatments (after 15 days, bee survival was 61%, 44% and 36% for bees that had ingested control solution, faeces or pollen, respectively; Figure 2.3.1.1). No control bumblebees became infected by parasites, but bumblebees fed sucrose solution contaminated with either faeces or pollen from commercially produced bumblebee colonies became infected by *A. bombi*, *C. bombi*, *N. bombi* or *N. ceranae*, with the first and last of these parasites being most prevalent across the treatment group (Figure 2.3.1.1). There was no difference between the faeces and pollen treatments in the prevalence of bees which developed *C. bombi* or *N. ceranae* (Table A2.3.2.1), but significantly more of the bees which had ingested pollen developed *N. bombi* and *A. bombi* infections (*P* = 0.031 and χ^2 = 7.39, d.f. = 1, *P* = 0.007, respectively). None of the bees which died within 6 days of exposure had parasite spores visible in their hind guts, and neither *A. bombi* nor *C. bombi* were

visible in the bees which died later. However, 45% of the bees which died 7 or more days after exposure and had ingested pollen, and 11% of those which had ingested faeces, had *Nosema* spores visible in their guts, containing respectively 1.8 x 10^4 and 9 x 10^3 *Nosema* spores on average in the small 0.2 cm^3 tissue samples examined. There was also evidence of a trait effect of exposure to the pollen or faeces, with a significant interaction between the effects of treatment and day of testing on the sucrose response threshold of bumblebees (χ^2 = 15.3, d.f. = 6, P = 0.018), due to bumblebees that ingested faeces or pollen having a lower sucrose response threshold than the control bumblebees on day 5 (Figure 2.3.1.2).

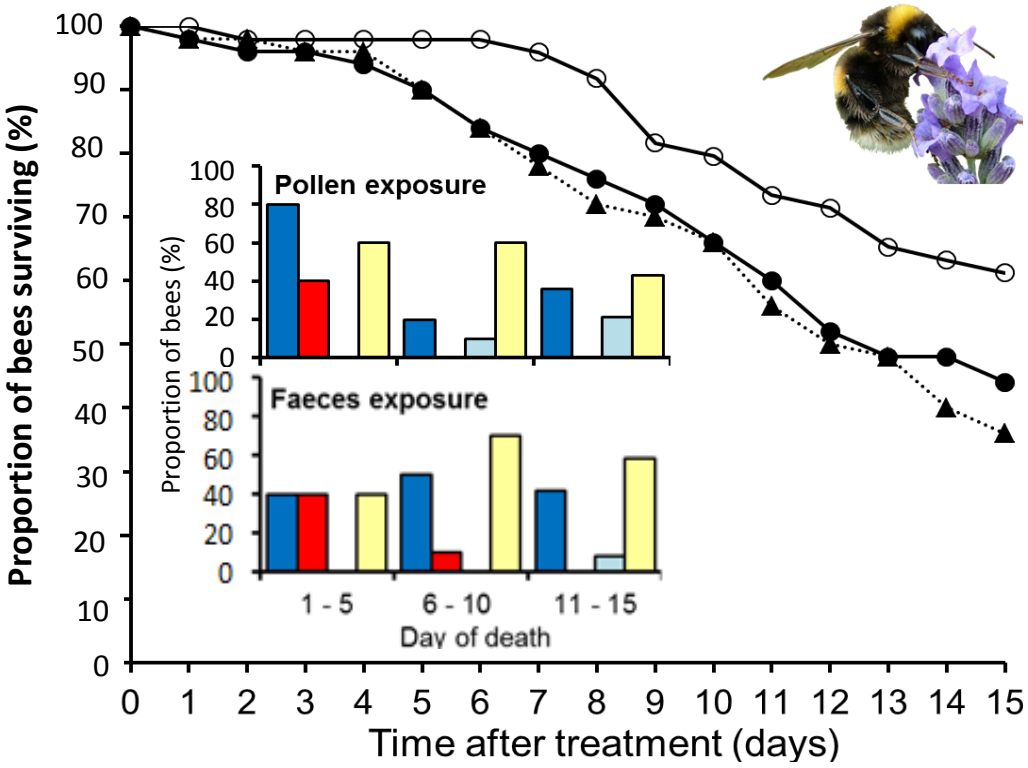


Figure 2.3.1.1 Ingestion by bumblebees of pollen or faeces from commercially produced bumblebee colonies leads to parasite infections. The effect on the survival of *Bombus terrestris audax* bumblebees of ingestion of either faeces (black circles, solid line) or pollen (triangles, dashed line) from parasite-infected, commercially produced bumblebee colonies, compared to ingestion of control solution (open circles, solid line; n = 50 for each treatment). Different letters beside lines indicate treatments that differed significantly (P < 0.05) from one another in Kaplan–Meier pairwise comparisons. Inset graphs show the proportion of bumblebees that had died either 1–5, 6–10 or 11–15 days after ingesting either pollen (top graph) or faeces (bottom graph), and which were then found by PCR to be positive for either the *Apicystis bombi* (dark blue columns), *Crithidia bombi* (red columns), *Nosema bombi* (light blue columns) or *Nosema ceranae* (yellow columns) parasites. No bees contained detectable deformed wing virus or *Nosema apis*, and control bees remained free of detectable parasite infections throughout the experiment.

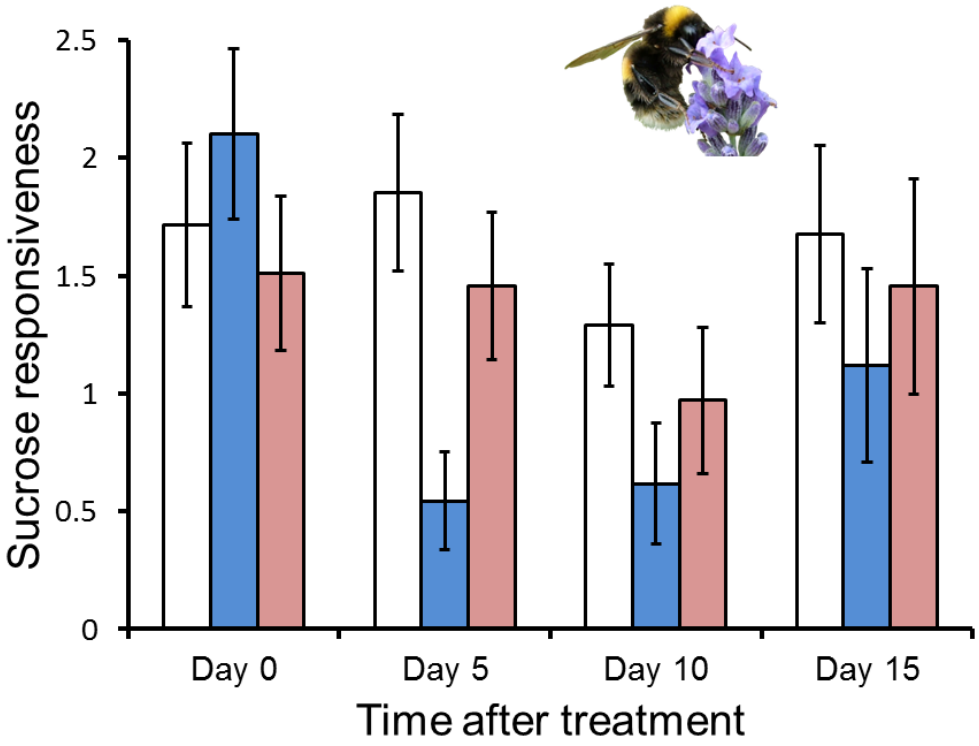


Figure 2.3.1.2 Ingestion by bumblebees of pollen or faeces from commercially-produced bumblebee colonies affects sucrose sensitivity. The effect on the mean ± s.e. sucrose responsiveness of *Bombus terrestris audax* bumblebees of ingestion of either faeces (red columns) or pollen (blue columns) from parasite-infected, commercially-produced bumblebee colonies, compared to ingestion of control solution (white columns). The sucrose responsiveness score indicates the percentage sucrose concentration (from 10-80%) at which the bee extended its proboscis to drink the solution, with 8 indicating that the bee extended its proboscis in response to all concentrations of sucrose and 1 indicating it only extended its proboscis in response to the 80% sucrose solution. There was a significant interaction between the treatment and the day of testing (χ^2 = 12.8, d.f. = 6, P = 0.046). The sucrose sensitivity of bees fed either control solution or faeces did not change significantly over time, but the sucrose sensitivity of bees fed pollen decreased, being significantly lower than that of bees fed the control solution on day 5 (P = 0.013 after sequential Bonferroni correction).

2.3.2 Experiment 2: Infection risk to adult honey bees

The survival of honey bees was significantly affected by treatment (*Wald* = 15.6, d.f. = 2, $P < 0.001$), being significantly reduced by the ingestion of bumblebee faeces from 70% to 40% of bees surviving after 14 days, but less affected by the ingestion of pollen (Figure 2.3.2.1). None of the control honey bees became infected by the various parasites, but substantial proportions of the honey bees which ingested either pollen or faeces from commercially produced bumblebee colonies became infected by *N. apis*, *N. ceranae* and *Apicystis bombi*, with 33–60% of bees becoming infected by *N.*

ceranae after ingesting bumblebee faeces and 20–27% after ingesting pollen (Figure 2.3.2.1). There was no significant difference between bees that ingested faeces or pollen in the numbers in which the *A. bombi* and *N. apis* parasites were subsequently detected (χ^2 = 0.296, d.f. = 1, P = 0.586, and χ^2 = 0.64, d.f. = 1, P = 0.424, respectively), but significantly more of the honey bees fed bumblebee faeces had *N. ceranae* than those fed pollen (χ^2 = 4.61, P = 0.032).

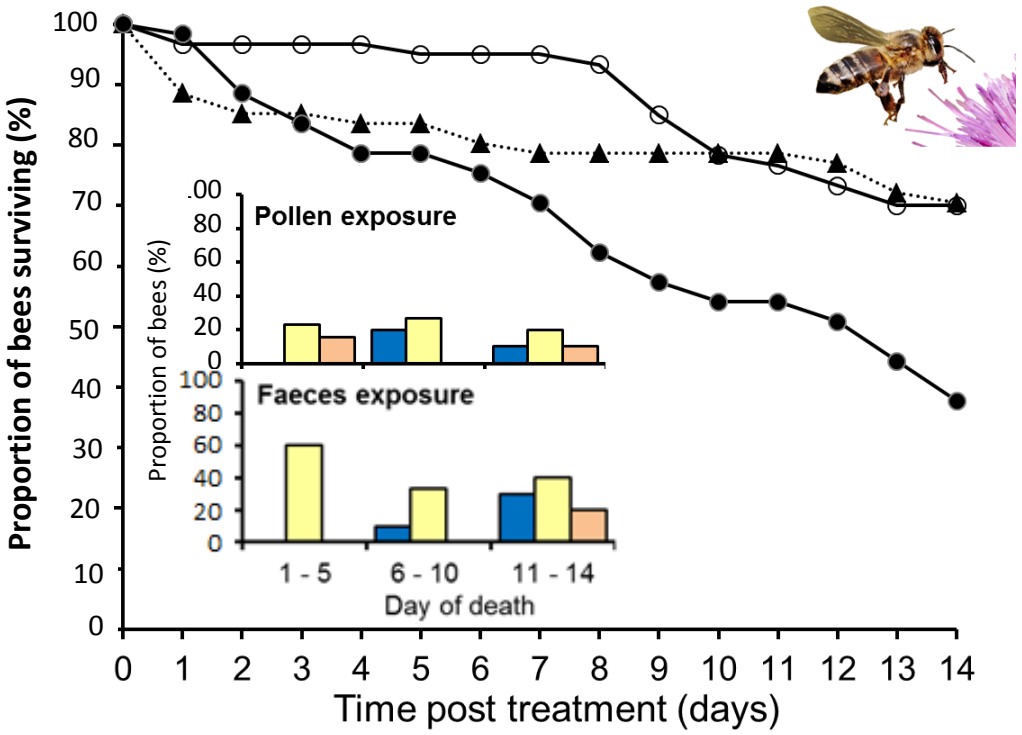


Figure 2.3.2.1 Ingestion by honeybees of pollen or faeces from commercially produced bumblebee colonies leads to parasite infections. The effect on the survival of *Apis mellifera* honeybees of ingestion of either bumblebee faeces (black circles, solid line) or pollen (triangles, dashed line) from parasite-infected, commercially produced bumblebee colonies, compared to ingestion of control solution (open circles, solid line; n = 60 for each treatment). Different letters beside lines indicate treatments that differed significantly (P < 0.05) from one another in Kaplan–Meier pairwise comparisons. Inset graphs show the proportion of honeybees that had died either 1–5, 6–10 or 11–14 days after ingesting either pollen (top graph) or faeces (bottom graph), and which were then found by PCR to be positive for either the *Apicystis bombi* (blue columns), *Nosema ceranae* (yellow columns) or *Nosema apis* (orange columns) parasites. No bees contained detectable *Nosema bombi* or *Crithidia bombi*, and control bees remained free of detectable parasite infections throughout the experiment.

2.3.3 Experiment 3: Infection risk to honey bee larvae

None of the larvae tested positive for the *M. plutonius* or *P. larvae* bacteria, and none of the control larvae developed infections of the fungal parasite *Ascosphaera apis*. However, larvae fed pollen from commercially produced bumblebee colonies had significantly lower survival than control larvae or larvae fed diet containing pollen that had been frozen and microwaved before ingestion to reduce the viability of parasites (*Wald* = 6.97, d.f. = 2, *P* = 0.031; Figure 2.3.3.1). Fifty-five percent of the pollen-fed larvae which died were found to be infected by the *Ascosphaera apis* fungal parasite, compared with 23% of those fed pollen that had been frozen and microwaved (χ^2 = 11.4, d.f. = 1, *P* = 0.001, Figure 2.3.3.1).

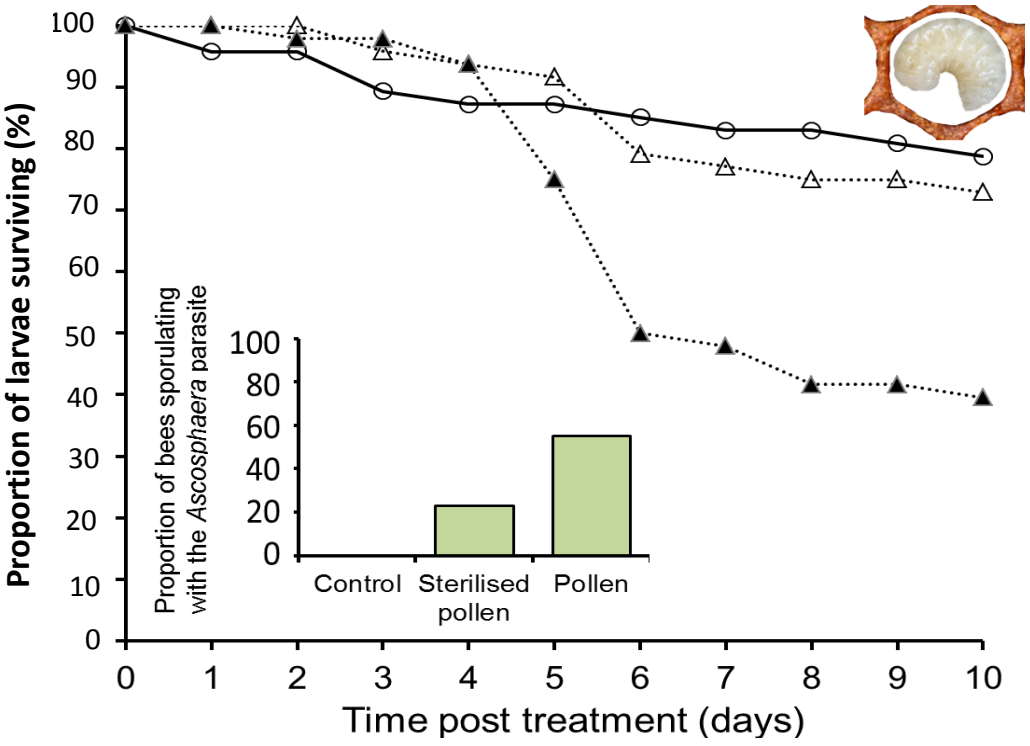


Figure 2.3.3.1 Ingestion by honeybee larvae of pollen supplied with commercially produced bumblebee colonies leads to parasite infections. The effect on the survival of *Apis mellifera* honeybee larvae of ingestion of either pollen from parasite-infected, commercially produced bumblebee colonies (black triangles, dashed line) or the same pollen sterilized by freezing and microwaving (white triangles, dashed line), compared to ingestion of control solution (open circles, solid line; n = 48 for each treatment). Different letters beside lines indicate treatments that differed significantly ($P < 0.05$) from one another in Kaplan–Meier pairwise comparisons. Inset graph shows the proportion of honeybees that died which then sporulated with the chalkbrood fungal parasite *Ascosphaera apis* after ingesting either pollen, sterilized pollen or control solution. No European foulbrood or American foulbrood was detected in the bees.

2.4 Discussion

Bumblebee colonies that were commercially produced as recently as 2011 and 2012 by all three of the producers that I investigated contained a number of faecal–orally transmitted parasites. Importantly, I sampled bees from colonies immediately upon receipt; therefore the parasites detected must have entered the colonies during their production. The parasites included three specialist parasites of bumblebees (*Apicystis bombi*, *Crithidia bombi* and *Nosema bombi*) which can negatively affect their health (Schmid-Hempel 2001), with the colony-level prevalence of these being similar to the prevalence reported in wild bumblebee populations (Gillespie 2010; Whitehorn *et al.* 2011; Goulson *et al.* 2012). There was also evidence of two parasites (DWV and *N. ceranae*) which can infect bumblebees and honey bees (Genersch *et al.* 2006), and three other honey bee-specific parasites, including *P. larvae* which is a notifiable disease in the UK and throughout the EU (European Commission 1992). *P. larvae* causes the highly virulent American foulbrood disease in honey bee larvae and colonies found with the disease in the UK have to be destroyed immediately, so the importation with commercially produced bumblebees of pollen carrying this parasite is of particular concern. The PCR and RT-PCR method I used detected the DNA of the parasites, but spores of both *Nosema* and *Apicystis* were clearly visible in the guts of the commercially produced bumblebees, and the number of *Nosema* spores observed was comparable to that found previously for *N. bombi* or *N. ceranae* infections of bumblebees (Rutrecht *et al.* 2007).

The results are consistent with various reports of parasites in bumblebee colonies produced up to 2008, and demonstrate that the problem is still present, in spite of the efforts and regulations designed to ensure that imported colonies are free of disease. Detection of parasites does not necessarily mean that they are infectious parasites which pose a risk to other bees. However, our controlled experiments confirmed that at least the *Nosema bombi*, *N. ceranae*, and *Apicystis bombi* parasites carried by commercially produced bumblebees and their pollen were infectious to other bumblebees, reducing survival and also having a trait effect on the sucrose response threshold of exposed bumblebees. Although the doses involved of *Nosema* were similar to those used in previous studies (Rutrecht *et al.* 2007), infection in the wild may well be lower or higher, and survival better or worse, than in our single inoculation laboratory experiment, but the results at a minimum demonstrate that some

parasites carried by commercially produced bumblebees are infectious. Pollen is an important component of bee nutrition that can enhance disease resistance (Foley *et al.* 2012), but our data show it can carry parasites and therefore be hazardous to bees as well. In contrast to the other parasites, the prevalence of *C. bombi* in the treated bees decreased over time, most probably due to the *C. bombi* having limited viability and being cleared effectively by the bees. The greater prevalence of *Nosema ceranae* and *Apicystis bombi* infections in bumblebees in Experiment 1 that had ingested pollen rather than bumblebee faeces is in keeping with the greater number of *Nosema* and *Apicystis* spores in the pollen exposure treatment, while the higher prevalence of *Nosema ceranae* in honey bees in Experiment 2 that were fed bumblebee faeces rather than pollen is not. Possibly this was because *Nosema* spores in pollen included many of the less virulent *N. apis* (Paxton *et al.* 2007), whereas the apparently limited ability of *N. apis* to infect bumblebees will have meant that *Nosema* spores in bumblebee faeces will have been only *N. ceranae* or *N. bombi*.

Concern about pathogen spillover from commercially produced bumblebees has been focused on the threat to native bumblebees. However, commercially produced bumblebees intermingle with many other managed and native pollinators as well, resulting in significant potential for interspecific transmission of parasites during shared flower use (Durrer & Schmid-Hempel 1994; Singh *et al.* 2010; Evison *et al.* 2012). Our results suggest that this danger is real, with commercially produced bumblebee colonies carrying at least five parasites of honey bees, of which *Nosema ceranae*, *N. apis* and *Apicystis bombi* were all infectious to adult honey bees. The pollen supplied with the bumblebee colonies also carried spores of the *Ascosphaera apis* fungal parasite which were infectious to honey bee larvae. Spores of this parasite are long-lasting and transmit between colonies by contaminating adult bees that then incorporate the spores accidentally in the food they feed to their larvae (Aronstein & Murray 2010).

These results suggest that a majority of the over a million commercially produced bumblebee colonies that are being imported globally each year still potentially contain a diversity of parasites that are viable, infectious and virulent. In some cases these parasites are highly likely to be different strains or species to those found in native populations in the areas to which they are imported, as observed in Japan and Argentina (Goka *et al.* 2001; Arbetman *et al.* 2012). Even when the parasite strains are the same, the importation of large numbers of infected hosts will increase

local parasite density and the probability of mixed parasite infections which can be particularly harmful to hosts. There is already correlational evidence of pathogen spillover from commercially produced bumblebees negatively affecting native bumblebee populations in North America and Argentina (Colla *et al.* 2006; Szabo *et al.* 2012; Arbetman *et al.* 2012). Our experimental results confirm that the parasites carried by commercially produced bumblebees are infectious to bumblebees and represent a threat to honey bees as well.

Implications for Management

Although the companies producing bumblebees have attempted in recent years to eliminate diseases from their operations, apparently with good success in the case of tracheal mites (Goka *et al.* 2006), the results show that far more robust measures are required. Eliminating parasites from the pollen fed to the bumblebees or replacing the pollen with a hygienic substitute is likely to be essential. In addition, the prevalence of parasites in bumblebee colonies that were sold and imported as being parasite-free, demonstrates that more robust checks are also required. Many of the parasites are difficult to detect visually and currently impossible to culture *in vitro*, so these checks will have to use sensitive molecular methods in order to be effective. The prevalence and intensity of parasite infections in animals can increase during shipping from the production facilities to the end-user, a phenomenon which is well known in vertebrates and sometimes termed ‘shipping fever’ (Barham *et al.* 2002), and which could be one possible explanation for why colonies had heavy parasite infections upon receipt. Either extremely rigorous parasite-screening at source or parasite screening on arrival, or probably both, would be needed to prevent this. A further problem relates to the regulations applied to bumblebees. In England, the importation licences requiring parasite-screening are limited to the non-native subspecies of *Bombus terrestris*. Our results show that native *B. t. audax*, as well as the non-native subspecies, carries parasites, so some form of regulation to prevent the import of parasites with a commercially produced native organism will also be needed if pathogen spillover is to be prevented. Given the ecological significance and vulnerability of many wild pollinator species, the economic importance of crop pollination with commercially produced bumblebees, and the substantial fitness effects of the parasites they currently carry, such measures to reduce the accidental importation of parasites with commercially produced bumblebees are urgently needed.

Chapter 3: A Hitchhikers Guide to Parasite Dispersal

Abstract

The dispersal of parasites around the environment is critical for understanding parasite epidemiology and, where species share resources, interspecific vectoring of parasites may play a major role in this. One of the best examples of such a situation is the shared use of flowers by a diversity of pollinator species, however the importance of flowers and interspecific vectoring for the dispersal of pollinator parasites is poorly understood. Here I use flight cage experiments to investigate the potential for the shared use of flowers to result in the vectoring of microbial parasites by non-host pollinators, using the bumblebee *Bombus terrestris* and the honey bee *Apis mellifera* as our model species. I show that during even short foraging periods of 3 h, three bumblebee parasites and two honey bee parasites are dispersed effectively on to flowers by their host bees, and that all five parasites are then vectored freely between flowers by non-host pollinator species. The results suggest that flowers are hotspots for the transmission of pollinator parasites that can be readily vectored by non-host species. Shared flower use by pollinator species is likely to be a significant mechanism for the dispersal of parasites in the environment.

3.1 Introduction

Parasites are of major ecological and evolutionary importance, with substantial effects on host fitness and population dynamics (Poulin 1999; Daszak *et al.* 2000; Prenter *et al.* 2004; Lafferty *et al.* 2008; Hatcher & Dunn 2011). The mechanisms of parasite dispersal are key to the epidemiology of parasite dynamics (Kamo & Boots 2006; McCoy 2008). Our understanding of host-parasite epidemiology comes primarily from studies of single host-parasite systems. However, all parasites exist in an environment in which they will encounter multiple other species, as well as their host, creating significant potential for non-host species to be important in the dispersal of the parasite (Rigaud *et al.* 2010). There are many classic cases of organisms vectoring parasites by acting as an intermediate host in which the parasite completes part of its life-cycle (Cox 2010; Coura & Viñas 2010). However, the incidental vectoring of parasites on the body surface of the vector, or following passage through the gut without infection

taking place, may be of much more importance, particularly for parasites which transmit via contact or faecal-orally.

The potential for vectoring of parasites will be particularly great when multiple species utilise the same food resource. An extreme example of this is plant-pollinator mutualisms. While some plant-pollinator systems are specific, in the vast majority of cases, flowers are visited by multiple pollinator species (Goulson & Darvill 2004; Fontaine *et al.* 2006). It then follows that vectoring of parasites by non-host species during shared flower use may be of great importance in pollinator-parasite interactions. There is currently great interest in the stress factors affecting pollinators, many of which are showing substantial population declines with knock-on effects on the plants that rely on them for pollination (Biesmeijer *et al.* 2006; Potts *et al.* 2010b; Pauw & Hawkins 2011; Burkle *et al.* 2013). Parasites are well established as being an important factor in at least some of these declines, with several bumblebee species showing population declines, often correlated with pathogen spillover from commercially-produced bumblebees (Chapter 2; Meeus *et al.* 2011; Szabo *et al.* 2012), and honey bee colony losses in many countries being associated with various emerging parasites, such as the *Varroa* mite and the microsporidian *Nosema ceranae* (Kraus & Page 1995; Genersch & Aubert 2010; Higes *et al.* 2010). Importantly, there is increasing evidence of parasite transmission between pollinator taxa being far more important than has generally been appreciated. Several honey bee viruses have been detected in bumblebees with at least one of these viruses causing clinical symptoms (Chapter 2; Genersch *et al.* 2006; Evison *et al.* 2012), the microsporidian parasite of honey bees, *Nosema ceranae*, has also been detected commonly in bumblebees and shown experimentally to infect them readily (Chapter 2 & 5; Plischuk *et al.* 2009a), and the neogregarine parasite of bumblebees, *Apicystis bombi*, has been shown experimentally to infect honey bees (Chapter 2; Lipa *et al.* 1996).

Remarkably, however, the epidemiology and transmission of pollinator parasites is still very poorly understood and the potentially profound role of shared flower use in particular, little investigated. Several studies have detected the presence of parasites in bee collected pollen (Chapter 2; Flores *et al.* 2005; Singh *et al.* 2010), however, it is unclear if these parasites were on the flowers and collected along with the pollen or, if they originate from the foraging bee (Copley & Jabaji 2012). Bumblebees have been shown to avoid flowers contaminated with high doses of parasite (Fouks & Lattorff 2011), implying that the threat is present and sufficient for

them to have evolved this capability. However, the only direct experimental evidence of parasite transmission via flowers comes from a study by Durrer & Schmid-Hempel (1994), in which bumblebees were shown to become infected by the trypanosome *Crithidia bombi* after feeding on flowers contaminated with the parasite. Here I investigate the potential for flowers to act as dispersal platforms for pollinator parasites and for non-host species to play a role in vectoring them across the environment.

3.2 Methods

3.2.1 Dispersal

The study used three colonies of *Apis mellifera carnica* honey bees and three colonies *Bombus terrestris audax* bumblebees. The honey bee colonies each consisted of three frames of bees, brood and food, in a mini-nucleus box. The bumblebee colonies were obtained from a commercial producer and contained approximately 60-80 workers at the time of the experiment. The honey bee colonies were found by PCR screening (see below) to be infected by *Nosema apis* and *Nosema ceranae* parasites, while the bumblebee colonies were infected by *Apicystis bombi*, *Crithidia bombi* and *Nosema bombi*; neither bee species had the parasites of the other bee species. The flowers used were a mixed group of 50 *Campanula cochleariifolia* which have bell shaped flowers and 30 *Viola tricolor* which have flat, platform-like flowers. The experiment was run using honey bees as the parasite provider and bumblebees as the vector, and repeated using bumblebees as the parasite provider and honey bees as the vector. In each case, three hives of the species providing the parasites were placed in a flight cage (6 m x 4 m x 1.5 m, L x W x H), and left for a day to acclimatise. A first group of mixed flowers was then placed in the flight cage, and the bees allowed to forage for 3 h. After this time, the colonies of the parasite provider were excluded from the foraging area, a second group of mixed flowers was placed in the foraging arena, and three colonies of the vector species allowed to forage on both the previously foraged upon flowers (shared flowers) and the new group (vector-only flowers) for 3 h (Figure 3.2.1.1). Ten bees per hive (both parasite provider and vector species) and 30 flowers per species were collected immediately prior to the start of the experiment to act as naïve controls. Following the experiment, a further 10 bees per colony and all flowers were collected for parasite screening.

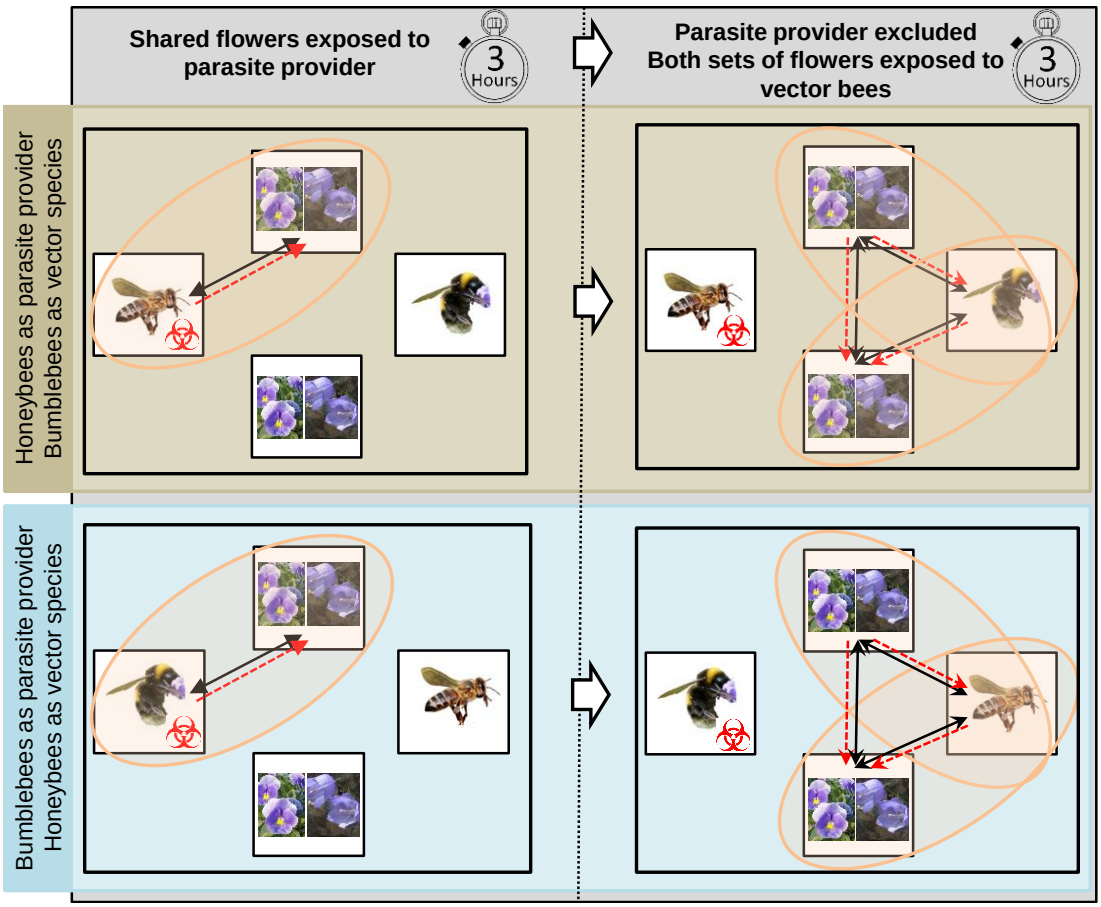


Figure 3.2.1.1 The movement of bees (black, solid arrows) and potential movement of parasites (red, dashed arrows) during experiments in which either honey bees provided parasites and bumblebees were the vectors (top graphs), or vice versa (bottom graphs). Initially the bees providing parasites were allowed to forage on a set of flowers (left graphs). The parasite provider bees were then excluded, and the vector bees allowed to forage on both sets of flowers (right graphs). Flowers consisted of a mix of *Viola tricolor* flat flowers and *Campanula cochleariifolia* bell-shaped flowers.

3.2.2 Screening

Bees and flowers were screened for the presence of pathogens. Ten bees from every bee hive used were screened before and after each experiment. To prepare samples for extraction, each bee had its individual tissues dissected, the malpighian tubules, fatbody and gut were homogenised with a micropestle. For flowers, every flower was removed from the stem and vortexed in 1 ml of 100% ethanol for 2 min. The flower was then removed from the eppendorf and the resulting solution of ethanol and particles from the flower (including any parasites present) was placed in a centrifuge at 14,000 g for 5 min, before the upper 800 µl of solution was discarded and the remaining 200 µl was then homogenised with a micropestle. The homogenised sample was then washed by adding 800 µl of TE buffer, vortexed for 30 seconds and placed in

a centrifuge at 14,000 g for 5 min, 800 µl of the supernatant was then discarded. This wash procedure was repeated two further times, the final time, 950 µl of supernatant was removed, resulting in 50 µl of sample. The DNA from each bee sample was then extracted using 5% Chelex solution before PCR screening for the *18S* Apidae host control gene to confirm DNA quality (Meeus *et al.* 2010) using 0.4 mM dNTP, 1.5 mM $MgCl_2$, 3 µl buffer, 1.25 U Taq, 0.2 µM each primer and 1 µl template, giving reaction volume of 10 µl in total. The PCR conditions were: 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 56°C, 45 s at 72°C before a final elongation stage of 3 min at 72°C. All samples were also screened for *Apicystis bombi* and *Crithidia bombi* using 0.4 mM dNTP, 1.5 mM $MgCl_2$, 2 µl (3 µl for *C. bombi*) buffer, 1.25 U Taq, 0.5 µM each primer and 1 µl template, giving 10 µl in total volume. The PCR conditions for these parasites were: 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 60°C (56°C for *C. bombi*), 45 s at 72°C before a final elongation stage of 3 min at 72°C (Meeus *et al.* 2010). Additionally, samples were screened for *N. ceranae* and *N. apis* (Chen *et al.* 2008), with reactions containing 0.25 mM dNTP, 3.75 mM $MgCl_2$, 2 µl buffer, 0.25 U Taq, 0.2 µM each primer and 1 µl template, giving 10 µl in total for each reaction. Reaction conditions were 2 min at 94°C, 35 cycles of 30 s at 94°C, 45 s at 61°C (63°C for *N. apis*), 120 s at 72°C before a final elongation stage of 7 min at 72°C. Finally, the samples were screened for *N. bombi* (Klee *et al.* 2006), with reactions containing 0.3 mM dNTP, 3.75 mM $MgCl_2$, 2 µl buffer, 0.25 U Taq, 0.2 µM each primer and 2 µl template, giving 10 µl in total for each reaction. Reaction conditions were 4 min at 94°C, 35 cycles of 60 s at 95°C, 60 s at 50°C, 60 s at 72°C before a final elongation stage of 4 min at 72°C. Each parasite screening was run with a positive and negative control and positive identification was confirmed by the presence of the appropriate sized amplicon following gel electrophoresis, with a 100bp DNA ladder.

3.2.3 Statistical analysis

The frequency of samples (bees or flowers in which each parasite was detected were compared between the treatments using generalized linear models (GLM) with binomial distribution, logit link function and the likelihood ratio χ^2 statistic. Colony of origin was included in the GLM models. Nonsignificant terms were removed stepwise in all cases to obtain the minimum adequate models. All analyses were carried out in PASW Statistics 19 (IBM, Armonk, NY, USA).

3.3 Results

No parasites were found to be present on the naïve flower samples taken prior to the experiment, all of the vector bees were also negative prior to the experiment of the parasites which were present in the parasite provider bees.

3.3.1 Honey bees as parasite providers

The prevalence of parasites in honey bee hives after the foraging period was not significantly different from the prevalence prior to the foraging period with prevalence's of 16-20% for *N. apis* (χ^2 = 0.01, P > 0.99) and 46-53% for *N. ceranae* (χ^2 = 0.01, P > 0.99). After the experiment, the prevalence of shared flowers exhibiting the presence of *N. apis* and *N. ceranae* had increased from 0% to 14% (χ^2 = 13.01, P < 0.001) and 59% (χ^2 = 70.24, P < 0.001) respectively (Figure 3.3.1.1).The prevalence of flowers with *N. apis* and *N. ceranae* present upon them following visitation by the vector agents (bumblebees), increased during the experiment from 0% to 6% (χ^2 = 5.76, P = 0.017) and 52% (χ^2 = 60.34, P < 0.001) respectively (Figure 3.3.1.1). Dispersal of *N. apis* and *N. ceranae* was equally likely regardless of the flower type (χ^2 = 0.29, P = 0.598, and χ^2 = 2.06, P = 0.151, respectively). At the end of the experiment, the prevalence of *N. ceranae* in the vector bumblebee colonies had increased from 0% to 23% (χ^2 = 10.63, P = 0.001), whilst *N. apis* remained undetected (Figure 3.3.1.1).

3.3.2 Bumblebees as parasite providers

The prevalence of parasites in bumblebee hives after the foraging period was not significantly different to the prevalence prior to the foraging period, with 33-36% of bees having *A. bombi* (χ^2 = 0.07, P = 0.79), 70-73% *C. bombi* (χ^2 = 0.08, P = 0.77) and 7% *N. bombi* (χ^2 = 0, P > 0.999). None of the control flowers removed prior to the experiment were positive for any parasite. Following visitation by both bees, the prevalence of flowers harbouring *A. bombi*, *C. bombi* and *N. bombi* however, was significantly higher than the 0% of contaminated flowers at the start of the experiment, with 48% (χ^2 = 22.11, P < 0.001), 75% (χ^2 = 44.63, P < 0.001) and 10% (χ^2 = 8.21, P = 0.017) respectively (Figure 3.3.2.1). At the end of the experiment, the prevalence flowers harbouring *A. bombi* and *C. bombi* following foraging activity by the vector

agents (honey bees) had also increased significantly from 0% to 22% (χ^2 = 8.97, P = 0.003), 43% (χ^2 = 19.84, P < 0.001) respectively, whilst *N. bombi* was present on 3% of these flowers though was non-significant (χ^2 = 2.26, P = 0.133). Dispersal was more likely to occur on the bell-shaped *C. cochleariifolia* flowers for the *A. bombi* and *C. bombi* parasites (χ^2 = 10.42, P = 0.001 and χ^2 = 22.53, P < 0.001; Figure 3.3.2.1 respectively), but for *N. bombi* (χ^2 = 2.07, P = 0.15; Figure 3.3.2.1) was equally likely regardless of the flower type. By the end of the experiment, the within hive prevalence of *A. bombi* and *C. bombi* in the vectoring honey bee colonies had increased from 0% to 7% (χ^2 = 0.73, P = 0.09) and 30% (χ^2 = 0.82, P < 0.001) respectively, whilst *N. bombi* remained undetected (Figure 3.3.2.1).

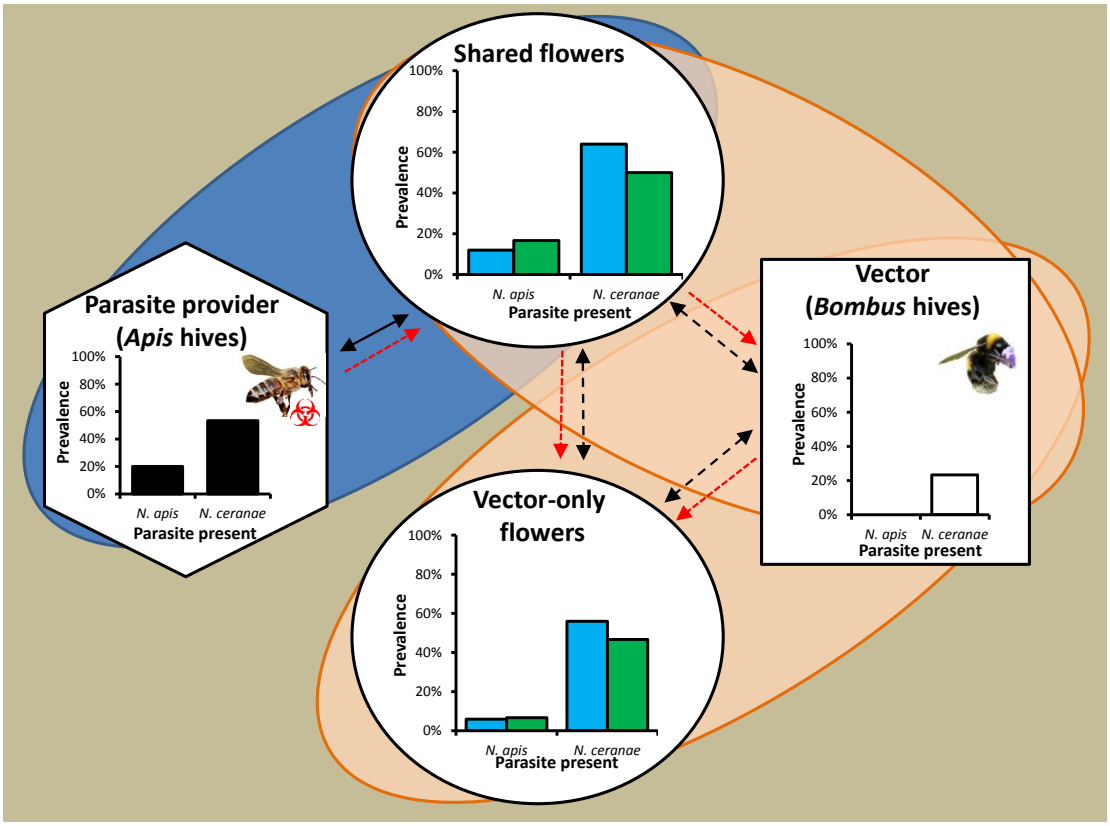


Figure 3.3.1.1 Bumblebees vector honey bee parasites. The prevalence of the honey bee parasites *Nosema apis* and *Nosema ceranae* within the honey bee colonies acting as the parasite providers (black columns), or after the experiment on the bell shaped *C. cochleariifolia* and flat formed *V. tricolor* flowers (middle graphs, blue and green columns respectively), or within bumblebee colonies that acted as vectors (open columns). All flowers and bumblebees were free of the two parasites prior to the experiment. One set of flowers (top graph) was initially exposed to honey bees for 3 h while the bumblebees were excluded from the foraging arena. The honey bees were then excluded from the arena, and the bumblebees allowed to forage freely on the same set of shared flowers, and also on a new set of clean, vector-only flowers (bottom graph). Solid black arrows represent movement of the parasite provider, dashed black arrows represent movements of vector species between flowers and hive and red arrows indicate possible dispersal routs of the parasites.

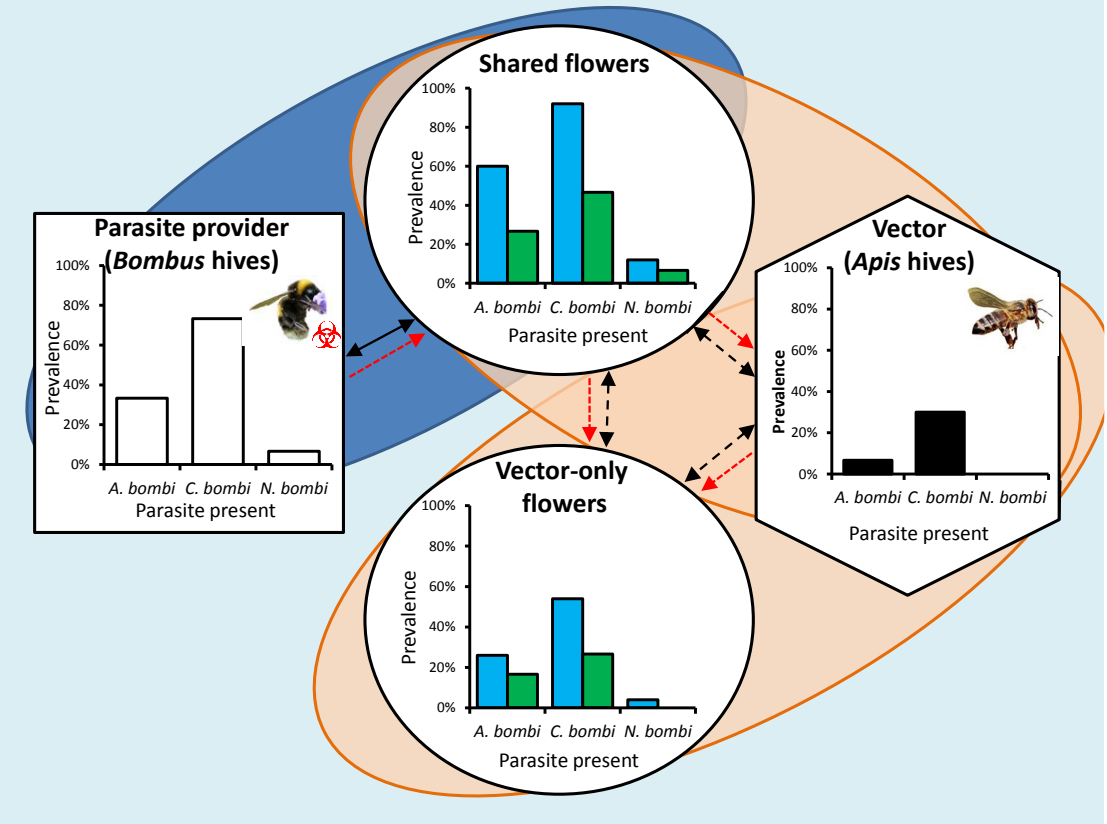


Figure 3.3.2.1 Honey bees vector bumblebee parasites. The prevalence of the bumblebee parasites *Apicystis bombi*, *Crithidia bombi* and *Nosema bombi* within the bumblebee colonies acting as the parasite providers (open columns), or after the experiment on bell shaped *C. cochleariifolia* and *V. tricolor* flowers (middle graphs, blue and green columns respectively), or within honey bee colonies that acted as vectors (black columns). All flowers and bumblebees were free of the three parasites prior to the experiment. One set of flowers (top graph) was initially exposed to bumblebees for 3 h while the honey bees were excluded from the foraging arena. The bumblebees were then excluded from the arena, and the honey bees allowed to forage freely on the same set of shared flowers, and also on a new set of clean, vector-only flowers (bottom graph). Solid black arrows represent movement of the parasite provider, dashed black arrows represent movements of vector species between flowers and hive and red arrows indicate possible dispersal routs of the parasites.

3.4 Discussion

The results show that a variety of pollinator parasites disperse from their bee hosts onto flowers. Parasites on flowers were then dispersed further by non-host bees to new flowers and back to their colonies. This was the case both for bumblebees and honey bees. Flower form effected the dispersal of some parasites, but once contaminated, flowers become hotspots for disease dispersal via vectoring bees.

The bumblebee parasites *A. bombi*, *C. bombi* and *N. bombi*, plus the honey bee parasites *N. apis* and *N. ceranae*, were all rapidly dispersed from infected individuals to flowers within 3 hours of foraging. Although the two flower species require different methods of flower handling by the bees (Dafni 1992), the three *Nosema* species showed no evidence of a relationship between flower-form and dispersal. *Apicystis bombi* and *C. bombi*, however, dispersed onto the bell-shaped *C. cochleariifolia* flowers more frequently than the flat-formed *V. tricolour* (with 21% and 36% greater dispersal respectively). This is likely to be a result of the increased physical contact that bees have with the bell-shaped flowers during foraging. This demonstrates not only that shared flowers are sites for parasite dispersal in all five of the pollinator parasites tested, but also that some flower forms may provide a more effective transmission platform for parasites than others. Furthermore, the restricted time-span of the experiment makes it probable that parasite dispersal in the natural environment is much greater. It may be expected that infected bees may have increased flower handling times and foraging demands due to the costs of infection which could drive parasite dispersal rate even higher (Otterstatter *et al.* 2005; Gegear *et al.* 2005, 2006; Tyler *et al.* 2006; Riddell & Mallon 2006; Alghamdi *et al.* 2008). The propensity of a parasite to transfer between pollinator and flower is remarkably high, with clear vectoring between foraging sites taking place very rapidly.

After honey bees had foraged on both shared and exclusive flowers, the bumblebee parasites *A. bombi* and *C. bombi* were detected within honey bees from the hives. As the bee samples screened consisted only of internal tissues, this demonstrates that the honey bees had ingested the parasites during the collection and processing of nectar and pollen from the contaminated flowers. Or the subsequent grooming of contaminated body surfaces by the bees. *Apicystis bombi* has been detected in honey bees previously, though its virulence in this host is unknown (Lipa & Triggiani 1996). In bumblebees it reduces the fatbody and survival of workers and over-wintering queens (Chapter 6; Liu *et al.* 1974; Macfarla *et al.* 1995). *Crithidia bombi* has not been shown to have an effect on honey bees, although the parasite is able to develop within honey bee hosts for a short time (Ruiz-González & Brown 2006). This suggests that honey bees could act as reservoir hosts for bumblebee parasites. After exposure to flowers contaminated with *Nosema ceranae* by honey bees, 23% of bumblebee workers in their hives shared *N. ceranae* within them. This means the bumblebees had ingested the parasite during foraging, food processing or grooming. *Nosema ceranae* is

traditionally thought of as being a honey bee parasite and has been implicated in colony losses across Europe (Higes *et al.* 2006, 2008b, 2010). However, *N. ceranae* has more recently been identified as an emerging pathogen in several bumblebee species, causing both lethal and trait effects (Chapter 5; Plischuk *et al.* 2009a). The results presented in this study highlight shared flower use as a likely mode of transmission, facilitating parasite spillover between different pollinators.

These results provide strong evidence that many parasites may benefit from the shared use of flowers by multiple pollinator species, with non-host as well as host pollinators dispersing the parasites around the environment. The frequent, polylectic contact that bees and pollinators in general have with flowers provides the ideal transmission platform for parasites to spread between host species and landscapes. This added dynamic highlights the need to widen parasite screening regimes for imported/exported bees and flower products to include parasites that may be vectored by the bees or flowers, and which may pose a potentially devastating threat to naïve pollinator communities. By taking a wider view of pollinators as hosts, I find a greater level of interaction between them and their parasites, suggesting that on a community level, parasite vectoring and spillover may be far more widespread that previously realised.

Chapter 4: The effects of managed bees on the prevalence of parasites in local bumblebee populations

Abstract

Honey bees have been domesticated for centuries, and are now managed commercially to pollinate crops and hive products. Recently, bumblebee colonies have also been reared commercially and transported worldwide to farms for crop pollination. Mounting evidence suggests that use of managed honey bees or commercially reared bumblebees may affect the health of local bumblebees. The increase in pollinator density and the mixing of managed and wild pollinator populations may augment parasite prevalence in wild bumblebees. Here, I screened 764 bumblebees from around greenhouses that either used commercially reared bumblebees or did not, as well as bumblebees from 10 colonies placed at varying proximities to an apiary, for the parasites *Apicystis bombi*, *Crithidia bombi*, *Nosema bombi*, *N. ceranae*, *N. apis* and deformed wing virus. In this preliminary study, I found increased parasite prevalence in bumblebees in close proximity to managed honey bees or commercially reared bumblebees. *Apicystis bombi* and *C. bombi* were particularly prevalent around greenhouses using commercially reared bumblebees, while bumblebees from near to the honey bee apiary had an 18% greater prevalence of *C. bombi* compared to bumblebees far from the apiary. Whilst these results support previous reports of parasite spillover from commercial bumblebees, they also suggest the elevated *C. bombi* prevalence may be due to increased pollinator density and/or bumblebee stress rather than a direct spillover from commercial bumblebees. The use of managed bees clearly comes at a cost of increased parasites in native bumblebees, which is not only a concern for bumblebee conservation, but also a phenomenon that is likely found in other sympatric pollinators around managed bees.

4.1 Introduction

In recent years several bumblebee species have suffered range declines in parts of Europe, the Americas and Asia (Williams 1982; Fitzpatrick *et al.* 2007; Kosior *et al.* 2007; Goulson *et al.* 2008; Xie *et al.* 2008; Martins & Melo 2010; Cameron *et al.* 2011). Changes in anthropogenic land-use is a major contributing factor to these

declines, with a proliferation of large monoculture crops and a reduction of species-rich hedgerows, reducing floral diversity and nesting habitats from many pollinators (Goulson *et al.* 2005, 2006; Biesmeijer *et al.* 2006; Ricketts *et al.* 2008; Goulson 2010). This has left some bumblebee species fragmented and vulnerable to parasites, particularly small populations with low genetic diversity, as is the case for many declining bumblebees species (Daszak *et al.* 2001; Goulson *et al.* 2008; Whitehorn *et al.* 2011).

The incidence of bumblebee parasites in the environment has been shown to fluctuate for a variety of reasons including the climate, parasite lifecycles and host-parasite dynamics (Macfarlane *et al.* 1995; Goulson 2010; Cameron *et al.* 2011). In addition to these, the deployment of commercially reared and imported bumblebees may be another driver of parasite increase in local bumblebee population. Commonly, commercially reared bumblebees are deployed within glasshouses to enhance the yields of soft fruit crops (Lye *et al.* 2011). Despite this, they are frequently found foraging beyond their glasshouses (Morandin *et al.* 2001; Murray *et al.* 2013) and native bees have been found foraging inside commercial greenhouses (Kraus *et al.* 2010; Lye *et al.* 2011). By freely mixing with wild bumblebees, the deployment of commercial bumblebees effectively increases the local density of bumblebees. Bumblebee parasites can be dispersed between bumblebees following shared flower usage (Chapter 3; Durrer *et al.* 1994). As a result, the rate of parasite transmission between bees will predictably rise with increased pollinator density (Anderson *et al.* 1986; Arneberg *et al.* 1998). In areas utilising commercial bumblebees, higher parasite prevalence may be expected as a result of parasite spill back from wild bumblebees, parasite spillover of introduced parasites from the commercial bumblebees, or stress related to the high pollinator density.

Spillback: Theory suggests that a high density of uninfected, commercial bumblebees could succumb to native parasites upon mixing with native pollinators. Following this, the high density of commercially reared bumblebees will drive unnaturally high parasite prevalence in the local area. This high density of infected commercially reared bumblebees may then become parasite reservoirs, with native parasites ‘spilling back’ to surrounding wild bumblebees populations (Kelly *et al.* 2009).

Stress: With increased competition for resources bumblebees will need to travel further afield for resources, possibly foraging on a greater number of flowers. It has been shown that increased competition can affect bumblebees by reducing their individual

and colony growth, potentially forcing them to produce small queens, which will be less likely to survive the winter (Goulson & Sparrow 2008; Elbagrmi et al. in prep). In addition, despite the lack of research into the effect of such stress on parasite prevalence, it is clear that stress in many forms can affect the physiology, behaviour and even survival of bees, and that a reduction in the condition of bees can further affect their ability to withstand infection by parasites (Brown *et al.* 2000; Even *et al.* 2012). The strength of immunity is the result of a resource trade-off between other body functions such as growth and learning (Frank 1996; Schmid-Hempel & Ebert 2003; Mallon *et al.* 2003). If bumblebees in high competition areas exhibit developmental retardation, we may expect they will also have a higher susceptibility to infection (Lafferty & Gerber 2002; Hedtke *et al.* 2011). This stress could be initiated via the increase in bumblebee density or with the increase in density of generalist pollinator, *Apis mellifera*.

Spillover: If commercially reared and managed bees are already hosting parasites, they may transmit them to wild bumblebees in the local area (Power & Mitchell 2004; Almberg *et al.* 2012). Parasite spillover has been well documented in other managed animal populations; such as managed American minks (*Neovision vision*) spreading a parvovirus to local wild populations, and domesticated dogs spreading canine distemper virus to Serengeti lion populations (Carpenter *et al.* 1996; Nituch *et al.* 2011). It is also suggested that bumblebee parasites may be spilling over from commercially reared bumblebees (Colla *et al.* 2006; Otterstatter & Thomson 2008; Murray *et al.* 2013). These studies are based on visual screening for parasites so should be interpreted with caution. Without knowing how bumblebee density or stress, effect parasite prevalence it's impossible to be certain if parasite spillover is being shown in such correlative studies around sites deploying commercially reared bumblebees. Additionally, wild bumblebees are increasingly found to harbour diseases such as deformed wing virus and *N. ceranae*; parasites that have a long history with honey bees and in many areas are implicated in honey bee colony collapse (Genersch *et al.* 2006; Plischuk *et al.* 2009). It is now believed these 'honey bee parasites' have spilt-over to bumblebees, with some exhibiting virulence and the ability to complete their lifecycles within bumblebee hosts, thus posing a significant conservation risk (Chapter 2, 5 & 6). To date, no bumblebee surveys have screened for this increased diversity of parasites in wild bumblebees.

Recently, commercially reared bumblebees have been found to carry a range of bumblebee parasites yet current regulations do not prevent their use on farms (Chapter 2; Natural England 2012). Past studies investigating parasite spillover from commercial bumblebees fail to address spillback and pollinator competition/density-dependent mechanisms driving parasite prevalence's. Here I investigate the effect of commercially reared bumblebees and managed honey bees on the prevalence of a range of parasites in bumblebees. I first examine the relationship between the prevalence of parasites in wild bumblebees at 5 sites in England. Parasite distribution is measured against the local presence and proximity to glasshouses, either using or not using commercially reared bumblebees. The effect of apiary proximity on bumblebee parasite prevalence is also tested with 10 bumblebee colonies sited near, and 10 far from an apiary. These results will help inform interpretation of past studies and provide directions for further research.

4.2 Methods

4.2.1 Sample collection to assess parasite prevalence due to:

4.2.1.1 The effect of commercially reared bumblebees

To determine the prevalence of parasites at sites either using commercial bumblebees or not, five greenhouse farm sites in England were selected. Sites were selected based on the presence of large scale commercial fruit farms that utilise glasshouse and/or polytunnels for crop growing. In addition, sites chosen were of comparable size and all located in areas of open farmland with no other sites known to be deploying bumblebees in the area (within 10 km). In addition, no farms in the selected sites were known to be treating their crops with abnormally high or low levels of pesticides. By ensuring all sites satisfied these conditions I ensured bees at each site would be subject to similar stresses and resource availability. Three of these in Cambridgeshire, Kent and Essex, were a focal greenhouse in which commercial bumblebees were used for the pollination of the greenhouse crops (from here on referred to as commercial sites) and two from sites in Merseyside and Oxfordshire (from here on referred to as non-commercial sites) where the focal greenhouse did not deploy commercial bumblebees. Bumblebees were collected with a sweep net at points 1, 3 and 5 km from the focal greenhouse sites, with approximately 50 bumblebees collected at each of the three

distances for each of the five sites. All bees were collected within a three week period in the summer of 2011. A total of 471 bumblebees were collected from around the sites using commercially reared bumblebees and a total of 293 bumblebees from around the sites not using commercially reared bumblebees. All of these 764 bumblebees were screened for parasites (as detailed in 4.2.2).

4.2.1.2 The effect of managed honey bees

Ten commercial *Bombus terrestris audax* bumblebee colonies, purchased from Biobest, and which each contained approximately 80-100 workers at the time of the experiment were used to determine the effect of proximity to managed honey bee colonies on parasite prevalence within bumblebee colonies. Five of the bumblebee colonies (from here on referred to as 'apiary colonies') were situated in an apiary in Yorkshire, consisting of 50, full-size honey bee hives, and the remaining five bumblebee colonies were sited > 1 km away from the apiary (from here on referred to as the 'non-apiary colonies'). The bumblebee colonies remained at these sites for a one-month period, during which they could forage freely. After this period, 20 bumblebee workers were taken from each colony and screened by PCR or RT-PCR for the presence of the parasites (see below).

4.2.2 Molecular screening for parasite presence

An approximately 0.5 cm^3 sample of midgut, malpighian tubules and fatbody were dissected and homogenised from each bee, and DNA was extracted from the homogenate using 5% Chelex. All DNA samples were amplified for the *18S* Apidae host control gene to confirm the quality of the DNA extraction before subsequently screening for parasites (Meeus *et al.* 2010). The molecular and thermal conditions for the Apidae control PCR, and parasite presence PCR/RTPCR, are described in table A2.2.1.1. Products were run alongside a size standard on a 1% agarose gel stained with ethidium bromide to confirm amplicon size. Each assay included a negative and positive control. All samples were screened for the presence of the parasites *Apicystis bombi*, *Crithidia bombi*, *Nosema bombi*, *N. ceranae*, *N. apis* and deformed wing virus (DWV).

4.2.3 Statistical analysis

Data from sites around glasshouses were grouped into areas using commercial bumblebees or not. Data from sites using honey bees were grouped as colonies near or far from the apiary. The parasite richness (defined as the number of parasite species detected in a single host) was compared between areas using a generalised linear model (GLM) with linear distribution, logit link function and the likelihood ratio χ^2 statistic. Changes in individual parasite prevalence were analysed using a GLM with binomial distribution, logit link function and the likelihood ratio χ^2 statistic. When looking at the effect of managed bumblebees, site type, transect distance, and site location nested within site type were the factors. When looking at the effect of managed honey bees a GLM, with location (near or far), and colony nested within location, were used as factors. Nonsignificant terms were removed stepwise in all cases to obtain the minimum adequate models. All analyses were carried out in PASW Statistics 20 (IBM, Armonk, NY, USA).

4.3 Results

4.3.1 The effect of commercially reared bumblebees on parasite prevalence in locally captured bumblebees

Overall, most captured bumblebees had either no infections (40.7%) or a single infection (40.3%) with cases of dual and tri infections being rare (16.8% and 2.1% respectively). The pathogen richness per bee was higher at commercial sites and within these sites, richness increased with increased proximity to the focal glasshouse (χ^2 = 60.18, d.f. = 1, $P < 0.001$; χ^2 = 21.11, d.f. = 2, $P < 0.001$; Figure 4.3.1.1A). Driving this trend, *A. bombi* was found at a higher prevalence in bumblebees captured from around commercial sites (χ^2 = 14.14, d.f. = 2, $P < 0.001$), and within these sites displayed a proximity effect towards focal glasshouse, increasing in prevalence from 8% at 5 km, to 46% when <1 km (χ^2 = 44.46, d.f. = 2, $P < 0.001$; Figure 4.3.1.1B). *Crithidia bombi* was more prevalent in bumblebees caught from around commercial than non-commercial sites (34% compared to 19%) but displayed no proximity effect (χ^2 = 19.22, d.f. = 1, $P < 0.001$; χ^2 = 0.844, d.f. = 2, $P = 0.656$; Figure 4.3.1.1C). The prevalence of *N. ceranae* in bumblebees caught from around commercial sites was

28% compared to 19% at non-commercial sites (χ^2 < 0.001, d.f. = 1, P = 0.995; Figure 4.3.1.1D). Whilst this was not significant, this is largely due to wildly different within-site prevalences (range from 0% to 46% between sites; χ^2 = 151.1, d.f. = 3, P < 0.001). The prevalence of *N. bombi, N. apis* and DWV in bumblebees caught were all under 1% and displayed no interaction between site and proximity to the greenhouse (χ^2 = 1.01, d.f. = 2, P = 0.602, Figure 4.3.1.1E; χ^2 = 1.03, d.f. = 2, P = 0.597, Figure 4.3.1.1F; χ^2 = 4.29, d.f. = 2, P = 0.117, Figure 4.3.1.1G; respectively).

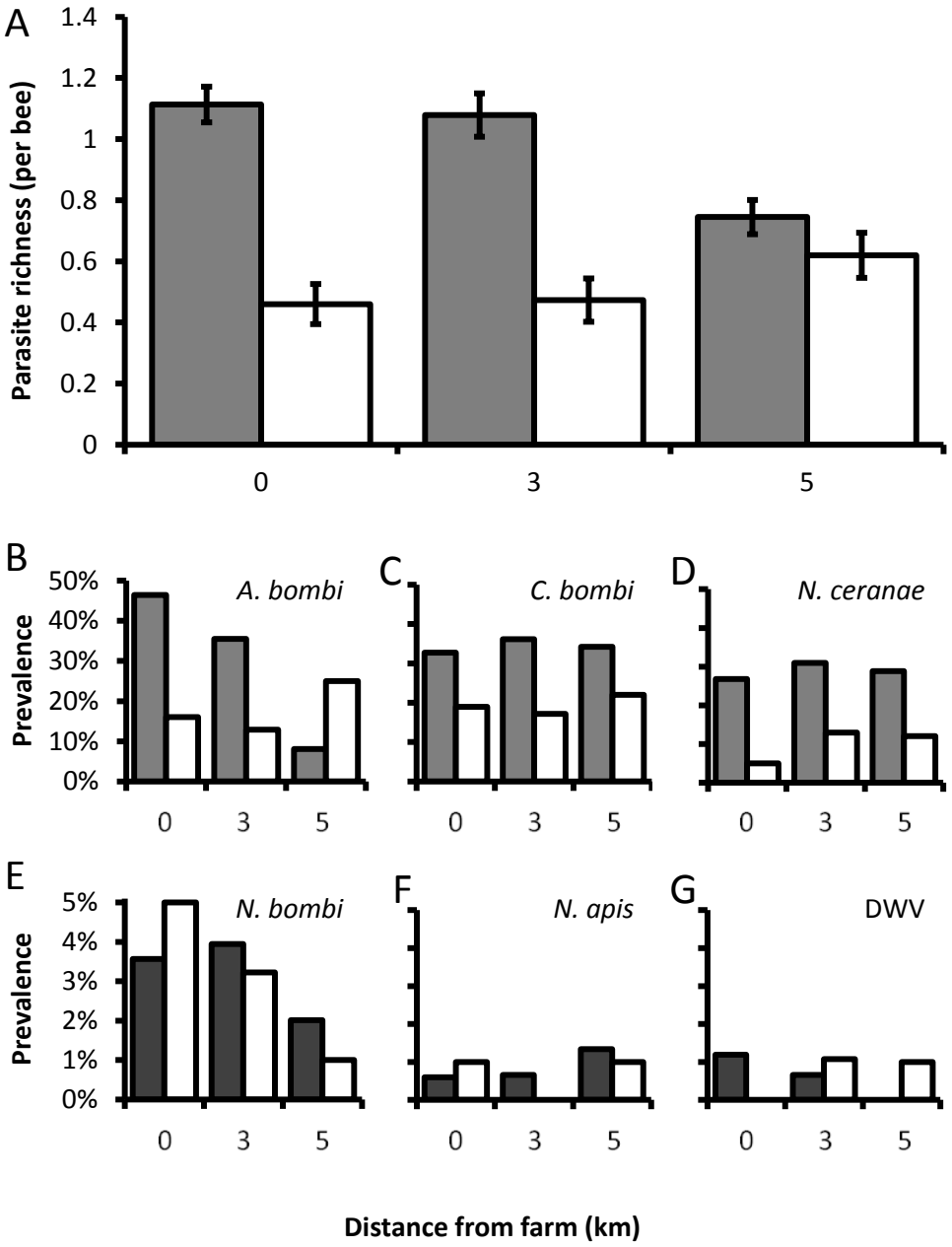


Figure 4.3.1.1 Occurrence of parasites in bumblebees sampled 0, 3 or 5 km from greenhouses that were either using (grey columns) or not using (white columns) commercially-produced bumblebee colonies. A) The mean ± s.e. parasite richness (number of species) infecting individual bees. B-G) The proportion of bumblebees sampled which were positive for the *A. bombi*, *C. bombi*, *N. ceranae*, *N. bombi*, *N. apis* and deformed wing virus (DWV) parasites.

4.3.2 The effect of managed honey bees on parasite prevalence within bumblebee colonies

The mean parasite richness varied between bumblebee colonies but was overall higher in colonies sited in close proximity to honey bees (χ^2 = 5.66, d.f. = 1, P = 0.017; Figure 4.3.2.1A). The average prevalence of *C. bombi* in bumblebee colonies near honey bees was 58%; significantly higher than the 30% found in colonies far from honey bees (χ^2 = 17.9, d.f. = 1, P < 0.001; Figure 4.3.2.1B). The prevalence of *A. bombi* and *N. ceranae* in colonies located near honey bees averaged 30% and 43%, respectively, which did not differ from the prevalence of these parasites in colonies far from honey bees (χ^2 = 0.83, d.f. = 1, P = 0.36; χ^2 = 0.27, d.f. = 1, P = 0.61). *N. ceranae* prevalence did however differ between colonies within sampling sites (χ^2 = 25.07, d.f. = 8, P = 0.002). *N. apis* had very low prevalence in general, and was only found in bumblebee colonies located near to honey bee hives (χ^2 < 0.01, d.f. = 1, P = 0.993). *Nosema bombi* and DWV were not detected in any of the 200 bumblebees sampled.

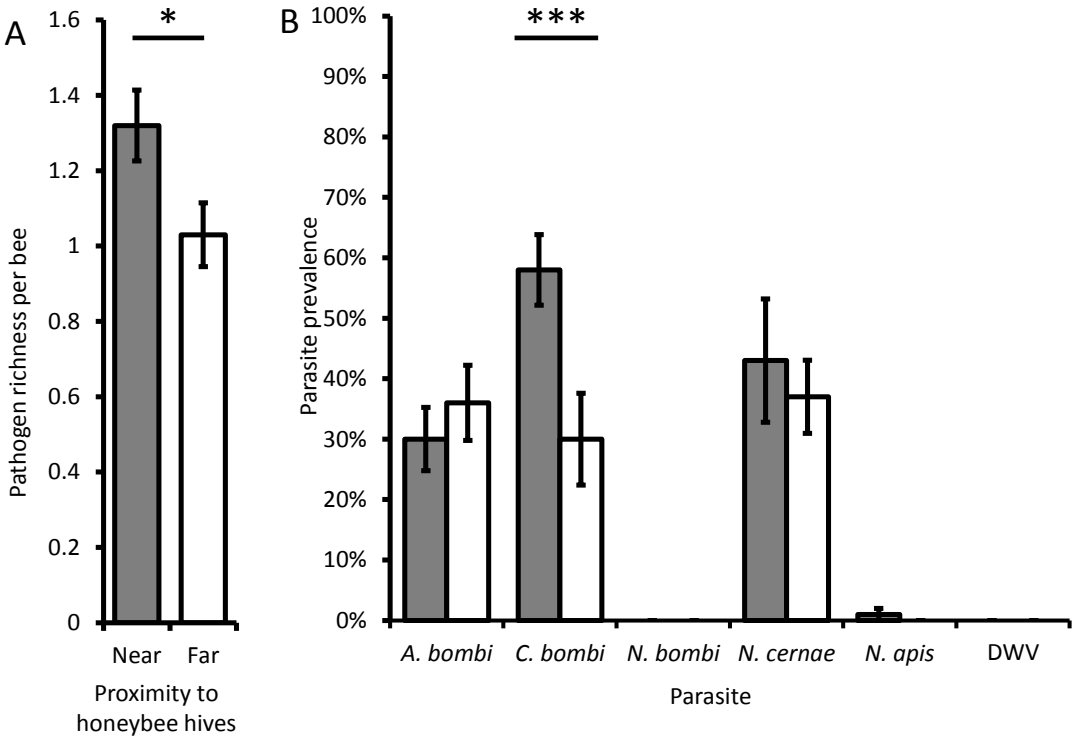


Figure 4.3.2.1 The mean ± s.e. parasite richness (number of species) per bumblebee (A), and the prevalence of six parasites per bumblebee colony (B), that were located either near (dark grey bars) or far (white bars) from managed honey bee hives. Asterisks and bars above columns indicate significant pairwise differences (* when P < 0.05; *** when P < 0.001).

4.4 Discussion

The results show that the parasite prevalence in bumblebees is affected by the presence of both commercially reared bumblebees and managed honey bees. The prevalence of *A. bombi* and *C. bombi* was, on average, 12% and 15% respectively higher in bumblebees caught at commercial sites. *Apicystis bombi* also shows a proximity effect with 46% of bumblebees caught within 1 km of a commercial site being infected. Bumblebees in colonies located close to a managed honey bee apiary had higher levels of the parasite *C. bombi* compared to bumblebees in colonies that were located further from the apiary. The deployment of managed colonies of either bumblebees or honey bees therefore appears to increase the prevalence of parasites in local bumblebees.

A wide diversity of parasites were detected in the wild caught bumblebees, including the honey bee parasites *N. ceranae, N. apis* and DWV. Recently, these three parasites were also identified in commercial bumblebee hives (Graystock *et al* 2013). *Nosema ceranae*, is an emergent honey bee parasite causing type-C nosemosis (no overt symptoms, progressive reduction of colony members until point of colony collapse, increased susceptibility to other disease) and responsible for the collapse of honey bee colonies in some areas (Higes *et al.* 2010; Botías *et al.* 2011). In addition, it has now been identified as a virulent parasite of bumblebees (Chapter 2 & 5; Plischuk *et al.* 2009a). Deformed wing virus is another honey bee parasite that is being increasingly found in bumblebees (Chapter 2; Genersch *et al.* 2006; Evison *et al.* 2012). The virus is almost ubiquitous in honey bee populations with only heavy infections causing significant colony collapse (Highfield *et al.* 2009; de Miranda & Genersch 2010). The full pathology and route of transmission in bumblebees is still unknown though it has been shown to cause significant worker mortality (Chapter 6). Whilst *N. apis*, doesn't seem able to infect bumblebees, it has been detected and found viable inside commercial bumblebees suggesting that it may be able to be vectored by bumblebees (Chapter 2 & 3). These three honey bee parasites, in addition to the bumblebee parasites *A. bombi, C. bombi* and *N. bombi,* have all been found in commercially reared bumblebee colonies (Chapter 2). In general, the parasite richness within each wild caught bumblebee host increased with proximity to glasshouses utilising commercial bumblebees.

Bumblebees caught from around greenhouses using commercially reared bumblebees had a higher prevalence of *A. bombi, C. bombi* than those caught around

greenhouses not using commercially reared bumblebees. Whether through parasite spillover, enhanced transmission, or stress of increased competition, commercially reared bumblebees appear to be increasing the prevalence of parasites in local bumblebees. These findings support previous studies that found, albeit using less sensitive non-molecular screening methods, a higher parasite prevalence near sites using commercially reared bumblebees (Colla *et al.* 2006; Fitzpatrick *et al.* 2007; Otterstatter & Thomson 2008). The effect of farm sites using commercially reared bumblebees on the prevalence *A. bombi* appears to be influenced by proximity to the focal glasshouse site. This perhaps suggests either a recent introduction from a point at the glasshouse sites or that transmission of this parasite is density dependent, occurring less readily and thus doesn't extend well; prevalence reduces from 46% at glasshouses to only 8% in bumblebees caught 5 km away. No studies have been performed on the horizontal transmission of this parasite, though it is commonly found, visually, at low prevalence (Shykoff & Schmid-Hempel 1991b; Goulson *et al.* 2012), suggesting the high density of bumblebees and frequent flower sharing within and immediately around these sites is helping drive the high prevalence found. Worryingly this parasite has been found to reduce bumblebee survival and fatbody reserves (Chapter 6). *Crithidia bombi* was also found to be more prevalent at commercial sites. Unlike with *A. bombi*, there was no proximity effect found. *Crithidia bombi* is known to readily transmit between bumblebees (Durrer & Schmid-Hempel 1994). Commercial sites had no effect on the prevalence of other parasites tested. For the parasites *N. bombi*, *N. apis* and DWV, this seems to be due to the low prevalences detected (less than 1% prevalence). *Nosema ceranae* however was abundant at some sites but completely absent at other sites. Whilst commercial bumblebee colonies have been found to contain *N. ceranae*, it is reassuring that the parasite, known to be largely abundant in honey bees, is not primarily determined by the presence of commercially reared bumblebees (Chapter 2).

The proximity to managed honey bee hives also had an effect on parasite prevalence in bumblebee colonies. Although the levels of *N. bombi*, *N. apis* and DWV were too low to conclude anything, and *A. bombi* and *N. ceranae* were not affected by proximity to the honey bee hives, *C. bombi* was clearly more prevalent in bumblebee colonies sited near to the honey bee hives. This effect on *C. bombi* prevalence cannot be due to spillover, because honey bees do not act as a suitable host to this parasite species (Ruiz-González & Brown 2006), It could however be due to stress from

competition leading to the bumblebees close to the honey bee apiary being more susceptible to infection (Elbagrmi *et al. in prep*). Alternatively, the effect could be due to vectoring. It has been shown previously that *C. bombi*, as well as *A. bombi, N. bombi, A. apis, N ceranae* and DWV have the capacity to be dispersed via shared flowers by both honey bees and bumblebees (Chapter 3; Durrer *et al.* 1995; Singh *et al.* 2010). It could therefore be that honey bee vectoring of *C. bombi* is more efficient than of the other parasites screened for in this study, thereby increasing its transmission. Ultimately, the mechanism driving increased *C. bombi* prevalence in bumblebees foraging near honey bee hives remains uncertain, and previous findings of *C. bombi* spillover from commercially reared bumblebees (Otterstatter & Thomson 2008; Murray *et al.* 2013; Whitehorn *et al.* 2013), might be artefacts of the higher density of foraging bees in these areas as shown here when placed near honey bees. This highlights the much ignored process of density driven spillback as another possible cause for elevated parasite prevalence in wild bumblebees in areas around managed bee populations.

Here I show that managed colonies of either bumblebees or honey bees can increase the parasite prevalence in local bumblebees. Our results suggest that the mechanisms may be three-fold: the direct effects of spillover and spillback, both of which are intrinsically tied to shared-flower use, and the indirect effect of increased *C. bombi* prevalence in bumblebees competing with honey bees. The increase in parasite prevalence associated with managed bees could either have direct lethal effects on bumblebees or indirect effects on bumblebee fitness through an increase in stress via competition for pollen and nectar resources. It is clear that as long as there is mixing between managed and native bumblebees, local population densities will inflate, placing wild populations at risk from potential parasite spillover, spillback and stress (Goka 2010). This could prove to be a major conservation threat to bumblebees.

Chapter 5: Emerging dangers: deadly effects of an emergent parasite in a new pollinator host

Abstract

There is growing concern about the threats facing many pollinator populations. Emergent diseases are one of the major threats to biodiversity and a microsporidian parasite, *Nosema ceranae*, has recently jumped host from the Asian to the Western honey bee, spreading rapidly worldwide, and contributing to dramatic colony losses. Bumblebees are ecologically and economically important pollinators of conservation concern, which are likely exposed to *N. ceranae* by sharing flowers with honey bees. Whilst a further intergeneric jump by *N. ceranae* to infect bumblebees would be potentially serious, its capacity to do this is unknown. Here I investigate the prevalence of *N. ceranae* in wild bumblebee populations in England and determine the infectivity of the parasite under controlled conditions. I found *N. ceranae* in all seven wild bumblebee species sampled, and at multiple sites, with many of the bees having spores from this parasite in their guts. When I fed *N. ceranae* spores to bumblebees under controlled conditions, I confirmed that the parasite can infect bumblebees. Infections spread from the midgut to other tissues, reduced bumblebee survival by 48% and had trait effects on behaviour. Although spore production appeared lower in bumblebees than in honey bees, virulence was greater. The parasite *N. ceranae* therefore represents a real and emerging threat to bumblebees, with the potential to have devastating consequences for their already vulnerable populations.

5.1 Introduction

Pollinators are of major ecological and economic importance, being essential for the reproduction of at least two thirds of flowering plant species (Ollerton *et al.* 2011) and pollinating crops with an estimated value of $153 billion pa globally (Gallai *et al.* 2009; Potts *et al.* 2010b). However, the populations of many pollinator species have declined substantially in recent years due to a multitude of factors, with some species going extinct and many more species now being vulnerable (Biesmeijer *et al.* 2006; Potts *et al.* 2010b). In the UK, for example, 8 out of 25 species of bumblebees have decreased substantially in abundance since 1940 and another two have gone extinct,

while 13 species have gone extinct in at least one European country and four across the entire continent (Goulson *et al.* 2008).

One of the major threats to biodiversity in general, and vulnerable species in particular, are emergent diseases (Daszak *et al.* 2000). Disease emergence occurs when a parasite infects a novel host population either through translocation or by chance development within hosts previously incompatible for pathogen replication (Daszak *et al.* 2001). The likelihood of this 'pathogen spillover' varies between host and pathogen, with closely related, sympatric hosts having a greater potential to transmit pathogens between them (Perlman & Jaenike 2003). Recently, the microsporidian parasite *Nosema ceranae* has emerged as an important disease of honey bees (*Apis* spp.). It is a gut parasite of adult bees, transmitted horizontally via the faecal-oral route, with infection occurring following the ingestion of spores which germinate in the midgut of the host insect and infect the epithelial cells (Gisder *et al.* 2011). Successful infections produce spores which are excreted in the faeces, and which are persistent, remaining viable on the bees, pollen and hive materials that they contaminate for periods in excess of a year (Fenoy *et al.* 2009; Higes *et al.* 2010). The parasite originated in the Asian honey bee, *Apis ceranae,* but following translocation of honey bees for apiculture, has successfully jumped host in recent decades to multiple other *Apis* species, most notably the Western honey bee, *Apis mellifera*, in which it has now spread worldwide to be the most common *Nosema* species found in many areas (Klee *et al.* 2007). The parasite can negatively affect lifespan, immunocompetence, learning and flower handling ability in its new *A. mellifera* host (Paxton *et al.* 2007; Higes *et al.* 2007, 2008b; Mayack & Naug 2009; Naug & Gibbs 2009; Antúnez *et al.* 2009). However, while in some areas, particularly Spain, the parasite appears to have a major effect on bee health and has been implicated in substantial colony losses (Higes *et al.* 2006, 2008b, 2010), in other areas, such as North America and Germany, the impact of the parasite appears to be less significant (Paxton *et al.* 2007; Klee *et al.* 2007; Cox-Foster *et al.* 2007; VanEngelsdorp *et al.* 2009; Fries 2010; Genersch *et al.* 2010; Gisder *et al.* 2010). The effects of the parasite therefore appear to be strongly dependent on context, other stresses or perhaps host-parasite strains (Paxton *et al.* 2007; Klee *et al.* 2007; Fenoy *et al.* 2009; Fries 2010; Vidau *et al.* 2011; Aufauvre *et al.* 2012; Chaimanee *et al.* 2013).

Alarmingly, two recent studies have found molecular evidence of *N. ceranae* in some bumblebee (*Bombus*) species, suggesting a spillover from honey bees to

bumblebees may be occurring (Plischuk *et al.* 2009; Li *et al.* 2012). This spillover could be a recent event brought about by the rapid geographic spread of *N. ceranae*, exposing naive bumblebee species from Europe and America which will not previously have encountered the parasite. However, it is currently unclear whether the *N. ceranae* detected molecularly in bumblebees represents infections or simply vectoring of ungerminated spores. Equally, it is not known how virulent *N. ceranae* is to bumblebees if it can infect them. Bumblebees are economically and ecologically important pollinators in a variety of ecosystems and many species are of conservation concern (Goulson 2010). Therefore the emergence of a new, virulent pathogen could have significant ramifications, particularly for populations that are already threatened by other causes such as habitat loss. Here I determine the prevalence of *N. ceranae* in wild British bumblebees and test experimentally whether *N. ceranae* from honey bees is able to infect the most widely distributed European bumblebee, *Bombus terrestris*. I also examine the lethal and trait effects of exposure to the parasite, and compare the virulence of the parasite across its host range.

5.2 Methods

5.2.1 Prevalence of *Nosema ceranae* in wild bumblebees

A total of 764 *Bombus sp*. were captured with sweep nets at five sites in England (Cambridgeshire, Merseyside, Oxfordshire, Kent and Essex), and stored immediately in 100% ethanol (see supplementary table). The midgut, malpighian tubules and fatbody were dissected from each bee (as these are the tissues in which *Nosema bombi* and *Nosema ceranae* infections have been reported in bumblebees and honey bees respectively; (Fries *et al.* 2001; Chen *et al.* 2009; Gisder *et al.* 2011)), homogenised together, and screened for *Nosema* by PCR. DNA was extracted using 5% Chelex and all samples were amplified for the *18S* Apidae host control gene to confirm the quality of the DNA extraction (Meeus *et al.* 2010) using 0.4 mM dNTP, 1.5 mM $MgCl_2$, 3 µl Buffer, 1.25 U Taq, 0.2 µM each primer and 1 µl template, giving 10 µl in total. This PCR was then subject to 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 56°C, 45 s at 72°C before a final elongation stage of 3 min at 72°C. Samples were also screened for *N. ceranae* and *N. apis* based on 16S rRNA sequences (Chen *et al.* 2008), with reactions containing 0.25 mM dNTP, 3.75 mM $MgCl_2$, 2 µl Buffer, 0.25 U Taq, 0.2 µM each primer and 1 µl template, giving 10 µl in total for each reaction. Reaction

conditions were 2 min at 94°C, 35 cycles of 30 s at 94°C, 45 s at 61°C (63°C for *N. apis*), 120 s at 72°C before a final elongation stage of 7 min at 72°C. Finally, the samples were also screened for *N. bombi* (Klee *et al.* 2006), with reactions containing 0.3 mM dNTP, 3.75 mM $MgCl_2$, 2 µl Buffer, 0.25 U Taq, 0.2 µM each primer and 2 µl template, giving 10 µl in total for each reaction. Reaction conditions were 4 min at 94°C, 35 cycles of 60 s at 95°C, 60 s at 50°C, 60 s at 72°C before a final elongation stage of 4 min at 72°C. Negative and positive controls were included in every assay. To ensure accuracy when determining the species of *Nosema* detected, all *Nosema* findings were double checked with additional primers targeting species specific regions of RPB1 gene (Gisder & Genersch 2013). In addition, the number of spores present in the tissue samples was counted using a compound microscope and haemocytometer. The three *Nosema* species are similar morphologically, so this was done only for bees that tested positive for *N. ceranae* but negative for *N. apis* and *N. bombi* by PCR.

5.2.2 Infectivity of *Nosema ceranae* from honey bees

The abdomens of 20 *Apis mellifera* honey bees from *N. ceranae* infected hives (confirmed by species-specific PCR (Chen *et al.* 2008)) were homogenised and the resulting lyse was slowly poured onto the surface of an isotonic solution of 95% Percoll gradient in PBS. This solution was then centrifuged for 45 min at 11,000g and 15°C to separate *Nosema* spores from other particles of different sizes (e.g. viruses or other parasites (Seleznev *et al.* 1995; Pertoft 2000)), with the resulting pellet of spores then extracted with a pipette. Spores were washed by first centrifuging at 14,000g for 15 min, removing the supernatant, and replacing with water before vortexing for 10 sec. This wash process was repeated three times to remove traces of Percoll and produce a clean suspension of *Nosema* spores, with the suspension then confirmed with a compound microscope to contain only *Nosema* spores and no other observable parasites (e.g. *Crithidia, Apicystis, Ascosphaera*). The identity of the suspended *Nosema* spores was confirmed as *N. ceranae* following PCR using 3 primer pairs specific to *N. ceranae*, *Nosema apis* and *Nosema bombi*, as above (Klee *et al.* 2006; Chen *et al.* 2008).

One hundred *Bombus terrestris audax* were collected from three parasite-free colonies (confirmed by screening 15 bees per colony by PCR for the three *Nosema* species) and placed into cohorts of 10 bees in 10 x 6 x 6 cm plastic boxes. All

bumblebees were starved for 8 hours before being individually hand-fed a single 5 µl dose of either a 40% sucrose solution containing approximately 6,500 Percoll-purified *N. ceranae* spores or a 40% pure sucrose control solution (n = 50 bees in each case). As pollen is often contaminated with *Nosema* and other parasites (Chapter 2), this was not fed to the bees. The bees were then replaced in their groups of 10 like-treated, nest-mate bees, provided with 40% sucrose solution *ad libitum*, and their survival checked daily for 14 days. In addition, 100 *Apis mellifera* workers from three *Nosema*-free colonies were also treated in the same way and their survival was monitored for 7 days to provide comparative data on parasite virulence.

The sensitivity of a bee to low sucrose concentrations has been linked to hunger and learning ability (Scheiner *et al.* 2001; Naug & Gibbs 2009) making it a good indicator of non-lethal effects of parasite infection. The sensitivity of the bumblebees to differing sucrose concentrations (10-80% in increments of 10) was therefore tested for the bees every 5 days using the proboscis extension response (Riveros & Gronenberg 2009), with the responses of the individual bees in each group of 10 being averaged.

After the experiment, all surviving bumblebees, as well as those that died during the experiment, were screened for infection by *N. ceranae*. To avoid cross-contamination of tissues, a tergite with attached fatbody was first removed carefully from the bee, prior to opening the abdomen and dissecting out a small section of midgut. This prevented entirely the fatbody sample becoming contaminated with any material from the digestive tract. The section of midgut was homogenised in 100 µl of deionised water and the number of *Nosema* spores counted using a compound microscope and haemocytometer. To confirm spores were *N. ceranae*, and also if an infection had spread to the fatbody, the samples of midgut and fatbody from each bee were screened separately by PCR as before. In addition, a subset of the samples that were positive for *Nosema* were sequenced to confirm the PCR amplicons were indeed *N. ceranae*.

5.2.3 Statistical analysis

Differences in bumblebee and honey bee survival were analysed using a Cox proportional hazards regression model, with colony-of-origin and cohort included to account for the structured nature of the data, and non-significant terms removed stepwise to obtain the minimum adequate model. Pairwise comparisons were made

using Kaplan-Meier models with the Breslow χ^2 statistic. The sucrose sensitivity data were analysed using Mann-Whitney *U* tests to compare the sensitivity of bees fed *N. ceranae* or control solution on 0, 5, 10 and 15 days after treatment. For comparison with other studies, the relative risk of exposure to *N. ceranae* was calculated on day 7 for both the bumblebee and honey bee data, and for comparable studies (caged bees, n ≥ 50 per treatment, known spore dose), as: $Relative\ Risk = \frac{\mathcal{P}\ exposed}{\mathcal{P}\ non-exposed}$ where $\mathcal{P}$ is the probability of death for either individuals exposed or not to *N. ceranae*.

5.3 Results

5.3.1 Prevalence of *Nosema ceranae* in wild bumblebees

Bumblebees from 7 different *Bombus* species across three of the five sites sampled had *Nosema ceranae* based on PCR. In total, 21% of the 764 bumblebees screened were positive for *N. ceranae* whilst also being negative for *N. bombi* and *N. apis*. The result was identical for both the Chen et al. (2008) and Gisder & Genersch (2013) protocols. Of these individuals, 19% had infections intense enough to produce observable *Nosema* spores under a microscope, with these bumblebees having on average 6,628 ± 1,261 spores in the small samples of their tissue (see table A5.3.1.1).

5.3.2 Infectivity of *Nosema ceranae* from honey bees

Bumblebees which ingested *N. ceranae* spores had significantly lower survival over the 15 day period than bumblebees fed control solution (χ^2 = 15.94, $P < 0.001$), with the greatest mortality (38% of those ingesting spores) occurring between 3 and 7 days after exposure (Figure 5.3.2.1). None of the control bumblebees had *Nosema* spores in their midguts or were positive for *N. ceranae* by PCR, but many of the bumblebees which had ingested *N. ceranae* spores were found to be infected by the parasite (Figure 5.3.2.1). A low proportion (0-25%) of the treated bumblebees which died up to 4 days after exposure were positive for *N. ceranae* by PCR, but this proportion was close to 100% for bumblebees which died from day 5 onwards. Approximately a third of the bumblebees which survived to the end of the 15 day experimental period were positive for *N. ceranae* in their midgut and also in their fat body, indicating that the parasite had moved between tissues in these bees (Figure 5.3.2.1). Interestingly, the proportion

of bumblebees which had *Nosema* spores detectable by microscopy in their midguts was much lower than the proportion positive by PCR, and spores were only seen in the bumblebees which died between days 4 and 7 after exposure (Figure 5.3.2.1).

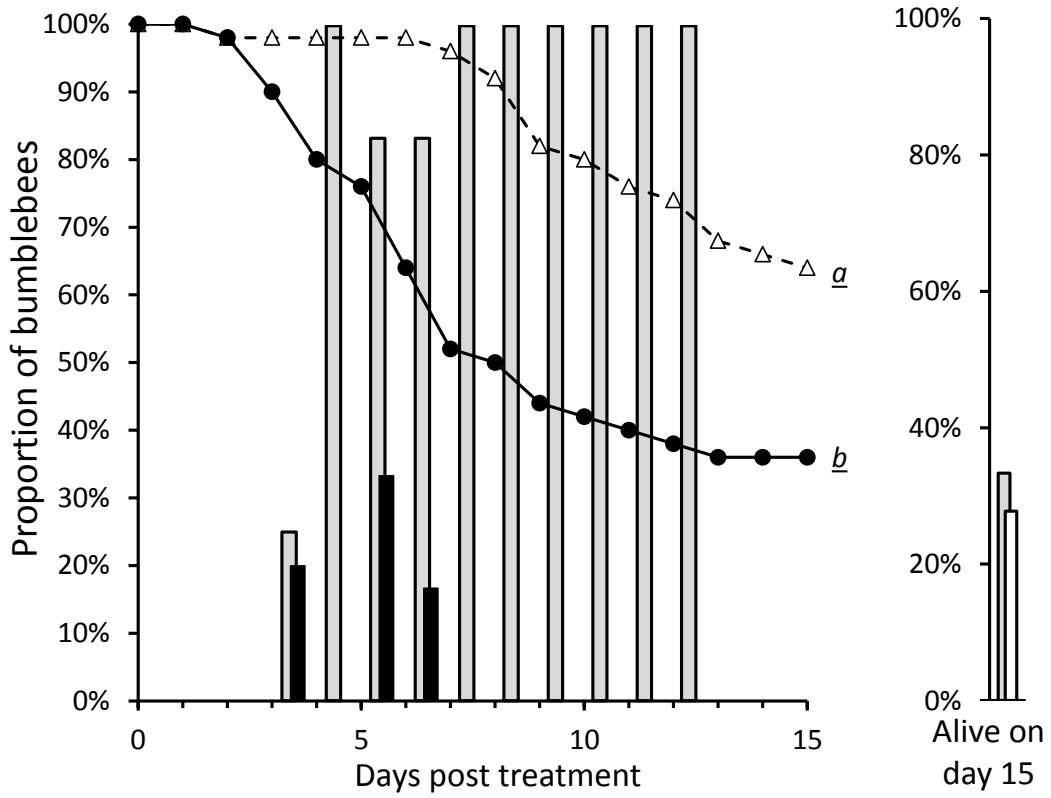


Figure 5.3.2.1 The proportion of bumblebees surviving after ingestion of either a sucrose solution control (dashed line) or a sucrose solution with 6500 spores of the *N. ceranae* parasite (solid line). Bars represent the proportion of dead bumblebees from the *Nosema*-treated group that had visible spores in the midgut (black bars), PCR-detectable *N. ceranae* in their midgut (light grey bars) or fatbody (white bars; only checked for bees that survived to the end of the experimental period). The parasite was never detected in the control bees.

These bees, which had originally been fed 6,500 *N. ceranae* spores, were found to have > 11,400 spores in just the small sample of midgut at the end of the 15 day period, indicating that substantial spore production had occurred. Sucrose sensitivity was similar on day 0 for bumblebees fed *N. ceranae* or control solution (U = 1220, N = 99, P = 0.969), but was significantly lower 5 and 10 days after exposure for bees that had ingested *N. ceranae* (U = 655, N = 85, P = 0.013, and U = 309, N = 60, P = 0.049, respectively; Figure 5.3.2.2). On 15 days after exposure, the sucrose sensitivity of surviving bees was again similar for bees fed *N. ceranae* or control solution (U = 242, N = 49, P = 0.394; Figure 5.3.2.2).

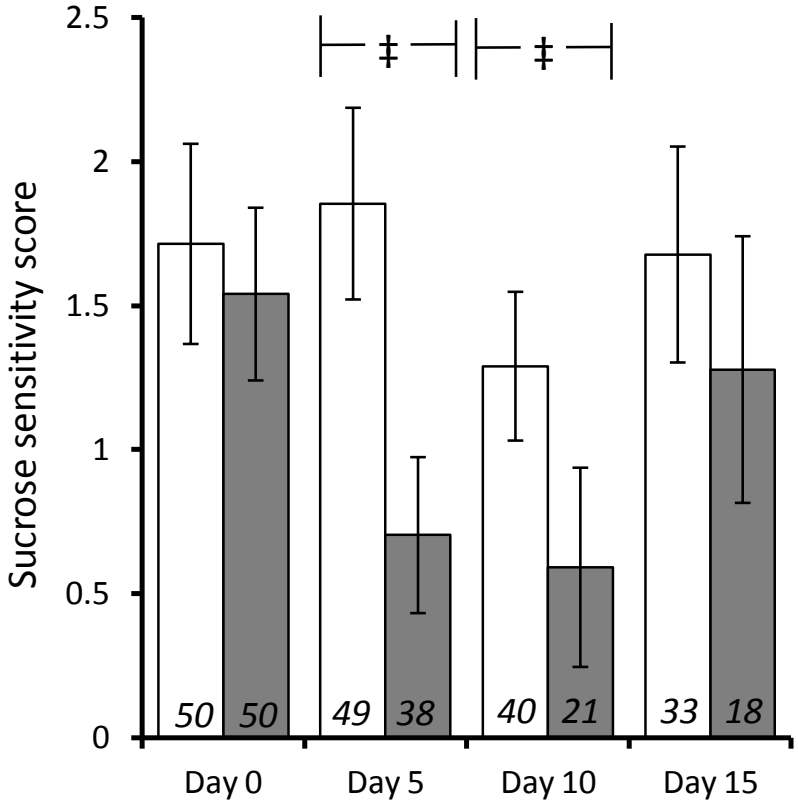


Figure 5.3.2.2 Mean ± s.e. sucrose sensitivity of bumblebees on 0, 5, 10 and 15 days following ingestion of either the *N. ceranae* parasite (dark grey) or a sucrose solution (white bars). Sample size is indicated at the base of each column. Significant pairwise differences ($P < 0.05$) are indicated by the ± symbol.

In contrast to the bumblebee results, the ingestion of *N. ceranae* spores had little effect on the survival of honey bees in our experiment at the dose tested, with survival being > 95% both for bees that ingested the control solution or the solution containing *N. ceranae* spores ($\chi^2 = 0.003$, $P = 0.953$; this also confirms that our washing protocol was successful at removing traces of Percoll). Based on mortality 7 days after treatment, the relative risk from exposure to *N. ceranae* for the honey bees treated here was broadly similar to that found in previous studies, with the relative risk associated with interspecific exposure between *Apis* spp. being somewhat greater (Figure 5.3.2.3). However, the relative risk from exposure to *N. ceranae* calculated from the bumblebee data was substantially higher (Figure 5.3.2.3).

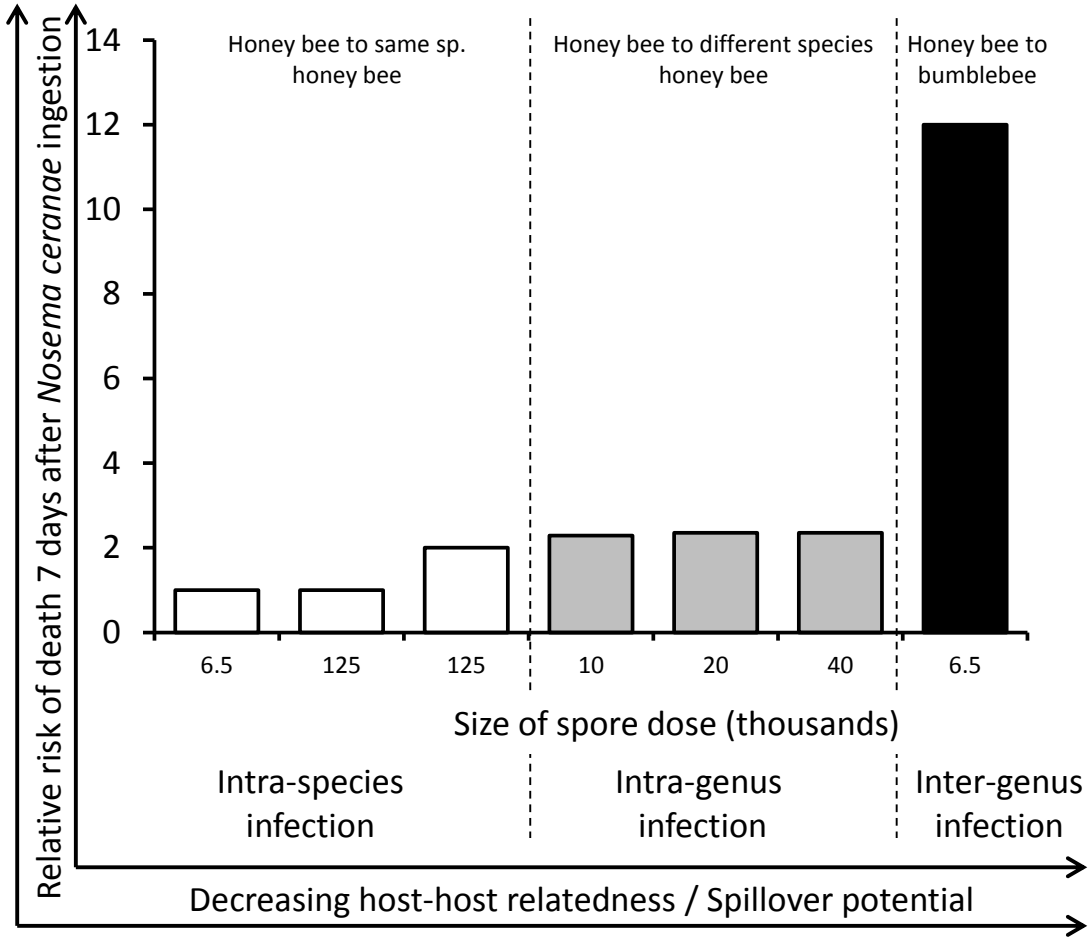


Figure 5.3.2.3 The relative risk that *N. ceranae* poses to either a host of the same species (intra-species infection), a different *Apis* species (intragenus infection) or to bumblebees (intergenus infection). The relative risk is calculated on day 7 for all comparable laboratory studies where N > 50 per treatment (Vidau *et al.* 2011; Suwannapong *et al.* 2011; Aufauvre *et al.* 2012).

5.4 Discussion

The results show conclusively that *N. ceranae* from honey bees is capable of infecting bumblebees and is already circulating in wild populations. Between 20-47% of wild-caught bumblebees at three out of the five sites I sampled in England were positive for *N. ceranae*, and in many cases these bees had *N. ceranae* spores in their guts. Furthermore, the presence of *N. ceranae* was not restricted to the common *B. terrestris* bumblebee but was found in a total of 7 different species (all the species tested). There have been previous reports of *N. ceranae* in wild bumblebees in Argentina and China based on PCR screening (Plischuk *et al.* 2009; Li *et al.* 2012), and our screening shows both that it is also present in a variety of UK bumblebees and that it is actually

infecting them. Ingestion of *N. ceranae* spores in our experiments resulted in 62% of bumblebees becoming infected, with the dose used being less than that in many infection studies of *N. ceranae* in honey bees (Higes *et al.* 2007, 2008a; Vidau *et al.* 2011), and an order of magnitude less than that in infection studies of bumblebees by their natural *N. bombi* parasite (Schmid-Hempel & Loosli 1998; Rutrecht & Brown 2009). *N. ceranae* therefore appears to be at least as, and quite probably more, infective to bumblebees than to honey bees. Many bumblebee species in the UK, as well as elsewhere are declining and now highly vulnerable to new stresses (Goulson *et al.* 2008), so the emergence of a new, virulent disease has significant implications for their conservation.

Infections by *N. ceranae* of bumblebees were highly virulent, with 48% of exposed bees dying within 7 days of exposure compared to 4% of unexposed bees, and the risk from exposure being substantially higher than for infections of honey bees. This was in spite of the bumblebees being provided with *ad libitum* food and a constant, benign environment, and the mortality may be even greater under natural conditions (Brown *et al.* 2000; Mayack & Naug 2009). It has been suggested that *N. ceranae* may be more virulent in *Apis mellifera* than the natural *Nosema* species, *N. apis* (Paxton *et al.* 2007), and it also seems to be far more virulent in bumblebees than their natural parasite *N. bombi* (Schmid-Hempel & Loosli 1998). Importantly, most of the mortality associated with *N. ceranae* infections occurred 3-7 days after exposure, which coincided with the period during which bees that died had spores in their midguts. *Nosema ceranae* spores are produced when infected epithelial cells rupture, releasing the spores into the gut, and this is a major component of the virulence expressed by the parasite (Dussaubat *et al.* 2012). However, many infected bees died without producing spores, suggesting that the virulence of the parasite is expressed in other ways as well. The surviving bees were either uninfected or had *N. ceranae* present in their fat body, as well as midgut. Whilst there is some debate regarding tissue tropism of *N. ceranae* in *Apis* hosts (Huang & Solter 2013), our dissection protocol specifically excluded contamination of the fat body sample with material from the digestive tract, so it appears that *N. ceranae* can move between tissues, in a similar way to that of the closely related *N. bombi* in *Bombus sp.* (Fries *et al.* 2001). The results suggest that there are at least four possible outcomes of the host-parasite interaction with bumblebees either: 1) resisting the parasite and remaining uninfected; 2) succumbing to infection, producing spores and concurrently suffering high

mortality; 3) tolerating infection, with the parasite moving into the fat body and not subsequently producing spores, or 4) dying due to some other effect of the infection.

In addition to the lethal effects of *N. ceranae* on bumblebees that it infected, there was also evidence of the parasite having trait effects on its host. The sucrose sensitivity of infected bees was substantially lower on 5 and 10 days after exposure, with these bees only extending their proboscis in response to a higher concentration of sucrose than control bees. Such reduced sucrose sensitivity has been correlated with impaired learning and flower handling ability in honey bees, which in turn reduces the efficiency, productivity and pollination services provided by the bee (Scheiner *et al.* 2001; Gegear *et al.* 2005, 2006; Iqbal & Mueller 2007). Even a small reduction in the growth of bumblebee colonies can substantially reduce the production of new reproductives (Muller & Schmid-Hempel 1992; Whitehorn *et al.* 2012), so these trait effects on behaviour may be significant. The effect of *N. ceranae* may well be further compounded by other trait effects, such as the reduction in immunocompetence and increased susceptibility to pesticide stressors which have been found when *N. ceranae* infects honey bees (Antúnez *et al.* 2009; Alaux *et al.* 2010).

The results demonstrate that the direct spillover of *N. ceranae* from honey bees to bumblebees can occur, and that its high virulence in bumblebees means that it poses a significant risk to them. Infected bumblebees can subsequently shed *N. ceranae* spores that are infective to other bumblebees and back to honey bees (Chapter 2). This provides evidence of the successful spillover and establishment of *N. ceranae* from honey bees to bumblebees. In addition to our own findings of *N. ceranae* in wild bumblebee populations in England, field surveys using genetic methods suggest that these infections are also taking place in Argentina and China (Plischuk *et al.* 2009; Li *et al.* 2012), meaning that there is now evidence of spillover on three different continents. Given the declines and vulnerability of many bumblebee populations, the effects of *N. ceranae* as an emergent, virulent disease may be serious. I sampled only relatively common bumblebee species, but given that all seven species were infected it seems probable that species of conservation concern will also be affected. Indeed, there is some evidence that small, isolated bumblebee populations which lack genetic diversity have higher prevalence of parasites (Whitehorn *et al.* 2011), so they may be affected more strongly.

The ability of *N. ceranae* to transmit between genera also raises concern about whether it may pose a threat to other genera of bees as well, many of which are also

showing declines (Biesmeijer *et al.* 2006). We have a very poor knowledge of the natural geographic distributions and host associations of bee diseases, and hence of the risks posed by transport of honey bee and bumblebee species for pollination (Goulson 2003b). More research on the potential intergeneric spillover of parasites, and the threats they may pose, to pollinators in general is urgently needed. However, it now appears clear that *N. ceranae* represents a real threat to bumblebees, and consideration of the potential spillover of the parasite from honey bees to bumblebee populations of conservation concern is necessary.

Chapter 6: The effects of single and mixed infections of *Apicystis bombi* and deformed wing virus parasites in the bumblebee *Bombus terrestris*

Abstract

As pollinators continue to decline, many species will become fragmented and face an increased risk of local extinction from virulent parasites. Only in recent times with the accessibility of sensitive molecular screening is the diversity and abundance of potentially lethal parasites being discovered in many pollinator populations. It is critically important to detect and record such parasites and their possible emergence. However, the significance of such findings is hard to assess without knowing the virulence, and therefore the mortal risk these parasites pose to bumblebees. Recently, the deformed wing virus (DWV), known to be ubiquitous in honey bees, has been detected in wild and commercial bumblebees. In addition the neogregarine *Apicystis bombi* has been discovered molecularly to be more prevalent than previously thought, and is an emergent parasite in South America. Whilst the increased detection of these parasites in bumblebees may be alarming, their virulence in bumblebees is unknown. Here, I assess for the first time the lethal and trait effects of these parasites during single and mixed infections of bumblebees (*Bombus terrestris*). Fifteen days after experimental exposure, 22% of bees exposed to *A. bombi*, 50% of bees exposed to DWV and 86% of bees exposed to both parasites had died, compared with 4% of control bees. After 5 days *B. terrestris* that had been fed *A. bombi* had increased sucrose sensitivity and upon death *A. bombi* fed bees had a lower lipid: body size ratio than control and DWV treated bees. Trait effects of deformed wing virus on infected bumblebees, or of co-infections of *A. bombi* and DWV, were not detected. The results show that both parasites can have significant, negative effects on bumblebee health, making them potentially of conservation concern. This may be particularly important in naïve populations where these parasites are emerging.

6.1 Introduction

Approximately 250 species of bumblebee exist worldwide, being responsible for the pollination of a variety of both wild flowers and economically important crops such as

tomatoes and sweet bell peppers (Velthuis & Van Doorn 2006; Goulson 2010). Recent evidence has highlighted declines of important pollinators in many areas worldwide and the potentially devastating consequences of its continuation (Potts *et al.* 2010b). Bumblebee declines have so far been reported across Europe, North America, South America and Asia (Fitzpatrick *et al.* 2007; Kosior *et al.* 2007; Goulson *et al.* 2008; Xie *et al.* 2008; Martins & Melo 2010; Cameron *et al.* 2011). These declines are particularly evident in bumblebee populations in the UK, where records date back to the mid-nineteenth century (Williams 1982; Goulson 2003a). It is argued that the impact of declining pollinators may be augmented by an increase in some generalist pollinators (Winfree *et al.* 2007). Some bumblebee species however, are intrinsically linked to plants requiring pollination via sonication (such as tomatoes) and with deep corollas that have co-evolved with long tongued bumblebees. As a result, declines in bumblebee species have been linked with the declines of some plants (Biesmeijer *et al.* 2006). A main cause of bumblebee declines is believed to be the intensification of farming in developed countries, reducing floral diversity and desirable nest sites (Goulson 2003a). Also contributing to declines are traditional and emerging bumblebee parasites, some of which are strongly implicated in the reduction of several bumblebee species (Williams & Osborne 2009; Meeus *et al.* 2011). In addition, as bumblebee populations decline, the threat faced by parasites will increase due to their populations becoming fragmented and losing their genetic diversity (Daszak *et al.* 2001; Goulson *et al.* 2008; Whitehorn *et al.* 2011).

Since the late nineteenth century, hundreds of thousands of bumblebee colonies have been reared under controlled conditions for the purposes of farming (Velthuis & Van Doorn 2006). This has allowed much research to be done on these pollinators and their common parasites (Goulson 2010; Meeus *et al.* 2011). The resulting development of bumblebees as a model system in host-parasite evolutionary ecology has allowed us to gain a very good understanding of two bumblebee parasites in particular, *Crithidia bombi* and *Nosema bombi* (Schmid-Hempel 1998). Our knowledge on the virulence of other bumblebee parasites though remains comparatively poor (Macfarlane *et al.* 1995; Schmid-Hempel 1998; Meeus *et al.* 2011). Recent advances in the molecular detection of parasites have highlighted that bumblebees are in contact with a greater diversity of parasites than previously recognised (Chapter 2 & 4; Singh *et al.* 2010; Evison *et al.* 2012).

Apicystis bombi is a neogregarine parasite found infecting bumblebees in Europe, North America and more recently, South America and Asia (Liu *et al.* 1974; Shykoff & Schmid-Hempel 1991b; Lipa & Triggiani 1992; Durrer & Schmid-Hempel 1995; Macfarlane *et al.* 1995; Colla *et al.* 2006; Cankaya & Kaftanoglu 2006; Rutrecht & Brown 2008; Plischuk & Lange 2009; Kissinger *et al.* 2011; Goulson *et al.* 2012; Morimoto *et al.* 2013; Murray *et al.* 2013). The emergence of this parasite, particularly in South America is believed to be due to spillover from invasive bumblebees, particularly commercially reared colonies (Chapter 2; Arbetman *et al.* 2012). *Apicystis bombi* is transmittable via spores shed from the faeces of infected hosts (Shykoff & Schmid-Hempel 1991b; Lipa & Triggiani 1996). The prevalence of bumblebees with visually detectable *A. bombi* spores is often low, however sensitive molecular screening of this parasite suggests it has a higher, and more widespread prevalence than previously found (Chapter 4; Plischuk *et al.* 2009a). Recently there have been reports that *A. bombi* may be playing a role in declines in Argentinean bumblebees (Plischuk & Lange 2009; Plischuk *et al.* 2011). Currently there is a lack of knowledge on the virulence of this unstudied parasite to assess how real a threat it poses to bumblebees (Chapter 4). Another parasite with an emerging incidence of detection in bumblebees is the picornovirus, deformed wing virus (DWV) (Chapter 2 & 4; Meeus *et al.* 2011). Considered a 'honey bee parasite', DWV has recently been discovered in commercial and wild bumblebees (Chapter 2; Li *et al.* 2011; Evison *et al.* 2012). This virus is ubiquitous in some honey bee populations, with little effect on the host unless in the presence of an additional parasite, *Varroa destructor* which acts synergistically with DWV, increasing the virulence and often leading to phenotypic honey bee wing deformities and subsequent collapse of the colony (de Miranda & Genersch 2010). Whilst bumblebees do not suffer from the parasitic mite *V. destructor*, DWV has now been detected in wild and commercially reared bumblebees, infecting a range of tissues (Chapter 2; Singh *et al.* 2010; Li *et al.* 2011; Evison *et al.* 2012). The virulence of this virus in bumblebees is unknown, however wild bumblebees with DWV have been seen to exhibit deformed wings, suggesting the virus can express the same high level of virulence to bumblebees as seen in honey bee hosts, even in the absence of *V. destructor* (Genersch *et al.* 2006). The increased detection of both DWV and *A. bombi* in wild and commercially reared bumblebee populations in combination with a shortage of knowledge regarding their virulence places a great and urgent need to understand the virulence of these parasites on

bumblebee hosts. Studies often focus purely on a single parasite-host interaction, yet parasites often occur as mixed infections, having profound effects on the outcome of the interactions (Rigaud *et al.* 2010; Dunn & Perkins 2012; Alizon *et al.* 2013). Here, for the first time, I investigate lethal and trait effects of single and mixed infections of these two little-studied parasites of bumblebees.

6.2 Methods

6.2.1 *Apicystis bombi* extraction for ingestion treatments

The fatbody of 40 *Bombus terrestris* bumblebees from *A. bombi* infected hives (confirmed by PCR; see below) were homogenised in 1000 μl of 30% sucrose solution and the resulting lyse was slowly passed through a syringe filter to remove bumblebee tissue fragments. This solution was then centrifuged for 30 min at 9,000g and 15°C and the resulting pellet of spores extracted with a pipette. Spores were washed by first centrifuging at 10,000g for 20 min, removing the supernatant, and replacing with 30% sucrose solution before vortexing for 10 sec. This wash process was repeated three times. The resulting solution was confirmed with a compound microscope to be a solution of *A. bombi* spores, free from bumblebee tissue membrane and other parasite spores. The suspended spores were confirmed as being only *A. bombi* by PCR amplification of the suspension with specific primers for *A. bombi*, and for the two *Nosema* species known to be found in bumblebee fatbody, *Nosema bombi* and *N. ceranae* (Chapter 5; Klee *et al.* 2006). The spore solution was then diluted in 30% sucrose solution to obtain a concentration of 5×10^5 spores per ml

6.2.2 DWV extraction for injection treatments

This extraction protocol was adapted from Iqbal & Mueller 2007. The fatbody of 50 *Bombus terrestris* bumblebees from DWV infected hives (confirmed by RT-PCR; see below) was frozen in liquid nitrogen, and homogenised with 2.5 ml phosphate-buffered saline (PBS, pH 7.4), then centrifuged at 3000 r.p.m. for 30 min at 10°C. The resulting solution was confirmed to be positive for DWV by RT-PCR, before being diluted 1:1000 in PBS.

6.2.3 Experimental infection

A total of 350 *Bombus terrestris audax* workers were collected from five colonies that had been obtained from Biobest and were confirmed to be parasite-free by screening 15 bees per colony by PCR and RT-PCR for *Crithidia bombi*, *A. bombi, Nosema bombi, Nosema ceranae* and DWV (see below; Chapter 2). The 350 bees were divided into groups consisting of 5 nestmate bees and placed in 10 x 6 x 6 cm plastic boxes. Each of the seven treatments was given to 2 groups from each of the 5 colonies, such that 50 bees received each treatment in total. The seven combinations of ingestion|injection treatments were: *A. bombi*|DWV, *A. bombi*|control, control|DWV, control|control, *A.bombi*|no injection, no ingestion|DWV, and no ingestion|no injection. For the *A. bombi* treatment, each bee was placed into a holding harness and individually fed a 5 μl dose of the parasite suspension, which contained 2,500 *A. bombi* spores. Bees receiving the control ingestion treatment were treated similarly, but fed 5 μl of pure 30% sucrose solution, while bees in no ingestion combinations were not hand-fed at all. For the DWV treatment, a 5 μl dose of the DWV solution was injected into the ventral side of the abdomen, between the 2nd and 3rd sternites. Bees receiving the control injection were injected with 5 μl of PBS, while those in the no injection combinations were not injected with anything. The bees received their injection treatments first and were subsequently starved for 5 h before receiving their ingestion treatments. Following treatment the bees were replaced in their cohorts of 5 like-treated nestmates, provided with 50% sucrose solution *ad libitum*, and their survival checked daily for 15 days.

6.2.4 Sucrose sensitivity and lipid extraction

The sensitivity of a bee to low sucrose concentrations has been linked to hunger and learning ability (Scheiner *et al.* 2001; Naug & Gibbs 2009) making it a good measure of sub-lethal trait effects of parasite infection. The sucrose sensitivity (SS) of bumblebees to differing concentrations was therefore tested for every bee in the experiment using the proboscis extension response (PER). Every 5 days a PER experiment was performed, in which each bee was harnessed in a modified eppendorf tube with moist cotton wool under red-light conditions. Whilst harnessed, bees were hand-fed to satiation with 30% sucrose solution before being left for a starvation period of 5 hours. After starvation, each bee had its antenna touched with varying

concentrations of sucrose solution (40-80% in increments of 10). Between each concentration trial, antenna were touched with H_20 after 60 s to prevent bees becoming conditioned; the next sucrose concentration was then applied following a further 60 s interval. Individuals that were responsive to a particular concentration extended their proboscis, resulting in a SS score of 1, and, as each bee was individually presented with 5 different concentrations, each bee could therefore score a maximum SS of 5 (Riveros & Gronenberg 2009). The responses of the each bee was measured, with high SS scores indicating bees responding to high and low sugar concentrations, and a low SS score indicating bees responding only to high sugar concentrations.

The leanness of each bee was calculated by determining their lipid content relative to their body size (Brown et al. 2000). For this, each abdomen (minus two removed tergites – see below) was dried at 70°C for 5 days, weighed and then immersed in ether for 24 hrs to dissolve the lipids. After rinsing the ether/lipid solute with fresh ether, the remaining abdominal tissues were dried for a further 5 days at 70°C before being reweighed. Based on the resulting weight loss (mg) and taking the length of the left hind tibia (mm) as an index of body size, the worker lipid: body size ratio was calculated.

6.2.5 Molecular screening

Any bees that died during the experiment, and all those surviving to the end of the 15 day experimental period were placed in 100% ethanol. All 350 bumblebees were removed from ethanol and each had their 5^{th} and 6^{th} tergites removed. These tergites are more posterior and are on the opposite side of the abdomen to the site that the injection treatments were administered. The fatbody attached to these 2 tergites was homogenised in 100 µl of 5% Chelex, for DNA and RNA extraction, by incubating at 100°C for 15 min. The fatbody extracts were then briefly vortexed before centrifuging at 4680 rpm for 15 minutes and collecting the supernatant.

Samples were first screened for the *18S* Apidae host control gene to confirm DNA quality using 0.4 mM dNTP, 1.5 mM $MgCl_2$, 3 µl Buffer, 1.25 U Taq, 0.2 µM each primer and 1 µl template, giving 10 µl in total (Meeus *et al.* 2010). The PCR was then subject to 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 56°C, 45 s at 72°C before a final elongation stage of 3 min at 72°C. Samples were screened for *Apicystis bombi* using 0.4 mM dNTP, 1.5 mM $MgCl_2$, 2 µl Buffer, 1.25 U Taq, 0.5 µM of each primer and 1 µl template, giving 10 µl total volume. This was then subject to 2 min at

94°C, 35 cycles of 30 s at 94°C, 30 s at 60°C, 45 s at 72°C before a final elongation stage of 3 min at 72°C (Meeus *et al.* 2010). Samples were screened for DWV using reverse transcription PCR whereby 2 µl of sample was added to 5 µl TaqMan® Fast Virus 1-Step Master Mix, 650nM of each primer and molecular grade water giving a total volume of 10 µl. The sample then underwent thermal cycling of 5 min at 50°C, 20 s at 95°C, 40 cycles of 3 s at 95°C, 180 s at 60°C before a final elongation stage of 10 min at 72°C (Chen *et al.* 2005). PCR conditions for the other parasites that were screened for when preparing parasite suspensions or when confirming the parasite-free status of the experimental bees are as in Chapter 2. PCR products were run on a 1% agarose gel stained with ethidium bromide to confirm amplicon size. Every assay included negative and positive controls.

6.2.6 Statistical analysis

Differences in bumblebee survival were analysed using a Cox proportional hazards regression model, with ingestion treatment, injection treatment and their interactions included in the model. Pairwise comparisons were made between individual treatments using Kaplan-Meier models with the Breslow χ^2 statistic. The interactive and singular effect of injection and ingestion treatments on sucrose sensitivity and lipid: body ratio was compared using generalized linear models (GLM) with linear distribution, logit link function and the likelihood ratio χ^2 statistic. Colony of origin and cohort was included in the GLM models. Nonsignificant terms were removed stepwise in all cases to obtain the minimum adequate models. Pairwise comparisons have been made using Mann-Whitney U tests and P values modified using the Bonferroni correction. All analyses were carried out in PASW Statistics 20 (IBM, Armonk, NY, USA).

6.3 Results

Injection treatments had no effect on sucrose sensitivity on either days 0, 5, 10 or 15 (χ^2 = 0.24, P = 0.88; χ^2 = 3.52, P = 0.17; χ^2 = 0.15, P = 0.93; and χ^2 = 3.08, P = 0.22). Ingestion treatments however did effect sucrose sensitivity on day 5 (χ^2 = 7.41, P = 0.025), but not days 0, 10 or 15 (χ^2 = 0.74, P = 0.69; χ^2 = 5.53, P = 0.06; and χ^2 = 1.69, P = 0.43). On day 5 the SS of bumblebees given just *A. bombi* spores were significantly higher than the Control | Control treated bumblebees, though similar to

the SS of bees given *A. bombi* | Control which were also high. Although not significant, there was a similar pattern on both days 10 and 15 (Figure 6.3.1.1; Table A6.2.4.1).

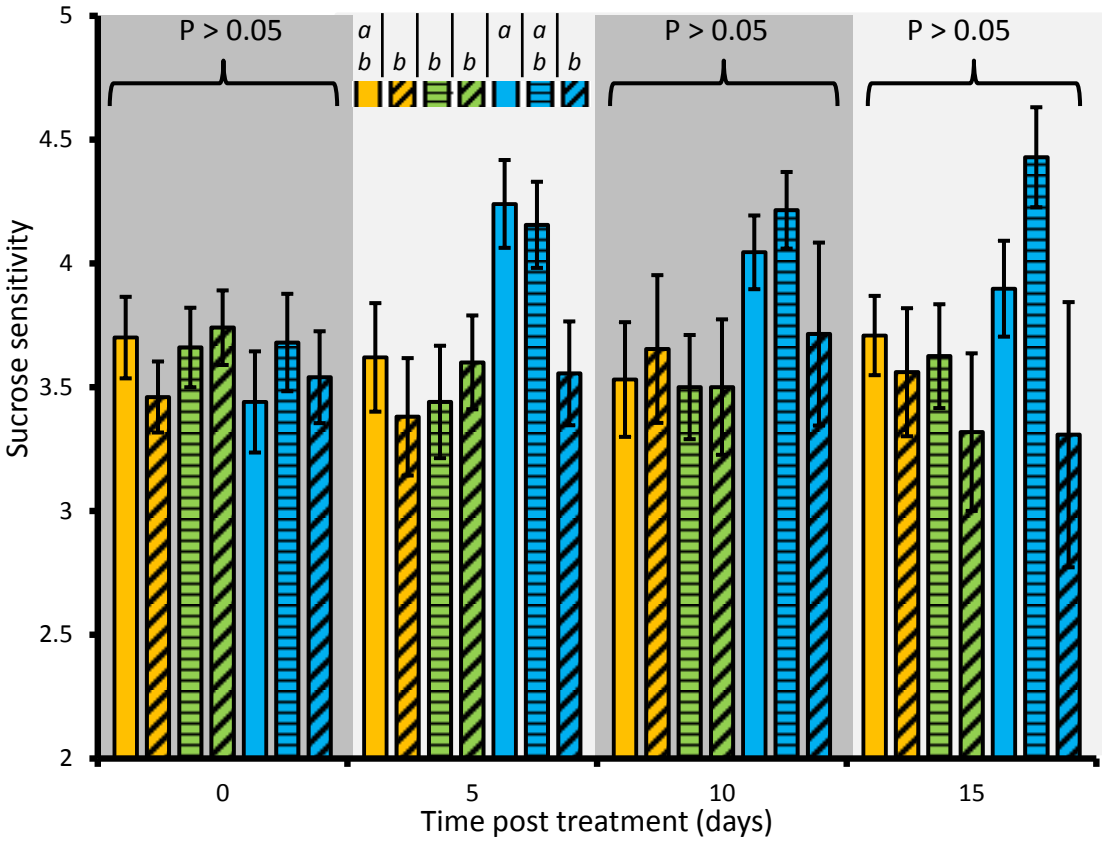


Fig 6.3.1. Bumblebee responsiveness to sucrose over 15 days following either hand feeding with *Apicystis bombi* spores (blue bars), sucrose control (green bars) or no hand feeding (orange bars) whilst also being injected with DWV (bars patterned with diagonal lines), PBS control (bars patterned with horizontal lines) or no injection (bars not patterned). Pairwise differences indicated by different italicised letters.

Over time the average sucrose sensitivity of bees treated with just *A. bombi* increased significantly over time (χ^2 = 11.07, d.f. = 3, P = 0.011). The sensitivity of bumblebees treated with *A. bombi* and no injection rose significantly from day 0 to day 5 (U = -41.8, P < 0.001), before it plateaued from day 5 onwards (Figure 6.3.1.1; Table A6.2.4.2). The SS of bees treated with nothing, *A. bombi* | DWV, *A. bombi* | control, DWV, control | DWV, or control | control did not change (χ^2 = 0.54, d.f. = 3, P = 0.91 ; χ^2 =0 .57, d.f. = 3, P = 0.92 ; χ^2 = 5.61, d.f. = 3, P = 0.13 ; χ^2 = 0.77, d.f. = 3, P = 0.86 ; χ^2 = 1.81, d.f. = 3, P = 0.61; χ^2 = 0.79, d.f. = 3, P = 0.85, respectively).

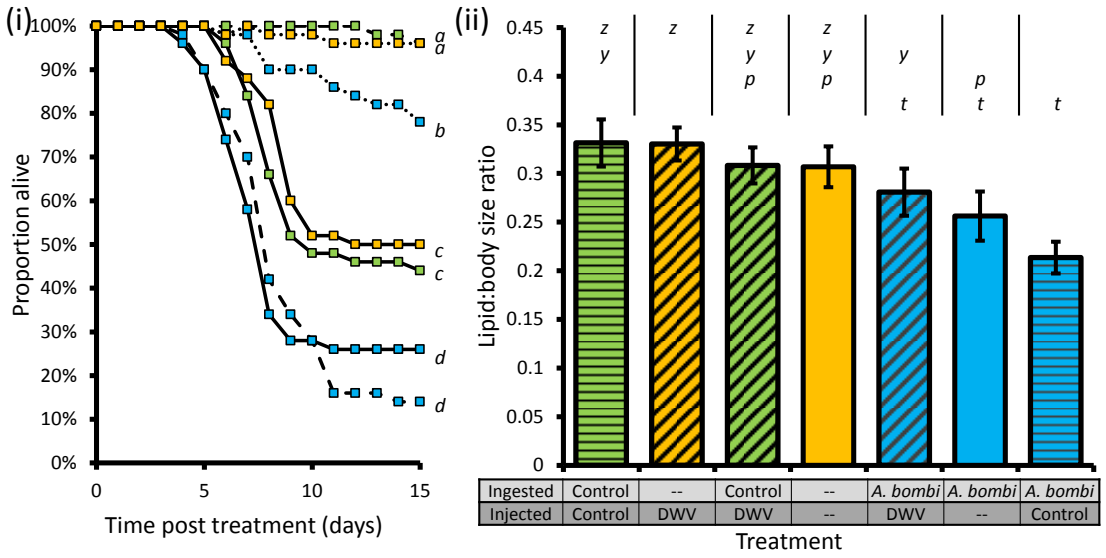


Fig 6.3.1.2. Survival of bees over 15 days (i) and subsequent lipid: body ratio (ii) following hand feeding with either *Apicystis bombi* spores (blue points/bars), sucrose control (green points/bars) or no hand feeding (orange points/bars) whilst also being injected with DWV (solid lines/diagonal bar pattern), PBS control (dashed lines/horizontal pattern) or no injection (dotted lines/no pattern). Significant pairwise differences are indicated by different letters.

There was a significant interaction between the effects of the ingestion and injection treatments on bumblebee survival (*Wald* = 16.5, d.f. = 2, $P < 0.001$). The bees which received either the control | control or no ingestion | no injection treatment combinations had very high survival over the 15 day period, and the survival of bees was reduced significantly when they had received the *A. bombi* | no injection combination (Figure 6.3.1.1i). Survival was significantly lower for bees which received either the control | DWV or, no ingestion | DWV combinations, and was very low for bees which received the *A. bombi* | control injection or *A. bombi* | DWV combinations (Figure 6.3.1.1i).

The lipid: body size ratio was affected by the ingestion treatment (*Wald* = 14.6, d.f. = 2, $P = 0.001$), but not by the injection treatment and there was also no interaction between ingestion and injection treatments (*Wald* = 1.1, d.f. = 2, $P = 0.6$; *Wald* = 4.7, d.f. = 2, $P = 0.1$, respectively). Bees in the control | control treatment had the highest average lipid: body size ratio and none of the treatments that included DWV differed significantly from this. The 3 treatments that included *A. bombi* ingestion all had significantly lower lipid: body size ratios than the control treatment (Figure 6.3.1.1ii; Table A6.2.4.3).

6.4 Discussion

Apicystis bombi, DWV and co-infections of these parasites are virulent in infected bumblebees, with infections by either or both parasites causing mortality to increase by 18%, 50% and 70% respectively. *Apicystis bombi* significantly reduces the fat body of bumblebee workers, increased their chances of death following injury and also increased their sensitivity to sucrose after 5 days. Deformed wing virus, and co-infections did not show any trait effects on bumblebees, but all still caused significant mortality.

It had previously been noted anecdotally that the fat body of bumblebees infected with *A. bombi* appeared 'much reduced' (Liu *et al.* 1974). The results here provide the first empirical evidence of this, with fat body being reduced by 17% on average by *A. bombi* infections. The fat body is essential for overwintering queen bumblebees and any reduction in their fat body would lower their chances of founding successful colonies (Fliszkiewicz & Wilkaniec 2007). Workers could also be negatively affected by reduced fat body as it is the site for many biochemical reactions that are important for their immunity and metabolism (Arrese & Soulages 2010). After five days, the SS of *A. bombi* infected bumblebees is elevated, presumably due to an increased demand for carbohydrates to compensate for reduced fatbody. In the wild this would increase the workers need to forage for nectar, reducing their pollen foraging efficiency for the colony and developing larvae. The number of *A. bombi* spores fed to bumbles was low compared to the numbers found in infected bees; despite this, a high mortality was witnessed over 15 days. This mortality increased three-fold if bees had been injected in their abdomen with either PBS or DWV prior to spore ingestion, suggesting that *A. bombi* infected bees also have reduced ability to cope with the effects of wounding. Whilst DWV was detectable in fatbody tissue, I found no change in the lipid mass of DWV-infected bumblebees, with bumblebees co-infected by DWV and *A. bombi* having an intermediate lipid: body mass ratio. In honey bees, DWV infection increases the hosts SS (Iqbal & Mueller 2007), however there was no evidence here of such a response in infected bumblebees. The sucrose sensitivity of DWV-infected bumblebees was similar to control treated bees, but interestingly, co-infected bumblebees also had a SS similar to controls, suggesting that DWV may inhibit the effects of *A. bombi* infections which drive increased SS.

The results demonstrate that DWV and *A. bombi* each have a high virulence and pose a significant risk to infected bumblebees. Whilst single infections of *A. bombi* impose only a moderate risk to bumblebee workers, the reduced ability to cope with the effects of wounding and the significant risks this parasite may pose to hibernating queens makes this parasite potentially high risk to infected bees. This supports recent correlating evidence that the emergence of *A. bombi* in South America may be responsible for rapid declines in some native bumblebee species. In addition, the high mortality of bumblebees infected with DWV makes it clear that both these parasites should be monitored more frequently in bumblebee populations (Imhoof & Schmid-Hempel 1998; Plischuk & Lange 2009). Both *A. bombi* and DWV are significantly understudied given the virulence they exhibit in bumblebees. Also the rates of transmission amongst bumblebees, and between honey bees and bumblebees are still unknown. This taken with the ubiquitous prevalence of DWV in honey bees, the growing evidence that *A. bombi* is highly prevalent in bumblebees, and the discovery of both parasites in commercially-produced and internationally distributed bumblebee colonies, highlights the need to understand more about these parasites; Including their effects on colony growth, reproduction and bumblebee declines.

Chapter 7: General Discussion

When assessing the parasite risks bumblebees may face, it is important to not simply look at parasites considered 'bumblebee parasites' but to identify the true range of parasites that bumblebees will frequently make contact with (Chapter 2; Evison *et al.* 2012). Honey bees have been widely studied for hundreds of years, and have more than 70 associated parasites. This compares with approximately 14 recognized bumblebee parasites (Macfarlane *et al.* 1995; Schmid-Hempel 1998; Coffey 2007). However, this does not represent an increased susceptibility of honey bees to parasites, rather it represents a bias in research towards the honey bee compared to the less studied bumblebee. Research into bumblebees and their parasites is now increasing, in part due to their commercial availability, allowing them to be a more accessible model organism for researchers. Despite this, much bumblebee parasite research is still based on simple microscopy, a method that whilst effective, has limited sensitivity and a high error rate. Additionally, by only distinguishing parasites morphologically, oversights may be present. Following molecular confirmation in 2009, *Nosema ceranae* was detected in bumblebees (Plischuk *et al.* 2009). Morphologically similar to *N. bombi,* it could have been present in South America (and elsewhere), yet misidentified for a considerable time (Teixeira *et al.* 2013). Indeed, a study in China has identified a large diversity of *Nosema* species within bumblebee guts (not confirmed infections), including *N. ceranae, N. bombi, N. apis* and some as yet unnamed *Nosema* subgroups (Li *et al.* 2012). Additionally, whilst there have been no reports of *N. ceranae* in European bumblebees prior to my survey, I molecularly identified its sole presence in 21% of 767 individual bumblebees, compared to just 4% that had visibly detectable spores and no detection of other *Nosema* species (Chapter 5).

Until March 2013, the regulations that control bumblebee imports into England stipulated that the bumblebees must be disease free. This is self-regulated in accordance with the rules outlined by Natural England (Natural England 2009; Report 2009). These rules required only microscopy screening of the malpighian tubules and fatbody (gut not required) from only 2 bees per colony at a frequency of 200 colonies per year (in the case of *N. bombi*) and 200 colonies every 10 weeks in the case of *C. bombi*. *Apicystis bombi* screening was not required at all, and DWV screening required only 'visual inspection'. From an estimated 45,000 hives imported per year (each with a conservative average of 100 bees inside), only 0.04% of the bees were

screened for *N. bombi*, *C. bombi* and DWV; 0% for *A. bombi*, 0% for American Foul Brood. No other internal parasites are listed as requiring inspection. Given the poor sensitivity (for example, the tissues known to be regularly inhabited by *C. bombi* were not required to be screened) of these screening methods it is unsurprising that when I screened these 'disease free' colonies using molecular techniques I detected that 77% of colonies contained parasites, and that in addition to finding the parasites mentioned above, I found evidence of *N. apis*, *N. ceranae* and chalkbrood in commercially reared and imported bumblebee hives (Chapter 2).

Parasites, by definition, are detrimental to their individual hosts, yet on a wider, colony, community or population scale their effects may differ, in some cases even being beneficial in maintaining ecological stability (Hatcher & Dunn 2011; Poulin 2011; Johnson & Hoverman 2012). In addition, focusing purely on one parasite and one host is extremely restrictive, especially when considering biological questions of applied value. Commonly parasites will infect a range of hosts, and hosts will be infected by a range of parasites (Chapter 2 & 3; Rigaud *et al.* 2010). Parasite virulence and success can be effected by the host species and the presence of co-infecting parasites (May & Nowak 1995; Poulin *et al.* 2011; Johnson & Hoverman 2012). For this reason, when approaching questions regarding conservation and/or the introduction of a host/parasite to an environment, a wider and more holistic view to the impact this may have should be adopted (Poulin 2011; Dunn & Perkins 2012). Ideally, entire communities and their parasite fauna would be monitored over generations, though this is impractical. By determining a parasite's sub-lethal trait effects on important life-history characteristics, in addition to its host range and lethality, a much more informed view can be gathered. It has been frequently shown, particularly when dealing with eusocial insects, that virulence on an individual level may not translate to the virulence imposed on a colony and/or reproductive level. In ants for example, an entomopathogen may be particularly lethal to an individual, producing many transmittable particles, but that same pathogen may have reduced lethality and transmission success when infected individuals are in social groups (Cremer *et al.* 2007; Graystock & Hughes 2011). In bumblebees, the relatively benign parasite *C. bombi*, is not considered to be particularly harmful on an individual level, but can cause trait effects that have cascading negative implications on a colony level (Brown *et al.* 2003). By assessing the trait effects of a parasite, reasonable estimations of the parasites effect on a higher population-level can be made. For bees, sucrose sensitivity

is a predictor of learning ability, which in turn is correlated with flower handling and foraging efficiency (Scheiner *et al.* 2001; Gegear *et al.* 2005, 2006; Iqbal & Mueller 2007). Learning ability is thus extremely important, as colonies with efficient resource acquisition will be able to produce more offspring and a higher number and quality of reproductive individuals (Muller & Schmid-Hempel 1992; Goulson 2010; Whitehorn *et al.* 2012). As a result, studies often take measures of a bees foraging or learning capacity as an indicator of bee health, making it an excellent sub-lethal trait measure to take when identifying the effects of parasites such as *A. bombi*, *N. ceranae* and DWV (Chapter 5 & 6; Iqbal *et al.* 2007; Mayack *et al.* 2009). Unlike honey bees, bumblebee queens also need to hibernate alone every year and in doing so utilise fatbody deposits formed whilst in the natal colony. When bumblebee queens have had insufficient fatbody reserves, they have been found to have reduced fitness, measured through reproductive success. The presence of *A. bombi* residing within the fatbody thus makes fatbody measures from bees infected with *A. bombi* particularly relevant to studies of this parasite (Chapter 6; Rutrecht *et al.* 2008).

The transmission of the trypanosome *C. bombi* via shared flowers has long been established (Durrer & Schmid-Hempel 1994), and recent studies show that bumblebees avoid contaminated flowers when given the choice (Fouks & Lattorff 2011). *Crithidia bombi* is largely considered to be a benign parasite, but the between colony transmission of other more virulent bumblebee parasites is largely believed to be via colony drifting of infected workers, or through the robbing of resources from weaker colonies. Possibly as a result of these assumptions, efforts by commercial bumblebee suppliers to stop mixing have focused more on preventing escape of reproductives, rather than parasite spillover. Having identified that infective parasites are being imported into England, it is necessary to identify if these parasites have a route to infect native bees. The parasite *C. bombi* can disperse via shared flower use, and several other bee parasites have been found in the pollen baskets of foraging bees (Chapter 2; Durrer *et al.* 1994; Flores *et al.* 2005; Singh *et al.* 2010). This suggests that flowers may have an important role for parasite dispersal. I found that all five of the parasites found in commercial bumblebees that were infective to adult bees, were able to be dispersed by both bumblebees and honey bees via shared flowers (Chapter 3). Not only does this have serious implications regarding pathogen spillover from commercially reared bumblebees, this finding changes our assumptions on pollinator parasite dispersal. Foraging pollinators may frequently be in contact with a diversity of

pollinator parasites, creating a high potential for parasite spillover and vectoring (see Figure 7.1). EU Directives 142/2011 and 92/65/EEC impose strict conditions on the transport of honey bee hive products such as honeycomb, royal jelly or honey within the EU due to a perceived risk of pests or disease transmission, but honey bee pollen, and hence commercially produced bumblebee colonies, are not subject to such scrutiny. Here I have identified numerous disease risks residing within honey bee collected pollen that pose a clear risk to our two most valuable pollinator groups, honey bees and bumblebees (Chapter 2 & 3).

Studies that have shown increasing parasite prevalence in bumblebees caught closer to farms using commercially reared bumblebees have been open to interpretation (Chapter 4; Otterstatter *et al.* 2008; Murray *et al.* 2013; Whitehorn *et al.* 2013). Competitive stress in bumblebees can cause marked decreases in worker sizes (Goulson & Sparrow 2008). Similar stress may be found in native bumblebees, competing with the artificially high density of pollinators around sites using commercial bumblebees. When provided with increased competition (via honey bees) I found that there were no difference in prevalence of *A. bombi* and *N. ceranae* in the bumblebees. Only *C. bombi* increased in prevalence in the stressed bees, suggesting that the marked increases of *A. bombi* and *N. bombi* found near commercial sites may not be a result of competition (Murray *et al.* 2013). The finding that commercially reared and imported bumblebee colonies contain a cocktail of infective parasites that can disperse via shared flower use, helps further inform our interpretation of such correlative studies on parasite prevalence around farms utilising commercially reared bumblebees (Chapter 2 & 3). Even when in 'enclosed greenhouses' there is still evidence of mixing between populations, encouraging shared flower use (Whittington *et al.* 2004; Murray *et al.* 2013). Had no parasites been found in commercially reared bumblebees on arrival, the detection of high parasite prevalence's near sites using commercial bumblebees could be due to spillback from native bumblebees via shared flower use. Taken together however, the correlative evidence of high parasite levels around sites using commercially reared bumblebees, imported bumblebees being infected with parasites on arrival, the free mixing of bumblebee populations and dispersal of parasites via shared flowers, spillover seems an extremely likely explanation.

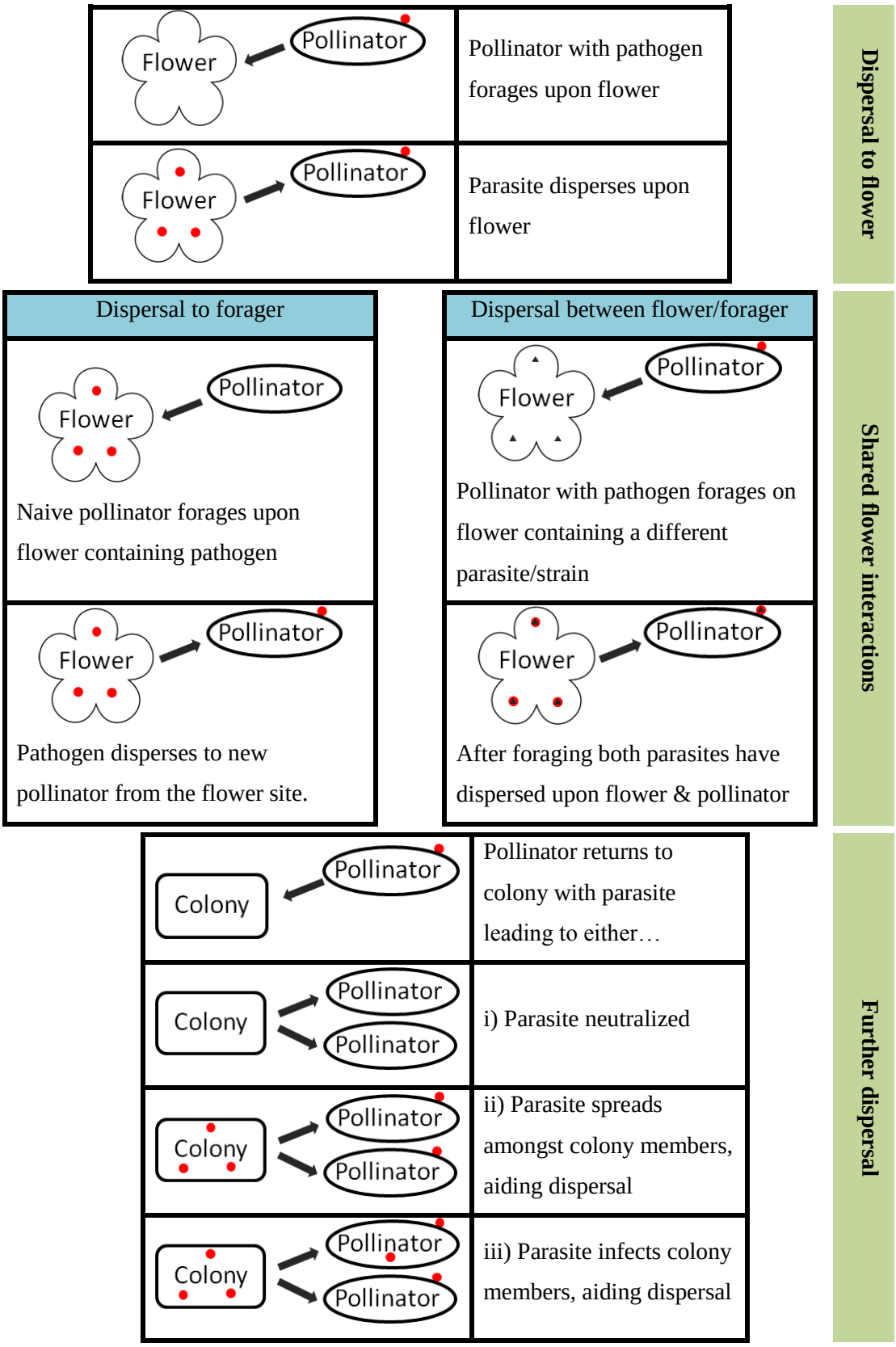


Figure 7.1 Diagram showing the mechanisms of parasite dispersal via shared flower use. Red circle and black triangles indicate different parasite species/strains

Considering the findings as a result of this thesis, in the light of the current knowledge of parasite epidemiology in bumblebees, I conclude the following:

1. In addition to anecdotal and correlative findings (Whittington & Winston 2003; Gegear *et al.* 2005; Colla *et al.* 2006; Otterstatter & Thomson 2007; Manson *et al.* 2010; Singh *et al.* 2010; Meeus *et al.* 2011; Murray *et al.* 2013), there is now significant evidence that up to 77% of commercially reared bumblebees and the pollen they are reared on contain a cocktail of infective parasites including *N. bombi*, *A. bombi*, *N. ceranae*, *N. apis*, DWV and *Ascosphaera* (Chapter 2)
2. It's already established that *N. bombi* can cause bumblebee declines and *C. bombi* makes bumblebees vulnerable to stress (Brown *et al.* 2000; Otti & Schmid-Hempel 2007; Meeus *et al.* 2011). New insights into the pathology of 'honey bee parasites' *N. ceranae* and DWV in addition to the little studied cosmopolitan parasite *A. bombi* show that these three parasites, found in commercially reared bumblebees can also cause significant bumblebee mortalities and behavioural changes (Chapter 2, 5, 6).
3. Commercially reared bumblebees commonly mix with native bumblebees (Whittington *et al.* 2004; Fitzpatrick *et al.* 2007; Kraus *et al.* 2010).
4. The mixing and shared use of flowers is a route of parasite dispersal on conspecific or inter-genera hosts (Chapter 3; Durrer *et al.* 1994).
5. Pathogen prevalence in wild bumblebees is increased around farms utilising commercial bumblebees (Chapter 4; Otterstatter *et al.* 2008; Murray *et al.* 2013; Whitehorn *et al.* 2013).
6. Though competitive stress may influence the prevalence of *C. bombi* in bumblebee populations, the higher prevalence of *A. bombi* around farms using commercial bumblebees is almost certainly the result of parasite spillover from commercial bumblebees (Chapter 4).

7.1 Synthesis

At the start of my PhD bumblebees imported into England were required to adhere to Natural England regulations that are based upon the importation of non-native species. These regulations required no molecular screening, and the only parasites that were microscopically screened for were *N. bombi* and *C. bombi*. Prior to publication of my

screening results where an additional 6 parasites were detected, Natural England were informed of the findings along with a correlative study identifying high parasite prevalence around farms using commercial bumblebees in Ireland, and they subsequently tightened up the import regulations (Natural England 2009, 2012; Murray *et al.* 2013; Graystock *et al.* 2013). These new regulations came into force on the 31st of March 2013. Suppliers are now required to molecularly screen for the 'bumblebee parasites' *N. bombi, C. bombi, A. bombi*, plus the traditionally termed 'honey bee parasites' that I detected in colonies produced in years 2011 and 2012: *N. apis*, *N. ceranae* and DWV. Now, 16 workers per colony are required to be screened every 10 weeks. This is still only *0.3%* of the estimated bees that are being produced, but the enhanced screening techniques will also greatly increase the detection sensitivity compared with the previous protocol. Whilst this may seem like good news, and a step in the correct direction, this is only half the story. These new regulations apply to all suppliers importing non-native *Bombus terrestris* subspecies into England. In 2010, Suppliers Biobest, Koppert, and later Syngenta, started the production and supply of *B. t. audax* into the UK. This is the native English subspecies of *B. terrestris,* and is therefore not regulated by the above 'non native species' regulations. Indeed, there are no regulations for checking the disease status of imported native subspecies *B. t. audax,* and this subspecies also is not required to be sited indoors. This is of huge concern since both subspecies supplied by commercial breeders have similar parasite fauna suggesting the current regulations in place are still not sufficient to prevent diseases being imported into England (Chapter 2).

Following evidence of commercial bumblebee escapes into Japan in 1996 and their increased invasion by 2004 (Matsumura *et al.* 2004; Goka 2010), the Japan invasive species act was established in 2004. This immediately set strict controls upon the importation and deployment of *Bombus terrestris* (Mizutani & Goka 2010) as a pollination aid. One of the main restrictions put in place was to impose strict, no mixing regulations, forcing all farm sites using commercial bumblebees to be heavily netted (Goka 2010; Mizutani & Goka 2010). Whilst netting doesn't always prove 100% effective, it will drastically reduce the chances of *B. terrestris* escape and minimise the likelihood of parasite spillover (Morandin *et al.* 2001; Koide & Yamada 2008; Dafni *et al.* 2010; Goka 2010). Such measures would be a highly beneficial here in England, helping prevent the pathogen spillover already identified in *A. bombi*, and minimising the threat from infectious *N. ceranae*, *N. apis*, *N. bombi*, chalkbrood and as

described by Singh *et al.* in 2010, DWV. Natural England however, is currently impotent towards the threat posed by native bumblebee subspecies, and netting just the non-native *B. t. dalmatinus* subspecies would be an inadequate compromise.

Over the course of the PhD, my work has helped identify the threats posed by the commercial rearing and importation of bumblebees, not only to native bumblebees but also to honey bees. The findings have been used to inform new import regulations and have highlighted the inadequacies of past and present regulations (or lack thereof) concerning native bumblebee subspecies. By also discovering parasites in pollen, not only do commercial suppliers have an idea regarding the source of their stock spoilage but also I have identified the need to monitor the disease risks associated with pollen transport. As a result, Natural England are now investing money into researching an effective screening protocol for both commercial suppliers and regulating bodies to use upon bumblebees. In addition some commercial bumblebee suppliers are also investing in research to discover a way to sterilize pollen, thus reducing the introduction of parasites into their breeding/rearing facilities. Finally, by identifying the role shared flowers play in parasite dispersal, I have highlighted not just the mechanism for parasite spillover between domestic and native bumblebee populations, but that this method of parasite dispersal may be far more widespread between pollinators than previously realised. Disease emergence via parasite spillover or spillback represents a serious threat to wildlife. Widespread bumblebee declines have been reported in North and South America following suspected parasite spillover. Having identified the mechanisms that could enable parasite spillover from commercial bumblebees, and regulations that could be improved to reduce this, it is hoped my results could help go towards further reducing the threat of parasite spillover in bumblebees.

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Appendix A

Primers & source	Assay mix								Thermal cycling			Amplicon size (bp)
	dNTP (nM)	$MgCl_2$ (nM)	5xbuffer (µl)	Taq (U)	Primer F (µM)	Primer R (µM)	Template (µl)	Total volume (µl)	1 Denaturing Min | Temp	2 Replication Sec | Temp	3 Elongation Min | Temp	
Nosema bombi (Klee et al. 2006)	0.3	3.75	2	0.25	0.2	0.2	2	10	4 | 95	35x 60 | 95 60 | 50 60 | 72	4 | 72	323
Nosema apis[Na] & *N. ceranae*[Nc] (Chen et al. 2008)	0.25	3.75	2	0.25	0.2 [Na] 0.2 [Nc]	0.2 [Na] 0.2 [Nc]	1	10	2 | 94	35x 30 | 94 45 | 63[Na] 45 | 61[Nc] 120 | 72	7 | 72	401[Na] 250[Nc]
Nosema apis[Na] & *N. ceranae*[Nc] (Gisder & Genersch 2013)	0.2	1.5	2	2.5	0.2 [Na] 0.2 [Nc]	0.2 [Na] 0.2 [Nc]	1	10	5| 95	35x 60 | 94 60 | 58 60 | 72	10 | 72	297[Na] 662[Nc]
Apicystis bombi (Meeus et al. 2009)	0.4	1.5	2	1.25	0.5	0.5	1	10	2 | 94	35x 30 | 94 30 | 60 45 | 72	3 | 72	260
Apidae[A] (internal) and *Crithida bombi*[Cb] (duplexed) (Meeus et al. 2009)	0.4	1.5	3	1.25	0.1 [A] 0.5 [Cb]	0.2 [A] 0.5 [Cb]	2	15	2 | 94	35x 30 | 94 30 | 56 45 | 72	3 | 72	130[A] 420[Cb]
Ascosphaera (James and Skinner 2005)	0.2	2.5	2	0.5	0.4	0.4	1	10	10 | 94	35x 45 | 94 45 | 62 60 | 72	5 | 72	550
Paenibacillus larvae (nested) external[Ex] and internal[In] (Lauro et al. 2003)	0.2	0.75[Ex] 1.0 [In]	2	0.5	0.5	0.5	2[Ex] 4[In]	10	10 | 94	35x 30 | 94 30 | 69~59[Ex] 30 | 59[In] 45 | 72	5 | 72	969[Ex] 525[In]
Melisococcus plutonius (Hemi-nested) primary[Py] and hemi[Hn] (Djordevic et al. 1998; McKee et al. 2003)	0.2	3.0 [Py] 1.5 [Hn]	2	1.5	0.6	0.6	2	10	2 | 95	40x 30 | 95 15 | 61[Py] 15 | 56[Hn] 60 | 72	5 | 72	486[Py] 276[Hn]

Figure A2.2.1.1

PCR chemical and thermal conditions used throughout

Table A2.2.2.1

Occurrence of parasites in adult workers from 48 bumblebee colonies of two subspecies, supplied by three commercial producers, in two years. In 34 colonies, 15 workers were pooled to give a single presence/absence for each colony; in 14 colonies, 15 workers were screened individually and the proportion of bees infected by the parasite is then indicated.

Yr	**Subspecies**	**Producer**	**ID**	***Apicystis bombi***	***Crithidia bombi***	***Nosema bombi***	**DWV**	***Nosema ceranae***	***Nosema apis***
2011	*audax*	Producer A	BBa1	0.47	0	0	0.13	0	0
2011	*audax*	Producer A	BBa2	0	0	0	0	0	0
2011	*audax*	Producer A	BBa3	0.73	0	0.47	0	0	0
2011	*audax*	Producer A	BBa4	0.47	0	0.2	0	0	0
2011	*audax*	Producer A	BBa5	0	0	0	0	0	0
2011	*audax*	Producer A	BBa6	absent	absent	absent	absent	absent	absent
2011	*audax*	Producer A	BBa7	absent	absent	absent	absent	absent	absent
2011	*audax*	Producer A	BBa8	present	present	absent	absent	absent	absent
2011	*audax*	Producer A	BBa9	absent	present	absent	absent	absent	absent
2011	*audax*	Producer A	BBa10	absent	absent	present	absent	absent	absent
2011	*dalmatinus/terrestris*	Producer A	BBt1	absent	absent	absent	absent	absent	absent
2011	*dalmatinus/terrestris*	Producer A	BBt2	present	absent	present	absent	absent	absent
2011	*dalmatinus/terrestris*	Producer A	BBt3	absent	absent	present	absent	absent	absent
2011	*dalmatinus/terrestris*	Producer A	BBt4	present	absent	absent	absent	absent	absent
2011	*dalmatinus/terrestris*	Producer A	BBt5	present	absent	absent	present	absent	absent
2012	*audax*	Producer A	BB1	present	present	absent	absent	absent	absent
2012	*audax*	Producer A	BB2	present	present	absent	absent	absent	absent
2012	*audax*	Producer A	BB3	present	present	absent	absent	absent	absent
2012	*audax*	Producer A	BB4	absent	absent	absent	absent	absent	absent
2012	*audax*	Producer A	BB5	absent	absent	absent	absent	absent	absent
2012	*audax*	Producer A	BB6	absent	present	absent	absent	absent	absent

Table A2.2.2.1 (Continued).

2012	*audax*	Producer A	BB7	present	present	absent	absent	absent	absent
2012	*audax*	Producer A	BB8	present	present	absent	absent	absent	absent
2012	*audax*	Producer A	BB9	0	0.67	0	0	0	0
2012	*audax*	Producer A	BB10	0	0	0	0	0	0
2012	*audax*	Producer A	BB11	absent	absent	absent	absent	absent	absent
2012	*audax*	Producer A	BB12	absent	absent	absent	absent	absent	absent
2011	*audax*	Producer B	KBa1	0	0	0	0.2	0	0
2011	*audax*	Producer B	KBa2	0	0	0.67	0.2	0	0
2011	*audax*	Producer B	KBa3	0	0.6	0	0	0	0
2011	*audax*	Producer B	KBa4	0	0	0	0.13	0	0
2011	*audax*	Producer B	KBa5	0.6	0.27	0	0.13	0	0
2011	*dalmatinus/terrestris*	Producer B	KBt1	present	present	absent	absent	absent	absent
2011	*dalmatinus/terrestris*	Producer B	KBt2	absent	absent	absent	absent	absent	absent
2011	*dalmatinus/terrestris*	Producer B	KBt3	0	0	0	0.13	0	0
2011	*dalmatinus/terrestris*	Producer B	KBt4	absent	present	present	absent	present	absent
2011	*dalmatinus/terrestris*	Producer B	KBt5	present	present	absent	absent	present	absent
2012	*audax*	Producer B	KB1	present	present	absent	absent	absent	absent
2012	*audax*	Producer B	KB2	present	present	absent	absent	absent	absent
2012	*audax*	Producer B	KB3	present	absent	absent	absent	absent	absent
2012	*audax*	Producer B	KB4	0.73	0.33	0	0	0	0
2012	*audax*	Producer B	KB5	present	absent	present	absent	absent	absent
2012	*audax*	Producer B	KB6	present	present	absent	absent	absent	absent
2011	*dalmatinus/terrestris*	Producer C	SBt1	absent	absent	present	absent	absent	absent
2011	*dalmatinus/terrestris*	Producer C	SBt2	absent	present	present	absent	absent	absent
2011	*dalmatinus/terrestris*	Producer C	SBt3	absent	absent	present	absent	absent	absent
2011	*dalmatinus/terrestris*	Producer C	SBt4	absent	absent	present	absent	absent	absent
2011	*dalmatinus/terrestris*	Producer C	SBt5	present	present	present	absent	absent	absent

Table A2.3.1.1

Occurrence of parasites in pollen supplied with bumblebee colonies as food by three commercial producers, in two years (n = 5 pollen samples screened per producer in each year).

Year	Producer	ID	*Apicystis bombi*	*Crithidia bombi*	*Nosema bombi*	DWV	*Nosema ceranae*	*Nosema apis*	EFB	AFB	*Ascosphaera* chalkbrood
2011	Producer A	B1A	absent	absent	absent	absent	absent	absent	absent	absent	present
2011	Producer A	B1B	present	present	absent	absent	present	absent	absent	absent	absent
2011	Producer A	B2A	absent	absent	absent	absent	absent	absent	absent	absent	present
2011	Producer A	B2B	present	present	absent	absent	present	absent	absent	absent	absent
2011	Producer A	B3	present	absent	absent	absent	present	absent	absent	absent	present
2012	Producer A	B1A	absent	present	absent	absent	absent	absent	absent	absent	present
2012	Producer A	B1B	present	present	absent	absent	present	absent	absent	absent	present
2012	Producer A	B2A	present	absent	absent	absent	absent	absent	absent	absent	present
2012	Producer A	B2B	absent	absent	absent	absent	absent	absent	absent	absent	present
2012	Producer A	B3	absent	present	present	absent	absent	absent	absent	absent	present
2011	Producer B	k1a	present	present	absent	absent	absent	absent	absent	absent	present
2011	Producer B	k1b	absent	absent	absent	present	absent	absent	absent	present	present
2011	Producer B	k2a	present	absent	present	absent	present	absent	absent	absent	present
2011	Producer B	k2b	present	absent	present	present	absent	present	absent	absent	present
2011	Producer B	k3	present	absent	absent	absent	absent	present	absent	absent	present
2012	Producer B	k1a	absent	absent	present	absent	absent	absent	absent	absent	absent
2012	Producer B	k1b	present	present	absent	absent	absent	absent	absent	absent	absent
2012	Producer B	k2a	absent	present	absent	absent	present	absent	absent	absent	present
2012	Producer B	k2b	present	present	absent	absent	present	present	absent	absent	absent
2012	Producer B	k3	present	absent	absent	absent	absent	absent	absent	absent	absent
2011	Producer C	S1A	present	absent	present	absent	absent	absent	absent	absent	absent
2011	Producer C	S1B	absent	absent	present	absent	absent	absent	absent	absent	absent
2011	Producer C	S2A	absent	present	absent	absent	absent	absent	absent	absent	absent
2011	Producer C	S2B	absent	absent	absent	absent	absent	absent	absent	absent	absent
2011	Producer C	S3	present	present	present	absent	absent	absent	absent	absent	present

Table A2.2.5.1

Results of generalized linear models or, where there was quasi-complete separation of data, Fisher's exact tests, examining the prevalence of five parasites in 48 commercially-produced bumblebee colonies of two subspecies, from three producers, in two years. The significant effects of year and subspecies on *Nosema bombi* are due to Producer C (which was only represented by 5 hives of the commercial subspecies from 2011) having 100% infection.

Parasite	**Term**	**Deviance/df**	**χ^2**	**df**	**P**
Apicystis bombi	Model fit	1	1.17	4	0.884
(over dispersed model)	Year		0.634	1	0.426
	Producer		0.466	2	0.792
	Subspecies		0.13	1	0.718
Crithidia bombi	Model fit	1.64	7.79	4	0.1
	Year		4.32	1	0.038
	Producer		3.14	2	0.209
	Subspecies		0.002	1	0.965
Nosema bombi	Model fit	Quasi-complete separation of data			
	Year	Fisher's			0.017
	Producer	Fisher's			0.002
	Subspecies	Fisher's			0.012
Deformed wing virus	Model fit	Quasi-complete separation of data			
	Year	Fisher's			0.036
	Producer	Fisher's			0.105
	Subspecies	Fisher's			1
Nosema ceranae	Model fit	Quasi-complete separation of data			
	Year	Fisher's			0.521
	Producer	Fisher's			0.188
	Subspecies	Fisher's			0.093

Table A.2.3.1.2

Results of generalized linear models or, where there was quasi-complete separation of data, Fisher's exact tests, examining the prevalence of eight parasites in 25 pollen samples from three producers in two years, that were provided as food with commercially-produced bumblebee colonies.

Parasite	**Term**	**Deviance/df**	**χ^2**	**df**	**P**
Apicystis bombi	Model fit	0.008	2.36	3	0.5
	Year		0.878	1	0.349
	Producer		2.12	2	0.346
Crithidia bombi	Model fit	0.26	2.11	3	0.55
	Year		1.87	1	0.172
	Producer		0.371	2	0.831
Nosema bombi	Model fit	1.98	4.19	3	0.241
	Year		0	1	1
	Producer		3.65	2	0.161
Deformed wing virus	Model fit	Quasi-complete separation of data			
	Year	Fisher's			0.229
	Producer	Fisher's			0.336
Nosema ceranae	Model fit	Quasi-complete separation of data			
	Year	Fisher's			1
	Producer	Fisher's			0.431
Nosema apis	Model fit	Quasi-complete separation of data			
	Year	Fisher's			1
	Producer	Fisher's			0.048
American foulbrood	Model fit	Quasi-complete separation of data			
	Year	Fisher's			1
	Producer	Fisher's			1
Ascosphaera	Model fit	1.002	0.579	3	0.901
(over dispersed model)	Year		0.095	1	0.758
	Producer		0.579	2	0.749

Table A5.3.1.1

***Nosema ceranae* presence in bumblebee spp. from various locations in England, UK.**

Location	Species	*N*	Molecular presence	Spore presence	Ave Spores per infected bee ±SE
Cambridgeshire	*B. hortorum*	6	6 (100%)	1	4000
	B. hypnorum	9	2 (22%)	0	-
	B. lapidarius	99	44 (44%)	8	7800± 4145.2
	B. lucorum	33	18 (55%)	4	11750± 4497.7
	B. pascuorum	15	8 (53%)	1	-
	B. pratorum	16	3 (19%)	0	-
	B. terrestris	44	23 (52%)	11	5409.1± 906.6
Merseyside	*B. hypnorum*	9	0	-	-
	B. lapidarius	17	3 (18%)	0	-
	B. lucorum	30	0	-	-
	B. pascuorum	2	0	-	-
	B. pratorum	12	2 (16%)	0	-
	B. terrestris	73	24 (33%)	4	4550± 2517.1
Oxfordshire	*B. lapidarius*	25	0	-	-
	B. lucorum	9	0	-	-
	B. pascuorum	12	0	-	-
	B. pratorum	16	0	-	-
	B. terrestris	88	0	-	-
Kent	*B. lapidarius*	21	4 (19%)	0	-
	B. lucorum	12	0	-	-
	B. pascuorum	5	2 (40%)	0	-
	B. pratorum	19	3 (16%)	0	-
	B. terrestris	94	22 (23%)	3	2666.7± 666.7
Essex	*B. lapidarius*	30	0	-	-
	B. lucorum	2	0	-	-
	B. pascuorum	38	0	-	-
	B. pratorum	25	0	-	-
	B. terrestris	3	0	-	-
Total freq.	7 diff Sp.	764	164	32	6628.1± 1261.3
Total %			21% (164/767)	4% (32/764) 19% (32/164)	

Table A6.2.4.1

Pairwise differences between **s**ucrose sensitivity scores 5 days post treatment. Mann-Whitney U tests with P values following Bonferroni correction.

Treatments **Ingest | Inject**	**Ctrl | Ctrl**	***A.b* | -**	***A.b* | Ctrl**	**- | DWV**	**Ctrl | DWV**	***A.b* | DWV**
Nothing	*U* = 14.3 *P* > 0.99	*U* = -51.5 *P* = 0.115	*U* = -34.4 *P* > 0.99	*U* = 15.3 *P* > 0.99	*U* = 12.9 *P* > 0.99	*U* = 13.6 *P* > 0.99
Ctrl | Ctrl		*U* = 65.8 *P* = 0.008	*U* = 48.6 *P* = 0.224	*U* = -0.97 *P* > 0.99	*U* = 1.4 *P* > 0.99	*U* = 0.922 *P* > 0.99
***A.b* | -**			*U* = 14.1 *P* > 0.99	*U* = 66.8 *P* = 0.007	*U* = 64.4 *P* = 0.011	*U* = 64.9 *P* = 0.014
***A.b* | Ctrl**				*U* = 49.6 *P* = 0.193	*U* = 47.2 *P* = 0.277	*U* = 47.8 *P* = 0.307
- | DWV					*U* = 2.4 *P* > 0.99	*U* = 1.89 *P* > 0.99
Ctrl | DWV						*U* = -0.498 *P* > 0.99

Table A6.2.4.2

Pairwise differences between sucrose sensitivity scores over the 15 days of the experiment in bees treated with *A. bombi* or *A. bombi* | PBS injection.

A. bombi treated bees			
	Day 5	Day 10	Day 15
Day 0	U = -41.8 P < 0.001	U = -21.7 P = 0.202	U = -17.2 P = 0.629
Day 5		U = -20.1 P = 0.296	U = -24.6 P = 0.125
Day 10			U = 4.5 P > 0.99

Table A6.2.4.3

Pairwise differences between the Lipid: body size ratio of treated bumblebees following a Bonferroni correction.

Treatments **Ingest | Inject**	**Ctrl | Ctrl**	***A.b* | -**	***A.b* | Ctrl**	**- | DWV**	**Ctrl | DWV**	***A.b* | DWV**
Nothing	*U* = -20.1 *P* > 0.99	*U* = 49.3 *P* = 0.310	*U* = 71.8 *P* = 0.008	*U* = -35.6 *P* > 0.99	*U* = -9.8 *P* > 0.99	*U* = 31.4 *P* > 0.99
Ctrl | Ctrl		*U* = -69.4 *P* = 0.013	*U* = -91.9 *P* <0.001	*U* = 15.6 *P* > 0.99	*U* = -10.3 *P* > 0.99	*U* = -51.4 *P* = 0.231
***A.b* | -**			*U* = 22.5 *P* > 0.99	*U* = 85.0 *P* = 0.001	*U* = -59.1 *P* = 0.073	*U* = -18.0 *P* > 0.99
***A.b* | Ctrl**				*U* = -107.5 *P* < 0.001	*U* = -81.6 *P* = 0.001	*U* = -40.5 *P* = 0.956
- | DWV					*U* = 25.8 *P* > 0.99	*U* = -67.0 *P* = 0.020
Ctrl | DWV						*U* = -41.2 *P* = 0.882

Table A2.3.2.1

Results of generalized linear models or, where there was quasi-complete separation of data, Fisher's exact tests, examining the prevalence of four parasites in bumblebees in Experiment 1 which had ingested either pollen or bumblebee faeces from infected colonies of commercially-produced bumblebees. No control-treated bees had any of the parasites and they were not included in the analyses. Day of death or last day of observation was included as a covariate in the models. The significant effect of time was largely due to none of the bees which survived to the end of the experiment being positive for *N. ceranae* or *C. bombi*.

Parasite	Term	Deviance/df	χ^2	df	P
Nosema ceranae	Model fit	1.87	22.6	5	<0.001
	Treatment		0.001	1	0.978
	Hive		1.29	2	0.73
	Day of death		20.8	1	<0.001
Nosema bombi	Model fit	Quasi-complete separation of data			
	Treatment	Fisher's			0.031
	Hive				
	Day of death				
Apicystis bombi	Model fit	1.66	12.7	5	0.026
	Treatment		7.39	1	0.007
	Hive		3.57	2	0.311
	Day of death		4.83	1	0.028
Crithidia bombi	Model fit	Quasi-complete separation of data			
	Treatment	Fisher's			1
	Hive				
	Day of death				