Bumble bees and their parasites across European communities: *Sphaerularia bombi* in native and non-native hosts

Thesis submitted for the degree of Doctor of Philosophy (PhD)
University of London

Royal Holloway, University of London
Egham Surrey
TW20 0EX

Catherine Mary Jones

September 2013

Declaration page

I, Catherine Mary Jones, declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, it is always clearly stated.

Catherine Mary Jones

Acknowledgments

First and foremost, I would like to thank my supervisor, Mark Brown, for selecting me as his first PhD student at Royal Holloway, University of London. Without his support and encouragement, the four years of my research would not have been possible. I would also like to thank my advisor, Vincent Jansen, and the technical and administration staff in the School of Biological Sciences, especially Tracey Jeffries, Rob Prouse and Sue Baldwin.

I am grateful to the BBSRC for the DTG to fund my research and the YES and Vitae courses that I attended.

My thanks also go to Joe Colgan for sharing his knowledge about Irish bumble bees and nematodes, to Roland Loosli, Paul and Regula Schmid-Hempel for helping with my fieldwork in Zurich, to Joe Colgan, Jim Carolan, and Nicola Marples for helping with my fieldwork in Dublin, and to Heather Rumble for looking after my bees in the lab while I was away.

I would like to thank all the members of the Brown Lab at Royal Holloway, for their support in the Bee Room and the Ecology Lab, especially Matthias Fürst and Gemma Baron - and for organising so many fabulous social events.

My thanks also go to Matthias Fürst, for showing me how to extract DNA from tissue samples and prepare it to send for sequencing, to Frances Medaney, for always being willing to help in the Molecular lab and to Selena Brace for spending days (weeks?) helping me to edit, align and analyse my DNA sequences and building the phylogenetic tree.

My thanks and love to Christine and Eric Jones for reading my first manuscript and asking questions about my research, and to Oscar, Percy, Daisy and Millie (who is no longer with us) for providing support as only a dog or cats can.

Finally, many thanks, and lots of love, to Lindsay Pilling, for financial support, for practical advice and always being there to give me encouragement and a hug.

Abstract

My research investigated the host-parasite relationships between *Bombus* species and their generalist parasites, considering both the host community and the parasite community. This research was based in Europe and thus the focus was on European *Bombus spp.* particularly *B. terrestris* and *B. hypnorum*, and their parasites species, particularly the nematode *Sphaerularia bombi.*

Bumble bees are important pollinators and are in global decline. Thus investigating their parasites, one of the factors that may be driving the decline, is vital both for the continued provision of this ecosystem service and for the conservation of bumble bees.

Initially I investigated the generalist endoparasites, particularly *S. bombi,* across three European populations of *B. terrestris*, collected in England, Switzerland and Ireland. I found that parasite prevalence differed across the European populations sampled e.g. the prevalence of *S. bombi* in *B. terrestris* queens is highest in Ireland. I also provide details of the bee husbandry and dissection methods used throughout my research.

I focused on England to examine the prevalence and impact of parasite communities found in the non-native species, *B. hypnorum*, and in five native *Bombus* host species. I also estimated the genetic diversity of the non-native *B. hypnorum*, from both the invaded (i.e. England) and native range (i.e. continental Europe), and that of two native *Bombus* species, *B. terrestris* and *B. lucorum*. The invasive *B. hypnorum* had higher parasite prevalence and lower functional genetic diversity than native species. Although parasites had a higher impact on the invader's fitness than on native species, parasites and low genetic diversity have not prevented the rapid invasion of the UK by *B. hypnorum*.

Having observed that infected *B. hypnorum* queens do not appear to deposit *S. bombi* parasite larvae in their faeces, I quantified parasite reproduction in infected hosts to examine the competence of the non-native *B. hypnorum* as a host for this generalist parasite. I found that *S. bombi* larvae are not deposited in the faeces of infected *B. hypnorum* queens suggesting that *B. hypnorum* is not a competent host for *S. bombi*. The host–parasite relationship between *S. bombi* and this non-native

bumble bee may alter the relationships between *S. bombi* and congeneric native host species.

Using standard molecular techniques, I investigated the phylogeography of *S. bombi* across Europe and asked whether the *S. bombi* parasites, found in non-native *B. hypnorum* in England, originate from England or from Continental Europe: Did the non-native *B. hypnorum* acquire parasites in the UK or were they co-introduced with the invading host? I found that the *S. bombi* population did not appear to be structured across the European native and non-native hosts sampled and therefore I was unable to establish whether these parasites were acquired or introduced.

Finally, I discuss what I have discovered during my research, how has this work added to the current knowledge on the subject and which areas warrant further investigation.

Table of Contents

Table of Figures

Table of Tables

Table of Plates

Chapter 1. General introduction

Introduction

Climate change, the loss of global biodiversity and biological invasions threaten both the natural world and our ability to produce sufficient food to feed the growing global human population. Agriculture (including food production) depends on ecosystem services (e.g. pollination services provided by bees and other insects; pest control provided by predators and parasites: Cock *et al* 2012). Thus human well-being relies on a diverse natural flora and fauna that both provide and support these ecosystem services.

Climate change is likely to increase the rate of change of species distributions: species may expand, shift (e.g. latitudinal shifts, i.e. towards the North and South poles or altitudinal shifts i.e. to higher elevations) or contract. In addition species may go extinct, either from an area or globally. Changes to distributions of insects have been reported for Lepidoptera (butterflies and moths), where 30-75% of species investigated expanded northwards and 20% contracted southwards (Parmesan 2006, Parmesan & Yohe 2003, Warren *et al* 2001, Parmesan *et al* 1999). For Odonata (dragonflies and damselflies) Parmesan (2006) and Hickling *et al* (2006) found a northward shift. Climate change and changing insect distributions may have serious consequences for human well-being as the introduction of disease by insect vectors expanding their ranges can impact livestock. For example the range of the *Culicoides spp.* midge has extended northwards into north west Europe including the UK. *Culicoides spp.* is the insect vector for Bluetongue virus and has introduced this disease of ruminants to domestic livestock (Carpenter et al 2009, Wilson & Mellor 2009).

Species introductions and biological invasions

Historically, crops and livestock have been transported around the globe and some species introductions have been beneficial to human well-being (Elton 1958). The introduction of *Bombus spp.* to New Zealand, at the end of the 19th and beginning of the 20th century, to pollinate red clover to feed the introduced livestock is one such example (MacFarlane & Griffin, 1990). However some introduced species become

invasive and have a detrimental effect e.g. the American mink, *Mustela vison* introduced to the UK to farm for fur, escaped (or may have been released) and is a predator of the declining native water voles *Arvicola amphibius* (Bonesi & Palazon, 2007).

A biological invasion occurs when a non-native species is introduced to a new location, becomes established and rapidly expands its range (Elton 1958, Williamson 1998). Such invasions may cause ecological damage and economic costs (Pimentel *et al* 2005). Biological invasions affect species interactions (e.g. between hosts and their parasites), the diversity and abundance of species, and the provision of ecosystem services (such as pollination), which are important for human well-being (Pejchar & Mooney 2009, Vila *et al* 2010).

Parasites

Parasites exist within all ecological communities and are important in regulating host populations (Price 1980, Hudson *et al* 1998, Brown *et al* 2003). Over 50% of all organisms are parasites (Price 1980). The importance of interactions between parasites and their hosts is often overlooked in conservation (Henson *et al* 2009), but understanding host–parasite relationships can be crucial to the success of a project (e.g. the large blue butterfly, *Maculinea arion,* and their ant host, *Myrmica sabuleti,* in the successful re-introduction of the butterfly in Somerset: Thomas *et al* 1999).

The distributions of parasites, and their hosts, will be altered by biological invasions causing mismatches that may increase the parasite's impact, as either the host or the parasite, or both move into new areas (Dunn 2009). Host-parasite interactions are structured by local adaptation (Kawecki & Ebert 2004) and relative migration rates of hosts and parasites are one of the factors affecting local adaptation (Greischar & Koskella 2007) (see below).

The role of parasites in biological invasions

Parasites may facilitate invasion success in several ways: an invading host may introduce parasites, acquire parasites or lose its parasites (Prenter *et al* 2004, Dunn 2009, Hatcher & Dunn 2011). An invading species may co-introduce its parasites

and diseases, which have a greater impact on native hosts, than on the invading host, giving the invading hosts an advantage over the native host. Two familiar examples of this, in the UK, are the grey squirrel, *Sciurus carolinensis,* and the American signal crayfish, *Pacifastacus leniusculus,* (Rushton *et al* 2006, Haddaway *et al* 2012). In the UK the non-native grey squirrel, has introduced squirrelpox virus that causes mortality in the native red squirrel, *S. vulgaris*, but not in the invading host (Rushton *et al* 2000, 2006). The invasive American signal crayfish, *Pacifastacus leniusculus,* has introduced crayfish plague, *Aphanmyces astaci,* that affects native crayfish in both Japan and the UK (Ohtaka *et al* 2005, Haddaway *et al* 2012).

Invading species may also acquire parasites from native hosts and, if the invading species is a less competent host, dilute (or reduce) the parasite impact on native hosts by presenting an alternative host (Norman et al 1999, Ostfeld & Keesing 2000). Alternatively, invading species may acquire parasites and act as a reservoir (a host where the parasite can successfully reproduce) for those parasites. These parasites may then spill-back into native hosts, increasing the impact of the parasites on the native hosts (Daszak *et al* 2000, Kelly *et al* 2009).

Parasite spill-over and parasite spill-back

Parasite spill-over occurs when parasites, infecting a host, reproduce abundantly and their offspring infect, or spill over to, other host species (Dunn 2009). If an invading host species introduces non-native parasites, these may spill over to infect native hosts (Daszak *et al* 2000, Hatcher & Dunn 2011). Parasite spill-back occurs when parasites infect a new host, where they can successfully reproduce and infect or spill back to, the original host (Norman *et al* 1999, Daszak *et al* 2000, Dunn 2009, Kelly *et al* 2009). Host competence (see below), either the competence of the original host (spill-over) or the competence of the new host (spill-back), will determine the extent of 'spillage'.

Parasite dilution

A decrease in parasite abundance in native species occurs through parasite dilution, where invading hosts provide an alternative host for native parasites 'diluting' the parasite prevalence and/or abundance in native hosts (Norman *et al 1999*, Ostfeld & Keesing 2000, Johnson & Thieltges 2010). For example, the

prevalence of *Bartonella spp*. parasites (bacteria transmitted by fleas) in native wood mice, *Apodemus sylvaticus,* decreased when the density of introduced bank voles, *Clethrionomys glareolus,* increased (Telfer *et al* 2005).

Reservoir host

In a reservoir host, parasites can reproduce successfully and abundantly, and be transmitted onto other host species (Norman *et al* 1999, Daszal *et al* 2000, Dunn 2009, Kelly *et al* 2009). For example, the invasive grey squirrel, *Sciurus carolinensis*, is a reservoir of squirrelpox virus that infects the native red squirrel, *S. vulgaris* (Thomas *et al* 2003, Tompkins *et al* 2003, Rushton *et al* 2006).

Host competence

Host competence determines the parasite's ability to infect, reproduce and successfully transmit of parasite offspring. Thus in a competent host a parasite can infect and reproduce successfully (Combes 2004, Schmid-Hempel 2011). However in a non-competent host, or a 'dead-end' host the parasite is unable to reproduce successfully.

Enemy release hypothesis (ERH)

Invasion success of a species may be facilitated by a release from natural enemies (Elton 1958, Keane & Crawley 2002, Torchin *et al* 2003). The enemy release hypothesis (ERH) proposes that invasive species arrive in a novel location without their natural enemies (herbivores, predators and parasites) and such release can potentially lead to a rapid increase in distribution and abundance of the invasive species (Keane & Crawley 2002, Torchin *et al* 2003). However, the results of ERH studies in animals systems vary: Dunn & Dick (1998) and MacNeil *et al* (2003) found that the invasive amphipods (*Gammarus pulex* and *G. tigrius*) were released from some of their parasites when compared with the native *G. duebeni celticus*. In contrast, Wattier *et al* (2007) found no loss of microspridian parasites in invasive populations of *Dikerogammarus villosus* (a freshwater amphipod) and Slothouber Galbreath *et al* (2007) found no release from microsporidian parasites in invasive populations of *Crangonyx pseudogracilis* (another amphipod). However, Colautti *et al* (2005) found that, when the sub-sampling of the native Eurasian range of the

European starling, *Sturnus vulgaris*, was taken into consideration, the North American population were not released from their helminth parasites.

The mechanisms that underpin enemy release may be stochastic or selective: Founding populations may arrive in novel locations without parasites by chance, may not have co-evolved with native parasite species therefore the parasites impact is reduced, or both mechanisms may work in tandem. Drake (2003) proposed a model for the initial establishment of an invasive species if released from its natural enemies. Although initial arrivals of large invading population are more likely to establish (due to propagule pressure), if those populations are escaping from a virulent parasite, large populations are more likely to co-introduce the parasite. Thus smaller populations escaping virulent parasites are more likely to establish and less likely to co-introduce a virulent parasites (Drake 2003).

Evolution of increased competitive ability (EICA)

If an invading species has escaped from its natural enemies, and its resources are not used for defence against that enemy, the resources may be used elsewhere, such as increased reproductive output. This alternative use of resources may give the invading species a competitive advantage and is known as the evolution of increased competitive ability (EICA) (Blossey & Notzold 1995).

Local adaptation

Parasite adaptation to their local hosts may be affected by many factors, including relative generation time and relative migration rates. Parasite population size (relative to that of the host), vertical transmission to host offspring (suggesting a close host-parasite relationship) and specialisation (i.e. parasites that specialize on one or a few hosts) may also affect the ability of the parasite to adapt to its host Greischar & Koskella (2007). Gandon (2002) suggested that specialists and virulent parasites are likely to be adapted to their hosts, although Ebert (1994) suggested that horizontal transmission and virulence may affect adaptation of the host to the parasite.

Generation time

Parasites often have a shorter lifecycle or generation time than their hosts, and therefore have multiple generations during a single generation of the host. Thus the shorter generation time of parasites, compared with that of their host, should lead to increasing local adaptation. Gandon and Michalakis (2002) found that shorter generations may reduce the ability of the parasite to adapt to its host when the parasite's genetic diversity is limited. Gandon and Michalakis (2002) also suggested that generation time would have little effect compared to migration rates.

Migration rates

Migration of individuals into a population (either parasite or host) increases gene flow and introduces the genetic variation required for natural selection to select from, or to work on. However, high migration rates homogenize populations reducing local adaptation (Greischar & Koskella 2007). Gandon (2002) predicted that if the gene flow of the parasite was greater than that of the host local adaptation would occur. Thus relative migration rates of parasites and their hosts (dispersal of hosts versus the dispersal of parasites) are important factors. Morgan *et al.* (2005) found that migration of parasites led to local adaptation of the parasite (in a bacteria-bacteriophage system) but Nash *et al.* (2008) found that restricted gene flow leads to local coevolution, in a butterfly parasite (*Maculinea alcon*) and ant host *Myrmica spp*. system. Although high migration homogenises populations, some migration introduces gene variation, and no migration limits the amount of genetic variation, a level of migration and gene flow exists where local adaptation is likely to occur.

Virulence and transmission routes

Virulent parasites, obligate parasites and vertically transmitted parasites are also predicted to be locally adapted to their hosts as they have a close relationship with their host (Greischar & Koskella 2007). Virulent parasites have a greater impact on their hosts than less virulent parasites. Obligate parasites must interact with a host to complete their lifecycle. Vertically transmitted parasites are transmitted from parent to offspring. Although Greischar and Koskella (2007) predicted that vertically transmitted parasites would show more local adaptation than horizontally transmitted parasites, which are transmitted to other host individuals, they found no significant differences.

Population size and reproduction

Local adaptation may also be affected by large population size and sexual reproduction, as these are likely to increase genetic variation, therefore the ability to evolve (Greischar and Koskella, 2007). Furthermore, parasites that specialise on one host are more likely to be locally adapted to their host species than generalists that infect multiple hosts, as are parasites with a simple lifestyle (i.e. one host) compared with parasites with more complex lifestyles (e.g. multiple host species).

Study system

My research investigates the host-parasite relationships between *Bombus* species and their generalist parasites, considering the communities of both host species and parasite species. Bumblebees are an important study system as they are key pollinators (Waser & Price 1981, Thomson *et al* 1986, Thomson & Goodall 2001) and provide valuable ecosystem services, which are important to human well-being (Klein *et al* 2006, Kremen *et al* 2007, Brown & Paxton, 2009). This research has been based in Europe and thus the focus is on European *Bombus spp*. particularly (but not exclusively) *B. terrestris* and *B. hypnorum*, and on European parasite species, particularly (but not exclusively) *Sphaerularia bombi*, a nematode worm.

Host system: Bumble bees

Global bumble bee declines

Bumble bees are ecologically and economically important as they pollinate crops and wild plants (Waser & Price 1981, Thomson *et al* 1986, Thomson & Goodall 2001). Bumble bee populations have declined globally in the 20th century and have continued to decline into the 21st century (Europe: Williams 1982, Grixti *et al* 2006, Kosior *et al* 2007, Fitzpatrick *et al* 2007, Brown & Paxton 2009, Williams & Osborne 2009, Potts *et al* 2010; North America: Cameron *et al* 2011; South America: Arbetmann et al 2012, Morales et al 2013). As 30% of global crop production is from crops that require pollination (Klein *et al* 2006) these declines are likely to affect global food supply and therefore human well-being.

Factors driving these declines are the loss of food sources (pollen and nectar), loss of nesting sites and hibernation sites due to land use change and agricultural

intensification (Kearns *et al* 1998, Goulson *et al* 2008), use of pesticides, both herbicides that reduce 'weeds' in crops i.e. wild flowering plants and insecticides that reduce pest insects (Whitehorn *et al* 2012, Gill *et al* 2012) and parasites (*Nosema bombi* implicated in USA: Cameron *et al* 2011, *Apicystis bombi* implicated in South America: Arbetmann *et al* 2012, *Locustacarus buchneri* implicated in Japan: Goka *et al* 2006).

Bumble bee lifecycle

The life cycle of the bumble bee queen is annual: the mated queen hibernates through the winter and emerges in the spring. Initially she forages for nectar and pollen, to replenish her depleted reserves, then seeks a suitable nesting site, where she founds a new colony (Sladen, 1912; Alford 1969b, 1975) (Figure 1-1). The queen gathers nectar and pollen and forms a pollen ball on which she lays her eggs and builds a wax cup that she fills with nectar (Sladen, 1912; Alford 1969b, 1975). She broods the eggs, keeping them warm while the first brood develops, producing workers, then later males and new queens (gynes) which mate and hibernate (Sladen, 1912; Alford 1969b, 1975) (Figure 1-1).

Bumble bee global distribution

The native global distribution of bumble bees covers the northern temperate zone from Europe across Asia to Japan and across North America and North to the Arctic (Williams & Osborne 2009). The native range also extends into South America (Williams & Osborne 2009). Bumble bees were introduced to New Zealand at the end of 19th century and beginning of the 20th century to pollinate the red clover crop to feed livestock (MacFarlane & Griffin 1990), and these are thought to have invaded Tasmania (Schmid-Hempel *et al* 2007, Allen *et al* 2007).

Haplodiploidy

Bumble bees are haplodiploid and their sex is determined at a single locus by complementary sex determination i.e. if there are two different alleles at the sex-determining locus the individual will be female and if there is just one allele at the sex-determining locus the the individual will be male. Diploid (heterozygous) females are produced from fertilised eggs (with two different alleles at the sex determining locus) and haploid (hemizygous) males are produced from unfertilised

eggs (with just one allele at the sex determining locus). However, diploid (homozygous) males occur from fertilised eggs when there are two identical alleles at the sex-determining locus. For example a (diploid) queen has two alleles at the sex-determining locus (which I will call 'a' and 'b') mates with a (haploid) male with one allele at the sex-determining locus (which I will call 'c') produces (diploid) workers from fertilised eggs (i.e. 'ac' and 'bc') in the first brood, but if a queen ('ab') mates with a closely related male ('a') she will produce 50% diploid workers ('ba') and 50% diploid males ('aa') in her first brood. The presence of males in the first brood (which is usually just females) at a 50:50 sex ratio can be used as a standard protocol to identify diploid male production (Gerloff & Schmid-Hempel 2005) and such males indicate low genetic diversity (Duchateau *et al* 1994, Whitehorn *et al* 2009).

Commercial bumble bees

Bumble bees can be domesticated and used to enhance pollination in greenhouse crops such as tomatoes, cucumbers, raspberries and strawberries, and orchard crops such as apples, pears, cherries etc. (Velthuis & van Doorn 2006).

Commercial colonies of *B. terrestris* are shipped around the world to enhance pollination but may escape and become invasive (Goulson 2003, Japan: Matsumara *et al* 2004, South America: Torretta *et al* 2006). Escaping commercial bumble bees may adversely impact native flora and fauna (Matsumara *et al* 2004, Ings *et al* 2006) and introduce parasites (Graystock *et al* 2013, USA: Cameron *et al* 2011, South America: Arbetmann *et al* 2012, Japan: Goka *et al* 2006).

Commercial colonies are often *B. terrestris*, which may be a non-native species or subspecies (Ings *et al* 2006). In the UK the *B. terrestris* subspecies *B. terrestris terrestris* (from Continental Europe) and *B. terrestris dalmatinus* (from Eastern Europe) were commonly used although B*. terrestris audax,* which is endemic to British Isles, is now also available (Ings *et al* 2006).

British bumble bees

In England there are six common species of bumble bee (known as the 'big six' Williams 1982, BBCT): *B. terrestris, B. lucorum, B, pascuorum, B. pratorum, B. hortorum* and *B. lapidarius. B. terrestris*, *B. lucorum* and *B, pascuorum* are common

and widespread (Edwards & Jenner 2005) and *B. pratorum, B. hortorum, B. lapidarius* and *B. jonellus* are widespread (Edwards & Jenner 2005). But many species are declining such as *B. monticola* which is not found in South East England (Edwards & Jenner 2005) and several species have gone extinct over the last 150 years*: B. pomorum* has not been recorded since 1864 and is probably extinct (NHM, Alford 1975), *B. cullumanus* has not been recorded since 1941 and is probably extinct (NHM, Alford 1975) and *B. subterraneous* has not been recorded since 1988 and is probably extinct (NHM, BBCT). However, there is a current re-introduction project led by Dr. Nikki Gammans to re-introduce *B. subterraneous* from Sweden to Dungeness in Kent, where it was last recorded in England.

In my research, I have used five native species *B. terrestris*, *B. lucorum, B. pascuorum, B. pratorum and B. jonellus* and the non-native *B. hypnorum.* All of these species occur in Continental Europe although the subspecies *B. terrestris terrestris* (which has a white tail and other subspecies) occurs in Continental Europe whereas in the UK queens of our native subspecies *B. terrestris audax* have 'buff' tails (Widmer *et al* 1998).

B. hypnorum

B. hypnorum, the tree bumble bee, is a distinctive bumble bee with a ginger thorax, a black head and abdomen, and a white tail. This non-native species was first reported in the UK in Landford, Wiltshire in 2001 (Goulson & Williams 2001). It has successfully invaded and expanded its range across England and Wales (BWARS, BBCT). *B. hypnorum* was recorded in Lennoxtown, Scotland in 2013 (BWARS, BBCT) and been reported in Iceland in 2010 (Atlas Hymenoptera). Its native range extends across Europe, and Russia to Japan (NHM, Atlas Hymenoptera).

B. hypnorum is in the subgenus *Pyrobombus,* along with the closely related species: *B. pratorum, B. jonellus* and *B. monticola. B. pratorum* and *B. monticola* successfully invaded Ireland in the 1940s and 1970s respectively (Speight 1974, Fitzpatrick *et al* 2007). *B. hypnorum* is also closely related to the north American species *B. perplexus* and may be conspecific (Hines *et al* 2006, Cameron *et al* 2007). Previous studies of *B. hypnorum* have found that they have the ability to multiply mate, which is unusual in *Bombus* species (Pouvreau 1963, Estoup *et al* 1995, Schmid-Hempel & Schmid-Hempel 2000, Paxton *et al* 2001).

Röseler (2002) and Alford (1975) also reported that *B. hypnorum* queens infected with the castrating parasite *Sphaerularia bombi* were able to found a colony and produce offspring.

In the UK bumble bee queens usually are univoltine *i.e.* produce one colony *per annum* but some species, are bivoltine *i.e.* can produce two colonies *per annum* (Edwards & Jenner 2005, BWARS). Bivoltine species include three *Pyrobombus* species: *B. hypnorum, B. pratorum* and *B. jonellus.* Bivoltinism occurs more frequently in the south of England and in favourable conditions (e.g. *B. terrestris*: Stelzer *et al* 2010).

Bee husbandry in the laboratory

Bumble bees are an excellent study system as some species have been domesticated and produced commercially. Thus colonies can be reared in the laboratory from wild caught queens, or commercial colonies (produced for pollination) can be purchased and experimentally infected (Velthuis & van Doorn 2006, Kelly 2009).

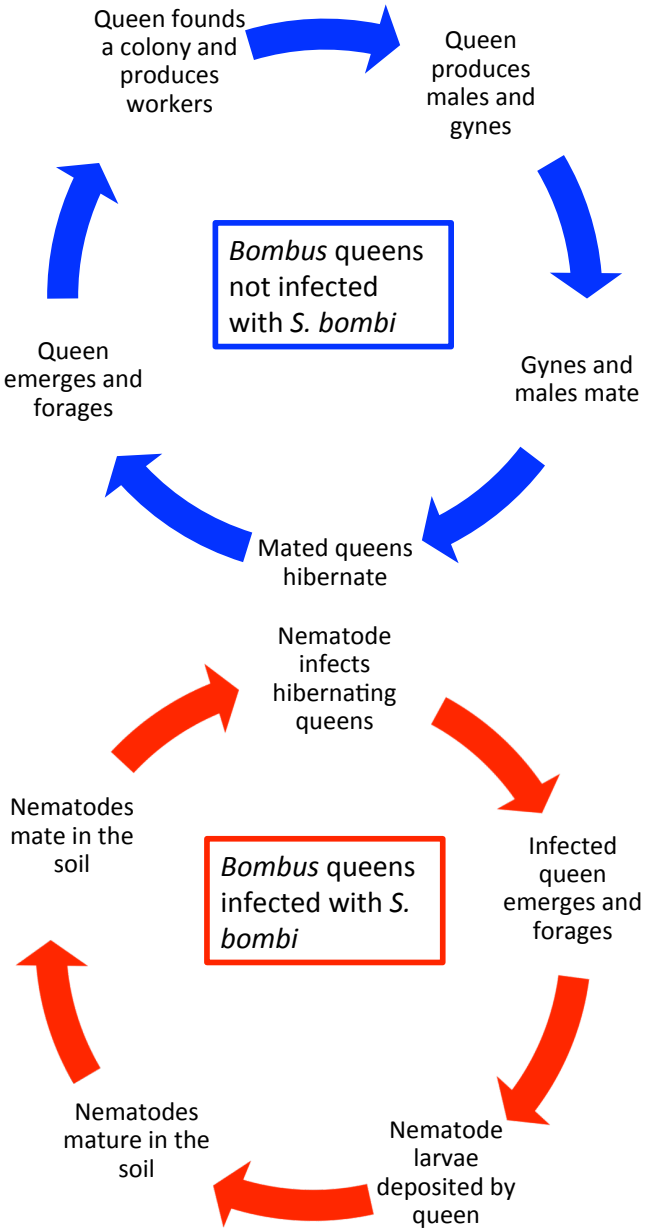

Figure 1-1 The lifecycles of *Bombus* queens and *Sphaerularia bombi* infecting *Bombus* queens

The lifecycle of a typical *Bombus* colony is shown in blue and the lifecycle of *S. bombi* infecting *Bombus* queens is shown in red.
The *Bombus* queen emerges, in the spring, forages and a nesting site where she founds a colony and produces workers. Later, in the summer, the queen produces gynes (new queens) and males that mate. The 'old' queen, the workers and the males die and only the mated queens hibernated and survive the winter.
Infected *Bombus* queens emerge from hibernation and forage, while the nematode inside everts her uterus and produces eggs. The nematode castrates the host and alters her behaviour: infected queens deposit nematode larvae, in their faeces, at *Bombus* hibernation sites. The nematode larvae mature and mate in the soil, then infect queens hibernating in the soil.
Modified from Poinar and van der Laan 1972

Parasite systems

Sphaerularia bombi

Sphaerularia bombi (Dufour) is an entomopathogenic nematode (round worm) in the order Tylenchida. It was first described by Dufour in 1837 (Alford 1975) and is unusual as it only infects bumble bee queens (Schmid-Hempel 1998). The only other member of the *Sphaerularia* genus is *S. vespae*, a parasite of hornets, *Vespa spp*. (Sayama *et al* 2007). *S. bombi* is an obligate (i.e. it requires a host to complete its lifecycle), highly virulent parasite that castrates bumble bee queens.

S. bombi infects bumble bee queens while they are hibernating in the soil (Poinar & van der Laan 1972) (Figure 1-1). When the queen emerges in the spring, the female *S. bombi* nematode everts her uterus inside the queen. The nematode is about 2mm long and the everted uterus, when fully extended can be approximately 20mm long. The nematode produces thousands of eggs that develop into stage 1, stage 2 and stage 3 larvae (L1, L2 and L3 respectively) inside the bee (Alford 1975, Kelly 2009). The queen deposits stage 3 (L3) nematode larvae into the soil, where they mature and mate. Only the mated female parasites infect the next generation of hibernating bumble bee queens.

The impact of *S. bombi* on *Bombus* queens is two-fold: firstly the parasite alters their behaviour and secondly, the parasite castrates the queens. When infected queens emerge from hibernation, instead of seeking nesting sites, the parasite alters their behaviour and they deposit nematode larvae at hibernation sites (Lundberg & Svensson 1975, Schmid-Hempel, 1998). This behaviour change ensures that parasite offspring are deposited in a suitable location to infect new hosts. The parasite also prevents the corpora allata inside the queen from developing. Therefore the queen's ovaries do not develop and she is unable to found a colony, lay eggs or produce any offspring, and thus she is lost to the bumble bee population as a foundress queen. However, some studies have reported that some species of bumble bee are resistant to castration by *S.bombi,* and can found a colony despite infection (Alford 1975, Röseler 2002). Two species, reported to have shown some resistance are *B. hypnorum* and *B. hortorum.* Both are found in Britain – the former a recent and successful arrival from continental Europe and the latter a widespread species described by the Bumblebee Conservation Trust (BBCT) as 'one of the big six'. Both the successful

establishment of *B. hypnorum* in England and its reported resistance to *S. bombi* are unusual and warrant investigation.

In England studies have reported prevalence levels of *S. bombi* in *Bombus* queens from 16% to 80% (Cumber 1949, Alford 1969a). Cumber (1949) found 23% prevalence in *B. terrestris*, 69% in *B. lucorum* (in April and May) and >80% in *B. agrorum* (=*B. pascuorum*) at Putney Heath in Surrey. Alford (1969a) found 16% to 33% across *Bombus spp.* in Englefield Green, Surrey and Tring, Hertfordshire with the highest levels of prevalence in *B. lucorum.* In continental Europe parasite prevalence of 34% in Sweden and 37-93% in France has been reported (summarised in MacFarlane *et al* 1995). MacFarlane & Griffin (1990) reported prevalence levels of 7% in *B. hortorum* and 56% in *B. terrestris* in New Zealand. In North America, Medlar (1962, cited in Goldblatt & Fell 1984) reported *S. bombi* prevalence in *Bombus* queens of 12% in Wisconsin, Fye (1966) reported 4-22% in Ontario, Poinar (1974, cited in Goldblatt & Fell 1984) reported 1-5% in California Goldblatt and Fell (1984) reported 1.7-7% in Virginia and recently Maxfield-Taylor *et al* (2011) reported prevalence of 30% in Oregon. In South America, Plischuk & Lange (2012) recently reported prevalence of 8-20% in *B. atratus*.

Apicystis bombi

Apicystis bombi (Lipa & Triggiani) is a protozoan parasite of bumble bees. *A. bombi* is a neogregarine, which are usually ingested, and infects adult bumble bees (Schmid-Hempel, 1998). The impacts are severe: Infected colonies fail to thrive and infected queens do not found colonies (Schmid-Hempel 1998). Durrer and Schmid-Hempel (1995) found that infected workers have degraded fat bodies.

Crithidia bombi

Crithidia bombi (Lipa & Triggiani), a trypanosome, is a single-celled gut parasite. It is horizontally transmitted via the faecal-oral route both within the colony and between colonies (Durrer & Schmid-Hempel 1994). It can also be transmitted vertically from foundress queens to their daughter queens, which may survive to found their own colonies (Ulrich *et al* 2011). The prevalence is usually high, between 10-35% of bees infected (Shykoff & Schmid-Hempel 1991, Korner & Schmid-Hempel 2005) but infected colonies are able to survive and reproduce but have a lower fitness (40%) (Brown *et al* 2000, Brown *et al* 2003a). A study of

Crithidia spp. from Switzerland and Alaska identified two separate lineages and it is now classified as two separate species, *C. bombi* and *C. expoeki*, with a distribution across Europe and North America (Schmid-Hempel & Tognazzo 2010).

Nosema bombi

Nosema bombi (Fantham & Porter) is a single-celled microsporidian parasite found in the gut, the malpighian tubules and the fat tissues of bumble bees. Although the prevalence of *N. bombi* is low it may be a cause of death in hibernating queens (Schmid-Hempel 1998).

Locustacarus buchneri

Locustacarus buchneri (Stammer) is a tracheal mite. The impact of *L. buchneri* on *Bombus* queens is currently unknown although studies by Husband & Sinha (1970) and Otterstatter & Whidden (2004) on males and workers show lethargy and reduced longevity in *B. bimaculatus* and *B. occidentalis*.

Thesis studies

My original intention in Chapter 2 was to investigate whether the parasite *S. bombi* was locally adapted to their *B. terrestris* hosts. However, as this was not possible I asked whether the parasite community varies across Europe: I investigated the generalist endo-parasites, across three European populations of *B. terrestris*, collected in England, Switzerland and Ireland. My primary focus was the nematode parasite, *S. bombi,* but I also record other generalist parasites. I also provide detailed methods, used throughout my research, for bee husbandry in the laboratory and bee dissection in Chapter 2.

In Chapter 3, I investigated whether an invading non-native species has been released from the parasites from its native range (the Enemy Release Hypothesis), or if parasites have played an alternative role in the invasion success of this species. I focused on the prevalence and impact of the parasite communities found in the non-native host species, *B. hypnorum* and in five native *Bombus* host species: Three common species *B. terrestris, B. lucorum* and *B. pascuorum* and two closely related species *B. jonellus* and *B. pratorum*. I also estimated the genetic

diversity of the non-native *B. hypnorum*, from both the invaded and native range, and that of two native *Bombus* species, *B. terrestris* and *B. lucorum.*

As I discovered in Chapter 3 that the non-native *B. hypnorum* infected by *S. bombi* parasites were able to produce offspring, in Chapter 4 I investigated whether *B. hypnorum* is a competent host for *S. bombi*. I also discuss the implications of the competence of a non-native host for the native *Bombus* populations.

Again following on from my findings in Chapter 3 on the prevalence of *S. bombi* in non-native hosts, Chapter 5 is a first investigation of the provenance of parasites in non-native hosts. Are the *S. bombi* parasites found in the non-native *B. hypnorum* queens in England likely to originate from England or from Continental Europe? Did the non-native *B. hypnorum* acquire parasites in the UK or were they co-introduced by the invading host?

I have included two pilot projects in the Experimental Appendices: the experimental infection of summer queens with *S. bombi* in 2010 and the experimental infection of *B. hypnorum* and *B. terrestris* queens with *S. bombi* in 2011.

Finally, I discuss what I have discovered over the years of my research, how this work has added to the current knowledge on the subject and which areas warrant further investigation.

Chapter 2. Parasites across three European populations

Abstract

The parasites and pathogens that affect wild animals may also affect domestic livestock. In addition, the impact of parasites and pathogens may limit the ecosystem services provided by wild populations, such as pollinators. As hosts, parasites and host-parasite interactions vary geographically, it is vital to understand the parasite community, and its impact, on economically important animals across their range.

I investigated the parasite community, and its impact, on a key pollinator, the bumble bee *Bombus terrestris,* across three European populations: England, Switzerland and Ireland.

I found that parasite prevalence, species richness and impact differed across the populations sampled. In *B. terrestris* queens prevalence of *Sphaerularia bombi* was highest in Ireland, the prevalence of *Apicystis bombi* and *Crithidia bombi* was highest in Switzerland. These parasites reduce the survival and colony founding success of *Bombus* queens, and therefore are likely to impact on bumble bee populations across Europe.

Introduction

Parasites exist within all ecological communities and are important in regulating host populations (Hudson *et al*, 1998; Brown *et al*, 2003). Parasites and pathogens that affect wild populations may also affect livestock (Daszak *et al* 2000, Jones *et al* 2008) and may cross-over from wild populations, via domestic livestock, to infect the human population directly (e.g. bird flu: Ferguson *et al* 2005). An understanding of host-parasite interactions and parasite ecology is necessary to manage outbreaks of parasites and diseases that threaten wildlife, livestock and our health.

Evolutionary processes

Evolutionary processes, such as natural selection, gene flow and mutation, occur in all biological organisms (Darwin 1859, Futuyma 2005). In nature, plants and animals must evolve more rapidly than their natural enemies (herbivores, predators and

parasites) to survive. The Red Queen hypothesis (van Valen 1973, Futuyma 2005) proposes that prey must run faster (i.e. evolve more quickly) than their predators (and predators must run faster to catch their prey) to survive. This 'race' between predators and prey, also applies to parasites and their hosts (e.g. Decaestecker 2007). Hosts are under selection pressure from parasites to adapt to parasites and, reciprocally, parasites are under selection pressure to adapt to their hosts leading to a pattern of local coevolution and adaptation (Thompson 2005, Greischar & Koskella 2007).

Local adaptation

Adaptation of parasites to their local hosts (or hosts to their local parasites) may be affected by many factors, including relative generation times, relative migration rates and the relative population size of hosts and parasites. Local adaptation may be affected by parasite virulence, whether the parasite is a generalist or a specialist, and whether parasite transmission is horizontal or vertical (see Chapter 1).

Parasites and social insects

Host-parasite interactions between social insects, such as bees, wasps and ants, and their parasites are particularly interesting due to the high levels of horizontal parasite transmission both within and between colonies (Schmid-Hempel, 1998). Despite, the within colony 'hygiene' behaviours (e.g. ants cleaning fungal spores from nest-mates: Cremer 2007, Ugelvig & Cremer 2012) and the altruistic behaviours of some individuals in social insect colonies (e.g. infected ants leaving the nest to die: Schmid-Hempel 1998), social insects are host to a wide range of parasite species (Schmid-Hempel, 1998). Some host behaviours, in social insects, are parasite manipulations to enhance the biological fitness of the parasite (e.g. a fungus that alters the behaviour of an infected ant to ensure onward transmission: Pontoppidan et al 2009). Thus from both a parasite and a host perspective social insects and their parasites provide a diverse study system.

Bumble bees

Bumble bees (*Bombus spp*.) are key pollinators of crops and wild flowers (Waser & Price 1981, Thomson *et al* 1986, Thomson & Goodall 2001) and provide valuable ecosystem services, which are important for human well-being (Klein *et al* 2006,

Kremen *et al* 2007, Brown and Paxton, 2009). Bumble bee populations have declined globally in the 20th century (Williams 1982, Kosior *et al* 1997, Fitzpatrick *et al* 2007, Colla *et al* 2008, Brown & Paxton 2009, Williams & Osborne 2009) and one of the potential factors driving these declines is parasites (Kosior *et al* 2007, Colla *et al* 2008, Cameron *et al* 2011, Arbetmann *et al* 2012, Graystock *et al* 2013).

The study system

Bombus terrestris is a common species, widespread throughout Europe, and host to many parasite species, therefore it is an excellent model system for investigating host-parasite interactions including local adaptation. The parasites of bumble bees include *Sphaerularia bombi,* a nematode worm; *Apicystis bombi,* a neogregarine; *Crithidia bombi,* a trypanosome; *Nosema bombi,* a microsporidian and *Locustacarus buchneri*, a tracheal mite. These are all generalist parasites, of *Bombus* species, with a global distribution (MacFarlane *et al* 1995, Schmid-Hempel 1998). The nematode parasite *S. bombi* only infects bumblebee queens and has a significant impact on bumble bee populations, castrating between 13% and 90% of queens (Alford 1969a, Poinaar & van der Laan 1972, Schmid-Hempel 1998, Rutrecht & Brown 2008, Kelly 2009). *A. bombi* kills bumblebee queens before they are able to found colonies (Rutrecht & Brown 2008). *C.bombi* has a lower impact reducing overall colony fitness by 40% (Brown et al 2003) and *N. bombi* causes similar effects to *C. bombi* (Otti & Schmid-Hempel 2007, Rutrecht & Brown 2009). The impact of *L. buchneri* on *Bombus* queens is currently unknown although studies on males and workers show lethargy and reduced longevity in *B. bimaculatus* and *B. occidentalis* (Husband & Sinha 1970, Otterstatter & Whidden 2004).

S. bombi is a highly virulent obligate parasite, which castrates its bumblebee queen hosts, preventing them from founding a colony. The nematode parasite is transmitted horizontally: *S. bombi* lays thousands of eggs inside an individual bumblebee host (Kelly 2009) that develop into larvae and are transmitted to the next host via a free-living stage in the soil at hibernation sites (Poinar & van der Laan 1972, Alford, 1969a). Consequently, these factors suggest that *S. bombi* would be locally adapted to *B. terrestris*.

MacFarlane and Griffin (1990) suggested that as annual migration rates are significantly higher in bumble bees than in nematodes this should lead to significant local adaptation of the parasite, *S. bombi* to the host, *B. terrestris*. As bumblebees

are robust flying insects they should disperse further than the free-living stage of the *S. bombi* parasite. However, it must be noted that the MacFarlane and Griffin study (1990) was in New Zealand where the bumble bee population was introduced and the nematode parasites were introduced within infected queens, therefore in this case migration refers to colonisation rather than migration into an existing population.

S. bombi to *B. terrestris* both have annual lifecycles and reproduce sexually, therefore these factors are unlikely to affect the local adaptation of the parasite to the host. However, *S. bombi* is a generalist parasite that infects all *Bombus* species, suggesting that the selection pressure is exerted between *S. bombi* and all *Bombus* species, rather than an individual species, potentially reducing its local adaptation to one species.

A study of local adaptation in Switzerland across three sites found that the virulence of *C. bombi* was dependent on the scale of the analysis: at a smaller scale the parasites were locally adapted (more virulent, measured by host mortality and body mass) to their hosts but at a larger scale this was not the case (Imhoof & Schmid-Hempel 1998). As *S. bombi* are more virulent than *C. bombi*, they are more likely to be adapted to their host. Thus the host *B. terrestris* and the parasite *S. bombi* provide an ideal host-parasite model system to investigate local adaptation.

There are two ways to investigate the importance of local adaptation on the reproductive success of the parasite: firstly, 'home *versus* away' where the nematode parasite is constant and the bumble bee host varies and secondly, 'local *versus* foreign' where the bumble bee host remains constant but the nematode parasites varies (Kawecki & Ebert 2004). I intended to examine whether parasites have different levels of success in infecting hosts from sympatric (local) and allopatric (foreign) populations and whether the level of damage (castration, reduced fecundity, reduced longevity) suffered by hosts differs between sympatric and allopatric parasites. As both hosts and parasites were collected from three separate populations, my fully crossed experimental design enabled the investigation of local adaptation using both the 'home *versus* away' and 'local *versus* foreign' approaches (Kawecki & Ebert 2004).

Project

My original intention was to investigate whether the nematode parasite, *S. bombi*, was locally adapted to one of its bumble bee hosts, *B. terrestris*, at the European-scale. To do this I collected bumble bees from three different populations and raised colonies from the three populations in a controlled laboratory environment to perform nine cross-infection experiments in a single environment, known as a common garden experiment. However, due to the low prevalence of *S. bombi* and limited production of sexual offspring in some of the *B. terrestris* populations sampled, common-garden cross-infection experiments were not possible. Consequently, the data reported represent the levels of infection (prevalence and infection intensity) and the impact of *S. bombi* on their *B. terrestris* hosts across the three geographically distinct European populations sampled. In addition, I record the dynamics of colony founding and success across the three European populations of *B. terrestris* in a common garden, which has not previously been reported. I asked whether parasite prevalence and parasite impact differed across Europe.

Methods

Bee collection

In March and April 2010 I collected at least 100 emerging spring *B. terrestris* queens from each of the three sites across Europe, England, Switzerland and Ireland. One hundred and fifty one emerging *B. terrestris* spring queens were collected from The Valley Gardens, Windsor Great Park, England (Lat. 51.42, Long. -0.60, Plate 2-1), with the permission of the Crown Estate, on 15th and 16th March 2010, and an additional 61 *B. terrestris* spring queens were collected between 14th and 28th April 2010. On 29th and 30th March 2010, 153 emerging *B. terrestris* spring queens were collected from Aesch, near Basle (Lat. 47.50 and Long. 7.59,) and Burghof near Winterthur (Lat. 47.48, Long. 8.86, Plate 2-2), Switzerland. A further 140 emerging *B. terrestris* spring queens were collected from The National Botanic Gardens (Lat. 53.37, Long. -6.27, Plate 2-3) and Merrion Square (Lat. 53.34, Long. -6.25), Dublin, Ireland on 8th and 9th April 2010.

Plate 2-1 Collection site in Windsor Great Park, England

The collection site (with collecting equipment) in The Valley Gardens, Windsor Great Park, England provided cultivated flowers (*Erica spp.* shown) as the main source of forage for emerging *B. terrestris* spring queens in an open park landscape.

The sites in England, Switzerland and Ireland were selected as they represent an appropriate geographic scale of analysis for local adaptation, of *S. bombi* to their bumble bee hosts, due to the distance and physical boundaries between them (Greischar & Koskella 2007, Imhoof & Schmid-Hempel 1998): Ireland and England are separated by the Irish Sea and England is separated from mainland Europe by the English Channel. Previous studies have shown that the selected sites harbour both host and parasite (Schmid-Hempel *et al* 1990, Kelly 2009) and contacts in Zurich and Dublin were able to provide information on bumble bee queen emergence to ensure that early emerging queens were sampled at each site and that sufficient queens were collected in a minimal amount of time, reducing any transportation stress to a minimum.

Plate 2-2 Collection site in Burghof, Switzerland

The collection site (with C.M. Jones holding an entomological net and a collecting vial) in Burghof, Switzerland provided wildflowers beneath a crop plant as the main source of forage for emerging *B. terrestris* spring queens in an agricultural landscape.

All *B. terrestris* queens seen were collected to ensure that the sample was representative of the population at that site. The bumble bee queens were collected using an entomological net and placed in individual plastic vials with holes in the plastic lids, placed within cool boxes with freezer packs. The queens collected in England were transported directly to Royal Holloway, University of London (RHUL) where they were housed in the Bee Room, a specially prepared laboratory (see below), following a faeces check. The queens collected in Switzerland and Dublin were kept in a chilled environment overnight (or for two nights) and transported to RHUL where they were housed in the Bee Room as quickly as possible to recover from any possible stress from transportation.

Plate 2-3 Collection site in the National Botanic Gardens, Dublin, Ireland

The collection site (with collecting equipment) in the National Botanic Gardens, Dublin, Ireland provided cultivated flowers (*Erica spp.* shown) as the main source of forage for emerging *B. terrestris* spring queens in a city garden landscape.

Faeces checks

Faecal samples were taken from live queens and examined using x400 phase contrast microscope for the following parasites: *Sphaerularia bombi, Apicystis bombi, Crithidia bombi,* and *Nosema bombi*. All these parasite species can be reliably identified using microscopic techniques (Rutrecht & Brown 2008). A faeces sample from each queen collected in England was checked on return to the laboratory and second sample approximately three weeks after collection. Following the second faeces check, queens that did not deposit *S. bombi* larvae were transferred to a queen rearing box to encourage colony founding (see below). For queens collected in Switzerland and Ireland a single faeces check was performed approximately three weeks after collection and queens were transferred to queen rearing boxes as they became available.

Plate 2-4 The Bee Room

The bee room is the laboratory where the bees were reared. The queens were initially kept in plastic boxes, shown in the foreground, and after faecal checks uninfected queens were kept in queen-rearing boxes, to the right. The picture was taken under white light although queens were kept in the dark and a red light used for working.

Bee husbandry

The three populations of queens were reared in the Bee Room (Plate 2-4), a specially prepared laboratory at RHUL where each queen was housed under optimal environmental conditions (50-60% humidity, 25-28^{o}C) to found a colony (or produce *S. bombi* larvae if infected). Queens were initially kept in plastic boxes (size: 120mm x 100mm x 70mm) on a layer of sand (washed play sand), with a pollen ball in a medium petri dish (diameter 58mm), to encourage egg-laying, and a 15ml falcon tube, with holes drilled in the tip and the lid firmly screwed on to prevent leakage, to dispense sugar-water (sugar-water tubes) (Plate 2-5). The sugar-water was made of 50% sugar syrup (Ambrosia, Thornes of Windsor) and 50% water. A small petri dish (diameter 37mm) was placed under the tip of the falcon tube to catch any drips and form a barrier between the tube and the sand. The sugar-water tubes were checked daily and replaced when the sugar-water level was low. All

sugar-water tubes were replaced once a week (on Wednesdays) to prevent fungal growth in the tubes. The pollen balls were produced using commercial frozen pollen (Agralan), ground into a powder, mixed with a small amount of sugar-water then formed into small balls. The pollen balls were checked for eggs twice a week (Monday and Friday) and if no eggs were present the pollen ball was replaced with a fresh one. If eggs were present these were recorded and an additional pollen ball added (or a small plastic container of loose pollen) (Plate 2-5). Queens received sugar-water and pollen *ad libitum.*

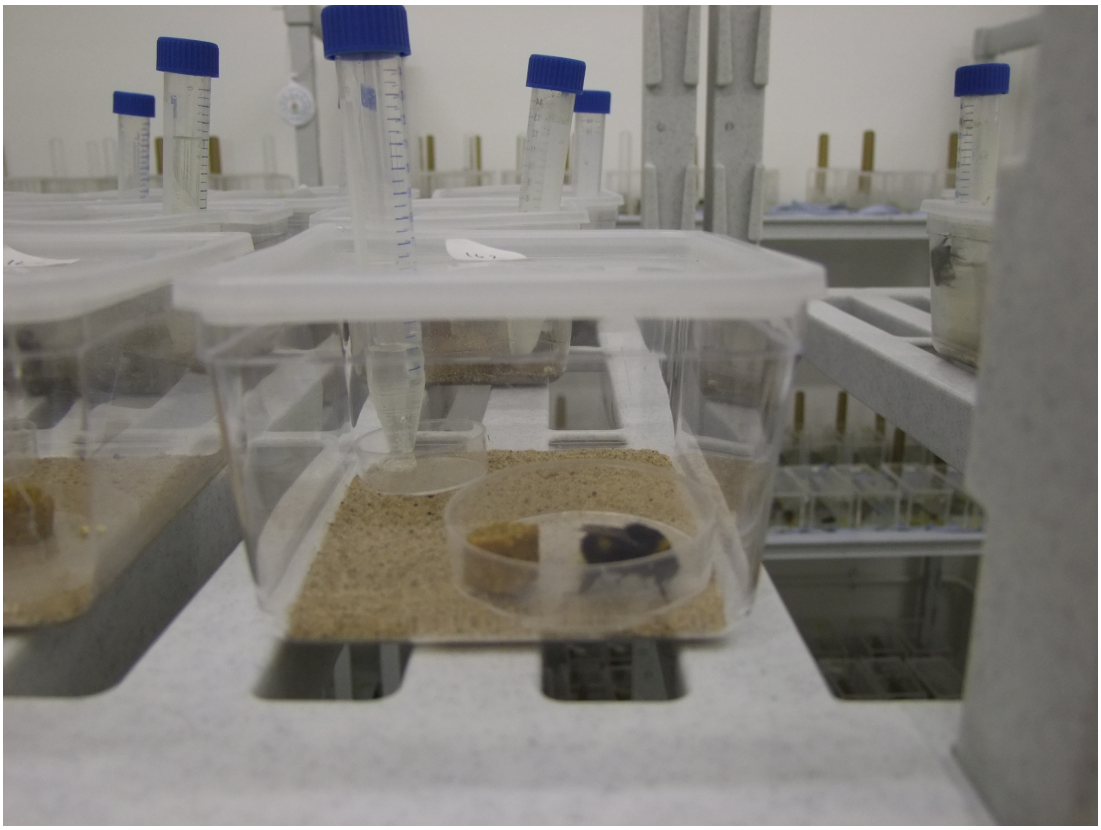

Plate 2-5 *B. terrestris* queen in a plastic box

Queens were initially kept in a plastic box on sand with pollen ball and falcon tube sugar-water dispenser.

Queens were kept in the dark and a red light used for working. Sterile procedures were used when handling the queens to avoid cross-contamination. Queens were transferred to clean sandwich boxes if the lid was wet, if the sand was wet or if the box was considered dirty. The queens were checked daily and any dead individuals were removed, transferred to a 2ml Eppendorf tube, labelled with the bee number and the date of death, and stored in a cryobox, in the freezer, at -80^{o}C, for dissection at a later date.

Plate 2-6 *B. terrestris* queen in a queen-rearing box

Following a faeces check, three weeks after collection, queens were kept in a queen-rearing box with a pollen ball and gravity feeder. This queen is brooding eggs in a pollen ball and a second pollen ball has been added.

Following a second faeces check for English queens and a first faeces check for Swiss and Irish queens, queens that were not depositing nematode larvae were transferred to a queen-rearing box (or a plastic box with recycled paper cat litter). A queen-rearing box is a specially constructed Perspex box, with a metal mesh base, with sugar-water dispensed from a gravity feeder and a pollen ball provided on a rectangular plastic sheet with a central hole with a round-ended 2ml Eppendorf tube pushed through to simulate brood (Plate 2-6). The pollen ball was placed adjacent to the plastic 'brood'. The set up of plastic boxes with cat litter was similar to the plastic boxes with sand, with the sand replaced by cat litter. Sugar-water and pollen ball checks and changes were as the sand boxes (see above).

Queens and colonies were raised and monitored in the bee room from March 2010 and details of the reproductive output of queens recorded, including eggs, larvae, pupae, workers, gynes (new queens) and males, and whether they brooded the pollen ball provided or produced and laid down wax or constructed a 'honey cup'. When a colony had 5 or more workers, the queen and the workers were transferred to 'colony' buckets (Plates 2-7 and 2-8). Colony buckets were white plastic buckets,

with a plastic colander inside, containing tissue paper, covered in black mesh and sealed into the bucket with silicon. A section of the lid was cut out and replaced with clear plastic (to see the colony inside) with a hole (for access) covered by a white plastic disc, weighted down by a rectangular piece of perspex. If the colony grew to more than 15 workers, a wooden foraging box was added to provide access to additional sugar-water tubes. Any sexuals (males and gynes) produced were transferred from the colony buckets to individual plastic boxes (for a single gyne or up to six males) or to wooden boxes (up to 20 males).

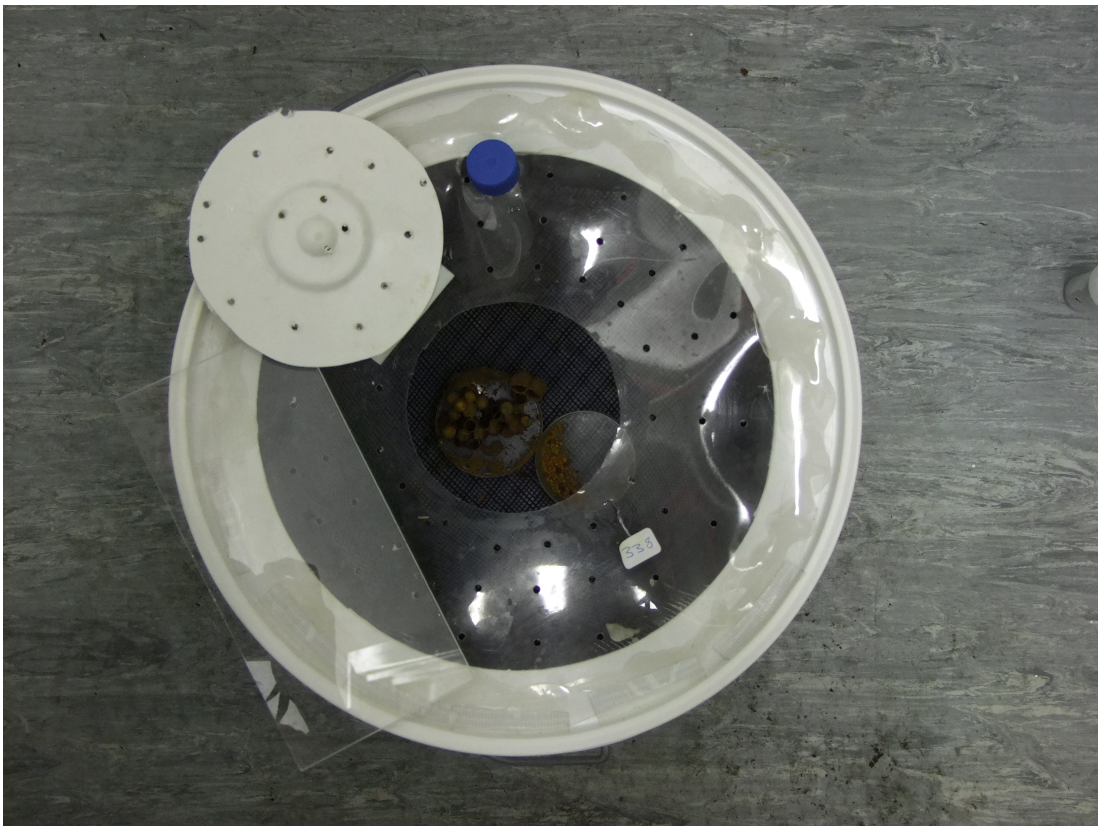

Plate 2-7 Colony bucket containing a *B. terrestris* queen and her colony.

When the colony grew to a queen and more than five workers, it was transferred to a colony bucket. Here the colony bucket is open to show the queen and brood inside, adjacent to a petri dish of loose pollen with a falcon tube sugar-water dispenser suspended from the top. A colony inside a colony bucket is shown in Plate 2.8.

Plate 2-8 *B. terrestris* queen and workers in a colony bucket

The colony of the queen, the developing brood and three workers is inside a colony bucket (shown in Plate 2.7).

Sand checks

Any queens with *S. bombi* larvae in their faeces check were kept in plastic boxes on a layer of sand with a sugar-water dispenser and received sugar-water *ad libitum*. The sand from the plastic boxes of all queens was checked for nematode larvae (see Experimental Appendices) and any nematode larvae were transferred into plastic boxes of clean damp sand, 'worm farms', to mature and mate. Although we were unable to perform the common garden cross-infection experiment, these nematode larvae were used in experimental infections (see Experimental Appendices).

Bee dissection

The queens were removed from the -80^{o}C freezer, transferred to a polystyrene container filled with ice and allowed to defrost. Each queen was removed from her

individual vial using forceps, her individual number was recorded and species identity checked.

The abdomen of each queen was separated from the thorax by twisting the abdomen and thorax in opposite directions or by cutting with small dissecting scissors. The head, thorax and legs were returned to the plastic vial. The abdomen was placed in a dissecting dish, with the ventral side uppermost, and the dissecting dish was placed under a dissecting microscope. The lights were switched on and directed at the bee's abdomen. The abdomen was pinned to the dissecting dish, just below where the abdomen was separated from the thorax, using a dissecting pin. The tip of the tail of the bee was held with forceps and the abdomen stretched slightly. The extended abdomen was pinned to the dissecting dish by the tip of the tail.

An incision was made laterally from the hole where the abdomen was separated from the thorax towards one side of the abdomen, then a second incision was made from the hole towards the other side. These incisions were both extended along the sides of the abdomen between the dorsal and ventral plates of the abdomen towards the tail, leaving the tail attached at the tip. The pin holding the tail was removed, the ventral section of the abdomen lifted, turned over away from the dorsal side, stretched slightly and the pin was then repositioned to hold the extended abdomen open.

The digestive system, from the honey crop to the faecal sac, was located within the abdominal section and if it was within the ventral portion it was carefully transferred using small tweezers to the dorsal portion. The entire digestive system was carefully transferred from the abdomen to a small drop of water in the dissecting dish, with faecal sac remaining attached. The faecal sac was carefully detached and the contents were emptied onto the left hand third of a glass slide, then a small amount of water was added and carefully covered with a cover slip. A small amount of Malphigian tubules, fine tubes attached to the gut, were detached and placed in the centre of a glass slide, then a small amount of water was added and carefully covered with a cover slip. A small amount of fat was removed from the exoskeleton of the abdomen and placed onto the right hand side of a glass slide, then a drop of water was added and carefully covered with a cover slip. The slide was placed under a microscope and inspected at x400 magnification for *A. bombi, C. bombi, N. bombi,* and the larvae of *S. bombi*.

The abdominal contents were investigated for the everted uteri of *S. bombi*; if located these were carefully removed and the number of uteri recorded. The ovaries, two sets of four ovarioles, were located within the abdominal cavity, and the developmental stage was recorded as no development (zero), some development (one) and fully developed (two). The tracheal tubes in the abdomen were also examined for *L. Buchneri* (adults and juveniles).

Data analysis

When the faeces check and dissection data were combined, if a queen was recorded as infected with a parasite at the faeces check, or at the dissection or both, it was recorded as infected by the parasite. I have included the 'early' English queens, the Swiss queens and Irish queens to compare the parasites of emerging spring queens across 3 European populations. I have also compared the 'early' and 'late' English queens to investigate the parasites of one population across two time periods. Parasite prevalence was calculated by dividing the number of infected queens by the total number of queens from each population (or time period for 'early' and 'late' English queens).

The parasite prevalence data and parasite impact data were analysed using Binary Logistic Regressions with the parasite (or parasite impact) as the dependent variable, bumblebee population (site) as the categorical variable set as the indicator, and the ENTER procedure. The impact of *S. bombi* on the longevity of queens in the laboratory was calculated comparing the longevity of queens infected with *S. bombi* with the longevity of uninfected queens using Mann-Whitney U tests. The impact of *S. bombi* on colony founding, ovarian development and egg laying were also calculated comparing the impact on infected with uninfected queens using Mann-Whitney U tests. A similar process was used to investigate the impact of *A. bombi* on the longevity of queens in the laboratory. The number of *B. terrestris* queens containing zero, one, two or three parasite species from each European population of *B. terrestris* hosts (parasite species richness) was compared using one sample t-tests. Statistical analyses of data were performed using IBM SPSS 19 for Windows.

Results

A total of 505 *B. terrestris* queens were collected: One hundred and fifty one from England, 153 from Switzerland and 140 from Ireland (a total of 444 early emerging spring queens) and 61 English queens that emerged later. Despite transporting queens from Switzerland or Ireland to RHUL as carefully as possible, a small number of *B. terrestris* queens did not survive and these were not included in the numbers of collected queens. However, the losses were relatively low (*ca*. 3%).

As faeces can only be collected from live queens, the faeces of 430 queens were checked (206 England, 137 Switzerland and 87 Ireland). The dissection data were collected from 434 queens (172 from England, 138 from Switzerland and 124 from Ireland): I was unable to record the parasite status of the remaining queens as the contents of their abdomens were decomposed. As five queens collected in Ireland had neither a faeces check nor dissection data, these were not included, reducing the number of Irish queens to 135 individuals and the total number to 500 queens.

Parasite prevalence

The prevalence of *S. bombi* differed significantly across the 3 European populations (Wald=55.055, df=2, p<0.001). The prevalence of *S. bombi* was significantly higher in Ireland (Wald=32.736, df=1, p<0.001, ExpB=0.046, 37%, N=135) than in England (3%, N=151) and significantly higher in Ireland than in Switzerland (Wald 26.729, df=1, p<0.001, ExpB=0.023, 1%, N=153) (Figure 2-1).

The prevalence of *C. bombi* differed significantly across the 3 European populations (Wald=14.791, df=2, p=0.001). The prevalence of *C. bombi* was significantly higher in Ireland (Wald=10.525, df=1, p=0.001, ExpB=0.213, 16%, N=135) than in England (4%, 6/151) not significantly higher in Ireland than in Switzerland (Wald0.530, df=1, p=0.466, ExpB=1.253, 20%, 30/153) (Figure 2-1).

A. bombi, *N. bombi* and *L. buchneri* were not present in all the European populations sampled. *A. bombi* was present in the emerging queens collected in Ireland (3%, 3/135) and in Switzerland (2%, 4/153), but not in England (Figure 2-1). The prevalence of *A. bombi* did not differ significantly across Europe (Wald=0.3, df=2, p=0.861). *N. bombi* was present in the emerging queens collected in Ireland (6%, 8/135) and in Switzerland (6%, 9/153), but not in England (Figure 2-1). The

prevalence of *N. bombi* did not differ significantly across the three European populations (Wald=0.000, df=2, p=1.000). *L. buchneri* was only recorded from three *B. terrestris* queens from Ireland with a prevalence of 2% (N=135) (Figure 2-1).

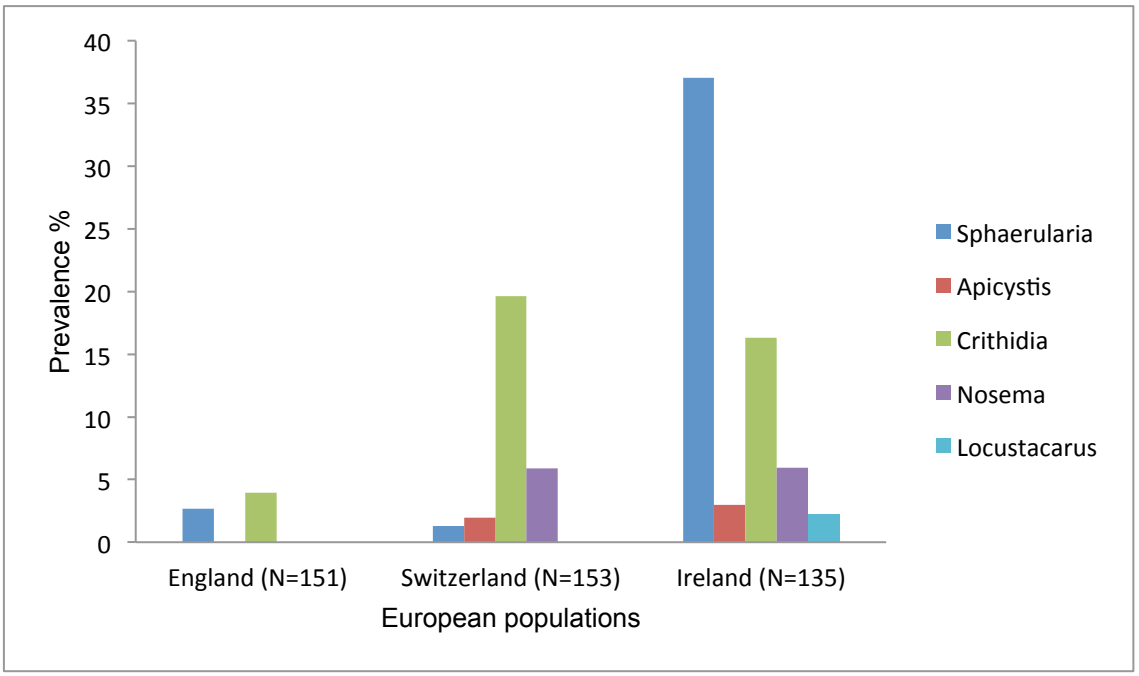

Figure 2-1 Parasite prevalence across three European populations

Parasite prevalence in early emerging *B. terrestris* spring queens varied across the three European populations sampled: The prevalence of *S. bombi* & *C. bombi* differed significantly (Wald=55.055, df=2, p<0.001 and Wald=14.791, df=2, p=0.001 respectively). *A. bombi*, *N. bombi* and *L. buchneri* were not present in all the populations sampled.

Across the two time periods for the English queens ('early' for queens collected in March and 'late' for queens collected in April), the prevalence of *S. bombi* was significantly higher in 'late' queens (18%, 11/61) than in 'early' queens (3%, 4/151) (Wald=11.879, df=1, p=0.001, ExpB=0.124) (Figure 2-2). The prevalence of *A. bombi* was higher in 'late' queens (5%, 3/61) than in 'early' queens where it was not recorded (N=151) (Figure 2-2). In 'late' queens, the prevalence of *C. bombi* was significantly higher (26%, 16/61) than in 'early' queens (4%, 6/151) (Wald=11.879, df=1, p=0.001, ExpB=0.124) (Figure 2-2). *N. bombi* and *L. buchneri* were not recorded from any of the English queens (Figure 2-2).

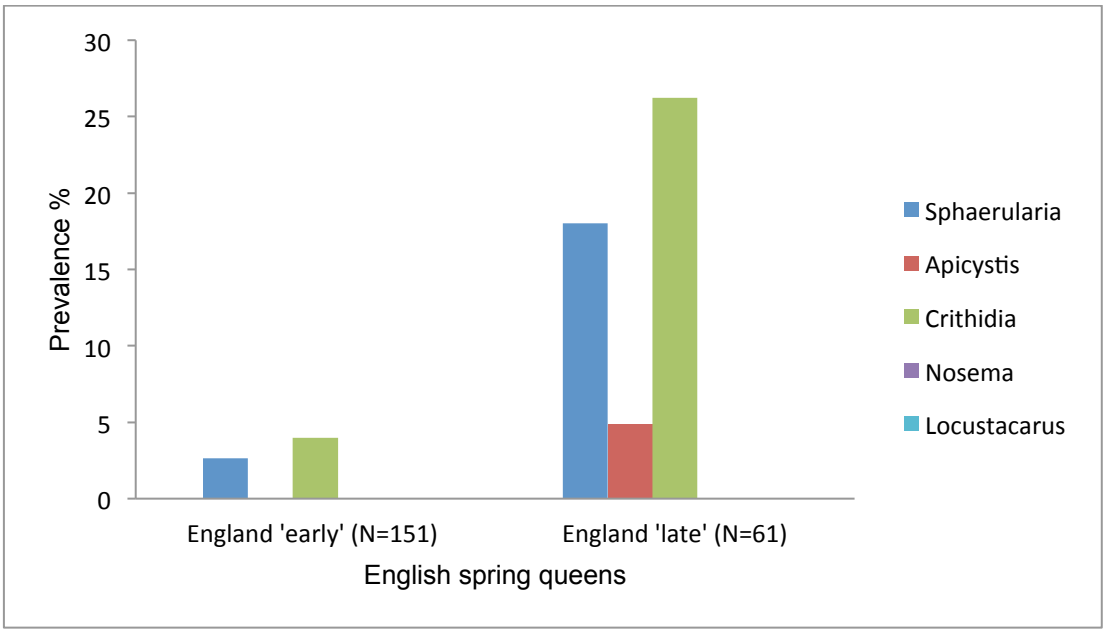

Figure 2-2 Parasite prevalence in English *B. terrestris* spring queens.

'Early' and 'late' spring queens were collected between 15th to 16th March 2010, and 14th to 28th April 2010 respectively. The prevalence of *S. bombi* and *C. bombi* were higher in 'late' queens (Wald=11.879, df=1, p=0.001, ExpB=0.124 and Wald=11.879, df=1, p=0.001, ExpB=0.124 respectively). *A. bombi* was present in 'late' queens (5%) but not in 'early' queens. *N. bombi* and *L. buchneri* were not found in any of the English queens.

Intensity of infection

The infection intensity for *S. bombi* infecting *B. terrestris* queens across the three European populations ranged from one to 27 nematode uteri per infected host with a mean infection intensity of 4.7 (±5.7SD) nematode uteri per infected queen. Queens from England ('early') contained between one and 20 adult female nematodes and a mean infection intensity of 7.3 (±9.0SD) nematode uteri per queen. Both infected queens from Switzerland contained only one adult female nematode (therefore a mean infection intensity of one nematode uterus per queen). Queens from Ireland contained between one and 27 adult female nematodes and a mean infection intensity of 5.0 (±3.3SD) nematode uteri per queen.

The infection intensity for *S. bombi* infecting English *B. terrestris* queens overall (i.e. early and late combined) ranged from one to 20 nematode uteri per infected host, with a mean of 4.3 (±5.4SD). Although the prevalence of *S. bombi* in English queens collected in April was higher than the queens collected in March (Figure 2-2), the queens collected in March contained more individual nematodes (April: 1-10 uteri with a mean of 3.1 ±3.3SD *versus* March: 1-20 uteri with a mean of 7.3±9.0SD).

Reproductive output

Across the three European populations, 50 of the *B. terrestris* emerging spring queens founded colonies (i.e. produced one or more live workers) but of these only 13 produced sexuals: 13 colonies produced males and only one produced a gyne (Table 2-1). Queens from England ('early') founded 25 colonies but only seven of these produced males. Queens from Switzerland founded 20 colonies, five of these produced males and one of these also produced a gyne. Queens from Ireland founded five colonies but only one of these produced males.

Table 2-1 Reproductive output of early emerging *B. terrestris* spring queens collected across three European populations

Country	Number of queens collected	Number of queens that founded a colony[b]	Number of colonies that produced males	Number of colonies that produced gynes
England	151	25	7	0
Switzerland	153	20	5	1
Ireland	122[a]	5	1	0
Total	426	50	13	1

[a] Although 140 Irish queens were collected, and 135 provided parasite data, 13 queens were not housed, therefore given the opportunity to found a colony and are omitted from this table
[b] A queen producing one or more live offspring (i.e. workers) was considered to have founded a colony

Parasite impact on colony founding

The early English, Swiss and Irish queens infected with *S. bombi* did not found colonies (i.e. produce 1 or more live offspring) and overall their reproductive output differed significantly to uninfected queens (Mann-Whitney U=8287.5, SE=459.9, $p<0.006$, N=426). However, separately the colony founding success of infected and uninfected early English queens (Mann-Whitney U=244.0, SE=55.6, p=0.368, N=151), Swiss queens (Mann-Whitney U=131.0, SE=36.3, p=0.761, N=153) and Irish queens (Mann-Whitney U=1620.0, SE=64.7, p=0.082, N=122) did not differ significantly. To calculate the impact of *S. bombi*, I omitted the data for 13 Irish queens that were not housed (therefore not given the opportunity to found a colony or lay eggs), thus the total number of queens reduced from 439 to 426, and the

number of Irish queens from 135 to 122. For late English queens there was also no significant difference (Mann-Whitney U=253.0, SE=22.9, p=0.336, N=61).

Parasite impact on egg-laying

The recorded egg-laying of queens infected or not infected with *S. bombi* also differed significantly (Mann-Whitney U=4854.000, SE=711.3, p<0.001, N=426). The overall difference in egg-laying was driven by the Irish queens (Mann-Whitney U=1215.0, SE=127.7, p<0.001, N=122), as the early English queens (Mann-Whitney U=161.5, SE=69.2, p=0.056, N=151) and the Swiss queens (Mann-Whitney U=85.0, SE=53.4, p=0.325, N=153) did not differ significantly. For late English queens there was also no significant difference (Mann-Whitney U=203.5, SE=37.8, p=0.590, N=61).

Parasite impact on ovarian development

The ovarian development of queens infected or not infected with *S. bombi* also differed significantly (Mann-Whitney U=2901.0, SE=667.9, p<0.001, N=414). Ovarian development was only recorded for queens that produced results from dissection therefore those that were decomposed were omitted. This overall difference in ovarian development was driven by the Irish queens (Mann-Whitney U=451.5, SE=112.2, p<0.001, N=98), as the early English queens (Mann-Whitney U=98.0, SE=50.3, p=0.197, N=122) and the Swiss queens (Mann-Whitney U=86.0, SE=47.6, p=0.410, N=138) did not differ significantly. For late English queens there was also no significant difference (Mann-Whitney U=45.5, SE=22.7, p=0.590, N=43).

Parasite impact on longevity

Across the three European populations, the laboratory longevity of the early English, Swiss and Irish queens infected with *S. bombi* was significantly lower than uninfected queens (Mann-Whitney U=4630.5, SE=886.7, p<0.001, N=439). The combined longevity for early English, Swiss and Irish infected queens (N=56) was 33.6 ($\pm$25.1SD) days compared with 67.5 ($\pm$34.0SD) for uninfected queens (N=383). The overall difference was due to the significant difference in longevity in Swiss (Mann-Whitney U=10.0, SE=62.2, p=0.006, N=153) and Irish (Mann-Whitney U=1486.0, SE=219.1, p=0.004, N=135) queens. For infected Swiss queens (N=2)

the longevity was 16.5 (±0.7SD), compared with 73.7 (±30.8SD) for uninfected Swiss queens (N=151). For infected Irish queens (N=50) the longevity was 32.1 (±24.0SD), compared with 48.7 (±30.4SD) for uninfected Irish queens (N=85). For early English queens there was no significant difference (Mann-Whitney U=239.0, SE=86.3, p=0.524, N=151): for infected queens (N=4) the longevity was 60.8 (±30.7SD), compared with 72.1 (±35.1SD) for uninfected English queens (N=147). For late English queens there was also no significant difference (Mann-Whitney U=179.0, SE=53.3, p=0.072, N=61).

Overall, the longevity in the laboratory of the early English, Swiss and Irish queens infected with *A. bombi* was significantly lower than uninfected queens (Mann-Whitney U=675.0, SE=332.9, p=0.012, N=439). The combined longevity for infected Swiss and Irish queens (N=7) was 31.0 (±19.3SD) days compared with 63.7 (±34.9SD) for uninfected early English, Swiss and Irish queens (N=432), although the difference in longevity in Swiss (Mann-Whitney U=79.5, SE=75.9, p=0.055, N=153) and Irish (Mann-Whitney U=185.0, SE=76.9, p=0.317, N=135) queens wa not significant. For infected Swiss queens (N=3) the longevity was 38.3 (±24.0SD), compared with 73.6 (±30.7SD) for uninfected Swiss queens and for infected Irish queens (N=4) the longevity was 25.5 (±16.3SD), compared with 43.1 (±30.4SD) for uninfected Irish queens. No early English queens were infected with *A. bombi.* For late English queens there was also no significant difference (Mann-Whitney U=36.0, SE=30.0, p=0.095, N=61).

Parasite species richness and parasite community structure

Across the three European *B. terrestis* populations, the number of parasite species found within individual queens differs significantly (G=88.217, df=6, p<0.001) (Table 2-2). The Swiss and Irish *B. terrestris* queens are host to more parasite species than the English *B. terrestris* queens. The Swiss queens are host to four parasite species, and I found up to three parasite species in an individual queen (Figure 2-3). Approximately 75% (115/153) of the Swiss queens were not infected by any parasite species. The Irish *B. terrestris* queens were host to a total of five parasite species, and once again, I found up to three parasite species in an individual queen. I only found the parasite *L. buchneri* in Irish *B. terrestris* queens. Less than half (*ca*. 47%, 63/135) of the Irish queens were not infected with any parasites (Figure 2-3).

In contrast, only *ca.* 7% (10/151) of 'early' English *B. terrestris* queens were infected by parasites, and the infected queens were only host to one of two parasite species (*S. bombi* n=4 and *C. bombi* N=6). For the 'late' English queens this falls to ca. 54% uninfected and the infected queens are host to one (*S. bombi* n=10, *C. bombi* N=14, *A. bombi* N=2) or two (*S. bombi* and *C. bombi* N=1, *C. bombi* and *A. bombi* N=1) parasite species (Figure 2-3).

Table 2-2 Number of *B. terrestris* queens infected with parasites across three European populations showing the number of parasite species, country, collection dates and total number of queens collected.

Country	Collection dates[a]	No parasites	1 parasite species	2 parasite species	3 parasite species	Total number of queens
England	15-16March	141	10	0	0	151
Switzerland	29-30March	115	33	4	1	153
Ireland	8-9April	63	58	13	1	135
England	14-28April	33	26	2	0	61

[a] All *B. terrestris* queens were collected in 2010

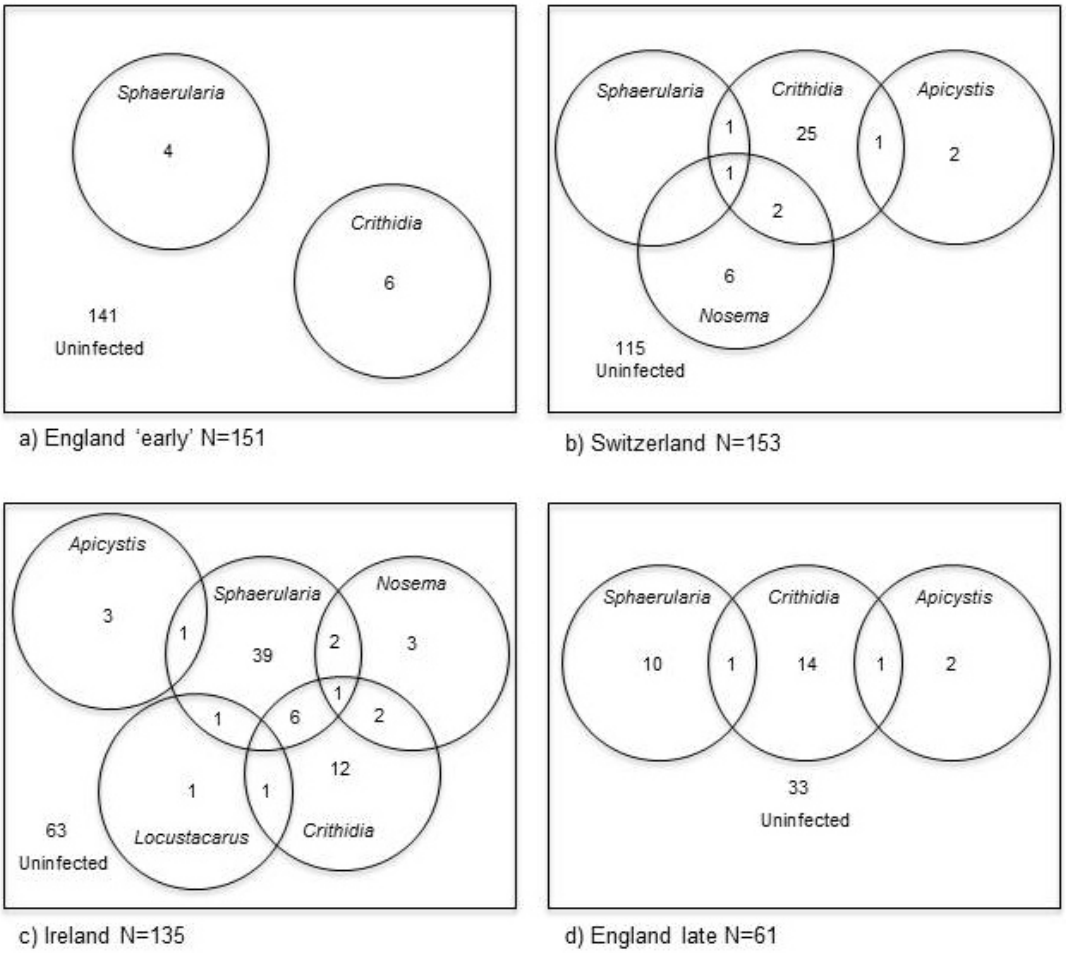

Figure 2-3 Parasite community structure

The parasite community of *B. terrestris* spring queens across three European populations showing overlaps where multiple infections occur: (a) England 'early' (collected 15-16 March), (b) Switzerland, (c) Ireland and (d) England 'late' (collected 14-28 April). (The size of circles is not representative of numbers.)

Discussion

Bumblebee queens collected in England and Ireland were the endemic buff-tailed *B. terrestris audax* subspecies but queens collected in Switzerland were the white-tailed Continental European subspecies *B. terrestris terrestris.* Thus parasites collected from English and Irish queens may have been locally adapted to English and Irish hosts and similarly, parasites collected from Swiss queens may have been locally adapted to Swiss hosts. However, as our Swiss hosts did not deposit any parasite larvae, I was unable to proceed with the common garden cross-infection experiment. Furthermore, I expected some of the queens collected across the three European populations to produce colonies, and that some of these would produce colonies that were sufficiently large to produce sufficient sexual offspring to proceed with the cross-infection experiments. However, this was not the case and neither our Swiss hosts nor our English and Irish hosts produced a sufficient number of sexual offspring to proceed with the cross-infection.

The collection and transportation of queens from the collecting sites, either directly to the laboratory, or via a temporary chilled storage site to the laboratory, was successful and most of the collected queens survived. However, 15 of the Irish queens were not housed on their return to the laboratory, and not checked the following day, therefore did not survive, probably due to lack of food and water. This emphasized the need for care processing samples, especially after a long trip, and for the requirement for daily checks.

To investigate the parasites across the three populations in Europe, I used faeces checks and dissection to establish the parasite status of the queens. However, when the faeces check and dissection data were combined, I made the assumption that if a queen was recorded as infected with a parasite at the faeces check, or at the dissection or both, it was included as infected by the parasite. This may over-estimate the parasite prevalence, as parasites recorded at dissection may only be present due to cross-contamination (i.e. transferred during handling in the laboratory) rather than acquired in the natural environment. However the use of sterile procedures should have minimised this risk.

The nematode *S. bombi* was present in the bumble bee populations sampled in England, Switzerland and Ireland, and I found that both parasite prevalence and infection intensity differed. I found the highest prevalence and infection intensity of

S. bombi in Ireland, and the lowest prevalence and infection intensity of *S. bombi* in Switzerland. The distance and physical barriers between the populations sampled and differing infectivity of parasites and/or susceptibility of hosts, and thus the local adaptation of the parasites to their hosts (and the hosts to their parasites) may explain the differing prevalence and infection intensity that we found. Although the three sites are physically separated *B. hypnorum* from continental Europe have been recorded in England (Goulson & Williams 2001) and *B. pratorum* and *B. monticola* from England recorded in Ireland (Speight 1974, Fitzpatrick *et al* 2007). Healthy *B. terrestris* queens can spread by up to 140km per annum but *S. bombi* have only spread 40km in 100 years in New Zealand (MacFarlane and Griffin 1990), but it should be noted that this refers to colonisation rather than migration. However other factors may also explain these findings (see below).

The physical attributes of the three sites selected may affect the differences found. The Swiss queens were foraging on wildflowers in sloping vineyards sites surrounded by an agricultural landscape adjacent to wooded areas (Plate 2-2). The English queens were mainly foraging on cultivated heathers (*Erica* spp.) in flowerbeds in an open parkland landscape (Plate 2-1). The Irish queens were foraging in cultivated flowerbeds in either a square completely surrounded by buildings or were mainly foraging on cultivated heathers (*Erica* spp.) in flowerbeds in a botanical garden in the city of Dublin (Plate 2-3). As we found higher parasite prevalence in Dublin, the limited area of forage availability may have condensed the bumble bee population into a restricted space, and as *S. bombi* are deposited by infected queens at bumble bee hibernation sites, the range of the parasites may be similarly condensed. If this is the case, increasing European urbanisation may have an impact on parasite prevalence and bumble bee populations, but connectivity between floral resources (and bee hibernation sites) could mitigate any impact.

As RHUL is close to the English collection sites, the plan to collect emerging *B. terrestris* spring queens in England in March to produce colonies and additional queens in April to produce *S. bombi* larvae was successful, although no gynes were produced. Unfortunately, due to both time and financial constraints, we were not able to revisit the Swiss and Irish sites to collect more queens to increase the number of colonies (a limiting factor for the Irish population) or queens infected with *S. bombi* (a limiting factor for the Swiss population).

With the number of infected Irish and English queens, and the number of colonies founded in the laboratory in 2010, I believe that a common garden cross-infection experiment to investigate local adaptation would be possible. A third population from a site, where many *B. terrestris* queens could be collected and parasite prevalence was high, would be required to replace the Swiss population, where the parasite prevalence was lowest and the infected queens did not produce nematode larvae.

As *S. bombi* larvae were required for the cross-infection experiments, the *B. terrestris* queens were initially housed in plastic boxes containing a layer of sand, to facilitate harvesting of nematode larvae. Although nematode larvae could be harvested from these boxes, these were not ideal for keeping the queens clean (some queens were covered in sand and/or sugar-water, Pers. Observ.) and encouraging uninfected queens to found colonies. In later projects infected (or potentially infected) queens were housed in plastic boxes with a sheet of paper (the size of the base of the box) in the boxes and it was still possible to harvest nematode larvae from these boxes.

Due to the number of queens collected, and the limited number of queen rearing boxes available, following the faecal check (second faecal check for English queens) uninfected queens were transferred to plastic boxes containing cat litter (recycled paper cat litter). These proved reasonably effective in absorbing excess moisture and keeping the queens clean, but queen rearing boxes were more effective at encouraging colony founding (Pers. Observ.).

The parasite communities across the three European populations varied in both the prevalence of the parasites species that were present in all three countries, and in the number of parasites species present.

The prevalence (3-18%) and mean infection intensity (3-7 uteri per queen) of *S. bombi* in *B. terrestris* queens in England was similar to the 17% and 5.1 uteri per queen reported in *B. terrestris* queens in the same area in 1960s (Alford 1969a). This compares with my findings of 11% in *B. terrestris* in England in 2011 (see Chapter 3). In Switzerland the prevalence of *S. bombi* I found was very low, with just two queens infected (ca. 1%) each with a single parasite. The prevalence of *S. bombi* in *B. terrestris* queens in Ireland was high (ca. 37%), although slightly lower than the 41.6% reported by Kelly (2009) in *B. terrestris.* Rutrecht & Brown (2008) found a lower prevalence of 15% in *B. pratorum* in Dublin. Dublin would be an excellent site to collect infected queens for future studies.

The prevalence of the high impact parasite *A. bombi* in England (0-5%) and was lower than in Switzerland (2%) or Ireland (3%). This compares with 4% reported by Rutrecht & Brown (2008) from *B. pratorum* in Ireland and my findings of 7% in *B. terrestris* in England (see Chapter 3).

The prevalence of *C. bombi* in B. terrestris ranged from overall 10% (4-22%) in England to 16% in Ireland and 20% in Switzerland. Shykoff & Schmid-Hempel (1991) reported the prevalence of *C. bombi* in Switzerland of 48% in *B. terrestris* queens and 80% in *B. terrestris* and *B. lucorum* workers. While *C. bombi* is a lower impact parasite than *S.bombi*, the prevalence is usually higher (Rutrecht & Brown 2008).

I found a fourth parasite species *N. bombi* in Switzerland and Ireland with a prevalence of 5% and 6% respectively. These levels fall between the prevalence of *N. bombi* in *B. pratorum* queens in Irleand of 0% (Rutrecht & Brown 2008) and in *B. terrestris* queens in Switzerland 14% (Shykoff & Schmid-Hempel 1991).

In Ireland I found a fifth parasite species *L. buchneri* in *B. terrestris* queens with a prevalence of *ca.* 2%, which was not found in *B. terrestris* queens from England or Switzerland. This compares with a prevalence of 34% found in *B. pratorum* in Ireland (Rutrecht & Brown 2008) and my findings of 19% in *B. pratorum* in England (see Chapter 3).

Although the common-garden cross-infection experiment planned was not possible, I was able to collect data on the host-parasite relationships of three populations of *B. terrestris* and five of its generalist parasites. Furthermore, the successes and challenges of this first project informed the bee husbandry protocols for later projects. In subsequent projects, instead of using the hand-made colony buckets, I used plastic colony boxes used by commercial producers of bumble bees and newly collected spring queens were immediately housed in queen-rearing boxes (or in plastic boxes on paper) instead of on sand to provide optimal conditions to improve colony founding success. I also recorded the prevalence of five generalist parasites of bumble bees in Windsor Great Park which, along with preliminary data from 2009 (Jones & Brown unpublished), represents the first records of these parasites in this area since Alford's work in the 1960s (Alford 1969a & b).

Chapter 3. Parasites and genetic diversity in an invasive bumble bee

Abstract

Biological invasions are one of the main threats to biodiversity. Climate change and the global transportation of species are likely to increase the ecological and economic damage caused by biological invasions. Therefore understanding the mechanisms behind invasion success is essential. Both the release of non-native populations from natural enemies, such as parasites, and the genetic diversity of these populations may play key roles in their invasion success.

I investigated the roles of parasite communities, through enemy release and parasite acquisition, and genetic diversity in the invasion success of the non-native bumblebee, *Bombus hypnorum*, in the United Kingdom.

The invasive *B. hypnorum* had higher parasite prevalence than native congeners, probably due to higher susceptibility and parasite acquisition. Consequently parasites had a higher impact on the invader's fitness than on native species. *B. hypnorum* also had lower functional genetic diversity at the sex-determining locus than native species. Higher parasite prevalence and lower genetic diversity have not prevented the rapid invasion of the UK by *B. hypnorum*. These data may inform our understanding of similar invasions by commercial bumble bees around the world. This study suggests that concerns about parasite impacts on the small founding populations common to re-introduction and translocation programs may be less important than currently believed.

Introduction

Biological invasions occur when non-native species successfully establish in a new location and rapidly expand their range (Williamson 1996). Such invasions may affect the diversity and abundance of native species, species interactions (e.g. symbioses) and the provision of ecosystem services (such as pollination), which are important for human well-being (Pimentel *et al* 2005, Pejchar & Mooney 2009, Vila *et al* 2010). The invasion success of a non-native species may be facilitated by a

release from natural enemies, such as herbivores, predators and parasites, potentially leading to a rapid increase in distribution and abundance of the invasive species (Elton 1958, Keane & Crawley 2002, Torchin *et al* 2003). Evidence supporting the enemy release hypothesis can be found from studies of plant-herbivore interactions (e.g. Agrawal & Kotanen 2003, Colautti *et al* 2004, Agrawal *et al* 2005, Liu & Stirling 2006) but the evidence from animal-parasite systems is less clear (e.g. Dunn & Dick 1998, MacNeil *et al* 2003, Georgiev et al. 2007). Given the historical, current and predicted global impact of invasive species (Elton 1958, Vitousek *et al* 1996, Wilcove *et al* 1998, Pimentel *et al* 2005) and the importance of species range expansion due to climate change (Parmesan *et al* 1999, Hickling *et al* 2006) understanding the mechanisms that facilitate these changes is a key challenge (e.g. Phillips *et al* 2010, White & Perkins 2012).

Previous studies of the role of parasites in enemy release, in both plant and animal systems, largely examine either parasite prevalence or the impact of individual parasite species (e.g. MacNeil *et al* 2003). However, parasites exist in communities (Cloutman 1975, Holmes & Price 1986) and invasive species may host multiple parasite species (e.g. Georgiev *et al* 2007). Interactions among parasite species, within a host, include competition for resources (Rigaud *et al* 2010) and alteration of transmission rates (e.g. castrating parasites reducing the transmission of other parasite species to the offspring of the host: Ben-ami *et al* 2011). The presence of multiple parasite species may also induce differing host immune responses, with differing impacts on individual parasite species (Schmid-Hempel 1998). In addition, the structure of parasite communities can have significant consequences for assessing the impact of individual parasites (e.g. Rutrecht & Brown 2008). Consequently, understanding the structure of parasite communities and their subsequent impact (Rigaud *et al* 2010) is essential to establish the role of parasites in invasions.

The role of parasites in biological invasions

While invading species may be released from parasites in their new location, the impact of parasites in the invaded communities may, in turn, be modified by invasive species, through parasite introduction (Prenter *et al* 2004, Dunn 2009) or parasite spill-over (Daszak *et al* 2000 where invading host species introduce non-native parasites and these spill-over to infect native hosts)(see Chapter 1). Invasive species may also acquire parasites from congeneric host species in the new

location (parasite acquisition: Dunn 2009) which may result in an increase (through invasive species acting as a reservoir for native parasites followed by parasite spill-back: Norman *et al* 1999, Daszak *et al* 2000; Dunn 2009, Kelly *et al* 2009) or a decrease in parasite abundance in native species (through parasite dilution, where invading hosts provide an additional or alternative host for native parasites 'diluting' the parasite prevalence and/or abundance in native hosts: Norman *et al* 1999, Ostfeld & Keesing 2000) depending on the competence of the invasive host at transmitting the infective stages of the parasite (see Chapter 1). These factors may occur individually or in concert, and thus investigating enemy release in the invaded range should take account of these complex interactions.

The role of genetic diversity in biological invasions

An additional factor that may play a key role in the host-parasite interactions of invasive species is the genetic diversity and provenance of the invasive host. Invasive species are likely to establish in a new location from only a few propagules or reproductive individuals, and therefore the founding population will have low genetic diversity (Dlugosch & Parker 2008). Low genetic diversity in natural populations is known to be associated with higher rates of parasitism (e.g. Whitehorn *et al* 2011) and thus genetically depauperate invasive species may be more likely to acquire parasites from congeners. In addition, as invading hosts have not co-evolved with native parasites, invading hosts may be maladapted to native parasites and the parasites may have a greater (or lesser) impact on such hosts (Thompson 2005). Relative to native hosts, if the non-native species is less susceptible to parasites and/or parasites have a smaller impact on fitness, non-native hosts are likely to benefit from enemy release despite the acquisition of generalist parasites from congeners.

Biology of the study system

Most bumblebees are annual eusocial species, passing through a solitary overwintering phase as queens. This makes the queen a key component of the annual lifecycle. Interestingly, bumblebee queens are particularly heavily impacted by parasites (Rutrecht & Brown 2008). Consequently, parasites that reduce the survival and fitness of the queen are likely to have a high impact on bumblebee populations and, therefore, in this study I focused on bumblebee queens. Bumblebee gynes (unmated new queens) disperse from their natal nests to mate in

late summer, prior to finding a hibernation site. Queens hibernate in individual hibernacula, which can be dispersed or aggregated, depending upon the species, and different species favour different hibernation sites (Alford 1969a, 1975, Sladen 1912). Post-hibernation queens disperse again, with estimates of aggregate dispersal of at least 5km (Lepais *et al* 2010), and congregate at florally-rich sites to forage for nectar and pollen. Parasites can be acquired from natal nests, interactions with males during mating, during hibernation and through foraging pre- and post-hibernation (Schmid-Hempel 1998).

While bumblebees (*Bombus spp*.) are generally considered to be beneficial, as they are important ecological and commercial pollinators, they can also be highly invasive (Dafni 1998, Goulson 2003). In Japan commercially introduced *B. terrestris* L. have escaped and threaten native congeners and their interactions with native plants (Matsumura *et al* 2004, Inoue *et al* 2008). Invasive *B. terrestris* have spread throughout Tasmania in the last 20 years (Allen *et al* 2007, Schmid-Hempel *et al* 2007) probably from New Zealand, where they were introduced in the 19th century (MacFarlane & Griffin 1990). Most recently, invasive *B. terrestris* has spread across Argentina and Chile, where it is blamed for rapid declines in the only native bumblebee species, *B. dahlbomii* Guérin-Méneville (Torretta *et al* 2006, Plischuk & Lange 2009, Goulson 2010; Arbetman *et al* 2012, Morales *et al* 2013). The increasing commercialisation of bumblebees as pollinators means that the dangers posed by such invasions are likely to increase (Williams *et al* 2012).

Project

Using the successful establishment of a non-native invasive bumblebee, *Bombus hypnorum* L., across England and Wales over the last decade (Goulson & Williams 2001, BWARS), I aim to identify the role of parasites and genetic diversity in this invasion. *B. hypnorum*, the tree bumblebee, has expanded across England, Wales and Scotland, northwards to Lennoxtown, Scotland (*ca.* 650 km), to Truro, Cornwall in the South West (*ca.* 300km) and Pembrokeshire in Wales (*ca.* 320km) since its first discovery in the New Forest, Wiltshire, England in 2001 (BWARS, Goulson & Williams 2001). The parasite community of bumblebees is composed of generalist parasites and has been well characterized (MacFarlane *et al* 1995, Schmid-Hempel 1998, Rutrecht & Brown 2008), making this an excellent opportunity to examine how enemy release and parasite acquisition may impact an invasive species, particularly

as recent work has suggested that parasites play a role in the dynamics of native bumblebee populations (Antonovics & Edwards 2011).

Enemy release can occur in two ways. First, an invading species, in the invaded range, may escape from the enemies it would have encountered in its native range (Hatcher & Dunn 2011). A model proposed by Drake (2003) suggests that such enemy release may be important for the establishment of small invading populations. Second, invading species may escape from enemies present in the invaded range, as those enemies are not adapted to exploit it (Dunn 2009). A comparison of enemies of invading species and those of congeneric native species, a community study, investigates the second mechanism (Hatcher & Dunn 2011). I take this approach because the origin of our focal species is currently unknown. To investigate the potential release from natural enemies of the non-native *B. hypnorum*, I determined the parasite community in queens of this invasive bumblebee species and compared it to those of five native bumblebee species with the expectation that *B. hypnorum* would have lower parasite prevalence and lower parasite species richness than native congeneric species, and thus it should be released from its parasite enemies. In addition to investigating parasite prevalence, parasite species richness and parasite community structure, I also investigated the parasite impact on host fitness, and functional genetic diversity at the sex-determing locus in, laboratory-reared colonies of the invasive *B. hypnorum*. I expected that parasites would have a greater impact on host fitness in *B. hypnorum* than in native congeneric species and that the genetic diversity of *B. hypnorum* would be lower than that of native *Bombus* species.

Methods

Sampling scheme

The sampling methodology was designed around the biology of the system (see above). Bumblebee queens were collected, between February and May 2011, from 2 primary, florally rich, sites in Surrey and Berkshire, Windsor Great Park (Lat. 51.41, Long. -0.60) and the Royal Horticultural Society (RHS) Garden, Wisley (Lat. 51.32, Long. -0.58). Additional queens were collected from florally rich sites at the Royal Botanic Gardens, Kew (Lat. 51.47, Long. -0.30), Royal Holloway, University of London (RHUL) (Lat. 51.43, Long. -0.56) and Horsell, Surrey (Lat. 51.32, Long. -0.57). My sampling area was geographically restricted due to the requirement to

catch sufficient queens within a limited time period. However, due to the rapid establishment of this invasive species in the UK, I believe that the population in South East England is likely to be representative of the UK *B. hypnorum* population as a whole. The non-native species *B. hypnorum* and five native species *B. jonellus*, *B. pratorum, B. lucorum, B. pascuorum* and *B. terrestris* were collected. The queens were collected using an entomological net and placed in individual plastic vials in a chilled container and transported to RHUL. On each day, sites were collected to exhaustion. The queens were spring queens, foraging after emerging from hibernation, and therefore from the first voltine generation. While abundant species may be the most obvious source of generalist parasites, such parasites are also more likely to infect related host species (Perlman & Jaenike 2003), and our sampling strategy was designed to cover both possibilities, with *B. jonellus* and *B. pratorum* being the phylogenetically closest relatives to the invasive *B. hypnorum* (Cameron *et al* 2007) and *B. lucorum, B. pascuorum* and *B. terrestris* being the most abundant native bumblebee species (Goulson & Darvill 2004, Goulson *et al* 2005, Williams 2005) in the UK.

Parasite – faecal check

Faecal samples were taken and examined using a x400 phase contrast microscope for the following parasites: *Sphaerularia bombi* Dufour, a nematode worm; *Apicystis bombi* Lipa & Triggiani, a neogregarine; *Crithidia bombi* Lipa & Triggiani, a trypanosome; and *Nosema bombi* Fantham & Porter, a microsporidian. All these parasite species can be reliably identified using microscopic techniques (Rutrecht & Brown 2008) except for *Crithidia spp*. for which molecular data show that only *C. bombi* occur in this area (MJF Brown unpublished data). These are all generalist parasites with a global distribution (MacFarlane *et al* 1995, Schmid-Hempel 1998). *S. bombi* infects bumblebee queens hibernating in the soil, castrating them and preventing them from founding colonies (Alford 1969, Poinar & van der Laan 1972) and *A. bombi* kills bumblebee queens before they are able to found colonies (Rutrecht & Brown 2008). Consequently both of these parasites have a high impact on spring queens. *C. bombi* reduces overall colony fitness by 40% (Brown *et al* 2003a), and *N. bombi* has similar effects (Otti & Schmid-Hempel 2007, Rutrecht & Brown 2009).

Parasite - dissection

The *B. jonellus, B. pratorum* and *B. pascuorum* queens were sacrificed by freezing after the faecal check and stored at -80^{o}C. They were later thawed, dissected and checked again for bumblebee parasites including *S. bombi, A. bombi, C. bombi, N. bombi* and *Locustacarus buchneri* Stammer, a tracheal mite. The impact of *L. buchneri* on queens is currently unknown although correlative studies on males and workers show lethargy and the cessation of foraging in workers of *B. bimaculatus* and reduced lifespan in *B. occidentalis* (Husband & Sinha 1970, Otterstatter & Whidden 2004).

Bee husbandry

B. hypnorum queens were reared in the laboratory at a controlled temperature (25-27^{o}C) and humidity (50-60%), and received sugar-water and pollen ad libitum. The queens were kept in the dark and a red light was used for working. Queens were kept in queen-rearing boxes, with a sugar-water dispenser and a pollen ball to encourage egg-laying. Records were kept of the reproductive output of *B. hypnorum* queens including eggs laid, number of workers, males and gynes (new queens) produced. Dead queens, either at natural death or at sacrifice, were stored at -80^{o}C. Queens with no offspring were sacrificed after a minimum of 10 weeks in the laboratory. The queens were thawed, dissected and checked for parasites as above.

Sterile procedures were used when handling queens in the laboratory, to prevent cross-contamination. Nevertheless, two *B. hypnorum* queens that were infected by *C. bombi* when dissected, but were not infected when the faeces samples were examined, were consequently rejected from the data set due to possible cross-contamination.

Queens of *B. terrestris* and *B. lucorum* were reared for other experiments by another researcher but I was still able to assess their parasite status (as described above) and whether they produced normal or diploid male colonies (see below).

Diploid males

Bumble bees are haplodiploid, females being diploid (heterozygous) and males haploid (hemizygous). However, diploid (homozygous) males occur in inbred or

genetically depauperate populations, and are indicative of low genetic diversity (Duchateau *et al* 1994) (see Chapter 1). A standard protocol for identifying diploid male production is through the presence of males in the first brood (which is usually just females) at a 50:50 sex ratio (Gerloff & Schmid-Hempel 2005) (see Chapter 1). Consequently, the timing of male production was recorded to assess whether colonies were producing diploid males (Duchateau *et al* 1994).

Analyses

Parasite prevalence was calculated by dividing the number of infected queens by the total number of queens of each species with Clopper-Pearson 95% confidence intervals. Here I report only parasite prevalence, as for the macroparasite *S. bombi* the impact of an individual worm is the same as the impact of multiple worms (Alford 1969a, Kelly 2009). The parasite prevalence data and parasite impact on colony founding data were analysed using Binary Logistic Regressions with the parasite (or parasite impact) as the dependent variable, bumblebee species and site as categorical variables with *B. hypnorum* set as the indicator species, and the forward log ratio procedure. All analyses were conducted twice, once with the entire dataset and once with just the two main sampling sites (Windsor and Wisley).

The number of parasites species in each of the parasite communities (where each bee species is a habitat that hosts a parasite community and each individual bee is a site within that habitat) and the similarity of those parasite communities were analysed using SPADE (Species Prediction And Diversity Estimation) software (Chao & Shen, 2010).

As a measure of genetic diversity at a functionally important locus, the sex-determining locus, I estimated the number of sex alleles in the native and invasive bumblebee populations, and in a continental European population of *B. hypnorum* (data from Brown, Schmid-Hempel & Schmid-Hempel 2003b), using the formula $\theta = 2/N$ where 'θ' is the probability of a diploid colony and 'N' is the number of sex alleles (Duchateau *et al* 1994; Adams *et al* 1977) (and differences tested using Fisher Exact tests). The minimum number of sex alleles was estimated by comparing the number of observed and expected diploid male colonies for a range of values and determining where they cease to be significantly different.

Statistical analyses of data were performed using IBM SPSS 19 for Windows and SPADE (Species Prediction And Diversity Estimation) software (Chao & Shen, 2010).

Results

A total of 378 bumblebee queens, collected in 225 hours across 45 days, were examined for parasites (59 *B. hypnorum*, 47 *B. jonellus*, 104 *B. pratorum*, 50 *B. pascuorum*, 61 *B. lucorum*, and 57 *B. terrestris*) and five parasite species were found (*S. bombi, A. bombi, C. bombi, N. bombi* and *L. buchneri*).

Parasite prevalence

The prevalence of *S. bombi* among bumblebee species differed significantly (Wald=25.584, df=5, p<0.001) and ranged from 29% in *B. hypnorum* to 0% in B. jonellus (Figure 3-1). The prevalence of *S. bombi* in *B. hypnorum* was significantly higher than its prevalence in *B. jonellus* (Wald=7.281, df=1, p=0.007, ExpB=0.058), *B. pratorum* (Wald=12.623, df=1, p<0.001, ExpB=0.156), *B. pascuorum* (Wald=10.051, df=1, p=0.002, ExpB=0.089) and *B. terrestris* (Wald=7.416, df=1, p=0.006, ExpB=0.205) but not significantly higher than in *B. lucorum* (20%, 12/61; Wald=0.921, df=1, p=0.337, ExpB=0.654). The prevalence of *S. bombi* across sites differed significantly overall (Wald=11.887, df=4, p=0.018) but in pairwise comparisons, the only significant difference was between Windsor and Horsell (Wald=8.633, df=1, p=0.003). The remaining sites, Wisley (Wald=3.147, df=1, p=0.076), Kew (Wald=0.697, df=1, p=0.404), and RHUL (Wald=1.457, df=1, p=0.227), did not differ significantly to our primary site (Windsor). The prevalence of *S. bombi* was not affected by collection date (this variable was not present in the final model). Qualitatively similar results were found when analyses were restricted to data from the two main sites (Windsor and Wisley; data not shown).

As with *S. bombi*, the prevalence of *A. bombi* among bumblebee species differed significantly (Wald=18.927, df=5, p=0.002) and ranged from 18% in *B. hypnorum* to 0% in *B. jonellus* (Figure 3-1). The prevalence of *A. bombi* in the non-native *B. hypnorum* was significantly higher than its prevalence in *B. jonellus* (Wald=4.841, df=1, p=0.028, ExpB=0.095), *B. pratorum* (Wald=9.216, df=1, p=0.002, ExpB=0.090), *B. pascuorum* (Wald=6.120, df=1, p=0.013, ExpB=0.108), *B. lucorum* (Wald=6.080, df=1, p=0.014, ExpB=0.072) and *B. terrestris* (Wald=4.416, df=1,

p=0.036, ExpB=0.244). The prevalence of *A. bombi* across sites did not differ significantly overall (Wald=6.454, df=4, p=0.168) and was not affected by the collection date. Again, results were qualitatively similar in the site-restricted analysis.

C. bombi was the only parasite found in all six bumblebee species and prevalence ranged from 5% in *B. pratorum* to 18% in *B. terrestris* (Figure 3-1). The prevalence of *C. bombi* among bumblebee species did not differ significantly (Wald=6.846, df=5, p=0.232). The prevalence of *C. bombi* across sites did not differ significantly overall (Wald=7.722, df=4, p=0.102) and, once again, was not affected by the collection date. Again, these results were qualitatively similar in the analysis restricted to the main sampling sites.

L. buchneri was only present in one of the six bumblebee species sampled, *B. pratorum*, with a prevalence of 16% (N=104), and *N. bombi* was only present in two *B. terrestris*, with a prevalence of 4% (N=57) and one *B. pascuorum* queen, with a prevalence of 2% (N=50).

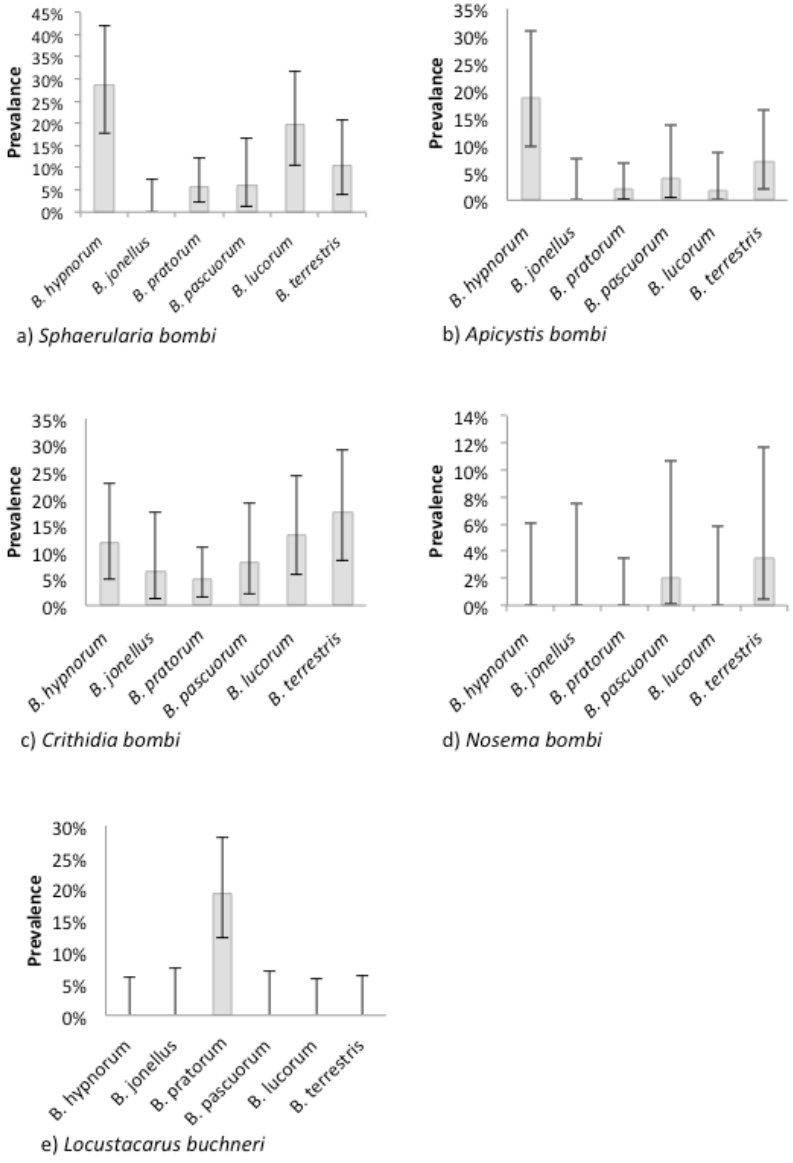

Figure 3-1 Parasite prevalence in non-native and native *Bombus* host species

The percentage prevalence of five parasite species: (a) *Sphaerularia bombi*, (b) *Apicystis bombi,* (c) *Crithidia bombi,* (d) *Nosema bombi* and (e) *Locustacarus buchneri* found in non-native (*B. hypnorum*) and native (*B. jonellus, B. pratorum, B. pascuorum, B. lucorum* and *B. terrestris*) hosts across the sites sampled. The percentages are calculated using the number of infected queens divided by the total number of queens for each *Bombus* species with Clopper-Pearson 95% confidence intervals. The prevalence of *S. bombi* (a) and *A. bombi* (b) differed significantly across species, but the prevalence of *C. bombi* (c) did not. *N. bombi* (d) was only found in two native host species (*B. terrestris* and *B. pascuorum*). *L. buchneri* (e) was only found in one host species (the native *B. pratorum*).

Parasite species richness

Observed parasite species richness differed among the sampled bumblebee species (Kruskal-Wallis H=24.764, df=5, p<0.001, N=378) and ranged from zero to three parasite species. The estimate of the number of parasite species in the non-native *B. hypnorum* (3, 95% CI +3.0-3.0) was between the estimate for *B. jonellus* (1.1, 95% CI+1.0-3.0) and *B. pascuorum* (4.5, 95% CI +4.0-9.0) (Figure 3-2). Nearly half of the *B. hypnorum* queens (45.76%, n=59) were infected by one or more parasites, with 20 infected by one parasite species, six infected by two parasite species and one infected with three parasite species. Only a single bumblebee queen was host to three parasite species, and this was a *B. hypnorum* (Figure 3.3).

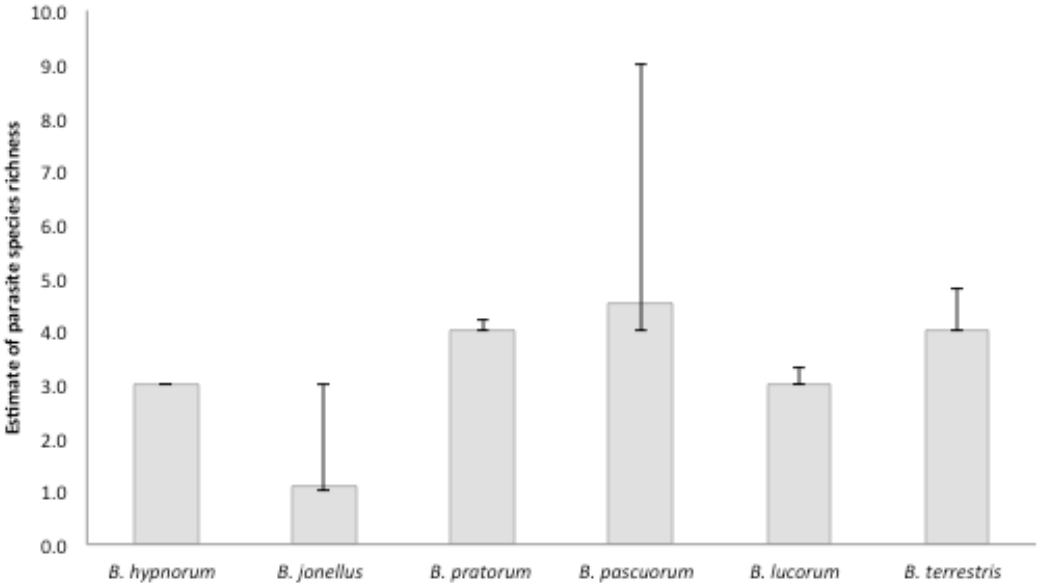

Figure 3-2 Estimate of parasite species richness for *Bombus* host species

The estimates of parasite species richness, across the sites sampled, and the 95% confidence intervals for non-native (*B. hypnorum*) and native (*B. jonellus, B. pratorum, B. pascuorum, B. lucorum* and *B. terrestris*) queens were calculated using SPADE software (Chao and Shen, 2010). The estimate for the non-native *B. hypnorum* (3.0 species, 95% CI = 3.0-3.0) was between the estimate for the native species *B. jonellus* (1.1 species, 95% CI = 1.0-3.0) and *B. pascuorum* (4.5 species, 95% CI = 4.0-9.0).

Parasite community structure

In contrast to my expectations that the invasive *B. hypnorum* may have escaped from its parasite enemies, the parasite communities across the non-native and native *Bombus* species were similar overall ('Morista similarity' multiple community measure = 0.597). Interestingly, in pairwise comparisons between the invasive species and the native species, *B. hypnorum* was more similar to the common species *B. pascuorum* (0.998), *B. lucorum* (0.917) and *B. terrestris* (0.898) than to the closely related species *B. jonellus* (0.295) or *B. pratorum* (0.360).

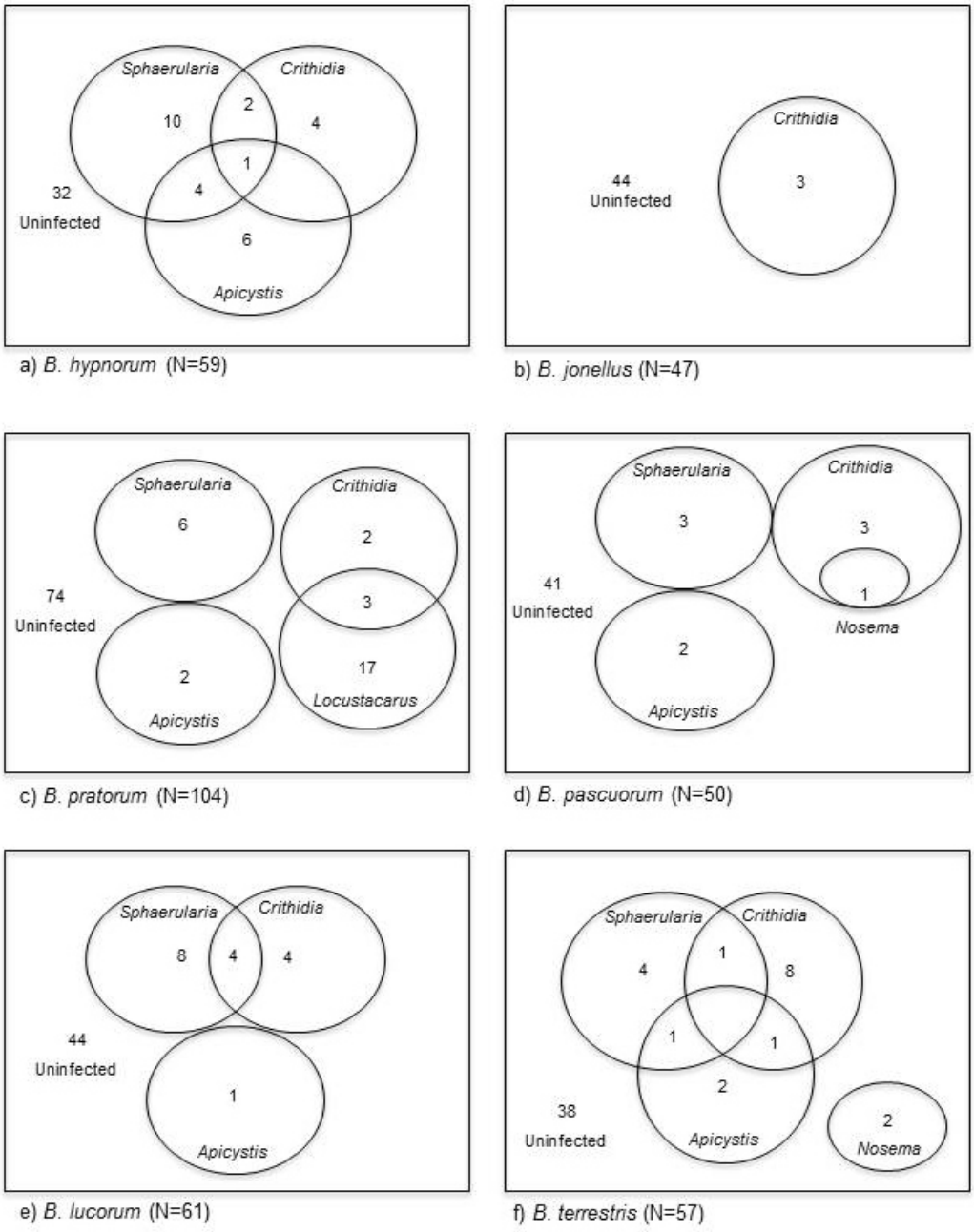

Figure 3-3 Parasite community structure

The parasite community in queens of non-native and native *Bombus* species showing overlaps where multiple infections occur: (a) *B. hypnorum* (non-native), (b) *B. jonellus* (native), (c) *B. pratorum* (native), (d) *B. pascuorum* (native), (e) *B. lucorum* (native), and (f) *B. terrestris* (native). (Size of ovals is not representative of numbers.)

Parasite impact on longevity and colony founding

As expected, *A. bombi* in *B. hypnorum, B, terrestris* and *B. lucorum* was associated with shorter longevity post-capture (U=464.000, p<0.001, N=177). As found in other studies, queens infected with *A. bombi* did not found a colony or produce any offspring (Rutrecht & Brown 2008). The mean post-capture lifespan of *Bombus* queens infected with *A. bombi* was 12.3 days (±7.8SD, N=16) and for uninfected queens 52.9 days (±35.6SD, N=161). *S. bombi* completely inhibited colony foundation in the two native species, as expected. However, the impact of *S. bombi* on *B. hypnorum* differed: five of the *B. hypnorum* queens (29%, N=17) infected with *S. bombi* laid eggs (two produced live offspring) and this differed significantly from the expectation that no queens infected with *S. bombi* would lay eggs ($\chi 2 = 5.8621$, df=1, p=0.0155). Due to sample sizes, I was not able to assess differences in the impact of the remaining, less abundant parasites. However, previous studies suggest that these have little effect on field caught spring queens (e.g. *C. bombi*, Shykoff & Schmid-Hempel 1991). Consequently, from hereon I focus on these two high-impact parasites, *A. bombi* and *S. bombi*.

Parasite community impact

The impact of individual parasites on a host population is modified by the structure of the parasite community (Rigaud *et al* 2010). Consequently, I determined the overall impact of *A. bombi* and *S. bombi* on our invasive and native hosts in the context of their parasite community structure. In contrast to an additive scenario, where the impact of parasites might be considered individually, the synergistic scenarios account for co-occurrence of parasite species within hosts. To be conservative, I calculate the community-level impact with and without our knowledge of the differential impact of *S. bombi* across species (see above). Under the additive scenario, where the prevalence of high impact parasites (*A. bombi*, approximately 19%; *S. bombi*, approximately 29%) was simply added, approximately 48% of our *B. hypnorum* queens would be lost from the population of queens potentially able to found a colony (Figure 3.4). Under the synergistic 'community' scenario, as 8% of *B. hypnorum* queens were infected by both *S. bombi* and *A. bombi* (Figure 3.3a.), approximately 40% of queens would be lost (11% with only *A. bombi*, 21% with only *S. bombi*, and 8% with both). As 8% (N=59) of our *B. hypnorum* queens infected with *S. bombi* were able to lay eggs they may have been able to produce a colony. Thus, under the synergistic 'probable' scenario

(Figure 3-4) approximately 32% of our *B. hypnorum* queens would be lost from the population of potential colony founding queens.

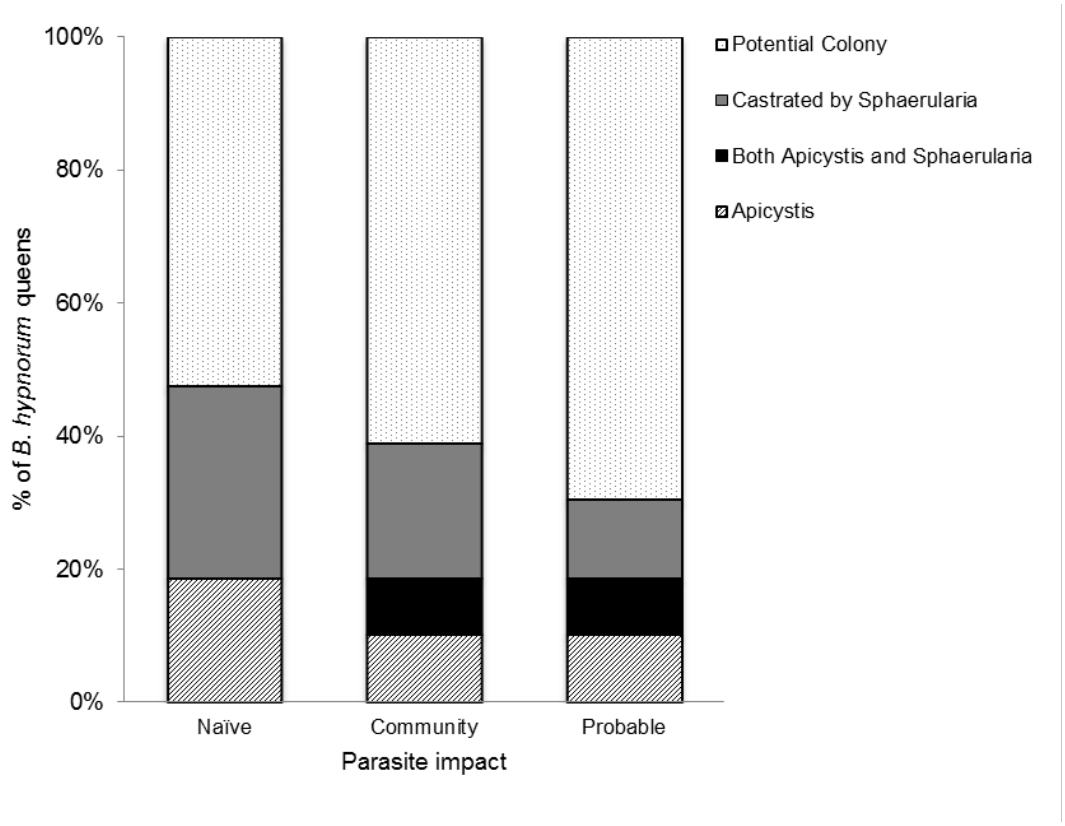

Figure 3-4 Percentage of *B. hypnorum* queens lost from the potential colony founding population as a result of 'high impact' parasites.

The high impact parasites, *Sphaerularia bombi* and *Apicystis bombi*, are shown additively in 'naïve' scenario (*ca*. 48% lost) and with parasite overlap in the 'community' scenario (*ca*. 40% lost). As *ca*. 8% of the 59 *B. hypnorum* queens were still able to found a colony (i.e. produce one or more live offspring), when the actual impact was taken into consideration, in the 'probable' scenario, only *ca*. 32% of the *B. hypnorum* queens were lost from the potential colony founding population.

If I consider the 'probable' impact of *S. bombi* and *A. bombi* on the non-native *B. hypnorum* and on the five native bumblebee species (Figure 3-5), I find the combined impact of *A. bombi* and/or *S. bombi* among bumblebee species differed significantly (Wald=21.668, df=5, p=0.001). The combined impact of *A. bombi* and/or *S. bombi* on the non-native *B. hypnorum* was significantly higher than the combined impact of *A. bombi* and/or *S. bombi* on *B. jonellus* (Wald=7.796, df=1, p=0.005, ExpB=0.053), *B. pratorum* (Wald=11.759, df=1, p=0.001, ExpB=0.197), *B. pascuorum* (Wald=8.138, df=1, p=0.004, ExpB=0.167) and *B. terrestris* (Wald=4.636, df=1, p=0.031, ExpB=0.346). The combined impact of *A. bombi* and/or *S. bombi* on *B. hypnorum* was not significantly higher than the impact on *B.*

lucorum (Wald=0.946, df=1, p=0.331, ExpB=0.658). The combined impact of *A. bombi* and/or *S. bombi* on non-native and native *Bombus* species across sites did not differ significantly (Wald=9.177, df=4, p=0.057). Thus, the number of queens lost from the population of queens potentially able to found a colony is higher for *B. hypnorum* (*ca.* 32%) than *B. lucorum* (*ca.* 23%), *B. terrestris* (*ca.* 18%), *B. pascuorum* (*ca*. 10%) and *B. pratorum* (*ca.* 8%). *B. jonellus* were not infected with either *A. bombi* or *S. bombi*.

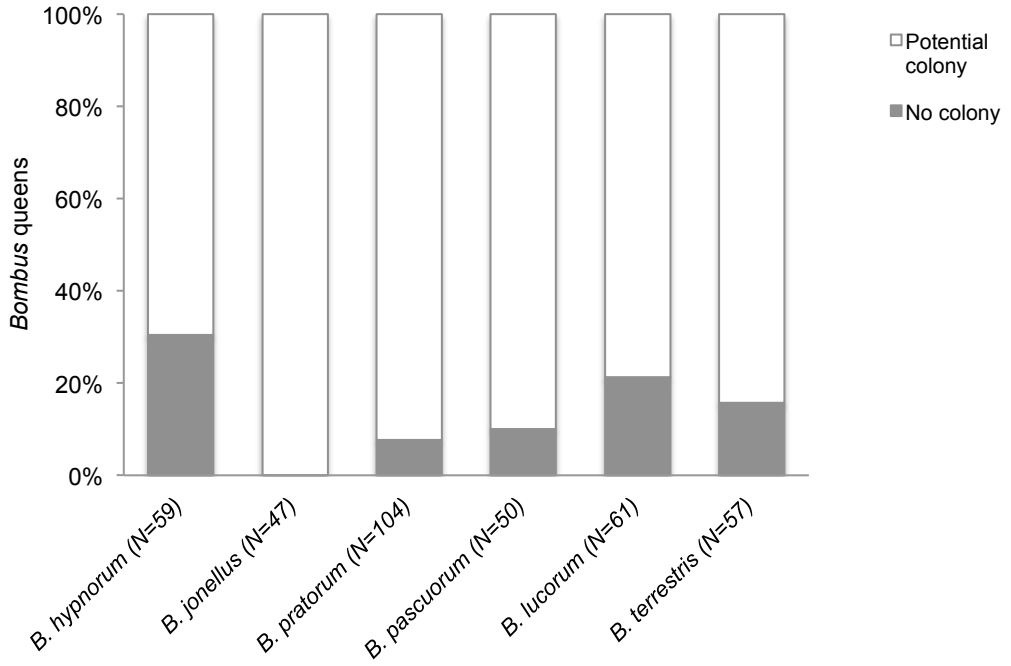

Figure 3-5 Percentage of *Bombus* queens lost from the potential colony founding population as a result of 'high impact' parasites.

The high impact parasites, *Sphaerularia bombi* and *Apicystis bombi*, are shown additively (as there were no multiple infections) for *B. pratorum*, *B. pascuorum* and *B. lucorum* (with *ca*. 8%, *ca*. 10% and *ca*. 21% lost respectively), with parasite overlap (as there were multiple infections) for B. terrestris (*ca*. 16% lost) and with the actual impact taken into consideration in for *B. hypnorum* (*ca*. 32% lost). *B. jonellus* was not infected with either of the high impact parasites.

Functional genetic diversity at the sex-determining locus

Of the 59 *B. hypnorum* queens, 13 produced a colony in the laboratory (i.e. produced one or more live offspring) but of these, three colonies produced both female (worker) and male offspring from the first brood with a ratio close to 50:50 (colony 227: two males and two workers, colony 172: two males and three workers, and colony 150: three males and three workers) indicating that they were producing diploid males (Duchateau *et al* 1994, Gerloff & Schmid-Hempel 2005). In total *59 B. terrestris* colonies and 57 *B. lucorum* colonies were reared in the laboratory but none of these produced males from the first brood (M Fürst, Pers. Comm.). Consequently the number of sex alleles in the *B. hypnorum* population was estimated to be four, compared to at least 32 for *B. terrestris* and at least 31 for *B. lucorum* (Table 3-1) This compares with an estimate of seven sex alleles for 10 Continental European (Scandinavian) *B. hypnorum* colonies (also shown in Table 3-1) using the same method and data from Brown *et al* (2003b). Although the estimates for *B. hypnorum* were calculated from a small number of colonies, these estimates suggest that the invading *B. hypnorum* population in the UK has lower genetic diversity than the native *B. terrestris* and *B. lucorum* populations, and appears to have lower genetic diversity than the Continental European *B. hypnorum* population.

Table 3-1 Estimated number of sex alleles based on the production of diploid males. The transition from significant to non-significant differences gives the minimum number of sex alleles in the population.

		Expected		Observed			
	N	Diploid	Not Diploid	Diploid	Not Diploid	χ^2	Significance
B. terrestris	30	3.9	55.1	0	59	4.0333	*
	31	3.8	55.2	0	59	3.9264	*
	32	**3.7**	**55.3**	**0**	**59**	**3.8198**	**n.s.**
	33	3.6	55.4	0	59	3.7133	n.s.
B. lucorum	30	3.8	53.2	0	57	3.931	*
	31	**3.7**	**53.3**	**0**	**57**	**3.8241**	**n.s.**
	32	3.6	53.4	0	57	3.7174	n.s.
	33	3.5	53.5	0	57	3.6109	n.s.
B. hypnorum – invasive UK	3	8.7	4.3	3	10	4.9983	*
	4	**6.5**	**6.5**	**3**	**10**	**2.0319**	**n.s.**
	5	5.2	7.8	3	10	0.8622	n.s.
	6	4.3	8.7	3	10	0.3361	n.s.
B. hypnorum – non-invasive Scandinavia	5	4.0	6.0	0	10	5	*
	6	3.3	6.7	0	10	3.9521	*
	7	**2.9**	**7.1**	**0**	**10**	**3.3918**	**n.s.**
	8	2.5	7.5	0	10	2.8571	n.s.

* = P<0.05

'N' is the number of sex alleles and each row refers to a given number of sex alleles for each species.

Shaded line shows the minimum estimate for the number of sex alleles in the population.

Discussion

The successful invasion of the non-native *B. hypnorum* suggested that this species may have escaped from its natural enemies, benefitting from a lower parasite load than native congeners. However, in sharp contrast, I found that not only was *B. hypnorum* infected by the same generalist parasite species as native congeners, but that the prevalence of the high impact species, *A. bombi* and *S. bombi,* was also higher (*A. bombi* 19% and *S. bombi* 29%) than in native bumblebee species (*A. bombi* 0-7% and *S. bombi* 0-20%). These results suggest that enemy release is not the main driver for the successful establishment, range expansion and invasion of this non-native species.

Assessing the impact of parasites on invasive species requires the host to be sufficiently established to provide a large enough sample size for analysis. Despite the fact that our sample area was invaded by this species in 2004, only 3 years after the start of the invasion, this was the first year sufficient spring queens could be caught to enable a comparison of parasite communities between it and native species (MJF Brown, unpublished data). While our samples of *B. hypnorum* were taken from only a portion of its invasive range, given the rapid expansion of this species I believe that our results are likely to be representative of the larger population and provide the first insight into the impact of parasites on invasion in this system.

Before discussing our results further, a number of caveats must be addressed. First, some of our sampled individuals may have been sisters, originating from the same natal colony. This has potential implications for both statistical independence and parasite status. However, given what is known about bumblebee nest density in the UK (Knight *et al* 2005) and queen dispersal (Lepais *et al* 2010), and the low rate at which sisters appear in samples of worker populations (Knight *et al* 2005), this seems unlikely to be a major concern. Secondly, queens may have emerged from closely aggregated hibernacula, with implications for infection by *S. bombi*. While little quantitative data on hibernacula exist (Alford 1969b, Sladen 1912), by sampling florally-rich sites (to which spring queens converge) across multiple locations, and collecting sites to exhaustion on sampling visits, our sampling design minimises this potential bias. Similarly, any unknown impacts of parasites that make queens more or less likely to be caught should have been avoided. Thirdly, by sampling spring queens I was unable to assess escape from social parasites (the cuckoo bumblebees). While the invasive population of *B. hypnorum* has definitely escaped

the social parasite *B. norvegicus*, which is absent from the UK, *B. sylvestris*, another social parasite of *B. hypnorum*, is present. It would be interesting to investigate this host/social-parasite interaction further.

The invading non-native population of *B. hypnorum* supported a similar parasite community at the species level, to that of congeneric native host species overall. I note that the potential for non-native parasite strains to be present still exists. Bumblebee parasites can be broadly classified as generalists (MacFarlane *et al* 1995, Schmid-Hempel 1998). The most parsimonious explanation, therefore, for this shared community is that *B. hypnorum* acquired its parasites from native hosts. Firstly, given the likely number of foundress queens in the non-native *B. hypnorum* population (based on the number of sex alleles in the population) and the prevalence of parasites in spring queens (MacFarlane *et al* 1995, Schmid-Hempel 1998, Rutrecht & Brown 2008) it is highly likely that *B. hypnorum* arrived parasite-free in the UK, although parasitized queens may have arrived and been unsuccessful in founding a colony. This is supported by the Tasmanian invasion where the low foundress population of *B. terrestris* had a low parasite load (Allen *et al* 2007). Secondly, the most prevalent parasites in *B. hypnorum* queens were those that either kill queens, or largely prevent colony establishment, thus preventing their potential spread from and within a non-native population (Rutrecht & Brown 2008). The shared parasite community and the hibernation-site transmission route of one of the parasites, *S. bombi*, suggest that the invading *B. hypnorum* acquired these parasites in the invaded environment. This matches the predictions of Drake's model (2003), where release from virulent parasites is important for the establishment phase of the invasion. Interestingly, the parasite community in the non-native *B. hypnorum* was very similar to that of the more abundant congeneric native hosts (*B. pascuorum, B. lucorum,* and *B. terrestris*) and much less similar to that of *B. hypnorum*'s closer relatives (*B. jonellus* and *B. pratorum*), suggesting that parasite acquisition was not phylogenetically constrained, but was driven by host abundance. Mechanistically, *B. hypnorum* has probably acquired its parasite community through overlap in the use of floral resources (Durrer & Schmid-Hempel 1994) and hibernation sites (Alford 1969b) with the native *Bombus* species. While the number of parasite species infecting *B. hypnorum* was similar to that of native congeners and the parasite community in *B. hypnorum* was similar to the parasite community of the native species overall, prevalence levels, particularly of the high impact parasites, were higher in the invasive species than in the native species. Higher prevalence could reflect higher susceptibility, which may relate to the low

levels of genetic diversity I found in *B. hypnorum* or to maladaptation to the parasites in its new range. Previous studies have shown that inbreeding in bumblebees correlates with higher parasite prevalence (Whitehorn *et al* 2011), but both mechanisms may be at play. Even though infections by one parasite species, *S. bombi*, had a reduced impact in *B. hypnorum*, this was outweighed by its higher prevalence. Nevertheless, the high prevalence and corresponding impact of acquired parasites does not appear to have constrained the spread of *B. hypnorum* across the UK. However, this high prevalence could still affect the native species. Firstly, higher prevalence in the invasive species may actually reflect a parasite dilution effect, where the presence of the new and possibly more susceptible host has lowered parasite prevalence in native species (Norman *et al* 1999, Ostfeld & Keesing 2000, Dunn 2009). In the absence of long-term records of parasite prevalence in these, or other bumblebee populations, it is not possible to test this idea. Secondly, the non-native host may also have a detrimental impact on the parasite by preventing transmission. *S. bombi* larvae are usually deposited in the soil at hibernation sites by infected queens, where hibernating queens are infected (Lundberg & Svensson 1975), but, if infected queens found colonies, as I found in this study, such deposition at hibernation sites will not occur, and therefore the parasite's lifecycle would be broken, making *B. hypnorum* a dead-end host for *S. bombi*. This lack of host competence (Ostfeld & Keesing 2000) is likely to reduce parasite prevalence in native congeners, again through the parasite dilution effect (Ostfeld & Keesing 2000, Dunn 2009). Further studies are needed to determine whether this is in fact happening, and, if so, what quantitative impact it is having on native host-parasite interactions.

In addition to assessing its impact on parasite prevalence, estimating functional genetic diversity at the sex-determining locus enables us to retrospectively assess the number of initial foundress queens in the invasive population (Lundberg & Svensson 1975, Schmid-Hempel *et al* 2007). *B. hypnorum* queens can be polyandrous and mate with between one and six males (Pouvreau 1963, Estoup *et al* 1995, Schmid-Hempel & Schmid-Hempel 2000, Paxton *et al* 2001), thus the *B. hypnorum* population in the UK may have been founded by as few as one or two multiply mated queens. Previous studies of both deliberately introduced populations of bumblebees in New Zealand (Lye *et al* 2011), and introduced *B. terrestris* in Tasmania also found that populations may have been established from as few as one or two mated queens (Schmid-Hempel *et al* 2007). Although *B. terrestris* (and *B. lucorum*) are usually monandrous, these studies show that bumblebees can

establish and become invasive from a small number of founding queens. Finally, diploid-male producing colonies of *B. terrestris* have been shown to have significantly lower fitness under semi-natural conditions (Whitehorn *et al* 2009), and consequently the high proportion of diploid-male producing *B. hypnorum* colonies found in this study should constrain population expansion.

Nevertheless, despite its high parasite prevalence and low diversity at the sex-determining locus*, B. hypnorum* has rapidly expanded its range in the UK. What factors might contribute to this success? One contributing factor may be its association with the 'urban' environment (urbanization is increasing in Europe, Eigenbrod *et al* 2011), and its use of resources rarely exploited by other bumblebee species such as nesting sites in trees, bird-boxes and buildings (BWARS, CM Jones Pers. Observ.). *B. hypnorum* is also a generalist forager that visits a wide range of flowers (BWARS) and generalists are often associated with biological invasion success (Williamson 1996). Furthermore, *B. hypnorum* has a bivoltine lifecycle (producing two generations per annum)(Edwards & Jenner 2005) and thus their population might increase more rapidly than univoltine species, such as *B. lucorum* or *B. pascuorum*. Additionally, a second generation *B. hypnorum* queen could mate and found a colony without hibernating, thus avoiding possible infection by *S. bombi* during hibernation.

A final possible explanation is that the bumblebee species assemblage in Great Britain is depauperate compared with that in Continental Europe, presumably due to the emergence of sea barriers to dispersal at the end of the last Ice Age. In some sense, then, *B. hypnorum* may simply be invading favourable habitat. Similarly, two related *Pyrobombus* species, *B. pratorum* and *B. monticola*, invaded Ireland, in the 1940s and 1970s, respectively where the bumblebee species assemblage is even more depauperate than Great Britain (Speight 1974, Fitzpatrick *et al* 2007) suggesting that bees from the *Pyrobombus* sub-genus, such as *B. hypnorum*, may be successful invaders. Unfortunately, no parasite or genetic data exist from the early stages of these invasions to compare with the current study.

Invasion by *B. terrestris* of South America (*ca.* 400km in eight years, Torretta *et al* 2006; Morales *et al* 2013), an area with a native bumblebee fauna, has proceeded at a similarly rapid rate as *B. hypnorum* in the UK (*ca.* 450km in 10 years, BWARS). In South America, parasites have been implicated in the invasion success through their impact on the native *Bombus* species (Torretta *et al* 2006, Plischuk & Lange

2009; Arbetman *et al* 2012). Our data from the *B. hypnorum* invasion suggest that it would be extremely valuable to examine the parasite communities and levels of genetic diversity in other invading and native populations to see whether our results are representative of a more general pattern. Unfortunately, whilst data exist for genetic diversity and parasites in invasive populations in New Zealand and Tasmania (Allen *et al* 2007, Schmid-Hempel *et al* 2007, Lye *et al* 2011), the absence of a native bumblebee fauna makes it difficult to extrapolate these results to other areas.

To conclude, this study shows that high parasite impact and low functional genetic diversity at the sex-determining locus have not prevented the invasion of a non-native bumblebee. This not only has implications for understanding economically important and ecologically devastating invasions (Inoue *et al* 2008, Plischuk & Lange 2009, Arbetman *et al* 2012), it also has implications for the successful design of re-introduction programs which begin with low founding populations and low parasite load (IUCN, Frankham, Ballou & Briscoe 2010). While the obvious next steps would be to investigate *B. hypnorum* in its native range, or the parasite community and genetic diversity of other invasive *Bombus* species in their invaded ranges, this work provides an important step in understanding the role of parasites and genetic variation in insect invasions. A recent study (Venesky *et al* 2012) suggested that captive breeding programs for re-introductions should select for tolerance to natural enemies, to avoid the impact of such enemies in small re-introduced populations with low genetic diversity. Our results, where a genetically depauperate, invasive population has expanded despite high parasite impact, suggest that such complex selection may not be required.

Chapter 4. Host-parasite interactions: Host competence

Abstract

Parasites may be one of the factors driving the global decline of bumble bees, and may be introduced by non-native bumble bee species. As bumble bees are key pollinators of crops, it is important to understand the host-parasite interactions between non-native bumble bees and their parasites.

I consider whether the non-native *B. hypnorum* is a competent host for the generalist parasite *S. bombi*, using parasite reproduction as a proxy for host competence. I investigated the production of parasite eggs and larvae, within this bumble bee host species, and the release of parasite larvae in the host's faeces.

I found that despite weekly faeces checks, over a period of up to 15 weeks, infected *B. hypnorum* queens did not deposit nematode larvae in their faeces and that *S. bombi* eggs rarely develop into *S. bombi* larvae in *B. hypnorum* hosts. Both of these findings suggest that *B. hypnorum* is not a competent host for *S. bombi* and may alter the relationship between *S. bombi* and congeneric native host species.

Introduction

More than 50% of organisms can be considered as parasitic, i.e. obtain resources for their survival from one (or a limited number of) other organisms (Price 1980). Parasites are represented by many taxa, from arthropods to fungi, bacteria and viruses (Schmid-Hempel 2011); have differing lifestyles, including parasites, with single or sequential host species, parasitoids (that kill their hosts) and pathogens (Schmid-Hempel 2011); and infect differing host species, from plants, insects and other arthropods, to birds and mammals including humans (Schmid-Hempel 2011). Furthermore, parasites vector diseases (e.g. malaria: Kovats *et al* 2001, Bluetongue virus: Carpenter *et al* 2009, Wilson & Mellor 2009). Extrinsic drivers of change in these relationships, such as changing climate, changing geographical distributions of species (including range expansions and biological invasions) and the increasing human population are likely to increase the impact of parasites and the diseases that they transmit. Thus is vital to understand host-parasite interactions, and the potential impacts that these changing relationships may have.

Parasites and biological invasions

Parasites may play an important role in the success of biological invasions: Ecological communities will be perturbed by the arrival of non-native species, which may introduce their non-native parasites. Non-native host species may also acquire parasites, from native host species, and act as a reservoir for these parasites which spill-back from the novel host to the native hosts (see Chapter 1) (Tompkins *et al* 2011, Dunn 2009, Daszak *et al* 2000). In addition the native host may act as a reservoir for parasites which then spill-over to non-native hosts (see Chapter 1) (Tompkins *et al* 2011, Dunn 2009, Daszak *et al* 2000). The ability of a host to become a reservoir host is determined by its competence. Thus host species that are more competent hosts are likely to become reservoir hosts and transmit parasites to other host species. In contrast, species that are less competent hosts are less likely to transmit parasites and may become 'dead-end' host for parasite transmission.

Parasite filters

The success of an organism is usually measured by its biological fitness: Its ability to produce offspring that survive to reproduce themselves. The same is true of parasites, and whilst parasites benefit from the protection and resources of their host during their lifetime, they also face the challenges of locating and infecting that host (and in the case of parasites with multiple host life-cycles, locating and infecting the original host) and then transmitting offspring to the next host (which may be a different species). These challenges can be categorised into parasite 'filters': The ecological and the physiological filters described by Schmid-Hempel (2011), is equivalent to the encounter and compatibility filters described by Combes (2004). The ecological (or encounter) filter is determined by biodiversity and behaviour. The parasite needs to exist in a suitable environment and geographic area where there is a sufficiently diverse community to support potential hosts. Furthermore, it must be active at an appropriate time of day and/or year to locate or 'encounter' the host (Combes 2004, Schmid-Hempel 2011). The physiological or compatibility filter is determined by the resources provided by the host and the hosts' defences against parasite attack (Combes 2004, Schmid-Hempel 2011). From the perspective of the host, biological fitness is determined by their ability to reproduce, therefore their susceptibility to parasites, their ability to avoid infection and defend against parasite attack (e.g. through immune responses) and the

virulence or impact of parasite are important factors. Highly virulent parasites include parasites that castrate their hosts and eliminate their reproductive output. From the parasite's perspective locating and infecting a host, avoiding the host's immune defences and obtaining sufficient resources to reproduce determine its biological fitness.

Host competence

A competent host is a host that the parasite can infect and in which the parasite can reproduce. Therefore, host competence determines infection success, reproductive success and the successful transmission of parasite offspring (or infective stages) to next host (or the next stage of the parasite's lifecycle). Thus the biological fitness of the parasite is determined by host competence. A host can be considered 'a dead-end host' when the parasite is able to infect the host but unable to transmit offspring (infective stages) to the next host.

Study system

Bumble bees, *Bombus spp*., are ecologically and economically important pollinators (Waser & Price 1981, Thomson *et al* 1986, Thomson & Goodall 2001), but are in decline globally, threatened by habitat loss, parasites and pesticides (e.g. Kearns et al 1998, Gill *et al* 2012, Morales et al 2013). Despite this general decline, the non-native *Bombus hypnorum* has rapidly expanded its range across England and Wales, and into Scotland, since its first discovery in England in 2001 (Goulson & Williams 2001, BWARS, BBCT). The arrival of *B. hypnorum* in Great Britain may alter the host-parasite interactions between both this non-native species and its generalist parasites, and native *Bombus* spp. and their generalist parasites.

Bumble bee queens

Bumble bees are eusocial species, which produce annual colonies. The queen is a key component of the annual lifecycle, as she is the only member of the colony that survives the winter by hibernating. Bumble bee gynes (unmated new queens) disperse from their natal colonies in late summer, to mate prior to finding a hibernation site, usually in the soil (Alford 1969b). Post-hibernation queens congregate at florally-rich sites to forage for nectar and pollen, before founding a colony, and producing offspring. However, bumble bee queens are host to many generalist parasites (Rutrecht & Brown 2008), including the nematode *S. bombi*,

which infects queens exclusively, and thus in this study I focused on bumble bee queens.

Nematode parasite

S. bombi is a high impact parasite that castrates its bumble bee host, preventing it from founding a colony and producing offspring (Alford 1969a, Poinar & van der Laan 1972, Schmid-Hempel 1998). Furthermore, *S. bombi* alters the behaviour of its host to ensure that parasite offspring are deposited at *Bombus* hibernation sites (Schmid-Hempel 1998). *S. bombi* has a simple life-cycle, with a free-living phase in the soil, where it infects the hibernating queens and a reproductive phase within the host. When the bumble bee queen emerges from hibernation in the spring, the *S. bombi* 'mother' everts her uterus inside the host and lays eggs that develop into larvae (Alford 1969a, Poinar & van der Laan 1972). The parasite larvae are then released into the soil, at *Bombus* hibernation sites, where they mature and mate. The mated females infect the next generation of *Bombus* queens hibernating at these sites.

Bombus hypnorum and *Sphaerularia bombi*

In my previous study of this host-parasite system (see Chapter 3), I found that *B. hypnorum* showed some resistance to the usual castrating impact of *S. bombi:* some infected *B. hypnorum* queens were able to found colonies, lay eggs and produce offspring. This raised several questions. Firstly, if infected *B. hypnorum* queens are able to found a colony, how are *S. bombi* larvae, which are usually deposited at hibernation sites by infected *Bombus* queens, transmitted to the next generation of *Bombus* queens? Does *S. bombi* reproduce successfully in *B. hypnorum* i.e. is *B. hypnorum* a competent host for *S. bombi*? Secondly, I asked if host and parasite reproduction could be mutually exclusive, i.e. do infected *B. hypnorum* queens successfully lay eggs when the parasite infecting them does not and does the parasite successfully lay eggs in its host when the infected *B. hypnorum* queen does not? Finally does infection intensity (i.e. the number of parasites infecting a single host) affect the reproductive success of the parasite and the host?

Using the host *B. hypnorum*, a non-native bumble bee in the United Kingdom, and the generalist *Bombus* parasite *Sphaerularia bombi*, a nematode, I asked whether *B. hypnorum* is a competent host for *S. bombi* and consider the implications of this

host-parasite interaction for both the parasite and for native *Bombus* species. I record the reproductive output of the parasite, *S. bombi,* in wild-caught, laboratory reared *B. hypnorum* queens to establish whether the parasite produces eggs and larvae within this host. I examine the faeces of the *B. hypnorum* queens on a weekly basis, to check if nematode larvae are deposited by this host. I dissect the *B. hypnorum* queens, *post-mortem,* to quantify the prevalence, the infection intensity and the development of the parasite within the host. I also record the reproductive success of the host, to investigate whether host and parasite reproductive success are mutually exclusive. To my knowledge this is the first study of the competence of *B. hypnorum* as a host for *S. bombi.*

Methods

Assessing the competence of *B. hypnorum* as a host for *S. bombi*

I investigated the competence of *B. hypnorum* queens as hosts for *S. bombi* in two ways. In 2012 I collected *B. hypnorum* queens, maintained them in the laboratory, checked their faeces weekly for *S. bombi* larvae then dissected queens to check for *S. bombi* 'mothers'. Dissections of queens collected in 2012 and those collected in 2011 (see Chapter 3) enabled a second test of this question, as I was able to record the presence or absence of nematode offspring in the haemocoel of host queens. The dissection data and records of host reproductive output in the laboratory enabled me to investigate whether the reproduction of the nematode parasite and the bumble bee host are mutually exclusive and whether infection intensity plays a role.

Bee collection

In 2012, from 12th January to 20th April, *B. hypnorum* spring queens were collected from florally rich sites in Windsor Great Park (Lat. 51.41, Long. -0.60), with the permission of the Crown Estate. Additional *B. hypnorum* queens were collected on the campus of Royal Holloway, University of London (RHUL, Lat. 51.43, Long. -0.56). At the beginning of the season (i.e. January and February), when queens started to emerge, I visited Windsor Great Park on every sunny day after 11am when the temperature reached 10^{o}C and spent a minimum of one hour searching for *B. hypnorum* queens. Later in the season (March and April), I searched for queens every dry working day from 8.30am when the temperature reached 10^{o}C, even if the weather was overcast, for at least 4 hours.

In 2011, *B.hypnorum* queens were collected from Windsor Great Park; the Royal Horticultural Society (RHS) Garden, Wisley; the Royal Botanical Garden at Kew; RHUL and from Horsell, Surrey (see Chapter 3).

The spring queens were collected and transported to the laboratory at RHUL, where faecal samples were taken and examined using a x400 phase contrast microscope for parasites (see Chapter 2). I checked for the target parasite *S. bombi,* and for parasites which might impact the laboratory longevity and reproductive success of the hosts: *Apicystis bombi,* a neogregarine; *Crithidia bombi,* a trypanosome; and *Nosema bombi,* a microsporidian. *A. bombi* kills bumble bee queens before they are able to found colonies (Rutrecht & Brown 2008), *C. bombi* and *N.* bombi reduce overall colony fitness (Brown *et al* 2003a, Otti & Schmid-Hempel 2007, Rutrecht & Brown 2009). These parasite species can be identified using a x400 phase contrast microscope (Rutrecht & Brown 2008).

Bee husbandry

The queens were then housed in individual 'queen-rearing' boxes in a specially prepared laboratory at RHUL. Each queen was housed under ideal environmental conditions (50-60% humidity, 25-28^{o}C) to found a colony. Queens were kept in queen-rearing boxes with a pollen ball to encourage egg-laying and a gravity feeder to dispense sugar-water (see Chapter 2). The pollen balls were checked for bumble bee eggs three times a week (Monday, Wednesday and Friday) and replaced with a fresh one if no eggs were present (see Chapter 2). Queens received sugar-water and pollen *ad libitum.* Queens were kept in the dark and a red light was used for working, and sterile procedures were used when handling the queens to avoid cross-contamination. The queens were checked daily and any dead individuals were removed and stored at -80^{o}C in the freezer for dissection at a later date.

Weekly faeces checks

I performed weekly faecal checks for all of the *B. hypnorum* queens collected in 2012 to check for larvae of the parasite *S. bombi.* These checks took place for 15 consecutive weeks, unless the queen died, in which case she was immediately frozen. *Bombus* queens deposit the first nematode larvae during the first three weeks following their emergence from hibernation (Kelly 2009) and the total expected period of larval deposition is six weeks (Kelly 2009). As *B. hypnorum* is resistant to castration by *S. bombi* (see Chapter 3), these hosts may delay parasite

larval production and deposition, I added six weekly checks to ensure that larval deposition was not missed. Therefore to check the potential period of nematode larvae deposition I checked the faeces for a total of 15 consecutive weeks. After 15 faecal checks the surviving queens were sacrificed by freezing.

Bee dissection

B. hypnorum queens were dissected to establish if they were infected with *S. bombi*, (see Chapter 2) and whether the parasite and the host were able to produce offspring (i.e. parasite eggs and larvae, and bumble bee eggs). The number of nematode uteri was recorded, each individual uteri was measured using a ruler and for comparison, the nematode uteri from *B. terrestris* and *B. lucorum* queens collected in 2011 were also measured (see Chapter 3). The haemocoel was checked for nematode eggs and larvae under both the dissecting microscope and the x400 phase contrast microscope, and their presence (or absence) was recorded. The queens were also checked for signs of ovarian development (i.e. bumble bee eggs in the ovarioles, ovarian tubes).

Data analysis

The prevalence of *S. bombi* was calculated by dividing the number of infected queens by the total number of queens from each species and/or year. The infection intensity was calculated by counting the number of *S. bombi* uteri in every infected queen. Statistical analyses of data were performed using IBM SPSS 19 for Windows.

Results

A total of 52 *B. hypnorum* queens were collected in 2012: 44 from Windsor Great Park and eight from RHUL, in 108 hours across 31 collecting days. All 52 queens were dissected but three of the queens examined (two from Windsor Great Park and one from RHUL) were too decomposed to record parasite status, therefore these were removed from the data set, and the analysis only includes the remaining 49 individuals. A further 59 *B. hypnorum* spring queens were collected in 2011 (see Chapter 3).

Parasite prevalence

A total of 108 *B. hypnorum* queens were examined for parasites: Forty nine in 2012 and 59 in 2011. The prevalence of *S. bombi* in *B. hypnorum* queens, collected in 2012, was *ca*. 22% (11/49) (Figure 4-1). Five *B. hypnorum* queens were infected with both *S. bombi* and *C. bombi*. A total of 25 queens were infected with *C. bombi* (*ca.* 51%). One queen was infected with both *C. bombi* and *N. bombi.* Four queens were infected with *N. bombi* (*ca.* 8%). Two *B. hypnorum* queens were infected with A. bombi (*ca.* 4%), and survived for 11 days and 15 days respectively in the laboratory. There was no pattern of seasonal variation in *S. bombi* prevalence in *B. hypnorum* (Figure 4-1).

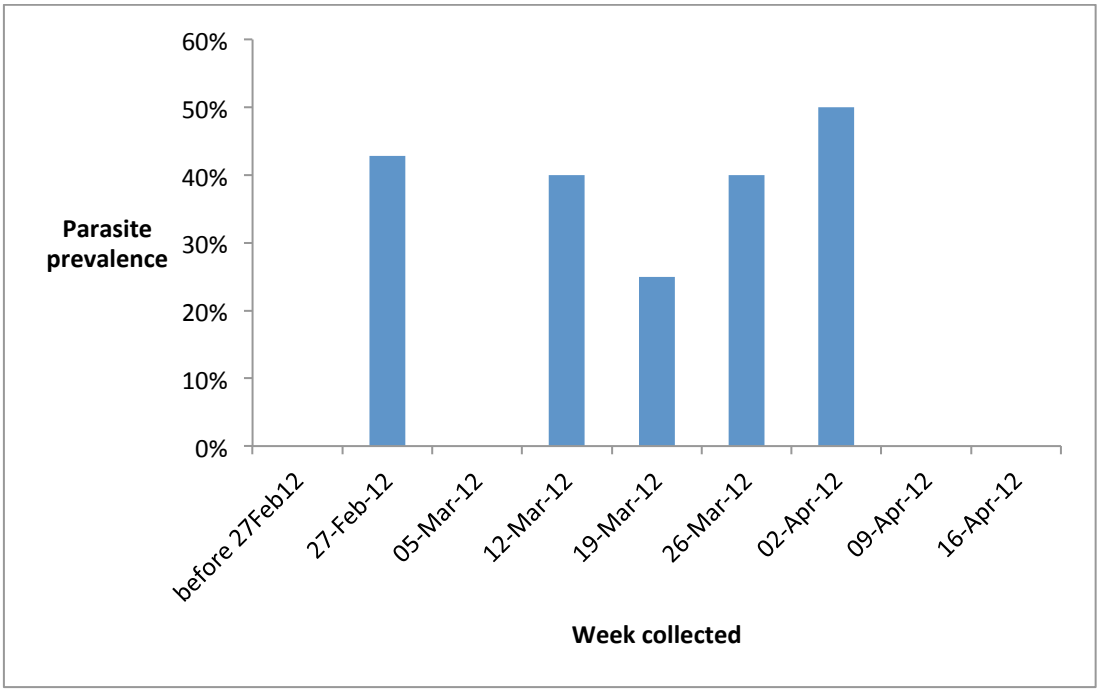

Figure 4-1 The prevalence of *S. bombi* in *B. hypnorum* queens collected in 2012

The percentage prevalence of *S. bombi* in *B. hypnorum* queens was calculated by dividing the number of infected queens, for each period, by the number of queens collected in that period. The dates shown indicate the start of the week the queens were collected. The prevalence varies across the collection period.

The prevalence of *S. bombi* in *B. hypnorum* queens, collected in 2011, was *ca*. 29% (17/59). Of these four queens were infected with S. *bombi* and *A. bombi*, two queens were infected with S. *bombi* and *C. bombi* and one queen was infected with *S. bombi, A. bombi* and *C. bombi* (see Chapter 3). A total of seven queens were infected with *C. bombi* (*ca.* 12%).

The combined 2012 and 2011 data gave me a total sample size of 108 *B. hypnorum* queens. The number of infected queens over the 2 year period was *ca.* 26% (28/108) confirming that *B. hypnorum* are susceptible to this parasite.

Parasite larvae in faeces checks

Despite weekly faeces checks, over a period of up to 15 weeks, the 11 *B. hypnorum* queens, that were later confirmed as infected by *S. bombi*, did not deposit nematode larvae in the faeces. The faeces of all 49 *B. hypnorum* queens collected in 2012 were checked at least once (Figure 4.2). An initial faeces check and three additional weekly checks (i.e. four faeces checks) were completed for 10 infected queens (91% of the total number of infected queens) and 33 uninfected queens (87%). The first expected record of parasite larval production is 21 days following queen emergence from hibernation (Kelly, 2009), therefore the first four weekly checks, cover the period when the first parasite larvae can be seen in faeces samples from wild caught queens (shown by the arrow labelled 'first parasite larvae production' on Figure 4-2). Further faeces checks were completed to cover the total period of parasite larval production of 21 days plus six weeks (Kelly, 2009), thus nine faecal checks (i.e. one initial check and eight subsequent weekly checks) were completed for eight infected queens (73%) and 23 uninfected queens (61%)(shown by the arrow labelled 'total parasite larvae production' on Figure 4-2). I completed a further six, i.e. total of 15, faeces checks on queens two infected (4%) and three uninfected (6%) queens that survived for 15 weeks, to ensure that any delay in parasite larval production or deposition was checked.

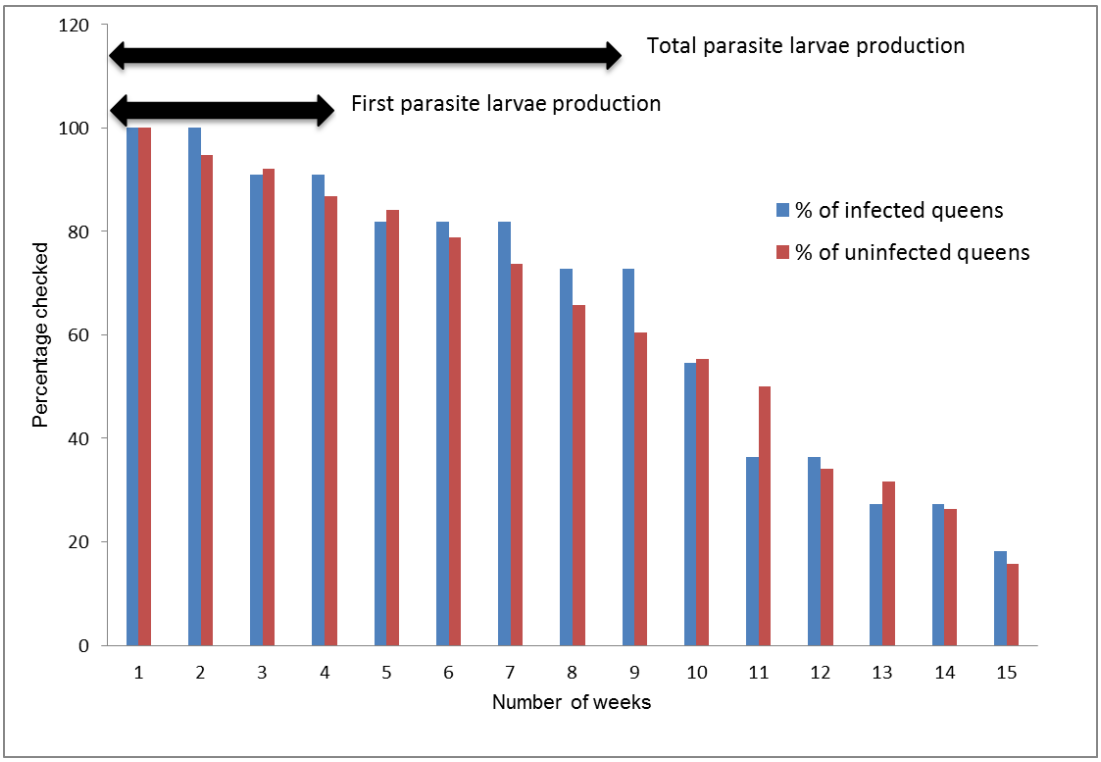

Figure 4-2 The proportion of *B. hypnorum* queens that completed up to 15 weekly faeces checks

The proportion of queens infected by *S. bombi* (in blue) and not infected by *S. bombi* (in red) that survived, in the laboratory, to complete between one and 15 weekly faeces checks to establish if they were depositing *S. bombi* larvae in their faeces. The first parasite larvae production period (three weeks) and the total parasite larvae production periods are shown with double ended black arrows. Over 90% of infected queens completed four faeces checks and over 70% of infected queens completed nine faeces checks.

Reproductive output of *S. bombi*

In 2012, the reproductive output of *S. bombi* in 11 infected *B. hypnorum* hosts was limited to three queens containing *S. bombi* eggs but no *S. bombi* larvae. The remaining eight infected queens contained no *S. bombi* eggs and no *S. bombi* larvae. Again in 2011, the reproductive output of *S. bombi* was limited to three of the 17 infected *B. hypnorum* queens: two queens contained *S. bombi* eggs but no *S. bombi* larvae and one queen contained both *S. bombi* eggs and *S. bombi* larvae. The remaining 14 infected queens contained no *S. bombi* eggs and no *S. bombi* larvae. While S. bombi females are able to infect *B. hypnorum* hosts over-wintering in the soil, and they are able to develop and evert their uterus, their ability to produce offspring (i.e. eggs and larvae) appears to be limited (Table 4-1).

Table 4-1 The reproductive output of *S. bombi* in *B. hypnorum* hosts

Year	Eggs[a]	Larvae[b]	
		No	Yes
2012	No	8	0
	Yes	3	0
2011	No	14	0
	Yes	2	1
Combined	No	22	0
	Yes	5	1

[a] *S. bombi* eggs produced by *S. bombi* parasites in infected *B. hypnorum* hosts

[b] *S. bombi* larvae produced by *S. bombi* parasites in infected *B. hypnorum* hosts

The eversion of *S. bombi* uteri

Overall the everted uteri from *B. hypnorum* queens were not a significantly different length to the everted uteri from either the *B. terrestris* queens or the *B. lucorum* queens (Kruskal-Wallis H=1.596, df=2, p=0.450, N=91) for *B. terrestris* and *B. lucorum* collected in 2011 and *B. hypnorum* collected in both 2012 and 2011 and (Kruskal-Wallis H=1.141, df=2, p=0.565, N=70) for *B. hypnorum*, *B. terrestris* and *B. lucorum* collected in 2011 (Figure 4.3). A total of 49 individual nematode uteri, from a total of 28 infected *B. hypnorum* queens, measured between 7mm and 22mm, with an overall mean of 12.9mm (SD3.2). Breaking this down into individual years, I found that in 2012, 21 nematode uteri (from 11 infected queens) measured 7-14mm with a mean of 11.4mm (SD1.9) and in 2011, 28 nematode uteri (from 17 infected queens) measured 9-22mm with a mean of 13.9mm (SD3.7). These measurements compare with between 10mm and 19mm with a mean of 12.8mm (SD3.2) from 11 nematode uteri from six infected *B. terrestris* queens and between 9mm and 19mm with a mean of 13.3mm (SD2.6) from 31 nematode uteri from 10 infected *B. lucorum* queens in 2011.

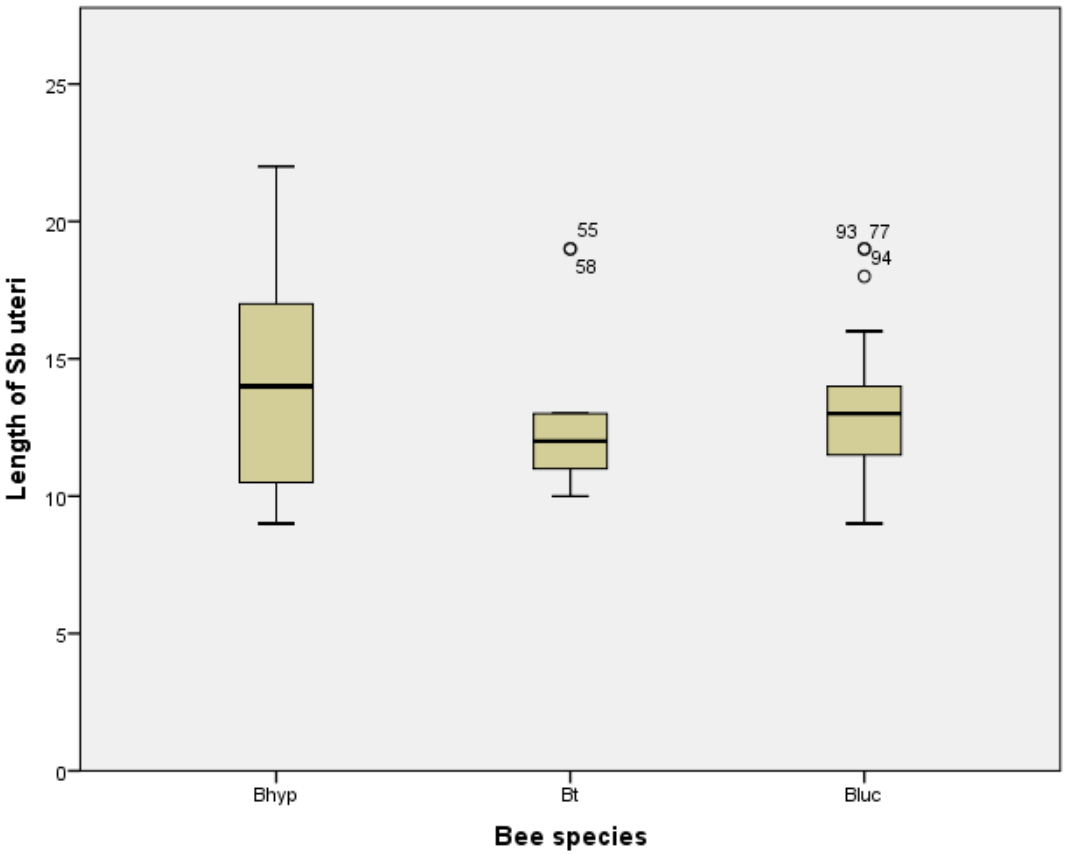

Figure 4-3 Parasite length in three species of *Bombus* queens

The length, in millimetres, of the everted uteri of *S. bombi* parasites found in three species of *Bombus* hosts is not significantly different: Kruskal-Wallis H=1.141, df=2, p=0.565 (Bhyp: parasites from *B. hypnorum* hosts N=33, Bt: parasites from *B. terrestris* hosts N=9 and Bluc: parasites from *B. lucorum* hosts N=28). The numbered circles indicate outliers. These data are from *Bombus* queens collected in 2011.

Infection intensity

The infection intensity of *S. bombi* parasites in *B. hypnorum* queens in 2012 ranged from one to eight parasites infecting an individual queen. Eleven infected *B. hypnorum* queens contained a total of 33 *S. bombi* individuals (overall mean 3.0 ±2.5SD). Five infected *B. hypnorum* queens contained just one *S. bombi* individual and the remaining six queens were multiply infected and contained a total of 28 *S. bombi* individuals (range 2-8, mean 4.7±2.2SD).

In 2011, 17 infected *B. hypnorum* queens contained a total of 59 *S. bombi* individuals (overall mean 3.5±3.9SD). Four infected *B. hypnorum* queens contained just one *S. bombi* individual and the remaining 13 queens were multiply infected and contained a total of 55 *S. bombi* individuals (range 2-17, mean 4.2±4.2SD).

Reproductive output of *B. hypnorum*

Of the 11 queens infected with *S. bombi* in 2012, three laid eggs, but no infected queens produced live offspring (Table 4-2). Of the 38 uninfected queens, 22 laid eggs and two produced live offspring (including one that produced both workers and males), but no queens produced gynes. Although the proportion of queens infected with *S. bombi* that laid eggs (*ca.* 27%, 3/11) is lower than the proportion of uninfected queens that laid eggs (*ca.* 58%, 22/38), the difference was not significant (G=3.289, df=1, 2-tailed p=0.070).

Table 4-2 Reproductive output of *B. hypnorum* queens collected in 2012

Infected queens (n=11)	Laid eggs	3
	Did not lay eggs	8
Uninfected queens (n=38)	Laid eggs	22
	Did not lay eggs	16

Of the 17 infected queens with *S. bombi* in 2011, five laid eggs, and two produced live offspring (Table 4-3). In 2011, 24 of the 42 uninfected queens laid eggs and 11 produced live offspring. Once again, the proportion of queens infected with *S. bombi* that laid eggs (*ca.* 29%, 5/17) is lower than the proportion of uninfected queens that laid eggs (*ca.* 57%, 24/42), and in this case close to significant (G=3.813, df=1, 2-tailed p=0.051).

Table 4-3 Reproductive output of *B. hypnorum* queens collected in 2011

Infected queens (n=17)	Laid eggs	5
	Did not lay eggs	12
Uninfected queens (n=42)	Laid eggs	24
	Did not lay eggs	18

The pattern for both 2012 and 2011 suggest that uninfected queens are more likely to lay eggs than infected queens (although not significantly). When the data from 2012 and 2011 are combined, the number of uninfected *B. hypnorum* queens that laid eggs is significantly higher the number of infected queens that laid eggs (G=7.120, df=1, 2-tailed p=0.008).

Mutually exclusive reproduction

In 2012, I found no significant relationship between the reproduction success of hosts and parasites (G=2.306, df=1, 2-tailed p=0.129). Although there is no significant difference, sample sizes are low, and the pattern suggests that *S. bombi* successfully lay eggs when B. *hypnorum* do not and B. *hypnorum* successfully lay eggs when *S. bombi* do not**.** 2011 data showed the same lack of a significant relationship (G=2.348, df=1, 2-tailed p=0.125) and the same pattern. When combining the data across both years there is a significant relationship (G=4.662, df=1, 2-tailed p=0.031) suggesting that host and parasite reproduction are mutually exclusive (Table 4.4).

Table 4-4 Reproductive output of *S. bombi* parasites and *B. hypnorum* hosts

The egg laying success of *S. bombi* parasites (in infected hosts) and (infected) *B. hypnorum* hosts in 2011 and 2012 suggest a pattern of mutually exclusive reproduction (i.e. the parasite lays eggs when the host does not and *vice versa*). When the data for both years are combined the result is significant and marked *

	B. hypnorum	*S. bombi*		Result
		No	Yes	
2012	No	5	3	G=2.306, p=0.129
	Yes	3	0	
2011	No	9	3	G=2.348, p=0.125
	Yes	5	0	
Combined	No	14	6	G=4.662, p=0.031*
	Yes	8	0	

Infection intensity and reproductive output of *S. bombi*

S. bombi are significantly more likely to lay eggs when the *B. hypnorum* queen is infected by more than one parasite (multiple infection) than in *B. hypnorum* queens infected by an individual parasite (single infection) in queens collected in 2012 (G=4.573, df=1, 2-tailed p=0.032). Although there is no significant difference in the reproductive output of *S. bombi* in queens with single *versus* multiple infections collected in 2011 (G=1.799, df=1, 2-tailed p=0.180). When the data across both years is combined, there is a significant difference (G=5.398, df=1, 2-tailed p=0.020) suggesting that infection intensity does affect the reproductive output of the parasite (Table 4.5). Parasites in multiple infections are more likely to lay eggs.

Table 4-5 Reproductive output of *S. bombi* parasites

The egg laying success of *S. bombi* parasites in single and multiple infections of *B. hypnorum* hosts suggests that parasites are more likely to lay eggs in multiple infections. In 2011 this is significant but not in 2012. When the data for both years are combined the result is also significant. The significant results are marked *

	Infection intensity	*S. bombi*		Result
		No	Yes	
2012	Single	5	0	G=4.573, p=0.032*
	Multiple	3	3	
2011	Single	4	0	G=1.799, p=0.180
	Multiple	10	3	
Combined	Single	9	0	G=5.398, p=0.020*
	Multiple	13	6	

Infection intensity and reproductive output of *B. hypnorum*

For queens collected in 2012, *B. hypnorum* queens infected by an individual parasite (single infection) are significantly more likely to lay eggs than *B. hypnorum* queens infected by more than one parasite (multiple infection) (G=6.161, df=1, 2-tailed p=0.013). However, for queens collected in 2011, this was not the case (G=0.050 df=1, 2-tailed p=0.823). When the data across both years is combined, there is no significant difference (G=1.581, df=1, 2-tailed p=0.209) suggesting that infection intensity may affect the reproductive output of the host (Table 4.6).

Table 4-6 Reproductive output of *B. hypnorum* hosts

The egg laying success of *B. hypnorum* hosts in single and multiple infections of *S. bombi* parasites suggests that hosts are more likely to lay eggs in single infections. In 2011 this is significant but not in 2012 or when the data for both years are combined. The significant result is are marked *

	Infection intensity	*B. hypnorum*		Result
		No	Yes	
2012	Single	2	3	G=6.161, p=0.013*
	Multiple	6	0	
2011	Single	3	1	G=0.050, p=0.823
	Multiple	9	4	
Combined	Single	5	4	G=1.581, p=0.209
	Multiple	15	4	

Discussion

I found *B. hypnorum* queens infected with *S. bombi*, in both 2012 (N=11, prevalence 22%) and 2011 (N=17, prevalence 29%). Although these prevalence levels were higher than the level of 9.1% in European *B. hypnorum* (N=44) reported by MacFarlane *et al* 1995, this confirmed that *B. hypnorum* in the UK are susceptible to *S. bombi*. Therefore *S. bombi* can pass successfully through the ecological (encounter) filter (Schmid-Hempel 2011, Combes 2004), probably via the same route as the infection of native species i.e. while hibernating in the soil, and thus are able to encounter and infect this non-native host species. Although these data confirm that a certain proportion of the hibernating *B. hypnorum* queens were infected by *S. bombi* parasites, many more parasites may have encountered hibernating *B. hypnorum* queens and failed to infect them. Experimental laboratory infections would be required to quantify the susceptibility of *B. hypnorum* hosts and determine whether, when parasites are known to be present, they succeed in infecting the host (see Experimental Appendices).

In both 2012 and 2011, eight (three and five respectively) of the 28 (11 and 17 respectively) infected *B. hypnorum* queens were able to lay eggs and 2 (2011 only) were able to produce live offspring, when raised in appropriate environmental conditions in the laboratory. Röseler (2002) reported that two *B. hypnorum* queens are able to found a colony and produce sexuals when infected with *S. bombi*. However, he did not quantify the likelihood of this occurring. Although the *B. hypnorum* in my study in 2012 did not produce sexuals, this lack of reproductive output may have been due to the regular disturbance caused by weekly faeces checks. Nevertheless, my data provides further evidence that *B. hypnorum* shows some resistance to the expected castrating impact of *S. bombi.* However, only 6 (3 in each year) of the 28 *B. hypnorum* hosts contained *S. bombi* eggs and only one infected queen (from 2011) contained *S. bombi* larvae. This suggests that whilst the parasite can infect the host and develop inside the host, its ability to reproduce (i.e. produce eggs and larvae) is limited and thus at this point the physiological (compatibility) filter (Schmid-Hempel 2011, Combes 2004) is restricted.

A total of 49 everted *S. bombi* uteri, from 28 *B. hypnorum* hosts (11 in 2012; 17 in 2011) did not differ significantly in length to those from *B. terrestris* and *B. lucorum* hosts. *S. bombi* in *B. hypnorum* hosts are able to develop and evert their uteri to a similar size to that of larger, native congeneric host species suggesting full eversion. However, as some of the sphaerules (spherical appendages) on the *S. bombi* uteri

appeared slightly flattened (pers. observ.), the uteri may not be fully developed. This possible lack of parasite development within this non-native host, and the possible resistance of this host to both castration and its impact on parasite reproduction warrant further investigation.

After faecal checks, that covered both the 'first parasite larvae production' period (initial 21 days) and the 'total parasite larvae production' period (subsequent six weeks), failed to record any *S. bombi* larvae in the faeces of the *B. hypnorum* queens, it appears unlikely that *B. hypnorum* hosts deposit *S. bombi* larvae either in the nest or at over-wintering sites. Poinar and van der Laan (1972) reported third stage *S. bombi* larvae in *B. hypnorum* in 1971 in the Netherlands although they do not state their sample size. However, my findings are for non-native *B. hypnorum* in England, i.e. outside its native range, and the host-parasite relationship between *B. hypnorum* and *S. bombi* may differ in its native range and in the invaded range. Furthermore, it is over 40 years since the Poinar and van der Laan study and therefore this host-parasite relationship may have altered over time. Decaestecker *et al* (2007) found that host-parasite relationships (*Daphnia* and its microparasites) could evolve over a period of years, thus the *B. hypnorum-S.bombi* relationship may have altered over the past 40 years.

Although *S. bombi* parasites may infect, and may produce offspring (eggs and rarely larvae) in *B. hypnorum* hosts, it appears that *B. hypnorum* do not deposit *S. bombi* larvae in their faeces. Thus the parasite *S. bombi* is unable to transmit larvae that will mature and mate in the soil and infect a new generation of hosts. Therefore the competence of *B. hypnorum* as a host for *S. bombi* is low – possibly so low that it may be a complete dead-end as a host for *S. bombi.* Although this nematode parasite passes successfully through the ecological filter, its passage through the physiological filter is restricted when infecting *B. hypnorum* hosts.

From my data, it is plausible that *B.hypnorum* queens, infected with *S. bombi,* do not deposit *S.bombi* larvae in their faeces, however it is possible that queens that died before the 21 days period of first larval deposition (Kelly, 2009), or before the nine weeks (21 days plus six weeks) of total expected larval deposition (Kelly, 2009), may have deposited *S. bombi* larvae. It would be interesting to repeat this study over multiple years and sites, to substantiate these findings.

In native *Bombus* hosts, *S. bombi,* is able to castrate the hosts and alter their behaviour, enabling parasite offspring (*S. bombi* larvae) to be deposited at host hibernation sites, where they are able to complete their life-cycle and infect the next generation of hosts (Poinar & van der Laan 1972). In *B. hypnorum*, the parasite's impact appears to be limited, as the host is not always castrated, and, as some hosts are able to found colonies, the parasite's ability to alter the host's behaviour is restricted. This suggests that *B. hypnorum* queens in England are resistant to this parasite or that the parasite is maladapted to this host. Perhaps this is because this non-native host acquired parasites from native congeners in the invaded range (see Chapter 3).

Previous studies of the *Bombus-S.bombi* host parasite relationship have shown that *Bombus* hosts infected by an individual nematode parasite are castrated by the parasite (e.g. Kelly 2009). However in the *B.hypnorum-S.bombi* system it appears that infection intensity may have an impact on both the reproductive output of the host and the parasite. From the queens collected in 2012, the host is significantly more likely to lay eggs when infected by a single *S.bombi* than when infected by multiple *S.bombi* individuals, and the parasite is significantly more likely to lay eggs in a multiple infection of a host than in a single infection. In 2011 the trend is similar but the differences are not significant. Despite the lack of a significant result, in the *B. hypnorum-S.bombi* system there appears to be a trend that *B. hypnorum* lay eggs when *S. bombi* do not, and *S. bombi* l ay eggs when *B. hypnorum* do not. If host reproduction and parasite reproduction are mutually exclusive, it is possible that the reproduction of either party is dependent on the same resource. Thus it is possible that the parasite requires resources from host, but is unable to obtain them suggesting constriction of the physiological filter.

My previous study of the *B.hypnorum-S.bombi* interaction found that the prevalence of *S. bombi* was higher in the non-native *B. hypnorum* than in native congeneric species, both in the closely related *B. jonellus* and B. *pratorum*, and in the abundant *B. terrestris* and *B. pascuorum* (see Chapter 3). As the non-native host, *B. hypnorum,* appears to be an incompetent host (or have low competence as a host for *S. bombi*) this unexpected finding in the relationship between a non-native species and a generalist parasite may have implications for the native congeneric species who share the parasite species. If *B. hypnorum* is a dead-end host, the presence of such a host may dilute the prevalence of parasites in native species. Furthermore, native species may act as a source of parasites that infect the non-

native species and are then unable to reproduce. This dilution effect will occur for two reasons. Firstly, the *Bombus* community has become more diverse as it has acquired a new potential host species for the parasite, therefore parasite impact on native *Bombus* species may be reduced (Johnson & Thieltges 2010). Secondly, the lack of competence of this new host species for the parasites, therefore lack of parasite offspring from parasites infecting these hosts, may reduce the parasites in the environment (the soil) to infect the next generation of hosts. From the parasites point of view, *B. hypnorum* appears to be an attractive host that it can infect, but due to the physiological filter imposed on this host-parasite system, *S. bombi* populations may decrease, unless the parasite is able to maintain its numbers in other *Bombus* species.

S. bombi is a generalist parasite that will infect both non-native and native *Bombus* host species, hibernating in the soil. If a *Bombus* gyne (new queen) mates and produces a colony without over-wintering in the soil, *S. bombi* does not have an opportunity to infect these queens. As *B. hypnorum* can be bivoltine, i.e. produce two generations in one year, this would enable an increase in the host population but the opportunity for the parasite to infect the host is missed due to the timing mis-match. Thus due to the behaviour of the host the ecological filter (Schmid-Hempel 2011) closes for the parasite.

If *Bombus* queens, such as *B. hypnorum*, are not castrated by *S. bombi* and are able to found a colony, they may not deposit nematode larvae at over-wintering sites that will infect the next generation of over-wintering queens. This is likely to disrupt the parasite's lifecycle, as if the parasites are able to produce nematode larvae, they are likely to be deposited in the nest rather than at over-wintering sites.
If nematode larvae are deposited in the nest, they may not have sufficient time to mature and mate before infecting the next generations of queens. However, if the host does not deposit parasite larvae or a limited number of parasite eggs develop into parasite larvae, perhaps the timing is irrelevant.

In addition to the potential disruption to the parasite's lifecycle due to possible deposition of nematode larvae in the nest rather than at over-wintering sites, this study found that despite successfully infecting *B. hypnorum* queens, *S. bombi* have limited reproductive success: they may produce eggs, they rarely develop into larvae, and they do not deposit nematode larvae in *B. hypnorum* faeces. This suggests that *B. hypnorum* is not a competent host for *S. bombi*.

Earlier studies reported *B.hortorum* foundress queens infected with *S. bombi* collected in England in 1964 (Alford 1969a) and in Germany in 1964 (Röseler 2002) this native species interaction with *S. bombi* would provide a useful comparison to the non-native *B. hypnorum* and *S. bombi* interaction. Once again these reports are over 40 years old and this host-parasite interaction may have altered.

There is increasing evidence that commercially reared non-native bumble bee colonies, introduced to enhance pollination of crops (such as tomatoes and raspberries) are introducing parasites (Graystock *et al* 2013, Morales *et al* 2013). However is the introduction of a new host species always detrimental to native congeneric species where parasites are concerned? In this study, despite a high prevalence of generalist parasites in the non-native hosts, the reproductive success of those parasites was limited and none of the infected hosts observed released parasite offspring. This suggests that the non-native *B. hypnorum* host may be a dead-end host for the parasite *S. bombi* and therefore may 'dilute' the parasite population, reducing the prevalence of *S.bombi* in native congeneric species. Therefore it could be argued that, with regards to this host-parasite interaction, the arrival of this non-native species may not necessarily be detrimental to native bumble bee populations.

Chapter 5. The provenance of parasites in non-native and native hosts

Abstract

Establishing the current distribution of a species is an essential first step in the understanding of range expansion of invasive species. Understanding the original distribution and consequent climatic niche can help us to predict the spread and impact of invasive species. Phylogeography uses phylogenetic trees, usually built from the genetic sequences of individuals sampled across the geographic range of the taxon, to examine population structure of the taxon.

Using standard molecular methods and mitochondrial gene CO1 sequences, I constructed a phylogenetic tree to investigate the population structure and provenance of parasites in native and non-native *Bombus* hosts. I asked whether invading hosts have introduced parasites from their native range, or whether these hosts have acquired parasites from native congeners in the invaded range.

From the DNA sequences obtained, I found that the population of the parasite *S. bombi* did not appear to be structured across the European native and non-native *Bombus* hosts sampled. This is the first study of the phylogeography of *S. bombi*.

Introduction

The geographic distribution of a species is not fixed, and naturally changes in response to biotic and abiotic factors. The rate of such change is increased by anthropogenic factors such as climate change and the global transportation of goods (Elton 1958, Parmesan & Yohe 2003). Natural changes in species distributions, in response to climate change, may introduce novel pests and diseases to areas (e.g. Carpenter *et al* 2009, Wilson & Mellor 2009) and may also cause a mismatch between parasites and their hosts, and parasites may have a greater impact on allopatric hosts than on sympatric hosts. The global transportation of goods introduces non-native species to novel locations, often resulting in the ecological impacts and economic costs of biological invasions (Vitousek *et al* 1996, Pimentel *et al* 2005).

Parasites and biological invasions

The success of biological invasions may be facilitated by host-parasite interactions (Daszak *et al* 2000, Dunn 2009, Kelly *et al* 2009). Non-native species may be parasites or pathogens, or may introduce novel parasites to native species. Introduced parasites may have coevolved with their invasive (sympatric) hosts, and thus may have a greater impact on native (allopatric) hosts. In addition, invasive hosts may act as a reservoir of non-native parasites that infect native hosts e.g. the Signal crayfish, *Pacifastacus leniusculus* (Haddaway *et al* 2012) and the Grey squirrel, *Sciurus carolinensi* (Rushton *et al* 2000, 2006, Tompkins *et al* 2003). In addition, generalist non-native parasites may establish in populations of hosts, such as rare species, that would otherwise be too small to support them, and thus have an additional impact on them (Dobson & Carper 1992).

Distribution and origin of species

Establishing the current distribution of a species is essential in our understanding of range shifts and expansions, invasive species and host-parasite systems. Understanding current species distributions is of particular importance for host-parasite systems where changes in distributions may have severe impacts on both the native fauna and the agricultural livestock of a region (e.g. Carpenter et al 2009, Wilson & Mellor 2009). Identifying the origins of invasive species is important as understanding the original distribution and consequent climatic niche can help us to predict the spread and impact of that species (e.g. Di Febbraro *et al* 2013). Understanding the origin of an invasive species is also important if biological control is to be undertaken, where natural enemies from the native range of the invader can be used to control invasive hosts in the invaded range (e.g. *Aphalara itadori*, a psyllid used to control the invasive weed Japanese Knotweed, *Fallopia japonica,* was identified from the native range of this plant species, Shaw *et al* 2009).

Phylogeography

The main method used to understand the species' current geographic distribution, and the origin of an invasive species, is phylogeography (Stepien et al 2002, Ficetola et al 2008). This approach uses phylogenetic (family) trees, usually built from the genetic sequences of individuals sampled across the geographic range of the taxon, to examine population structure and the history of the geographical distribution of the taxon (e.g. Nieberding *et al* 2005). While both nuclear and mitochondrial DNA (mtDNA: from the mitochondria in an animal's cells) can be used

in phylogeographic studies, mtDNA has the unique property of being transmitted via the maternal line only and mutating significantly faster the nuclear DNA sequences. The section of mtDNA often used is the mitochondrial gene cytochrome c oxidase subunit 1 (abbreviated to CO1) as it evolves relatively rapidly (Blaxter 2003, Hebert *et al* 2003). While trees built from such mtDNA can provide insight into the population distribution and history, the analysis and interpretation of such DNA sequences may be hampered by heteroplasmic sequences. Heteroplasmy occurs in DNA sequences when a single organism possesses multiple DNA sequences in a single gene (Magnacca & Brown 2010). The number of species where heteroplasmy occurs is currently not known (Magnacca & Brown 2010).

Bombus hypnorum and *Sphaerularia bombi*

The tree bumble bee*, B. hypnorum,* was first reported in England in 2001 (Goulson & Williams, 2001) and has rapidly expanded its range across England, Wales and Scotland (BWARS, BBCT) from its native range in Continental Europe. In my study of the non-native *B. hypnorum* and native *Bombus* species in England (Chapter 3), I found that the prevalence of *Sphaerularia bombi ,* a castrating nematode parasite (Poinaar & van der Laan 1972) was higher (29%) in the non-native species than in native congeneric species (0-20%), suggesting that escape from parasites could not explain the rapid expansion of this non-native species. However, although, the biology of the host-parasite system suggested that these nematode parasites were acquired from native congeneric species in the invaded range, the provenance of the parasites infecting the invasive species was not certain. Therefore, I sought evidence to support (or reject) the parasite acquisition hypothesis for *S. bombi* in non-native *B. hypnorum*. One way to achieve this was to conduct a phylogeographic study of this parasite across both multiple hosts species and its geographic range.

Range and population structure of *S. bombi*

Sphaerularia bombi has been reported from queens across a range of bumble bee species in Europe, New Zealand (an introduced population), North America and South America (Alford 1969a, Poinar & van der Laan 1972, MacFarlane *et al* 1995 Plischuk & Lange 2012). While it has been assumed to be a generalist parasite (Schmid-Hempel 1998, Kelly 2009), neither the population structure of this parasite across host species, nor across its geographical range have previously been investigated. From the perspective of the current study, there are a number of reasons to suspect that the parasite, assuming that it is a true generalist parasite

(and not a mix of cryptic species), should show population structure that would elucidate whether it had been acquired by *B. hypnorum* in its non-native range, or brought over with it from its native range in continental Europe.

Firstly the numerous physical barriers across Europe, such as mountain ranges (the Alps, the Pyrenees, the Vosges) and bodies of water (the English Channel, the Irish Sea) may impose spatial structure on the parasite population. Evidence for this comes from studies of host populations. Previous studies have demonstrated that the *Bombus spp.* host populations are structured across Europe both morphologically and genetically. *B. terrestris* is widely dispersed across Europe (including Northern Africa and islands in the Mediterranean and off coast of North Africa) and exhibits both morphological and genetic population structure. The various *B. terrestris* subspecies have a range of colour patterns across Europe e.g. *B. terrestris audax* the UK has a 'buff' tail, where continental *B. terrestris* subspecies mostly have white tails, indicative of underlying genetic structure in these populations. Indeed, previous studies of *Bombus* phylogeography (using mtDNA) have found that the host population is genetically structured across Europe by water bodies (*B. terrestris*: Estoup *et al* 1996, Widmer *et al* 1998) and mountain ranges (*B. pascuorum*: Widmer *et al* 1999). This host structure provides an *a priori* reason for supposing a similar structure in its parasite *S. bombi*.

Secondly, the dispersal ability of the *Bombus* host is likely to be far greater than that of the parasite. *Bombus* queens disperse from the natal nest to mate, after mating to find a hibernation site and possibly after hibernation (Alford 1975). The dispersal opportunities for *S. bombi* are probably limited to local dispersal during their free-living stage in the soil (Poinar and van der Laan 1972). Larger scale dispersal can only occur in infected hosts, but the dispersal of infected hosts is currently unknown. MacFarlane & Griffin (1990) reported that *S. bombi* had expanded their distribution in New Zealand by 30-40km over 100 years (*ca.* 0.5km *per annum*) compared with up to 140km *per annum* for their *Bombus* hosts. While, bumble bees were introduced to New Zealand, in the late 19th century (MacFarlane & Gurr 1995), along with their *S. bombi* parasites, and therefore these rates of dispersal are based on colonising a new area instead of dispersing within an occupied area, this nevertheless indicates a reduced dispersal ability of the parasite. Given that host populations are structured (see above), the lower dispersal rate of the parasite should result in even stronger spatial population structure.

While the phylogeography of *S. bombi* has not previously been investigated, studies of other nematodes indicate that the mtDNA CO1 gene may provide a suitable marker for such studies. Although the mtDNA CO1 gene has been used to identify nematode species, known as 'bar-coding' (e.g. soil nematodes: Blaxter 2003, marine nematodes: Derycke *et al* 2010), it has also has been used successfully in phylogeographic studies of nematodes (Nieberding *et al* 2005, Nieberding *et al* et al 2008. For example Nieberding *et al* (2008) found that the lineages of *Heligmosomoides spp.* (a nematode parasite) matched that of their mice hosts (*Apodemus sylvaticus* and *A. flavicolis*) and showed population structure across South West Europe for *Apodemus sylvaticus* and across the Balkan and the Middle East for *A. flavicolis*.

Project

In this chapter, using molecular methods and DNA from parasites collected across Europe, I investigated the provenance of *S. bombi* parasites in invasive *B. hypnorum* hosts to establish whether the invading hosts have introduced parasites from their native range, or whether they have acquired parasites from native congeners in the invaded range. I investigated the relatedness of *S. bombi* parasites collected from *B. hypnorum* hosts, both from the invaded range (England) and from their native range (Belgium), and *S. bombi* parasites collected from other *Bombus* hosts from England, Ireland, Switzerland and Belgium to determine the structure of the parasite population across Europe. Given the presence of physical barriers and host poplation structure, I expected to find genetic differentiation between the parasite populations in the British Isles (i.e. England and Ireland) and those from Continental Europe (i.e. Belgium and Switzerland). I also expected that the parasites from the non-native hosts would be closely genetically related to the parasites from native congeners in England.

Methods

Collection of parasite samples

Bumble bee queens were collected across Europe. *B. terrestris* were collected from England, Switzerland and Ireland in 2010 (see Chapter 2). In 2011, the invasive *B. hypnorum* and 4 native *Bombus spp.* (*B. terrestris, B. lucorum, B. pascuroum* and *B. pratorum*) were collected in England (see Chapter 3). In 2012, *B. hypnorum* were collected from England, the invaded range, (see Chapter 4) and Belgium, the native

range. *B. terrestris* and *B. pascuorum* queens were also collected from Belgium in 2012. All Belgian samples were collected by Dr. Thibaut DeMeulemeester. The English, Swiss and Irish *Bombus* queens were stored in the freezer -80^{o}C and thawed prior to dissection (as described in Chapter 2). The Belgian samples were stored in alcohol, and were liberally rinsed in water prior to dissection. The queens were examined for the everted uteri of *S. bombi*, and, if located, these were carefully removed and individually transferred to a labelled 0.2ml Eppendorf tube and stored in the -20^{o}C freezer. The DNA was extracted from only one *S. bombi* uteri from each infected queen; if the queen was infected with more than one *S. bombi*, the individual processed was randomly selected from the whole uteri (or largest section) available.

DNA extraction and purification

DNA extraction and purification were completed using a standard protocol using a DNeasy Blood & Tissue extraction kit (Qiagen Inc.) using a 'Spin-Column Protocol', following the manufacturer's instructions to produce the DNA template. All chemicals were provided in the kit except ethanol and double distilled H_2O (dd$H_2$0). The kit included various buffers (solutions made of H_2O, weak acid and related base) to buffer (control the speed of) various reactions at each step of the process.

The initial 'tissue lysis' step consisted of placing 180ul of Buffer ATL, 20ul of Proteinase K (an enzyme that breaks down proteins), the nematode sample, and a small metal ball-bearing in plastic tube (with a lid) in a rack in a 'TissueLyser II' machine set at a frequency of 30 Hz for two minutes to break up the sample. The tubes were then removed from the rack and placed in a TECHNE Dri-block DB 2D heating block and heated at 56^{o}C for three hours (the optimal temperature and time to break down the protein and release the genetic material). The samples were removed from the heating block and vortexed for 15 seconds.

A 'master' mix of 200ul Buffer AL and 200ul ethanol (96–100%) for each sample (plus 10% spare, in case of minor micropipettting errors) was prepared and thoroughly mixed using the micropipette. Then 400µl of the master mix was added to each sample. The solution was transferred, using a micropipette, into the DNeasy Mini spin column placed in a 2ml collection tube. The samples were evenly distributed in a Thermo Scientific Sorvall Legend Micro 17 centrifuge and then centrifuged at 6000g for one minute. This process forced the solution through the

filter in the spin column, leaving the DNA on the filter in the spin column. The collection tube and the solution were discarded.

The spin column was placed on a new 2ml collection tube and 500µl Buffer AW1 was added to each sample. These were centrifuged for one minute at 6000g and the collection tube and the solution were again discarded. The spin column was again placed on a new 2ml collection tube and 500µl Buffer AW2 was added to each sample. These were centrifuged for three minutes at 17,000g to dry the filter in the spin column. This time and speed ensure that all traces of ethanol are removed as ethanol may interfere with subsequent reactions following elution. The spin column was then carefully removed from the collecting tube to ensure that it did not come into contact with the solution that may contain ethanol.

The spin column was placed in a clean 1.5 ml tube (with a lid) and 200µl Buffer AE was transferred directly onto the filter using a micropipette. The sample was incubated at room temperature for 1 minute and centrifuged for 1 minute at 6000g to elute. The spin column was discarded. The 1.5ml tube containing the elutant , the 'ready-to-use' DNA, was either amplified immediately using polymerase chain reaction (PCR, described below) or was sealed and stored in the freezer at -20^{o}C to be processed later.

Polymerase Chain Reaction (PCR)

The target (CO1) section of the DNA template was amplified using a PCR protocol provided by Dr. James Carolan, National University of Ireland, Maynooth, using standard *Taq* (*Thermus aquaticus*) polymerase (Invitrogen) and LCOHym and NancyShort primers (Sigma Aldrich). Both 'LCOHym' and 'NancyShort' are general CO1 primers for mtDNA (Magnacca & Brown 2010, Folmer *et al* 1994, Simon *et al* 1994):
LCO_Hym (C1-J-1514): 5'-TATCAACCAATCATAAAGATATTGG-3'
Nancy_short (C1-N-2194): 5'-CCCGGTAAAATTAAAATATAAAC-3'

A master mix of ddH_2O, dNTPs (the nucleotides to make up the new DNA), 10x buffer, Magnesium Chloride ($MgCl_2$), forward and reverse primers (LCOHym and NancyShort) and *Taq* was prepared (Table 5-1).

Table 5-1 The PCR Master mix used to amplify DNA from nematode samples

Reagent	1 Reaction (ul)
ddH_2O	12.3
dNTPs (10mM mix)	0.4
10x buffer	2.0
$MgCl_2$	1.2
LCOHym primer	1.0
Nancyshort primer	1.0
Taq	0.1
DNA	2.0

A strip of tubes (one tube for each DNA sample, plus a positive control and a negative control) was set up in a rack and 18ul of master mix was added to each tube. 2ul template DNA (or the positive and negative controls) was then added to each tube. The positive control was a sample of DNA that had previously produced successful results (i.e. a band at the correct location in the gel – see below), and thus was only used from the second run onwards. The negative control was a sample of ddH_2O, which should not contain any DNA and therefore should not produce a band at the expected location in the gel – see below). The strip of tubes were loaded in the BioRad DNAEngine Tetrad 2 Peltier Thermal Cycler using settings provided by Dr. Jim Carolan although the last step (cooling) was adjusted from 4^{o}C to 10^{o}C (as our thermocycler is not designed to cool at low temperatures). The settings for the thermocycler were: an initial denature step at 94^{o}C for five minutes, followed by 32 cycles of: denature at 94^{o}C for one minute, annealing at 48^{o}C for 45 seconds and an extension period at 72^{o}C for one minute. This was followed by a final extension at 72^{o}C for seven minutes, then left to soak at 10^{o}C 'forever' i.e. until the program was stopped and the samples removed. This process produced my PCR product i.e. DNA for each sample.

Gel electrophoresis

Gel electrophoresis was used to determine that the PCR process had amplified the target sequence (the CO1 gene), in my DNA samples. Each sample was run through a 2% agarose gel for 40 minutes at 120V. The 2% agarose gel was made with 2g of agarose powder, mixed with 100ml of 100x Tris borate-EDTA (TBE) and heated in a microwave until the agarose has dissolved. 5ul of ethidium bromide (EtBr), which binds to DNA and fluoresces under UV light, was added and the

solution swirled to mix. The solution was then poured into a tray, with a comb to produce holes, or wells, and allowed to cool and set for approximately 20 minutes.

The set gel was then transferred into a bath of TBE buffer, where I loaded samples of PCR-amplified DNA mixed with loading buffer and a ladder. I used 5-10ul of DNA and 1.5-3ul of loading buffer for each sample (i.e. 5ul DNA and 1.5ul of loading buffer) but found that 8ul of DNA and 2ul of loading buffer, produced good results and was easier to load in the gel. The buffer includes a dye, which shows the movement of the DNA through the gel, and glycerol to increase the density of the sample, so that it sinks when loaded in the well. Using a micropipette, I loaded the DNA template and loading buffer mixture for each sample (usually 10ul in total, see above) into the wells in the gel. I loaded 2.5ul of ladder (Hyperladder 4, Bioline) into the well on the left hand side. The ladder creates a scale for DNA of different sizes: 100-1000bps. This ladder highlights at 300 base intervals, and thus the size that I was looking for (approximately 600 bases) could be identified.

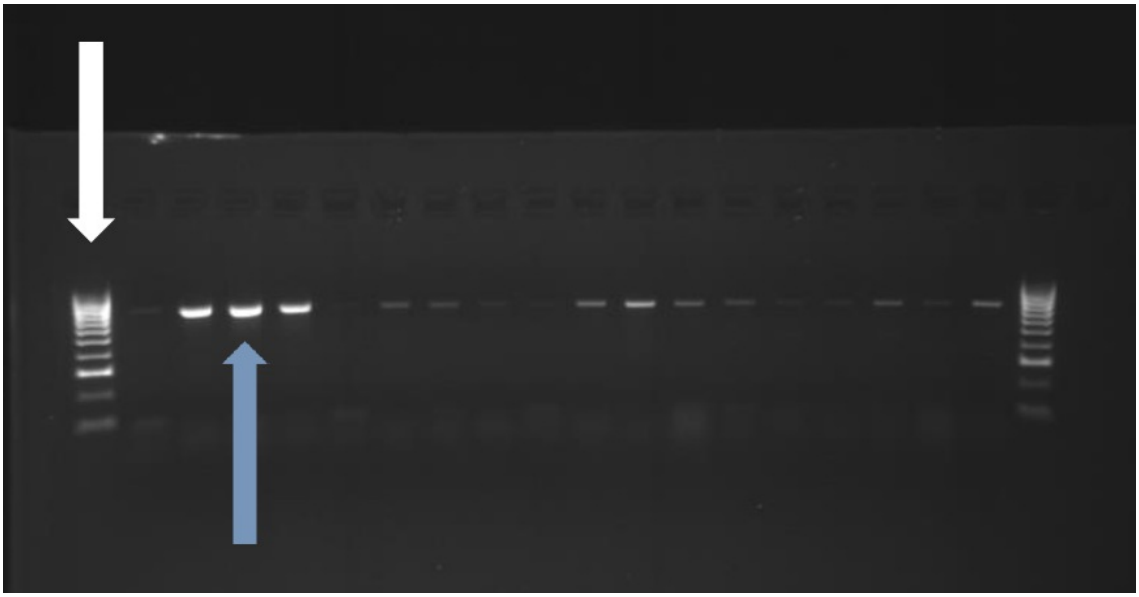

Plate 5-1 Image of the agarose gel after electrophoresis

The gel shows clear bands at approximately 600 bases (indicated by the blue arrow), faint bands and the ladder which creates a scale of DNA of 100 to 100 bases (indicated by the white arrow).

The electric current, passed through the gel in the bath of TBE, induces the negatively charged DNA (and loading buffer) to migrate through the gel towards the positive anode. The distance of the migration, at the specified power after the set time, indicates the size (i.e. number of base) of the amplified section of DNA.

After 40 minutes, I removed the gel and placed it under UV light in a GeneFlash Sygene Bio Imaging machine to check the bands and record an image. If the bands were visible and at the right position i.e. the right size (Plate 5.1), I proceeded to the DNA clean up step. If the bands were not clear, I repeated the PCR process (at least once).

DNA cleanup

For the DNA samples that produced visible bands in the gel, 5ul of the remaining PCR product was cleaned up using Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP-IT, http://www.affymetrix.com). ExoSAP-IT cleans up post PCR DNA samples by removing unused primers and nucleotides. I added 2ul of ExoSAP-IT to 5ul post PCR DNA in a 0.2ml tube and placed the tube in the thermocycler set at 37°C for 15 minutes to degrade the primers and nucleotides then at 80°C for 15 minutes to inactivate ExoSAP-IT.

The cleaned up post-PCR DNA samples were sent, with a sample of the LCOHym primer, to Source BioScience LifeSciences (SBSLS, http://www.lifesciences.sourcebioscience.com) for sequencing. Sequencing reactions were performed by SBSLS using Applied Biosystems 3730 series DNA Analyzers.

Sequence analysis

To check the DNA sequences received from SBSLS were from nematodes, I used the nucleotide blast (Basic Local Alignment Search Tool - BLAST) function (megaBLAST) on the National Center for Biotechnology Information website (NCBI, http://www.ncbi.nlm.nih.gov/). This website holds a database of DNA sequences from many organisms and can be used to establish which organism on the database most closely matches a DNA sequence. The results are available for differing levels of similarity: megaBLAST for ‘highly similar sequences’, ‘discontiguous megablast’ for ‘more similar sequences’ and ‘blastn’ for ‘somewhat similar sequences’. Using megaBLAST, I checked for ‘highly similar sequences’, and if this was unsuccessful, I tried the ‘discontiguous megablast’ and, finally, ‘blastn’. Only ‘highly similar’ nematode sequences were used to build the phylogenetic tree (see below).

Preliminary investigations, to confirm the success of the out-sourced sequencing, were conducted using Geneious (http://www.geneious.com/). The chromatograms, a visual display of the DNA sequences, were reviewed, and the ambiguous sections (i.e. ‘N’s and/or unclear peaks) at the start an end of the sequence were trimmed (Figure 5-1 and an untrimmed DNA sequences shown in Figure 5-4).

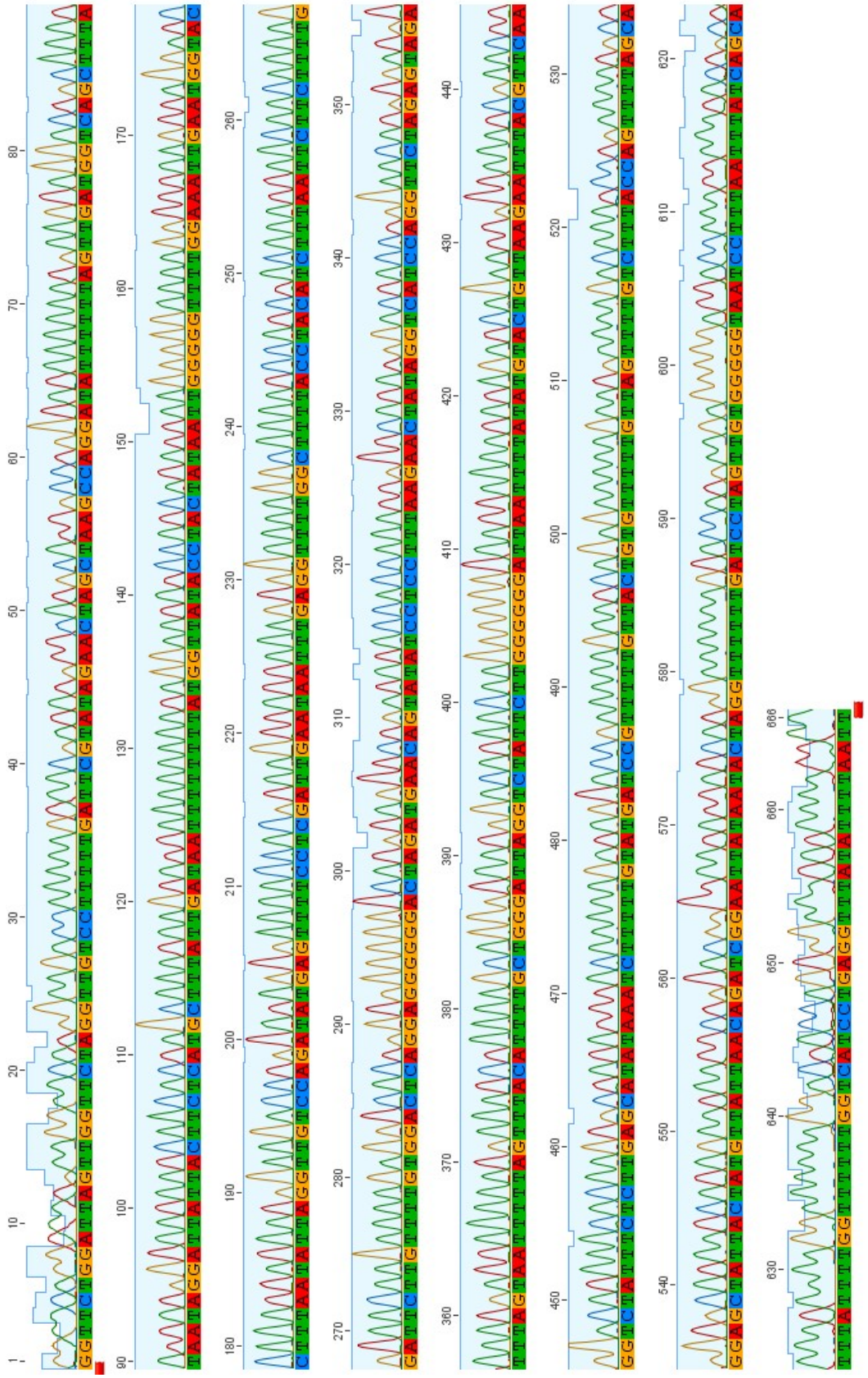

Figure 5-1 A chromatogram showing a good DNA sequence with clear peaks.

The chromatogram for nematode sample Sb2 with red blocks at the ends indicating where the sequence has been trimmed.

Sequencing chromatograms were reviewed, trimmed and edited using SEQUENCHER 4.0 analysis software (Gene Codes Corporation). For the two samples (Sb81 and Sb82), which appeared to be heteroplasmic (i.e. where multiple mtDNA haplotypes are present, Magnacca & Brown 2003), at heteroplasmic sites in the DNA sequences (i.e. where two peaks occur) the dominant peak was used to describe the character state (i.e. DNA base). If peaks were of equal or similar height (i.e. with lower peak more than two thirds of the height of the larger peak), the ambiguous state 'N' was used.

Sequences were manually aligned using Se-Al (http://tree.bio.ed.ac.uk/software/seal/). In addition to the samples that I processed, I included two *S. bombi* DNA sequences provided by Dr. James Carolan. The DNA sequences provided by Dr. James Carolan were from a *S. bombi* found in a *B. terrestris* queen collected in Dublin, Ireland in 2010 and from a *S. bombi* found in a *B. vosnesenskii* queen collected in the Willamette Valley, Oregon, United States of America also in 2010.

Prior to running the phylogenetic analysis to build a phylogenetic tree, I selected the best-fit partition and model of molecular evolution for nucleotide alignment under Bayesian Information Criterion using Partitionfinder (Lanfear 2012). This selects the best partition (breaking into parts) and model to use to build the tree. The Hasegawa-Kishino-Yano model (HKY, Hasegawa *et al* 1985) was selected for all partitions.

Bayesian trees were constructed using MrBayes 3.2 (Ronquist 2003). MrBayes uses Bayesian inference and Markov chain Monte Carlo (MCMC) methods to estimate the posterior distribution of model parameters, i.e. steps which use the most likely scenario and probabilities to estimate the evolutionary relationships between the samples and this is the standard method for constructing phylogenetic trees. I used the HKY substitution model, selected using PartitionFinder (see above), using a four chains run (i.e. four simultaneous runs) for 1 x 10^6 generations, which sample every 1x 10^3 generations with an initial 'burn-in' period, where 250 sampled trees are rejected.

The initial analysis used mtDNA CO1 sequences (available from the NBCI website) for *Bursaphelenchus mucronatus* and *B. xylophilus,* 2 nematode species from the

same order as *S. bombi* (Tylenchida), as outgroups, in order to root the tree (i.e. determine the most divergent or ancestral sequence) with the American *S. bombi* as part of the ingroup. This confirmed that the American *S. bombi* DNA sequence was sufficiently different to the European *S. bombi* DNA sequences to use as an outgroup.

The final analysis constructed a Bayesian phylogenetic tree using a single representative of each unique version of the European *S. bombi* DNA sequences (haplotype). The phylogenetic tree, constructed from the 19 haplotypes groups, separates the outgroup, the nematode DNA sequence (SphT1) from *B. vosnesenskii* collected in USA, from the other sequences. This separation is supported by a posterior probability measure of 1 (where: >0.9 is absolutely confident and <0.80 is limited confidence e.g. 0.76 means 24% of trees did not match this tree).

Results

Sample collection and processing success

I extracted DNA from a total of 90 nematode individuals. Approximately 24% (22/90) of these were from *B. hypnorum* queens collected in the invaded range (England) and *ca.* 6% (5/90) from their native range (Belgium) (Figure 5-2). Samples from native congeners from the invaded range (England) represented *ca.* 39% (35/90) and *ca.* 14% (13/90) were from Ireland where the invasive species is not currently present. A further *ca.* 17% (15/90) were from native congeners from the native range (Switzerland N=2 and Belgium N=13). Of the 90 nematode samples prepared, 76 produced visible bands in the PCR gel and were sent to SBSLS for sequencing (Table 5-2). Nematode samples, from *B. hypnorum* queens collected in the invaded range (England) represented *ca.* 26% (20/76) and those collected from their native range (Belgium) represented *ca.* 7% (5/76) (Figure 5.2). Approximately 42% (32/76) were from native congeners from the invaded range (England) and *ca.* 17% (13/76) were from Ireland. An additional *ca.* 20% (15/76) were from native congeneric hosts from the native range (Switzerland N=2 and Belgium N=13).

Table 5-2 The parasite samples collected and processed

The parasite samples are shown indicating the year, the country, the host species and the number of samples collected. The number of DNA sequences obtained and the number of nematode sequences obtained are also shown.

Year	Country	Host species	Samples	DNA Sequenced	Nematode sequences
2010	England	*B. terrestris*	11	9	8
	Switzerland	*B. terrestris*	2	2	2
	Ireland	*B. terrestris*	13	8	7
2011	England	*B. hypnorum*	11	11	7
		B. terrestris	4	3	2
		B. lucorum	11	11	5
		B. pascuorum	3	3	1
		B. pratorum	6	6	6
2012	England	*B. hypnorum*	11	10	2
	Belgium	*B. hypnorum*	5	5	2
		B. terrestris	12	7	4
		B. pascuroum	1	1	0
Total			**90**	**76**	**46**

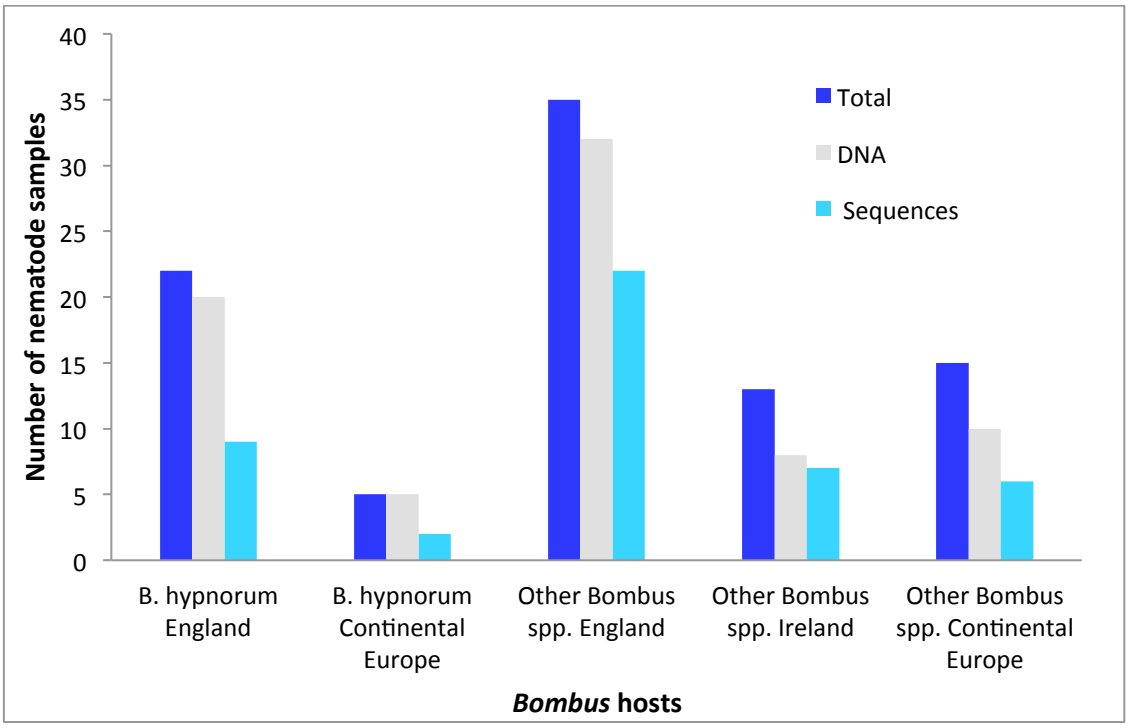

Figure 5-2 Parasite samples from *Bombus* host species in native and invaded ranges.

The number of parasite samples processed for the invasive *B. hypnorum* in both its invaded (England) and native range (Continental Europe) and other *Bombus spp*., in the invaded (England), the currently uninvaded (Ireland) and the native range (Continental Europe).

When the sequences received from SBSLS were compared to sequences in the NCBI website, using the nucleotide BLAST, 46 of the DNA sequences matched nematode sequences (Table 5.2, Figure 5.3). Approximately 20% (9/46) of these nematode sequences were from *B. hypnorum* queens collected in the invaded range (England) and *ca.* 4% (2/46) from their native range (Belgium) (Figure 5.2). Nematode sequences from native *Bombus spp*. collected from the invaded range (England) represented *ca*. 48% (22/46) of the samples. I also obtained *ca.* 15% (7/46) of the samples from Ireland, and *ca.* 13%, 6/46 were from native congeners from the native range (Switzerland N=2 and Belgium N=4). 11 of the final 46 (24%) nematode sequences were from *B. hypnorum* queens (England: 9/46, 20%; Belgium: 2/46, 4%).

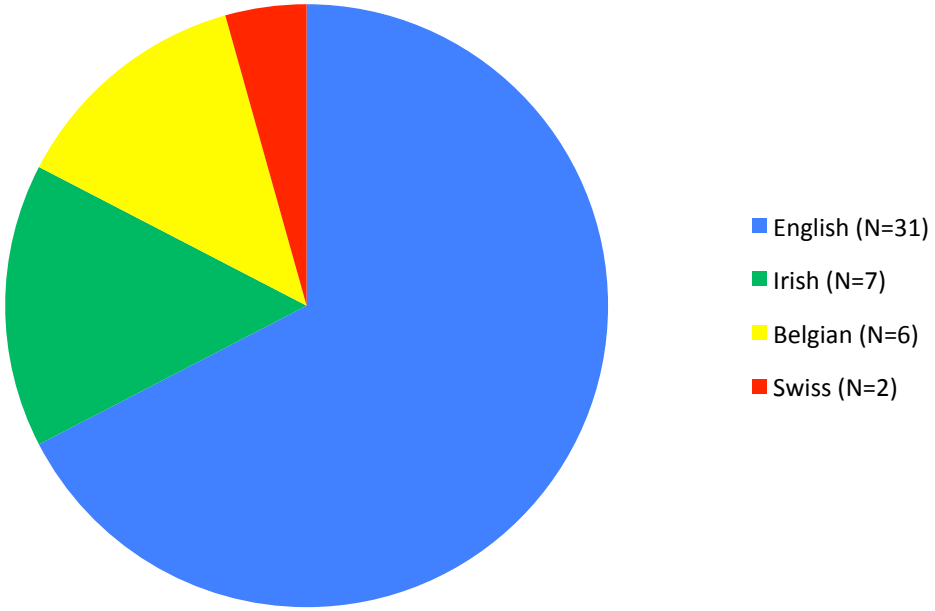

Figure 5-3 The proportion of DNA sequences collected from each country

The chart shows the proportion of the final 46 nematode DNA sequences collected from each country (England *ca.* 67%, Ireland *ca.* 15%, Belgium *ca.* 13% and Switzerland *ca*. 4%).

DNA sequence (BLAST) results

The most frequent top BLAST result, i.e. the most similar DNA sequence on the NCBI database, for the 46 nematode DNA sequences received from SBSLS was for the CO1 gene of *Bursaphelenchus sp*. (Table 5.3). Other top BLAST results included *Bursaphelenchus mucronatus* (a pinewood nematode), *Toxocara cati* (a feline roundworm), *Acrostichus sp.* CO1 (a nematode associated with sweat bees), *Metastrongylus sp* (lungworms) and *Pangrolaimus sp*.(a plant parasitic nematode).

Table 5-3 Top BLAST result for the DNA sequences (N=46) in the phylogenetic tree, showing the number of bases sequenced, matched and the percentage match.

Sample code	Host species	Country	Year	Top BLAST result	Bases sequenced	Max score	Percentage Identified
Sb1	*B. terrestris*	England	2010	*Bursaphelenchus sp.*	705	477	83
Sb2	*B. terrestris*	England	2010	*Bursaphelenchus sp.*	700	477	83
Sb3	*B. terrestris*	England	2010	*Toxocara cati*	698	462	83
Sb4	*B. terrestris*	Ireland	2010	*Bursaphelenchus sp.*	704	472	83
Sb5	*B. terrestris*	Ireland	2010	*Bursaphelenchus sp.*	699	481	83
Sb8	*B. hypnorum*	England	2012	*Acrostichus sp.* CO1	704	388	80
Sb9	*B. terrestris*	England	2010	*Bursaphelenchus sp.*	698	483	83
Sb13	*B. pratorum*	England	2011	*Bursaphelenchus sp.*	698	475	83
Sb14	*B. pratorum*	England	2011	*Bursaphelenchus sp.*	701	481	83
Sb17	*B. terrestris*	England	2010	*Bursaphelenchus sp.*	699	473	83
Sb20	*B. terrestris*	Ireland	2010	*Bursaphelenchus sp.*	708	466	83
Sb21	*B. pratorum*	England	2011	*Bursaphelenchus sp.*	708	416	80
Sb22	*B. terrestris*	England	2011	*Metastrongylus*	708	488	81
Sb24	*B. lucorum*	England	2011	*Bursaphelenchus sp.*	705	481	83
Sb27	*B. pratorum*	England	2011	*Bursaphelenchus sp.*	703	477	83
Sb30	*B. hypnorum*	England	2011	*Bursaphelenchus sp.*	700	481	83
Sb33	*B. terrestris*	England	2010	*Bursaphelenchus sp.*	701	481	83
Sb34	*B. terrestris*	England	2010	*Bursaphelenchus sp.*	708	486	83
Sb37	*B. pratorum*	England	2011	*Bursaphelenchus sp.*	699	481	83
Sb38	*B.pascuorum*	England	2011	*Bursaphelenchus sp.*	700	481	83
Sb40	*B. hypnorum*	Belgium	2012	*Bursaphelenchus sp.*	705	486	83
Sb41	*B. terrestris*	Ireland	2010	*Bursaphelenchus sp.*	713	475	83
Sb42	*B. terrestris*	Ireland	2010	*Bursaphelenchus sp.*	706	481	83
Sb45	*B. hypnorum*	England	2011	*Bursaphelenchus sp.*	701	481	83
Sb47	*B. hypnorum*	England	2012	*Bursaphelenchus sp.*	701	481	83
Sb49	*B. terrestris*	England	2011	*Bursaphelenchus sp.*	701	486	83
Sb54	*B. terrestris*	Belgium	2012	*Bursaphelenchus*	699	568	82
Sb55	*B. terrestris*	Belgium	2012	*Bursaphelenchus sp.*	704	486	83
Sb56	*B. terrestris*	Belgium	2012	*Bursaphelenchus sp.*	699	481	83
Sb57	*B. terrestris*	England	2010	*Bursaphelenchus sp.*	715	470	83
Sb58	*B. terrestris*	Ireland	2010	*Bursaphelenchus sp.*	703	481	83
Sb59	*B. terrestris*	Ireland	2010	*Bursaphelenchus sp.*	700	481	83
Sb60	*B. pratorum*	England	2011	*Bursaphelenchus sp.*	697	475	83
Sb62	*B. lucorum*	England	2011	*Bursaphelenchus sp.*	699	481	83
Sb63	*B. lucorum*	England	2011	*Bursaphelenchus*	697	586	82
Sb69	*B. lucorum*	England	2011	*Bursaphelenchus sp.*	704	486	83
Sb71	*B. lucorum*	England	2011	*Bursaphelenchus*	657	573	83
Sb73	*B. hypnorum*	England	2011	*Bursaphelenchus sp.*	699	486	83
Sb74	*B. hypnorum*	England	2011	*Bursaphelenchus sp.*	690	403	80
Sb75	*B. hypnorum*	England	2011	*Bursaphelenchus sp.*	705	483	83
Sb76	*B. hypnorum*	England	2011	*Pangrolaimus paetzoldi*	699	412	78
Sb79	*B. hypnorum*	Belgium	2012	*Bursaphelenchus sp.*	699	486	83
Sb81	*B. terrestris*	Switzerland	2010	*Bursaphelenchus*	694	477	81
Sb82	*B. terrestris*	Switzerland	2010	*Bursaphelenchus*	703	507	81
Sb84	*B. hypnorum*	England	2011	*Bursaphelenchus*	672	326	76
Sb90	*B. terrestris*	Belgium	2012	*Bursaphelenchus sp.*	698	486	83

The remaining 30 DNA sequences received from SBSLS are shown in Table 5.4. The top BLAST results for the sequences resulted in ‘no similar sequences’ but were subsequently checked using the ‘discontiguous megablast’ for ‘more similar sequences’ and ‘blastn’ for ‘somewhat similar sequences’ produced varied results. 14 were very small (only five bases), indicating that the sequencing process had failed and three others were too small to identify as nematodes (273 to 360 bases). 13 samples, which appeared to be a suitable size (600-700 bases), but did not match with nematode sequences, matched with *Bombus spp.* sequences in BLAST. Five of these matched with *B terrestris, B. lucorum* and *B. pascuorum* sequences and the nematode samples were collected from the host species that was matched in BLAST. Eight sequences matched with *B. perplexus* sequences and were from nematode samples collected from *B. hypnorum* queens.

Table 5-4 Top BLAST result for the DNA sequences (N=30) not in the phylogenetic tree, showing the number of bases sequenced and matched.

Sample code	Host species	Country	BLAST result	Bases sequenced	Bases matched
Sb6	*B. lucorum*	England	*B. lucorum* CO1	618	100%
Sb7	*B. hypnorum*	England	*B. perpexus* CO1	517	97%
Sb12	*B. terrestris*	Ireland	No similar sequences	5	n/a
Sb15	*B. pascuorum*	England	*B. pascuorum* CO1	694	84%
Sb16	*B. terrestris*	England	No similar sequences	5	n/a
Sb23	*B. lucorum*	England	No similar sequences	5	n/a
Sb25	*B. terrestris*	England	No similar sequences	5	n/a
Sb29	*B. lucorum*	England	No similar sequences	5	n/a
Sb32	*B. terrestris*	Belgium	*B. terrestris* CO1	582	88%
Sb39	*B. hypnorum*	Belgium	*B. perplexus* CO1	697	96%
Sb43	*B. lucorum*	England	No similar sequences	5	n/a
Sb44	*B. hypnorum*	England	*B. perplexus* CO1	700	93%
Sb46	*B. hypnorum*	England	*B. perplexus* CO1	698	87%
Sb48	*B. pascuorum*	Belgium	*B. pascuorum* CO1	703	99%
Sb50	*B. lucorum*	England	No similar sequences	5	n/a
Sb51	*B. hypnorum*	England	No similar sequences	273	n/a
Sb52	*B. hypnorum*	England	*B. perplexus* CO1	700	97%
Sb61	*B. pascuorum*	England	No similar sequences	5	n/a
Sb64	*B. hypnorum*	Belgium	No similar sequences	317	n/a
Sb65	*B. terrestris*	Belgium	No similar sequences	5	n/a
Sb70	*B. lucorum*	England	*B. lucorum* CO1	706	99%
Sb72	*B. hypnorum*	England	No similar sequences	5	n/a
Sb78	*B. hypnorum*	England	*B. perplexus* CO1	699	90%
Sb80	*B. hypnorum*	Belgium	No similar sequences	5	n/a
Sb83	*B. hypnorum*	England	*B. perplexus* CO1	702	94%
Sb85	*B. hypnorum*	England	No similar sequences	5	n/a
Sb86	*B. hypnorum*	England	No similar sequences	5	n/a
Sb87	*B. hypnorum*	England	No similar sequences	360	n/a
Sb88	*B. hypnorum*	England	*B. perplexus* CO1	701	91%
Sb89	*B. terrestris*	Belgium	No similar sequences	5	n/a

Heteroplasmy

I found two heteroplasmic DNA sequences (i.e. when a single organism possesses multiple DNA sequences in a single gene). These were Sb81 and Sb82, which were both from nematode samples collected from *B. terrestris* queens in Switzerland in 2010 (Sb82 is shown in Figure 5-4). Thirty-eight of 632 bases (*ca.* 6%) within the CO1 mtDNA sequence were heteroplasmic.

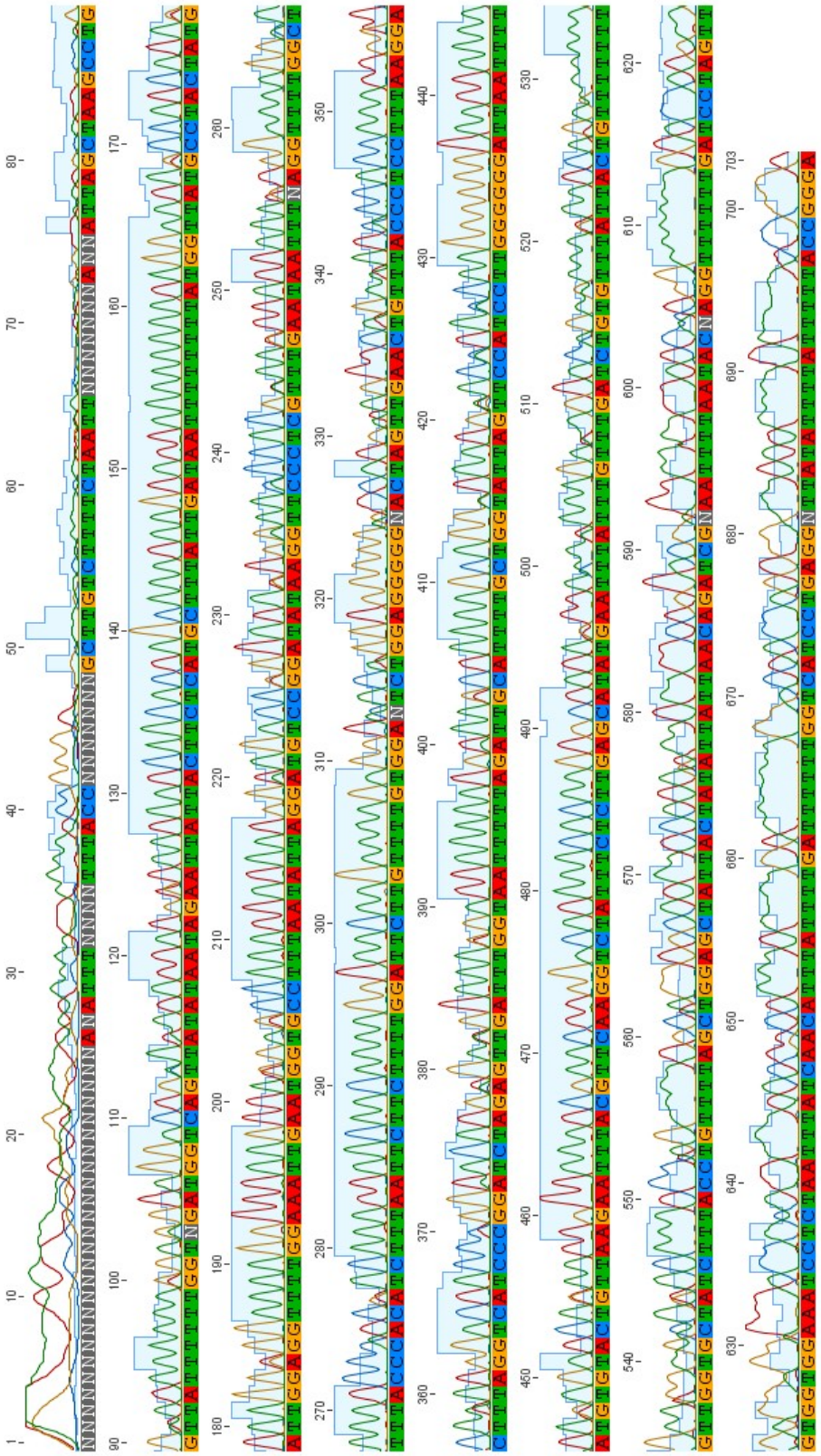

Figure 5-4 A chromatogram showing a heteroplasmic DNA sequence.

The chromatogram for nematode sample Sb82 which has many ambiguities (shown as 'N's), a lack of clear peaks (e.g. top row, from base 0-88) and many double peaks (e.g. 2nd row, bases 91, 100 and 103).

Phylogenetic tree

For simplicity, the final phylogenetic tree was constructed using a single representative of each unique version of the European *S. bombi* DNA sequences (haplotype), with the American *S. bombi* sequence included as the outgroup (Figure 5.5). The 46 DNA sequences were divided into 19 separate haplotype groups (Table 5.5). The largest haplotype group contained 22 of the 46 (*ca.* 48%) nematode DNA sequences and included nematodes collected from *B. terrestris* (from England, Ireland and Belgium), *B. lucorum* (from England), *B. pascuorum* (from England), *B. pratorum* (from England) and *B. hypnorum* (from England and Belgium). The 2nd largest haplotype group contained seven (*ca.* 15%) nematode DNA sequences and all of these were from *B. terrestris* hosts collected in Ireland. The final haplotype group with multiple DNA sequences contained two sequences from nematodes collected from *B. terrestris* collected in Belgium. The remaining 16 groups contained a single haplotype from a single nematode DNA sequence.

Table 5-5 Haplotype table showing the samples in each haplotype group, with the country and host species that the parasite sampled originated from.

Haplotype	Number of	Sequences in	Country	Host species
Sb2	22	**Sb2**	England	*B. terrestris*
		Sb1	England	*B. terrestris*
		Sb8	England	*B. hypnorum*
		Sb14	England	*B. pratorum*
		Sb22	England	*B. terrestris*
		Sb24	England	*B. lucorum*
		Sb30	England	*B. hypnorum*
		Sb33	England	*B. terrestris*
		Sb34	England	*B. terrestris*
		Sb37	England	*B. pratorum*
		Sb38	England	*B. pascuorum*
		Sb40	Belgium	*B. hypnorum*
		Sb45	England	*B. hypnorum*
		Sb47	England	*B. hypnorum*
		Sb49	England	*B. terrestris*
		Sb56	Belgium	*B. terrestris*
		Sb59	Ireland	*B. terrestris*
		Sb62	England	*B. lucorum*
		Sb69	England	*B. lucorum*
		Sb73	England	*B. hypnorum*
		Sb75	England	*B. hypnorum*
		Sb79	Belgium	*B. hypnorum*
Sb4	7	Sb4	Ireland	*B. terrestris*
		Sb5	Ireland	*B. terrestris*
		Sb20	Ireland	*B. terrestris*
		Sb41	Ireland	*B. terrestris*
		Sb42	Ireland	*B. terrestris*
		Sb48	Ireland	*B. terrestris*
		SphT2	Ireland	*B. terrestris*
Sb55	2	Sb55	Belgium	*B. terrestris*
		Sb90	Belgium	*B. terrestris*
Sb3	1	Sb3	England	*B. terrestris*
Sb9	1	Sb9	England	*B. terrestris*
Sb17	1	Sb17	England	*B. terrestris*
Sb21	1	Sb21	England	*B. pratorum*
Sb27	1	Sb27	England	*B. pratorum*
Sb54	1	Sb54	Belgium	*B. terrestris*
Sb57	1	Sb57	England	*B. terrestris*
Sb60	1	Sb60	England	*B. pratorum*
Sb63	1	Sb63	England	*B. lucorum*
Sb71	1	Sb71	England	*B. lucorum*
Sb74	1	Sb74	England	*B. hypnorum*
Sb76	1	Sb76	England	*B. hypnorum*
Sb81	1	Sb81	Swiss	*B. terrestris*
Sb82	1	Sb82	Swiss	*B. terrestris*
SphT1	1	SphT1	USA	*B. vosnesenskii*

There is a polytomy at the base of the tree (where the nematode DNA sequences from Europe separate from the American outgroup) meaning that there is a clear division between the main 2 groups but which of these is most closely related to the outgroup is uncertain (see Figure 5.5).

There is another polytomy at the base of the smaller group (lower section of Figure 5.5) which contains the two heteroplasmic Swiss sequences (Sb81 and Sb82), two English sequences (Sb71 and Sb63) and the Belgian sequence (Sb54). While the separation of the English and Belgian nematodes into a monophyletic group is clearly supported by a posterior probability of 1, how this monophyletic group and the two Swiss nematodes are related to each other is uncertain. One of the English nematode (Sb71) is a clear sister-group to the other English nematode (Sb63) and the Belgian sample (Sb54) is 0.99. The posterior probability for separation of the English sample (Sb63) from the Belgian nematode (Sb54) (posterior probability is 0.99). The posterior probability for separation of the English nematode (Sb63) from the Belgian nematode (Sb54) is only 0.76, and thus the relationship between these is less certain.

The larger group (in Figure 5.5) separates the Belgian samples (Sb55 grouped with Sb90 that is not shown) from the remaining samples in this group with a posterior probability of 1. The remaining samples in this large group form another polytomy with a posterior probability of 0.61. The largest haplotype grouping (Sb02, N=22) forms part of this polytomy. Nematode DNA sequences from England (Sb03) and Ireland (Sb04, N=7) form a subgroup within this polytomy and are separated by a posterior probability of 0.92. The Irish nematode DNA sequences, including SphT2 provided by Dr Jim Carolan, are mostly grouped within Sb04.

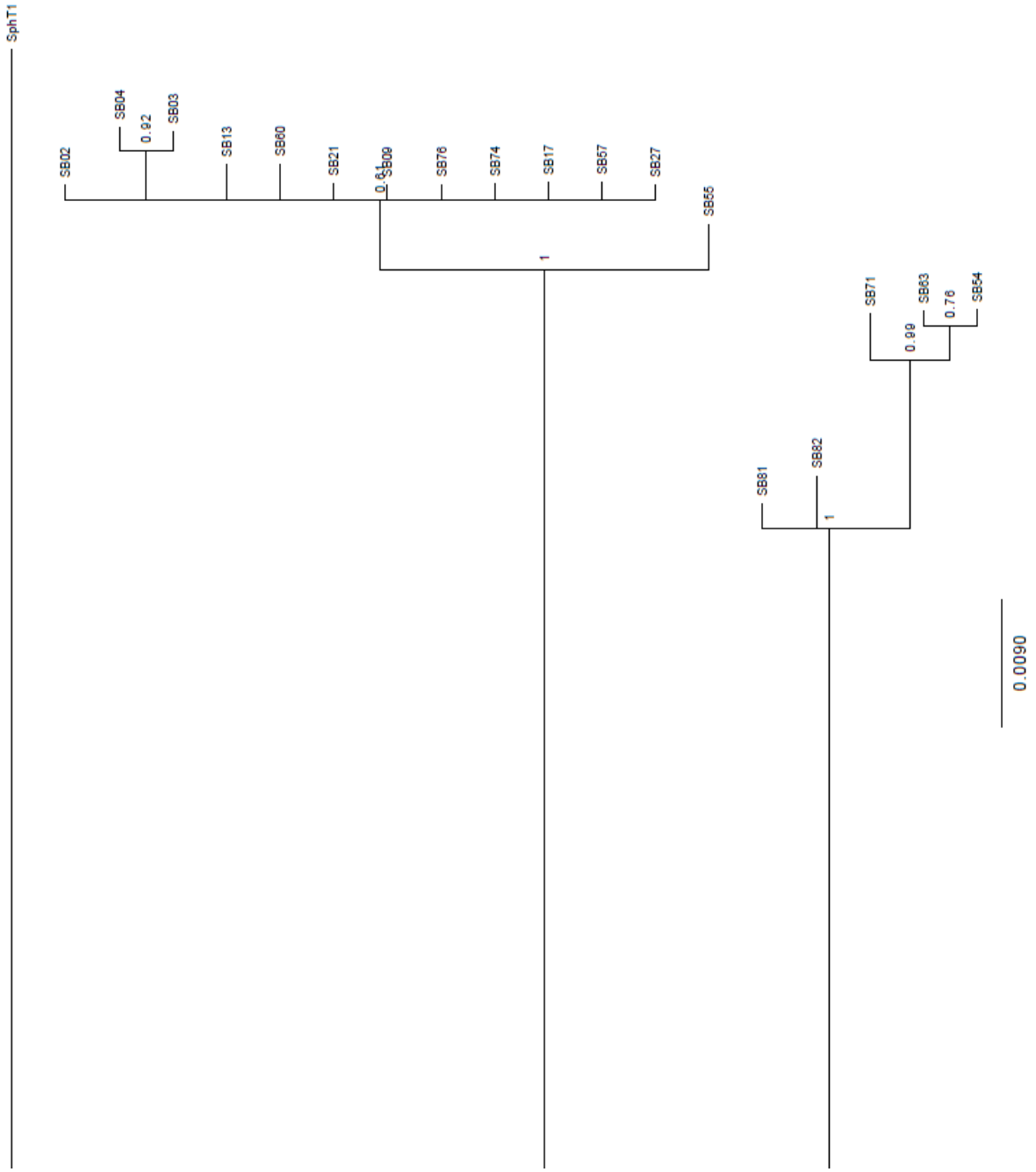

Figure 5-5 The phylogenetic tree of parasite DNA sequences

The phylogenetic tree of *Sphaerularia bombi* DNA sequences using haplotype groups (detailed in Table 5-5) shows the American sequence (SphT1) as the outgroup and the European sequences spread across two groups. SphT1 was from the U.S.A. from *B. voseneskii*. In the larger grouping Sb02 (N=22) was from England (E), Ireland (I) and Belgium (B) from *B. terrestris* (Bt), *B. hypnorum* (Bhyp), *B. pratorum* (Bprat), *B. pascuorum* (Bpas) and *B. lucorum* (Bluc). Sb04: N=7, I, Bt; Sb03: E, Bt; Sb13/Sb60/Sb21: E,Bt; Sb09: E, Bt; Sb76/Sb74: E, Bhyp; Sb17/Sb57: E, Bt; Sb27: E, Bprat; Sb55: B, Bt. In the smaller grouping Sb81/Sb82: Switzerland, Bt; Sb71/Sb63: E, Bluc; Sb54: B, Bt

Network

The network describes the genetic separation of the nematode DNA sequences from each other (Figure 5.6). The network has been simplified by omitting eight groups with a single haplotype (Sb3, Sb9, Sb17, Sb21, Sb27, Sb57, Sb74 and Sb76) from the polytomy in the larger group and Sb54 from the smaller group on the phylogenetic tree. The network splits the nematode DNA sequences into two groups. The larger group (highlighted in blue in Figure 5.6) contains a total of 33 sequences from England, Ireland and Belgium. Within the larger group, samples are separated by between one and four changes in DNA bases. The smaller group (highlighted in red in Figure 5.6) contains four sequences from England and Switzerland. Within the smaller group, samples are separated by between one and six changes in DNA bases. The two groups are separated by a minimum of 17 base changes (between Sb2 and Sb81).

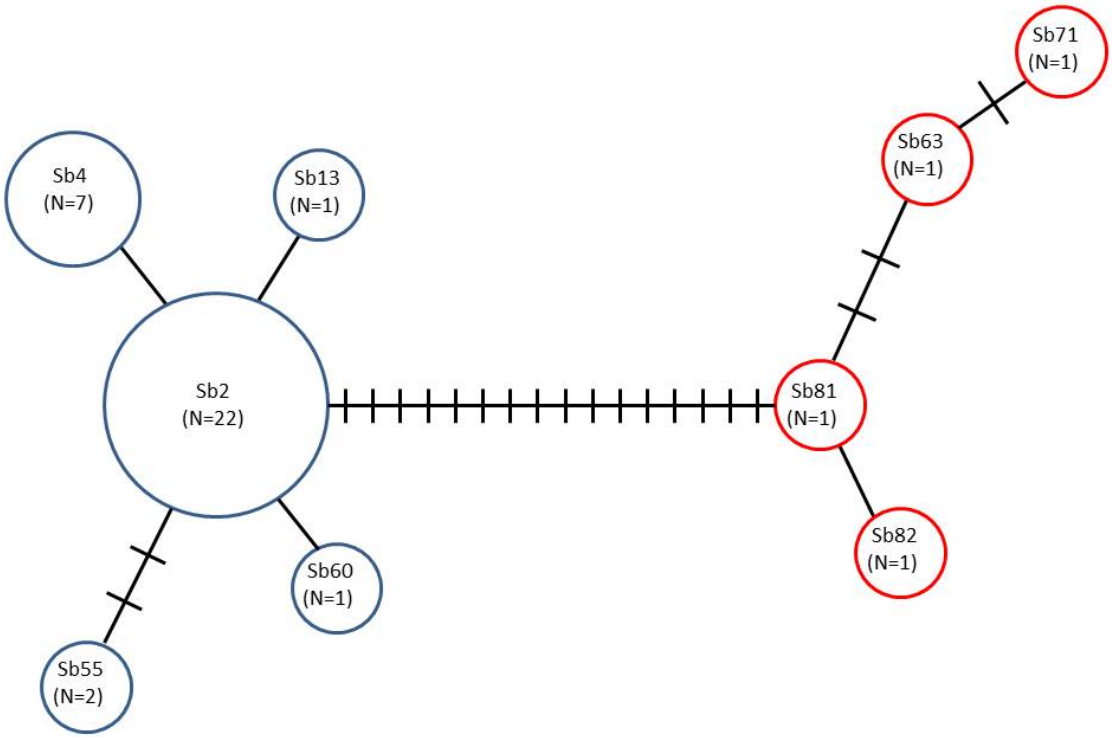

Figure 5-6 Network of parasite DNA sequences

The network shows the relatedness of the nematode sequences with the number of base changes separating the haplotypes indicated by the number of sections on each connecting line. In the larger group (in blue) Sb02 (N=22) was from England (E), Ireland (I) and Belgium (B) from *B. terrestris* (Bt), *B. hypnorum* (Bhyp), *B. pratorum* (Bprat), *B. pascuorum* (Bpas) and *B. lucorum* (Bluc). Sb04: N=7, I, Bt; Sb13: E,Bt; Sb60: E, Bprat; Sb55: B, Bt. In the smaller group (in red) Sb81 and Sb82 were from Switzerland, Bt; Sb71 and Sb63: E, Bluc. The parasite samples across European countries (and *Bombus* species) are spread acroos the two groups.

Discussion

I investigated whether *S. bombi* parasites, from non-native *B. hypnorum* queens collected in England were more closely related to *S. bombi* parasites from *Bombus* queens collected in Continental Europe (Switzerland and Belgium) than to *S. bombi* parasites from native *Bombus* queens collected in England (and Ireland). My goal was to establish whether *S. bombi* were introduced by invading *B. hypnorum* from their native range, or whether *S. bombi* were acquired by the invasive species from native congeners in the invaded range. Despite my expectation that the *S. bombi* population would be structured across Europe i.e. Continental European samples (from Switzerland and Belgium) would differ from English (and Irish) samples, which would have enabled me to answer these questions, the *S. bombi* DNA sequences that were analysed suggest that the *S. bombi* population is not structured across Europe, leaving my initial questions unanswered.

Before discussing the major results, there are a number of caveats. First, I was only able to use 46 of the 90 *S. bombi* from *Bombus* queens, that I collected in England Ireland and Switzerland and that Dr. Thibaut De Meeulemeester collected in Belgium, in my phylogenetic tree. This reduction in the number of samples occurred for several reasons: (i) Only 76 samples of the DNA, extracted from nematodes and purified, produced visible bands in the gel after electrophoresis, indicating a lack of DNA in the samples. This may have been due to sample degradation or errors in the manual process. (ii) Some of the samples produced short sequences: 14 samples produced very short sequences (5 DNA bases), and a further three were too short to match to nematode sequences (273 to 360 DNA bases), an indication of limited success sequencing. Initially, I experienced some issues obtaining sequences from the some of the samples that I sent to SBSLS for sequencing although the samples appeared to contain DNA (i.e. samples that showed clearly visible bands in the gel). SBSLS suggested that the sequencing issues may be due to the concentrations of the DNA in the samples: DNA might be excessively diluted or concentrated for the sequencing reactions (pers. comm. Dave Negus). SBSLS tried various alternative dilutions and I sent multiple repeat samples, with varying dilutions to obtain the 46 DNA sequences. (iii) Thirteen DNA sequences matched with *Bombus spp.*: five matched the host species that the nematode samples were collected from (*B terrestris, B. lucorum* and *B. pascuorum*) and eight matched *B. perplexus* sequences. *B. perplexus* is genetically very similar to *B. hypnorum*, and may be conspecific (Hines *et al* 2006, Cameron *et al* 2007) and, unsurprisingly, these sequences were from nematodes collected from *B. hypnorum* queens. As I

used general CO1 primers, rather than primers that were nematode specific, the host DNA must have been amplified instead of the parasite DNA. This was probably a result of contamination due to insufficient rinsing of the nematodes post-dissection. (iv) While I did not expect that almost half of my samples would fail to produce nematode DNA sequences, a 50% success rate is not unusual (pers. comm. M Fürst).

The second major caveat is the presence of mitochondrial heteroplasmy in the nematodes collected from two Swiss *B. terrestris* queens. While heteroplasmy can reduce the chance of correctly placing and identifying individual sequences on a tree (Magnacca & Brown 2010), this does not seem to have occurred in this study, as these sequences placed quite clearly with three non-heteroplasmic sequences from England and Belgium. It is unclear why nematodes from Switzerland produced heteroplasmic sequences – further sampling is clearly required. However, it is also possible that the hosts (which both appeared to contain an individual parasite) actually each contained more than one parasite and this showed in the DNA sequences received from SBSLS. Another possible explanation in that these samples were contaminated somehow, possibly due to human error.

Although the final tree contained sequences from Switzerland (2 of 46), Belgium (6 of 46), Ireland (7 of 46) and England (31 of 46), there was an almost complete absence of evidence for geographical structuring of the parasite population. In fact, the largest haplotype group represented almost half (*ca.* 48%, 22/46) of the nematode DNA sequences and included nematodes collected from England, Ireland and Belgium, indicating the geographic spread of this haplotype. My initial expectation, based on geographical features and host phylogeography, was for a tree where sequences from Ireland, England and continental Europe formed distinct groups (or clades). In contrast, the Swiss parasite sequences are closely related to both English and Belgian sequences, and the Belgian sequences appear in both major European clades. The Irish sequences are mostly restricted to a single group (Sb4), although a single Irish sequence (Sb59) appears in the large haplotype group, which includes sequences from parasites from England and Belgium. This suggests that the parasite DNA sequences from Ireland may be a sub-group of the English / Irish / Belgian group. Interestingly, whilst there is no evidence for geographic structure, there are two clearly distinct lineages of the parasite, as seen in both the tree and the network diagram, which may be indicative of two separate expansions of the parasite into post-glacial Europe from distinct refugia (Gassert *et*

al 2013). However, in the absence of nematode from potential refugia (i.e. the Iberian and Italian peninsulas) this remains speculation.

The original goal of this study was to establish whether the parasites found in *B. hypnorum* queens in England were introduced or acquired from native congeners. However, the DNA sequences of parasites from *B. hypnorum* in both the invaded range and their native range group together (in the large polytomy on the phylogenetic tree and in the large group on the network). Furthermore the DNA sequences of parasites from *Bombus spp.* in both the native range and the invaded range occur across the phylogenetic tree and the network. The distribution of DNA sequences of parasites, from non-native and native hosts, in both the native and invaded range, suggest that the parasite population is not geographically structured. Therefore it is not possible to establish if the parasites in the invading hosts were introduced or acquired with the current data.

There are several potential explanations for the lack of structure in the parasite population that I found. Firstly, the *S. bombi* population may lack geographic structure across Europe, due to an underlying absence of host population structure. However, both morphological and genetic differences exist in *B. terrestris* populations (Estoup *et al* 1996, Widmer *et al* 1998) and genetic differences exist in *B. pascuorum* populations (Widmer *et al* 1999). This suggests that similar population structure is likely in the remaining *Bombus* species in this study (*B. hypnorum, B. lucorum* and *B. pratorum*) and across the bumble bee assemblage as a whole. Thus an absence of host population structure does not appear to explain the lack of structure in the parasite population. Of course, it remains possible that a small subset of *Bombus* species has enhanced dispersal abilities in comparison to *B. terrestris* and *B. pascuorum,* and thus may act to disrupt population structure in the parasite. Further genetic studies across *Bombus* species are required to investigate this hypothesis.

Secondly, the population of *S. bombi* may have been homogenous (i.e. genetically similar) and panmictic (i.e. all individuals can mate, leading to gene transfer and mixing) across Europe, since the British Isles were connected to each other and to mainland Europe before the last glaciation (Svendsen *et al* 2004), and such panmixia might explain the results of this study. However, when the ice retreated from the Alps, the British Isles and northern Europe, the nematode would have expanded into unoccupied areas, and as physical barriers, such as the English

Channel, formed these populations would cease to be homogenous and panmictic. Studies of other taxa (e.g. Taberlet *et al* 1998) suggest that the time elapsed, since these physical events, is sufficient to have generated structure in the *S. bombi* population across Europe.

Thirdly, the *S. bombi* population in the British Isles (England and Ireland) may have become homogenised with the *S. bombi* population of Continental Europe, due to the human transportation of nematode parasites either in the soil or in infected *Bombus* queens from Continental Europe to the British Isles. This may be possible in soil transported in potted plants or on plant products, on the tyres of vehicle, even on the footwear or in transported infected queens hibernating in the soil. However, the nematode parasites, or the infected queens, would need to be collected at a hibernation site and deposited at a hibernation site, in an appropriate time-frame (both amount of time and suitable time of year/season) to allow the parasite to progress to the next stage of its lifecycle (Poinar & van der Laan 1972). Whilst this may be possible, due to such specific spatio-temporal requirements, it is probably an unlikely explanation.

Fourthly, the *S. bombi* population may be similar across Europe due to enhanced dispersal in infected *Bombus* queens. If parasitized queens disperse further than un-parasitized queens, and such dispersal extends across the English Channel (and the Irish Sea), then the parasite may exhibit less population structure that that of its host. However, if infected *Bombus* queens can disperse over longer distances than previously thought, introductions of Continental European *S. bombi* parasites to the British Isles would not be expected to have a detrimental or disruptive impact on the host-parasite interactions of native *Bombus spp.* and their nematode parasites, due to the phylogenetic proximity of British and Continental European *S. bombi* parasites. This would contrast with findings in New Zealand, where bees dispersed more rapidly than parasites (140km *per annum* and 0.5km *per annum* respectively: Macfarlane & Griffin 1990). The New Zealand study refers to introduced hosts, of which only a proportion were infected with the parasite, colonising new regions, that were parasite free, thus the prevalence of the parasite was low. Furthermore, as the hosts were dispersing into previously unoccupied areas, with no native *Bombus* competitors, the pressure to disperse over a great distance was limited.

Finally, *B. hypnorum* may indeed have introduced parasites, such as *S. bombi,* when it invaded Britain. However, while this could explain some of the lack of

structure in my phylogenetic tree, this cannot explain the proximity of the Irish nematodes (where *B. hypnorum* is currently absent) to the Belgian nematodes.

Previous molecular studies of other *Bombus* parasites differ in their conclusions regarding the structuring of parasite populations (Goka *et al* 2010, Schmid-Hempel & Tognazzo 2010). In Japan, several studies found the tracheal mite, *Locustacarus buchneri* in commercially produced *B. terrestris* colonies imported from Europe (Goka et al 2006, Yoneda et al 2008). Goka *et al* (2010) analysed the mtDNA CO1 gene from tracheal mites from native *Bombus* species and from commercially produced colonies from Europe and found that they differed, suggesting that the population of this parasite is structured across Europe and Asia. In contrast, Schmid-Hempel & Tognazzo (2010) found similar strains of both *Crithidia bombi* and *C.expoeki* in samples collected in Switzerland and Alaska suggesting a global panmictic population structure. This study provides preliminary data molecular data on another bumble bee parasite.

Bumble bees are in decline globally and among the factors driving these declines are parasites and commercially produced bumble bee colonies (Kosior *et al* 2007, Goulson *et al* 2008, Grixti et al 2009, Cameron *et al* 2011). Commercially produced bumble bee colonies, often *B. terrestris*, are introduced to enhance the pollination of greenhouse crops such as tomatoes (Japan: Matsumura *et al* 2004, South America: Morales *et al* 2013). The escape of sexual offspring from these colonies and the parasites they may carry are potential factors in the decline of *Bombus* species (Goulson *et al* 2008, Inoue *et al* 2008, Arbetman *et al* 2013, Graystock *et al* 2013). The parasite, *Nosema bombi*, has been implicated the declines of *Bombus spp,* in North America (Cameron *et al* 2011). In South America the introduction of *B. terrestris* is associated with range contraction of both *B. dahlbomii* and the previously introduced *B ruderatus* (Arbetman *et al* 2013, Morales *et al* 2013), and *B. terrestris* may have co-introduced the parasite, *Apicystis bombi* (Arbetman *et al* 2013). However, it is important is establish whether these parasites were already present, and acquired by the non-native host, or were introduced with the non-native host, and this can be investigated using molecular techniques.

The conclusions of this study are limited by the sample size and the use of a single mitochondrial gene, CO1. Consequently, further analysis of additional parasite samples, especially of parasites from *Bombus* queens collected in continental Europe, might provide greater clarification. However these findings may still have

implications for conservation projects such as the quarantine requirements for the re--introduction of *B. subterraneous* to England (pers. comm. Nikki Gammans, Mark Brown, Natural England, Hymettus, RSPB, BBCT & Swedish Government).

Chapter 6. Discussion

My research investigates the host-parasite relationships of bumble bees (*Bombus spp.)* and their generalist parasites at the community level of both host species and parasite species. As my research was based in Western Europe (England, Ireland, Switzerland and Belgium) the focus was on European *Bombus spp.* particularly *B. terrestris* and *B. hypnorum,* and on European parasite species, particularly *S. bombi*, a nematode worm. *B. terrestris* is common and widespread across Europe. As *B. terrestris* has been domesticated (Velthuis & van Doorn 2006) and is reared commercially, it is an excellent study system. *B. hypnorum* has recently arrived in the UK (Goulson & Williams 2001) and successfully expanded its range across England, Wales and Scotland (BWARS, BBCT) from continental Europe. I was interested in the parasite community of a non-native bumble bee, the parasite community of native *Bombus* species and how the arrival of a new species might alter the host-parasite interactions in the invaded ecosystem.

In my first research project, in Chapter 2, I intended to investigate whether the parasite *S. bombi* was locally adapted to its bumble bee hosts. Using both infected and uninfected *B. terrestris* queens from three European populations, I planned a cross-infection experiment in a common garden to investigate whether parasites were adapted (or maladapted) to their sympatric hosts compared with allopatric hosts. To do this, I required sexual offspring from all three host populations and larvae from all three parasites populations. Unfortunately, the number of host colonies that produced sexual offspring and the number of infected hosts, to produce parasite larvae, was insufficient to proceed with this project.

Despite the lack of success in my initial cross-infection project, there were some positive aspects. Firstly, I learned how to maintain both bumble bee hosts and nematode parasites in the laboratory. I spent many hours under red light, feeding over 500 queens and cleaning their boxes to ensure that the queens and their offspring were kept in optimal conditions. Secondly, I was able to investigate whether parasite prevalence differed across the three European populations that I sampled (i.e. England, Ireland and Switzerland). My focal parasite species was the nematode, *S. bombi*, and I found that the prevalence of this parasite was very different across Europe: prevalence ranged from *ca*. 1% in Switzerland to *ca.* 37% in Ireland. I also found that the number of parasite species infecting queens, in England, was only two parasite species in early queens (and three for later queens)

whilst in Switzerland four parasite species were recorded and in Ireland five parasite species were recorded. Furthermore, the prevalence of *S. bombi* was highest in Ireland, where previous studies have investigated the *Bombus*-*S. bombi* host-parasite system (Rutrecht & Brown 2008, Kelly 2009) and the prevalence of *C. bombi* was highest in Switzerland, where many studies have investigated the *Bombus*-*C. bombi* host-parasite system (e.g. Brown *et al* 2000, Schmid-Hempel & Tognazzo 2010).

In Chapter 2, I suggested that the differing prevalence and parasite community may be due to the differing habitats that the queens were collected from rather than the country they were collected from. In England, queens were collected from a maintained garden within a large park (2,000 hectares, Windsor Great Park). In Switzerland, queens were collected from vineyards, in open countryside or farmland. In Ireland the queens were collected from Merrion Square and the National Botanic Gardens, both in the city of Dublin. To investigate whether the habitat affects parasite prevalence in this study system, *Bombus* queens could be collected along a rural/urban gradient (i.e. from the centre of a city, across suburban areas and into the open countryside), across multiple (European) countries and examined for parasite prevalence to establish if the country (and geographic separation) or the habitat features (rural/urban) have a greater influence on these differences.

Another possible explanation for the high prevalence of *S. bombi* in Ireland (although lower in England and lowest in Switzerland) is that Ireland's fauna is depauperate compared to the England and continental Europe. Therefore, at the edge of their ranges, hosts and parasites from Ireland might have limited genetic diversity. Furthermore, the queens that I collected came from the city of Dublin, where Merrion Square and, to a lesser extent, the National Botanic Gardens are enclosed by roads and buildings, potentially reducing gene flow and leading to local adaptation. *S. bombi* parasites in Dublin may be locally adapted to their *Bombus* hosts and thus their ability to successfully infect their hosts would be high. It would be interesting to carry out the planned cross-infection experiments to investigate the local adaptation of Irish *S. bombi* parasites to their *Bombus* hosts. Although England would provide a suitable 2nd population of both infected and uninfected hosts, a 3rd population would be required, as an alternative to Switzerland, where the parasite prevalence from my samples was low.

I used DNA sequences from the parasites that I collected from *B. terrestris* queens in Dublin in 2010, and the additional sample provided by Dr Jim Carolan, in Chapter 5, to investigate the structure of the *S. bombi* population across Europe. The Irish samples were closely related on the phylogenetic tree suggesting that the genetic diversity of the parasite population in Ireland may be limited. However, I only used the MtDNA gene COI. Additional parasites samples collected from the rural areas surrounding Dublin, and from other host species, would provide further evidence of the diversity of the parasite's gene pool in Ireland. My findings from the MtDNA gene COI provide a starting point for further investigation either using this gene or also using other genes.

I asked whether the prevalence and impact of the parasite communities found in the non-native host species and native host species differed in Chapter 3. I reported the prevalence of *S. bombi* in 5 native *Bombus* species (*B. jonellus, B. pratorum, B. terrestris, B. lucorum* and *B. pascuorum*) and for the non-native *B. hypnorum* in 2011 in Chapter 3 and found that the prevalence of two high impact parasites (*S. bombi* and *A. bombi*) was higher in the non-native than in the native species. I also found that some *B. hypnorum* queens infected with *S. bombi* were able to produce offspring, although this parasite usually castrates queens. I also reported the prevalence of *S. bombi* in the non-native *B. hypnorum* in 2012 in Chapter 4. Although the biology of the parasite *S. bombi* suggested that this parasite was acquired in the invaded range, the provenance of S. bombi parasites in non-native *B. hypnorum* queens was unclear.

In Chapter 3, I also asked if the genetic diversity of non-native host species (in both the invaded and native range) and native host species (in the invaded range) differ? Using the production of diploid males to estimate functional genetic diversity, I found that *B. hypnorum* queens in England had limited genetic diversity. The functional genetic diversity of *B. hypnorum* queens collected in England was lower than *B. terrestris* and *B lucorum* queens collected in England and slightly lower than *B. hypnorum* queens collected in continental Europe. The level of genetic diversity found suggested that the invading population of *B. hypnorum* may have been founded by as few as one or two queens, and as *B. hypnorum* are polyandrous (Schmid-Hempel & Schmid-Hempel 2000, Paxton *et al* 2001) this could be just a single multiply mated queen.

I observed that non-native *B. hypnorum* infected with the parasite *S. bombi* did not appear to deposit nematode larvae in their faeces. Therefore in Chapter 4 I asked whether *B. hypnorum* was a competent host for *S. bombi*. I found that, despite many weeks of regular faeces checks, followed by dissection to establish parasite status, *B. hypnorum* do not deposit *S. bombi* larvae in their faeces. Furthermore, although *S. bombi* parasites infecting *B. hypnorum* queens may lay eggs, they rarely develop into larvae. Thus *B. hypnorum* is not a competent host for *S. bombi* and may be a dead-end host. This has implications for the native *Bombus* populations in the United Kingdom and raises many more questions. Why is *B. hypnorum* resistant to *S. bombi*? Does *B. hypnorum* prevent *S. bombi* from producing larvae? Does *B. hypnorum* castrate *S. bombi*? To investigate further would require an appropriate protocol for experimentally infecting queens (see Experimental Appendices). As Röseler (2002) and Alford (1975) reported that the native *B. hortorum* was also resistant to castration by *S. bombi*, I would have liked to have included this species but I was only able to catch a couple of *B. hortorum* queens and they died after a few days in the laboratory. If sufficient *B. hortorum* queens could be collected and reared in the laboratory, I would like to investigate if they are competent hosts for S. bombi, using a similar method to that used for *B. hypnorum* in Chapter 4.

Despite biological reasons for believing that the invading host, *B. hypnorum,* had acquired *S. bombi* parasites from native congeners (Chapter 3), I wanted to find evidence to support this. In a first attempt to identify the origin of the *S. bombi* parasites in an invading species, I investigated the population structure of *S. bombi* from non-native *B. hypnorum* and native *Bombus* queens in England, and from *B. hypnorum* and other *Bombus* queens from the native range. I did not find any population structure for this parasite across Europe, therefore I was unable to establish whether *S. bombi* were acquired or co-introduced by invading *B. hypnorum.* However the number of nematode samples from that produced DNA sequences, especially from continental Europe, were limited, therefore it would be useful to obtain additional nematode samples to include in the phylogenetic tree. Other studies have found population structure in some *Bombus* parasites (e.g. *L. buchneri*: Goka 2010) but not in others (e.g. *C. bombi* and *C. expoeki*: Schmid-Hempel & Tognazzo 2010). Thus there may (or may not) be structure to the *S. bombi* population across Europe.

To enhance the phylogenetic tree built using DNA sequences from *S. bombi* from England, Ireland, Switzerland and Belgium, I would like to obtain more samples

from continental Europe. *S. bombi* samples from France and the Netherlands would useful due to the proximity of these locations to England. Sweden would be another good location as *B. hypnorum* are common (pers. comm. Paul Schmid-Hempel).

The *B. subterraneous* re-introduction project team, led by Dr Nikki Gammans, collected *B. subterraneous* queens in Sweden in 2012 and 2013. The queens were kept in quarantine at Royal Holloway, University of London to check for parasites prior to the release of healthy (i.e. parasite free) queens at Dungeness in Kent, where they were last recorded. In 2013 I was responsible for was the care of these queens during their quarantine, and for the parasite (and physical) checks prior to their release. As some of the *B. subterraneous* queens from Sweden were infected with *S. bombi,* I would also like to include these *S. bombi* samples in the phylogenetic tree.

In the Experimental Appendices, I have included two pilot projects, where I attempted to experimentally infect *Bombus* queens. In 2010, I attempted in infect summer queens. In 2011, I asked if the non-native species, *B. hypnorum*, is more susceptible to infection by the parasite *S. bombi* than native species. I had limited success with both the experimental infection of *Bombus spp.* summer queens and of *B. hypnorum* and *B. terrestris* laboratory reared and mated queens. In 2010 the single *B. hypnorum* queen that I attempted to infect was successfully infected (with seven parasite uteri). Only one other queen was successfully infected (one of six *B. lucorum* queens that I attempted to infect) contained one parasite uterus. In 2011, three *B. hypnorum* queens and two *B. terrestris* queens were successfully infected. These results suggest that *B. hypnorum* may be more susceptible to *S. bombi* than native species. Therefore it would be useful to quantify this difference in susceptibility. As *B. hypnorum* may be a dead-end host for *S. bombi*, increased susceptibility may have further implications for the parasite population and for native *Bombus* species. To investigate this further, I would need to develop an enhanced experimental infection protocol for *S. bombi* in *Bombus* species.

My comparison of the parasite community in non-native *B. hypnorum* with that of native *Bombus* species found some unexpected results. I would like to extent this study to cover more sites across England, Wales and Scotland to confirm these findings apply to all invaded areas. I am particularly interested in whether the same pattern appears at the edge of the invaded range i.e. the North of England and Scotland. I believe that *B. hypnorum* is like to reach Ireland soon and as two other

species of the subgenus *Pyrobombus* (*B. pratorum* and *B. monticola*) have successfully invaded Ireland (Speight *et al* 2002, Fitzpatrick *et al* 2007), *B. hypnorum* is also likely to do so. The invasion of *B. hypnorum* and their parasite community could be monitored prior to their arrival (although data is available from previous studies e.g. Rutrecht & Brown 2008, Kelly 2009), during their establishment and range expansion. As *B. hypnorum* has net yet been reported in Ireland, the biological invasion process and the role of parasites in the invasion success could be studied.

B. hypnorum was first reported in the UK to the north of the New Forest in Wiltshire (Goulson & Williams 2001). It may have flown across the English Channel (possibly assisted by the wind) or been inadvertently transported by ship. However, the provenance of the non-native *B. hypnorum* in the UK is unknown. Molecular techniques could be used to investigate the population structure of *B. hypnorum* across Europe. Thus the origin of the *B. hypnorum* population in the UK could be clarified.

There are many other outstanding questions raised by the arrival of the non-native *B. hypnorum* that warrant further investigation: Is *B. hypnorum* an invasive species? It has arrived, established and rapidly expanded its range across England and Wales and into Scotland but is it causing ecological and economic damage? Is it outcompeting other native Bombus species? Is *B. hypnorum* introducing, acting as a reservoir and enabling parasite spill-over, or is it a dead-end host that acts as a sink for the parasites sourced from native Bombus species?

Another potential avenue of research using the *Bombus*-*S. bombi* study system relates to the expected changes in environmental conditions i.e. soil conditions (pH, moisture, temperature) and climatic conditions (precipitation, low/high temperatures, duration of periods of low or high temperatures) and how these will affect *Bombus* hosts, nematode parasites and host-parasite interactions.

Recent bumble bee research, including the projects under the Insect Pollinators Initiative (IPI), has enhanced our knowledge of the impact of pesticides on bumble bees (Whitehorn *et al* 2012, Gill *et al* 2012) and the impact of parasites and commercially produced bumble bees on wild native bee populations (Morales *et al* 2013, Graystock *et al* 2013). The preliminary success of the *B. subterraneous* re-introduction project has shown the success of habitat creation and conservation

projects, with the report of the first *B. subterraneous* worker at Dungeness in Kent since 1988 (pers. comm. Nikki Gammans).

Over the course of my research, the *Bombus*-*S. bombi* host-parasite system has yielded some interesting projects, some unexpected results and many ideas for further work. I believe that there are many opportunities to use this study system to investigate many other important ecological questions

References

Adams, J., Rothman, E.D., Kerr, W.E. & Paulino, Z.L. (1977) Estimation of the number of sex alleles and queen matings from a diploid male frequencies in a population of *Apis mellifera. Genetics,* 86, 583-596.

Agrawal, A.A. & Kotanen, P.M. (2003) Herbivores and the success of exotic plants: a phylogenetically controlled experiment. *Ecology Letters,* 6, 712–715.

Agrawal, A.A., Kotanen, P.M., Mitchell, C.E., Power, A.G., Godsoe, W. & Klironomos, J. (2005) Enemy release? An experiment with congeneric plant pairs and diverse above- and belowground enemies. *Ecology,* 86(11), 2979-2989.

Alford, D.V. (1969a) *Sphaerularia bombi* as a parasite of bumble bees in England. *Journal of Apicultural Research,* 8, 49-54.

Alford, D.V. (1969b) A study of the hibernation of bumblebees (Hymenoptera:Bombidae) in Southern England. *Journal of Animal Ecology,* 38(1), 149-170.

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

Allen, G.R., Seeman, O.D., Schmid-Hempel, P. & Buttermore, R.E. (2007) Low parasite loads accompany the invading population of the bumblebee, *Bombus terrestris* in Tasmania. *Insectes Sociaux,* 54, 56-63.

Antonovics, J. & Edwards, M. (2011) Spatio-temporal dynamics of bumblebee nest parasites (*Bombus* subgenus *Psythirus* ssp.) and their hosts (*Bombus* spp.). *Journal of Animal Ecology,* 80, 999-1011.

Arbetman, M.P., Meeus, I., Morales, C.L., Aizen, M.A. and Smagghe, G. (2012) Alien parasite hitchhikes to Patagonia on invasive bumblebee. *Biological Invasions (published online DOI:* 10.1007/s10530-012-0311-0).

Atlas Hymenoptera [online] available from: http://zoologie.umh.ac.be/hymenoptera/pagetaxon.asp?tx_id=3045 (accessed 1st September 2013).

Bee Wasps and Ants Recording Society (BWARS) [online] available from: http://www.bwars.com/index.php?q=bee/apidae/bombus-hypnorum (accessed 1st September 2013).

Ben-ami, F., Rigaud, T. and Ebert, D. (2011) The expression of virulence during double infections by different parasites with conflicting host exploitation and transmission strategies. *Journal of Evolutionary Biology,* 24, 1307-1316.

Blaxter, M. (2003) Counting angels with DNA. *Nature* 421, 122-124

Blossey, B. & Notzold, R. (1995) Evolution of Increased Competitive Ability in Invasive Nonindigenous Plants: A Hypothesis. *Journal of Ecology* 83(5) 887-889

Bonesi, L. & Palazon, S. (2007) The American mink in Europe: Status, impacts and control. *Biological Conservation*. 143, 470-483

Brown, M.J.F., Loosli, R. & Schmid-Hempel, P. (2000) Condition-Dependent Expression of Virulence in a Trypanosome Infecting Bumblebees. Oikos 91(3) 421-427

Brown, M.J.F., Schmid-Hempel, R. & Schmid-Hempel, P. (2003a) Strong context-dependent virulence in a host-parasite system: reconciling genetic evidence with theory. *Journal of Animal Ecology,* 72(6), 994-1002.

Brown, M.J.F., Schmid-Hempel, R. & Schmid-Hempel, P. (2003b) Queen-controlled sex ratios and worker reproduction in the bumble bee *Bombus hypnorum*, as revealed by microsatellites. *Molecular Ecology*, 12, 1599–1605.

Brown, M.J.F. & Paxton R.J. (2009) The conservation of bees: a global perspective *Apidologie* 40, 410-416

Bumblebee Conservation Trust (BBCT) [online] available from: http://bumblebeeconservation.org/about-us/case-study/short-haired-bumblebee-reintroduction/ (accessed 1st September 2013)

Cameron, S.A., Hines, H.M. & Williams, P. (2007) A comprehensive phylogeny of the bumble bee (*Bombus*). *Biological Journal of the Linnean Society,* 91, 161-188.

Cameron, S.A., Lozier, J.D., Strange, J.P., Koch, J.B., Cordes, N., Solter, L.F. & Griswold, T.L. (2011) Patterns of widespread decline in North American bumble bees. *Proceedings of the National Academy of Sciences of the United States of America*. 108 (2) 662-667

Carpenter, S., Wilson, A. and Mellor, P.S. (2009) *Culicoides* and the emergence of bluetongue virus in northern Europe. *Trends in Microbiology* 17 (4) 172-178

Chao, A. and Shen, T.-J. (2010) Program SPADE (Species Prediction And Diversity Estimation). Program and User's Guide published at http://chao.stat.nthu.edu.tw.

Cloutman, D.G. (1975) Parasite community structure of largemouth bass, warmouth, and blue gill in Lake Fort Smith, Arkansas. *Transactions of the American Fisheries Society,* 104, 277-283.

Cock, M.J.W., Biesmeijer, J.C., Cannon, R.J.C., Gerard, P.J., Gillespie, D., Jimenez, J.J., Lavelle, P.M. & Raina, S.K. (2012) The positive contribution of invertebrates to sustainable agriculture and food security. *CAB Reviews* 7, No. 043

Colla, S. & Packer, L. (2008) Evidence for decline in eastern North American bumblebees (Hymenoptera: Apidae), with special focus on *Bombus affinus* Cresson. *Biodiversity and Conservation.* 17, 1379-1391

Colautti, R.I., Ricciardi, A., Grigorovich I.A & MacIssac, H.J. (2004) Is invasion success explained by the enemy release hypothesis? *Ecology Letters,* 7, 721–733

Colautti, R.I., Muirhead, J.R., Biswas, R.N. & MacIssac, H.J. (2005) Realsied vs apparent reduction in enemies of the European starling. *Biological Invasions,* 7, 723–732

Combes, C. (2004) *Parasitism: The Ecology and Evolution of Intimate Interactions.* University of Chicago Press, Chicago

Cremer, S., Armitage, S.A.O. & Schmid-Hempel, P. (2007) Social immunity. *Current Biology* 17, R693-R702

Cumber R.A. (1949) Humble-bee parasites and commensuals found within a thirty mile radius of London. *Transactions of the Royal Entomological Society London A* 24, 119-127

Dafni, A. (1998) The threat of *Bombus terrestris* spread. *Bee World,* 79(3), 113–114.

Darwin, C. (1859) (1998) *The origin of species*. Wordsworth classics of world literature, Ware, Hertfordshire

Daszak, P., Cunningham, A.A. & Hyatt, A.D. (2000) Emerging infectious diseases of wildlife: threats to biodiversity and human health. *Science,* 287, 443.

Decaestecker, E., Gaba, S., Raeymaekers, J.A.M., Stoks, R., Van Kerckhoven, L., Ebert, D. & De Meester, L., (2007) Host-parasite 'Red Queen' dynamics archived in pond sediment. *Nature* 450, 870-873

Derycke, S., Vanaverbeke, J., Rigaux, A., Backeljau, T. & Moens, T. (2010) Exploring the Use of Cytochrome Oxidase c Subunit 1 (COI) for DNA Barcoding of Free-Living Marine Nematodes. *PLoSONE* 5 (10) e1371/journal.pone0013716

Di Febbraro, M., Lurz, P.W.W., Genovesi, P., Maiorano, L., Giradello, M., & Bertoliino, S. (2013) The Use of Climatic Niches in Screening

Procedures for Introduced Species to Evaluate Risk of Spread: A Case with the American Eastern Grey Squirrel. *PLoSONE* 8(7): e66559

Dlugosch, K.M. & Parker, M. (2008) Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Molecular Ecology,* 17, 431-449.

Dobson, A. & R. Carper (1992) *Global warming and potential changes in host-parasite and disease-vector relationships*. In: *Global warming and biodiversity* ed. R. L. Peters and T. E. Lovejoy. Yale University Press. New Haven

Drake, J.M. (2003) The paradox of the parasites: implications for biological invasion. *Proceedings of the Royal Society of London Series B (Supplement),* 270, S133-S135.

Duchateau, M.J., Hoshiba, H. & Velthuis H.H.W. (1994) Diploid males in the bumble bee *Bombus terrestris:* sex determination, sex alleles and variability. *Entomologia Experimentalis et Applicata*, 71, 263-269.

Dunn, A.M. & Dick, J.T.A. (1998) Parasitism and epibiosis in native and non-native gammarids in freshwater in Ireland. *Ecography,* 21(6), 593-59.

Dunn, A.M. (2009) Parasites and Biological Invasions. *Advances in Parasitology, Vol 68: Natural History of Host-Parasite Interactions* ed. J.P. Webster) pp 161-184.

Durrer, S. & Schmid-Hempel, P. (1994) Shared use of flowers leads to horizontal pathogen transmission. *Proceedings of the Royal Society of London Series B - Biological Sciences,* 258, 299-302.

Durrer, S. & Schmid-Hempel, P. (1995) Parasites and the regional distribution of bumble bee species. *Ecography* 18, 114-122

Ebert, D. (1994) Virulence and Local Adaptation of a Horizontally Transmitted Parasite. *Science* 265, 1084-1086

Edwards, M. & Jenner, M. (2005) *Field Guide to the Bumblebees of Great Britain & Ireland*. Ocelli

Eigenbrod, F., Bell, V.A., Davies, H.N., Heinemeyer, A., Armsworth, P.R. & Gaston, K.J. (2011) The impact of projected increases in urbanization on ecosystems services. *Proceedings of the Royal Society of London Series B - Biological Sciences,* 278, 3201-3208.

Elton, C.S. (1958) *The ecology of invasions by plants and animals.* The University of Chicago Press

Estoup, A., Scholl, A., Pouvreau, A. & Solignac, M. (1995) Monoandry and polyandry in bumble bees (Hymenoptera; Bombinae) as evidenced by highly variable microsatellites. *Molecular Ecology,* 4, 89-93.

Estoup, A., Slignac, M., Cornet, J.-M., Goudet, J. & Scholl A. (1996) Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in Europe. *Molecular Ecology* 5,19-31

Ferguson, N.M, Cummings, D.A.T., Cauchemez, S., Fraser, C., Riley, S. Meeyai, A., Iamsirithaworn, S. & Burke, D.S. (2005) Strategies for containing an emerging influenza pandemic in Southeast Asia. *Nature* 437, 209-214

Ficetola, G.F., Bonin, A. & Miaud, C. (2008) Population genetics reveals origin and number of founders in a biological invasion. *Molecular Ecology* 17, 773-782

Fitzpatrick, U., Murray, T.E., Paxton, R.J., Breen, J., Cotton, D., Santorum, V. & Brown M.J.F. (2007) Rarity and decline in bumblebees – A test of causes and correlates in the Irish fauna. *Biological Conservation*, 136, 185–194.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3(5) 294-299

Frankham, R., Ballou, J.D. & Briscoe, D.A. (2010) *Introduction to Conservation Genetics.* 2nd Edition. Cambridge University Press

Futuyma, D.J. (2005) *Evolution* Sinauer Associates, Inc. Sunderland

Fye, R.E. (1966) *Sphaerularia bombi* Duford Parasitizing *Bombus* queens in Northwestern Ontario. *The Canadian Entomologist* 98, 88-89

Gandon, S. (2002) Local adaptation and the geometry of host-parasite coevolution. *Ecology Letters* 5, 246-256

Gandon, S & Michalakis, Y. (2002) Local adaptation, evolutionary potential and host-parasite coevolution: interactions between migration, mutation, population size and generation time. *Journal of Evolutionary Biology* 15, 451-462

Gassert, F., Schulte, U., Husemann, M., Ulrich, W., Rodder, D., Hochkirch, A., Engel, E., Meyer, J. & Habel, J.C. (2013) From southern refugia to the northern range margin: genetic population structure of the common wall lizard, *Podarcis muralis. Journal of Biogeography* 40, 1475-1489

Georgiev, B.B., Sánchez, M.I. , Vasileva, G.P., Nikolov, P.N. & Green, A.J. (2007) Cestode parasitism in invasive and native brine shrimps (*Artemia*

spp.) as a possible factor promoting the rapid invasion of *A. franciscana* in the Mediterranean region. *Parasitology Research*, 101, 1647-1655.

Gill, R.J., Ramos-Rodriguez, O. & Raine, N.E. (2012) Combined pesticide exposure severely affects individual– and colony-level traits in bees. *Nature* 491, 105-108

Goka, K. (2010) Introduction to the Special Feature for the Ecological Risk Assessment of Introduced Bumblebees: Status of the European bumblebee, *Bombus terrestris*, in Japan as a beneficial pollinator and an invasive alien species. *Applied Entomology and Zoology* 45 (1) 1-6

Goka, K., Okabe, K. & Yoneda, M. (2006) Worldwide migration of parasitic mites as a result of bumblebee commercialization. *Population Ecology* 48, 285-291

Goldblatt, J.W. & Fell, R.D. (1984) Parasites and Parasitization Rates in Bumble Bee Queens, *Bombus spp.* (Hymenoptera: Apidae), in Southwestern Virginia. *Environmental Entomology* 13 (6) 1661-1665

Goulson, D. & Williams, P. (2001) *Bombus hypnorum* (Hymenoptera: Apidae), a new British bumblebee? *British Journal of Entomology and Natural History,* 14, 129–131.

Goulson, D. (2003) Effects of introduced bees on native ecosystems *Annual Review of Ecology, Evolution and Systematics*, 34, 1–26.

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

Goulson, D., Hanley, M.E., Darvill, B., Ellis, J.S. & Knight, M.E. (2005) Causes of rarity in bumblebees. *Biological Conservation*, 122, 1-8.

Goulson, D. Lye, G.C. & Darvill, B. (2008) Decline and Conservation of Bumble Bees. *Annual Review of Entomology* 53, 191-208

Goulson, D. (2010) Impacts of non-native bumblebees in Western Europe and North America. *Applied Entomology and Zoology,* 45(1), 7-12.

Graystock, P., Yates, K., Evison, S.E.F., Darvill, B., Goulson, D. & Hughes, W.O.H. (2013) The Trojan hives: pollinator pathogens, imported and distributed in bumblebee colonies. *Journal of Applied Ecology*

Greishar, M.A. & Koskella, B. (2007) A synthesis of experimental work on parasite local adaptation. *Ecology Letters* 10: 418-434

Grixti, J.C., Wong, L.T., Camerin, S.A. & Favret, C. (2009) Decline of bumble bees (*Bombus*) in the North American Midwest. *Biological Conservation* 142, 75-84

Haddaway, N.R., Wilcox, R.H., Heptonstall, R.E.A., Griffiths, H.M., Mortimer, R.J.G., Christmas, M. & Dunn, A.M. (2012) Predatory Functional Response and Prey Choice Identify Predation Differences between Native/Invasive and Parasitised/Unparasitised Crayfish. *PLoS ONE* 7(2): e32229

Hasegawa, M., Kishino, H., & Yano, T. (1985) Dating of human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 22 (2): 160–174

Hatcher, M.J. & Dunn, A.M. (2011) *Parasites in ecological communities: From interactions to ecosystems.* Cambridge University Press

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003) Biological identifications through DNA barcades. *Proceedings of the Royal Society of London Series B - Biological Sciences* 270, 313-321

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

Hickling, R., Roy, D.B., Hill, J.K., Fox, R. & Thomas, C.D. (2006) The distributions of a wide range of taxonomic groups are expanding polewards. *Global Change Biology*, 12, 450-455.

Hines, H.M, Cameron, S.A. & Williams, P.H. (2006) Molecular Phylogeny of the bumble bee subgenus Pyrobombus (Hymenoptera: Apidae: *Bombus*) with insights into gene utility for lower-level analysis. *Invertebrate Systematics* 20, 289-303

Holmes, J.C. & Price, P.W. (1986) Communities of parasites. *Community ecology: Patterns and processes*. (eds J. Kittawa & D. J. Anderson) pp 187-213. Blackwell Scientific Publications, New York

Hudson, P.J., Dobson, A.P., & Newborn, D. (1998) Prevention of Population Cycles by Parasite Removal. *Science* 282: 2256-2258

Husband, R.W. & SInha, R.N. (1970) A Revision of the Genus *Locustacarus* with a Key to Genera of the Family Podapolipidae (Acarina). *Annuals of the Entomological Society of America* 63(4) 1152-1162

Imhoof, B. & Schimid-Hempel, P. (1998) Patterns of local adaptation of a protozoan parasite to its bumblebee host. *Oikos* 82, 59-65

Ings, T.C., Ward, N.L. & Chittka L. (2006) Can commercially imported bumble bees out-compete their native conspecifics? *Journal of Applied Ecology* 43, 940-948

Inoue, M.N., Yokoyama, J. & Washitani, I. (2008) Displacement of Japanese native bumblebees by the recently introduced *Bombus terrestris* (L.)(Hymenoptera: Apidae). *Journal of Insect Conservation,* 12, 135-146.

Insect Pollinators Initiative (IPI) [online] available from: http://www.bbsrc.ac.uk/pollinators/ accessed 1st September 2013.

IUCN (1998) *Guidelines for Re-introductions*. Prepared by the IUCN.SSC Re-introductions Species Group, IUCN, Gland, Switzerland and Cambridge, UK 10 pp.

Janovy, J. Jr., Clopton, R.E., Clopton, D.A., Snyder, S.D., Efting, A. & Krebs, L. (1995) Species density distributions as null models for ecologically significant interactions of parasite species in an assemblage. *Ecological Modelling*, 77, 189-196.

Johnson, P.T.J. & Thieltges D.W. (2010) Diversity, decoys and the dilution effect: how ecological communites affect disease risk. *Journal of Experimental Biology* 213, 961-970

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., & Daszak, P. (2008) Global trends in emerging infectious diseases. *Nature* 541, 990-993

Kawecki, T.J. & Ebert, D. (2004) Conceptual issues in local adaptation. *Ecology Letters* 7, 1225-1241

Keane, R.M. & Crawley M.J. (2002) Exotic plant invasions and the enemy release hypothesis. *Trends in Ecology and Evolution* 17, 164-170

Kearns, C.A., Inouye, D.W. & Waser, N.M (1998) Endangered mutualisms: The conservation of plant-pollinator interactions. *Annual Review of Ecology and Systematics* 29, 83-112

Kelly, M.D. (2009) Investigation into the host-parasite relationship of Sphaerularia bombi and its host(s) *Bombus spp.* PhD Thesis submitted to Trinity College Dublin

Kelly, D.W., Paterson, R.A., Townsend, C.R., Poulin, R. & Tompkins, D.M. (2009) Parasite spillback: A neglected concept in invasion ecology? *Ecology* 90(8), 2047–2056.

Klein, A.-M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C. & Tscharntke, T. (2006) Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society of London Series B - Biological Sciences* Published online (doi:10.1098/rspb.2006.3721)

Korner, P. & Schimid-Hempel P. (2005) Correlates of parasite load in bumblebees in an Alpine habitat. *Entomological Science* 8, 151-160

Kosior, A., Celary, W., Olejniczak, P., Fijal, J., Krol, W., Solarz, W. & Plonka, P. (1997) The decline of the bumble bees and cuckoo bees (Hymenoptera:Apidae: Bombini) of Western and Central Europe. *Oryx* 41: 79-88

Kovats, R.S., Campbell-Lendrum, D.H., McMichael, A.J., Woodward, A. & Cox, J. St H. (2001) Early effects of climate change: do they include changes in vector borne disease? *Philosophical Transactions of the Royal Society of London Series B - Biological Sciences,* 356, 1057-1068

Kremen, C., Williams, N.M., Aizen, M.A., Gemill-Herren, B., Lebuhn, G., Minckley, R., Packer, L., Potts, S.G., Roulston, T., Steffan-Dewenter, I., Vazquez, D.P., Winfree, R., Adams, L., Crone, E.E., Greenleaf, S.S., Keitt, T.H., Klein, A.-M., Regetz, J. & Ricketts, T.H. (2007) Pollination and other ecosystem services produced by mobile organisms: a conceptual framework for the effects of land-use change. *Ecology Letters* 10: 299-314

Lanfear, R., Calcott, B., Ho, S. Y. W. & Guindon, S. (2012) PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* 29 (6), 1695-1701

Liu, H. & Stiling, P. (2006) Testing the enemy release hypothesis: a review and meta-analysis. *Biological Invasions,* 8, 1535-1545.

Lundberg, H. & Svensson, B.G. (1975) Studies on the behaviour of *Bombus* Latr. Species (Hym.,Apidae) parasitized by *Sphaerularia bombi* Dufour (Nematoda) in an alpine area. *Norwegian Journal of Entomology*, 22(2), 129-134.

Lye, G.C., Lepais, O. & Goulson, D. (2011) Reconstructing demographic events from population genetic data: the introduction of bumblebees to New Zealand. *Molecular Ecology*, 20, 2888–2900.

MacFarlane, R.P. & Griffin, R.P. (1990) New Zealand distribution and seasonal incidence of the nematode *Sphaerularia bombi* Dufour, a parasite of bumble bees. *New Zealand Journal of Zoology*, 17, 191-199.

MacFarlane, R.P & Gurr, L. (1995) Distribution of bumble bees in New Zealand. *New Zealand Entomologist* 18, 29-35

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

MacNeil, C., Dick, J.T.A., Hatcher, M.J., Terry, R.S., Smith, J.E. & Dunn, A.M. (2003) Parasite-mediated predation between native and invasive amphipods. *Proceedings of the Royal Society of London Series B - Biological Sciences* 270, 1309-1314

Magnacca, K. & Brown, M.J.F. (2010) Mitochondrial heteroplasmy and DNA barcoding in Hawaiian *Hylaeus* (Nesoprosopis) bees (Hymenoptera: Colletidae). *BMC Evolutionary Biology* 10: 174-184

Matsumura, C., Yokoyama, J. & Washitani, I. (2004) Invasion status and potential ecological impacts of an invasive alien bumblebee *Bombus terrestris* L. (Hymenoptera: Apidae) naturalised in Southern Hokkaido, Japan. *Global Environmental Research*, 8(1), 51-66.

Maxfield-Taylor, S. , Skyrm, K.M. & Rao, S. (2011) First record of *Sphaerularia bombi* (Nematoda: Tylenchida: Sphaerularidae), a parasite of bumble bee queens, in the Pacific Northwest. *The Pan-pacific Entomologist* 87(2),134-137

Morales, C.L., Arbetmann, M.P., Cameron, S.A. & Aizen M.A. (2013) Rapid ecological replacement of a native bumble bee by invasive species. *Frontiers in Ecology and the Environment.*

Morgan, A.D., Gandon, S. & Buckling, A. (2005) The effect of migration on local adaptation in a coevolving host-parasite system. *Nature* 437, 253-256

Nash, D. Als, T.D., Maile, R., Jones, G.R. & Boomsma, J.J. (2008) A Mosaic of Chemical Coevolution in a Large Blue Butterfly. *Science* 319, 88-90

Natural History Museum (NHM) website [online] available from: http://www.nhm.ac.uk/research-curation/research/projects/bombus/_key_colour_british/alphabetic_list.html (accessed 1st September 2013)

Nieberding, C., Libois, R., Douady, C.J., Morand, S. & Michaux, J.R. (2005) Phylogeography of a nematode (*Heligmosomoides polygyrus*) in the western Palearctic region: persistence of the northern cryptic populations during ice ages? *Molecular Ecology* 14, 765-779

Nieberding, C.M., Durette-Desset, M.-C., Vanderpoorten, A., Casanova, J.C., Ribas, A., Deffontaine, V., Feliu, C., Morand, S. Libois, R. & Michaux J.R. (2008) Geography and host biogeography matter for understanding the phylogeography of a parasite. *Molecular Phylogenetics and Evolution* 47, 538-554

Norman, R., Bowers, R.G., Begon, M. & Hudson, P.J. (1999) Persistence of tick-borne virus in the presence of multiple host species: tick reservoirs and

parasite mediated competition. *Journal of Theoretical Biology* 200, 111-118.

Ostfeld, R.S. & Keesing, F. (2000) Biodiversity and disease risk: the case of Lyme disease. *Conservation Biology* 14(3), 722-728.

Ohtaka, A., Gelder, S.R., Kawai, T., Saito, K., Nakata, K. & Nishino, M. (2005) New records and distributions of two North American branchiobdellian species (Annelida: Clitellata) from introduced signal crayfish, *Pacifastacus leniusculus*, in Japan. *Biological Invasions* 7, 149-156

Otterstatter, M.C. & Whidden, T.L. (2004) Patterns of parasitism by tracheal mites (*Locustacarus buchneri*) in natural bumble bee populations. *Apidologie*, 35, 351-357

Otti, O. & Schmid-Hempel, P. (2007) *Nosema bombi:* A pollinator parasite with detrimental fitness effects. *Journal of Invertebrate Pathology* 96, 118-124.

Parmesan, C., Ryrholm, N., Stefanescu, C., Hill, J.K., Thomas, C.D., Descimon, H., Huntley, B., Kaila, L., Kullberg, J., Tammaru, T. *et al.* (1999) Poleward shifts in geographical ranges of butterfly species associated with regional warming. *Nature* 388, 579-583

Parmesan, C. & Yohe, G. (2003) A globally coherent fingerprint of climate change impacts across natural systems. *Nature* 421:37-69

Parmesan, C. (2006) Ecological and Evolutionary Responses to Recent Climate Change. *Annual Review of Ecology, Evolution and Systematics* 37, 637-669

Paxton, R.J., Thorn, P.A., Estoup, A. & Teng, J. (2001) Queen-worker conflict over male production and the sex ratio in a facultatively polyandrous bumblebee, *Bombus hypnorum*: the consequences of nest usurpation. *Molecular Ecology* 10, 2489-2498.

Pejchar, L. & Mooney, H.A. (2009) Invasive species, ecosystem services and human well-being. *Trends in Ecology and Evolution* 24, 497-504

Perlman, S.J. & Jaenike, J. (2003) Infection success in novel hosts: An experimental and phylogenetic study of *Drosophila*-parasitic nematodes. *Evolution,* 57(3), 544–557.

Phillips, B.L., Keleear, C., Pizzatto, L., Brown, G.P., Barton, D. & Shine R. (2010) Parasites and pathogens lag behind their host during periods of host range advance. *Ecology*, 91(3), 872-881

Pimentel, D., Zuniga, R. & Morrison D. (2005) Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* 52, 273-288.

Plischuk, S. & Lange, C.E. (2009) Invasive *Bombus terrestris* (Hymenoptera: Apidae) parasitized by a flagelleate (Euglenozoa: Kinetoplastea) and a neogregarine (Apicomplexa: Neogregarinorida). *Journal of Invertebrate Pathology* 102, 263-265.

Plischuk, S. & Lange, C.E. (2012) *Sphaerularia bombi* (Nematoda: Sphaerulariidae) parasitizing *Bombus atratus* (Hymenoptera: Apidae) in southern South America. *Parasitology Research*. DOI 10.1007/s00436-012-2853-6

Poinar, G.O.J. & van der Laan, P.A. (1972) Morphology and life history of *Sphaerularia bombi. Nematology* 18, 239-252.

Pontoppidan, M.-B., Himaman, W., Hywel-Jones, N.L., Boomsma, J.J. & Hughes, D.P. (2009) Graveyards on the Move: The Spatio-Temporal Distribution of Dead Ophiocordyceps-infected Ants. *PLoSONE* 4(3) e4835

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

Pouvreau, A. (1963) Observations sur l'accouplement de *Bombus hypnorum* L. (Hymenoptere, Apidae) en serre. *Insectes Sociaux* X(2), 111-118.

Prenter, J., MacNeil C., Dick, J.T.A. & Dunn, A.m. (2004) Roles of parasites in animal invasions. *Trends in Ecology and Evolution* 19, 385-390

Price PW (1980) *Evolutionary Biology of Parasites*. Princeton University Press, Princeton

Rigaud, T., Perrot-Minnot, M-J. & Brown, M.J.F. (2010) Parasite and host assemblages: embracing the reality will improve our knowledge of parasite transmission and virulence. *Proceedings of the Royal Society of London Series B - Biological Sciences* 277, 3693-3702.

Ronquist, F. & Huelsenbeck, J. P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574.

Röseler, P.-F. (2002) A scientific note on the reproduction of two bumblebee queens (*Bombus hypnorum*) infested by the nematode *Sphaerularia bombi. Apidologie* 33, 423-424

Rushton, S.P., Lurz, P.W.W., Gurnell, J. & Fuller, R. (2000) Modelling the spatial dynamics of parapoxvirus disease in red and grey squirrels: a possible

cause of the decline in the red squirrel in the UK? *Journal of Applied Ecology* 37, 997-1012

Rushton, S.P., Lurz, P.W.W., Gurnell, J., Nettleton, P., Bruemmer, C., Shirley, M.D.F. & Sainsbury, A.W. (2006) Disease threats posed by alien species: the role of a poxvirus in the decline of the native red squirrel in Britain. *Epidemiology and Infection* 134, 521-533

Rutrecht, S.T. & Brown, M.J.F. (2008) The life-history impact and implications of multiple parasites for bumble bee queens. *International Journal for Parasitology* 38, 799–808

Rutrecht, S.T. & Brown, M.J.F. (2009) Differential virulence in a multiple-host parasite of bumblebees: resolving the paradox of parasite survival? *Oikos* 118, 941-949

Sayama, K. Kosaka, H. & Makino, S. (2007) The first record of infection and sterilization by the nematode *Sphaerularia* in hornets (Hymenoptera, Vespidae, *Vespa*). *Insectes Sociaux* 54, 53-55

Shaw, R.H., Bryner, S. & Tanner, R. (2009) The life history and host range of the Japanese knotweed psyllid, *Aphalara itadori* Shinji: Potentially the first classical biological weed control agent for the European Union. *Biological Control* 49, 105-113

Schmid-Hempel, P. (1998) *Parasites in Social Insects* Princeton University Press

Schmid-Hempel, R. & Schmid-Hempel, P. (2000) Female mating frequencies in *Bombus* spp. from Central Europe. *Insectes Sociaux* 47, 36–41

Schmid-Hempel, P., Schmid-Hempel, R., Brunner, P.C., Seeman, O.D. & Allen G.R. (2007) Invasion success of the bumblebee, *Bombus terrestris*, despite a drastic genetic bottleneck. *Heredity* 99, 414–422.

Schmid-Hempel, R. & Tognazzo M. (2010) Molecular Divergence Defines Two Distinct Lineages of *Crithida bombi* (Trypanosomatidae), Parasites of Bumblebees. *Journal of Eukaryotic Microbiology* 57(4) 337-345

Schmid-Hempel, P (2011) *Evolutionary Parasitology: The Integrated Study if Infections, Immunology, Ecology and Genetics.* Oxford University Press, Oxford.

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

Simon, C., Frati, F., Beckenback, A., Crespi, B., Liu, H. & Flook, P. (1994) Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene

Sequences and a Compilation of Conserved Polymerase Chain Reaction Primers. *Annals of the Entomological Society of America* 87(6) 651-701

Sladen, F.W.L. (1912) (1989) *The Humble-Bee: Its Life-History and How to Domesticate It* MacMillan & Co Ltd., London/Logaston Press

Slothouber Galbreath J.G.M., Smith, J.E., Becnel, J.J., Butlin, R.K. & Dunn, A.M. (2010) 'Reduction in post-invasion genetic diversity in *Crangonyx pseudogracilis* (Amphipoda: Crustacea): a genetic bottleneck or the work of hitchhiking vertically transmitted microparasites? *Biological Invasions*, 12, 191-209

Speight, M.C.D. (1974) *Bombus lapponicus, Parasyrphus lineola*, and *Phaonia exoleta*: insects new to Ireland. *Irish Naturalists' Journal*, 18, 123–124.

Stelzer, R.J., Chittka, L., Carlton, M. & Ings, T.C. (2010) Winter active bumblebees (*Bombus terrestris*) achieve high foraging rates in urban Britain. *PLoSONE,* 5(3), e9559.

Stepien, C.A., Taylor, C.D. & Dabrowska, K.A. (2002) Genetic variability and phylogeographical patterns of a nonindigenous species invasion: a comparison of exotic vs. native zebra and quagga mussel populations. *Journal of Evolutionary Biology* 15, 314-328

Svendsen, J.I., Alexanderson, H., Astakhov, V.I, Demidov, I, Dowdeswell, J.A., Funder, S., Gataullin, V., Henriksen, M., Hjort, C., Houmark-Nielsen, M., Hubberten, H.W., Ingolfsson, O., Jakobsson, M., Kjaer, K.H., Larsen, E., Lokrantz, H., Lunkka, J.P., Lysa, A., Mangerud, J., Matiouchkov, A., Murray, A., Moller, P., Niessen, F., Nikolskaya, O. Polyak, L., Saarnisto, M., Siegert, C., Siegert, M., Speilhagen, R.F., Stein, R. (2004) Late Quaternary ice sheet history of northern Eurasia. *Quaternary Science Reviews* 23, 1229-1271

Taberlet, P., Fumagalli, L., Wust-Saucy, A.-G., & Cossons, J.-F. (1998) Comparative phylogeography and post glacial colonization routes in Europe. *Molecular Ecology* 7, 453-464

Telfer, S., Bown, K.J., Sekules, R., Begon, I., Hayden, T. & Birtles, R. (2005) Disruption of a host-parasite system following the introduction of an exotic host species. *Parasitology* 130, 661-668

Thomas, J. A. (1999) The large blue butterfly, a decade of progress. *British Wildlife* 11, 22-27

Thomson, J. D., Price, M.V., Waser, N.M & Stratton, D.A. (1986) Comparative studies of pollen and fluorescent dye transport by bumble bees visiting *Erythronium grandiflorum*. *Oecologia* 69 (4), 561-566

Thomson, J. D. & Goodall K. (2001) Pollen removal and deposition by honey bee and bumble bee visitors to apple and almond flowers. *Journal of Applied Ecology* 38,1032-1044

Thompson, J.N. (2005) *The Geographic Mosaic of Coevolution.* The University of Chicago Press

Tompkins, D.M., White, A.R. & Boots, M. (2003) Ecological replacement of native red squirrels by invasive greys driven by disease. *Ecology Letters* 6, 189-196

Torchin, M.E., Lafferty, K.D., Dobson, A.P., McKenzie, V.J. & Kuris, A.M. (2003) Introduced species and their missing parasites. *Nature,* 421, 628-630.

Torretta, J.P., Medan, D. & Abrahamovich, A.H. (2006) First record of the invasive bumblebee *Bombus terrestris* (L.)(Hymenoptera, Apidae) in Argentina. *Transactions of the American Entomological Society*, 132(3+4), 285-289.

Ugelvig, L.V. & Cremer, S. (2012) Effects of social immunity and unicoloniality on host-parasite interactions in invasive insect societies. *Functional Ecology* 26,1300-1312

Ulrich, Y., Sadd, B. & Schmid-Hempel, P. (2011) Strain filtering and transmission of a mixed infection in a social insect. *Journal of Evolutionary Biology* 24, 354-362

Van Valen, L. (1973) A New Evolutionary Law. *Evolutionary Theory* 1, 1-30

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

Venesky, M.D., Mendelson J.R.III, Sears. B.F., Stilling, P. and Rohr, J.R. (2012) Selecting for tolerance against pathogens and herbivores to enhance success of reintroduction and translocation. *Conservation Biology,* 26(4), 586-592.

Vila, M., Basnou, C., Pysek, P., Josefsson, M., Genovesi, P., Gollasch, S., Nentwig, W., Olenin, S., Roques, A., Roy, D., Hulme, P.E. & DAISIE partners (2010) How well do we understand the impacts of alien invasive species on ecosystems services? A pan-European, cross-taxa assessment. *Frontiers in Ecology and the Environment,* 8(3), 135-144.

Vitousek, P.M., D'Antonio, C.M., Loope, L.L. & Westbooks, R. (1996) Biological invasions as global environmental change. *American Scientist,* 84, 468-478.

Warren, P., Hill, J.K., Thomas, J.A., Asher, J., Fox, R., Huntley, B., Roy, D.B., Telfer, M.G., Jeffcoate, S., Harding, P., Jeffcoate, G., Willis, S.G., Greatorex-Davies, J.N., Moss, D. & Thomas, C.D. (2001) Rapid responses of British butterflies to opposing forces of climate and habitat change. *Nature* 414, 65-69

Waser, N.M. & Price, M.V. (1981) Pollinator choice and stabilizing selection for flower color. *Evolution* 35 (2), 376-389

Wattier, R.A., Haine, E.R., Beguet, J., Martin, G., Bollache, L., Musko, I.B., Platvoet, D. & Rigaud, T. (2007) No genetic bottleneck or associated micro-parasite loss in invasive populations of a freshwater amphipod. *Oikos* 116, 1941-1953

White, T.A. & Perkins, S.E. (2012) The ecoimmunity of invasive species. *Functional Ecology*, 26, 1313-1323

Whitehorn, P.R., Tinsley, M.C., Brown, M.J.F., Darvill, B. & Goulson, D. (2009) Impacts of inbreeding on bumblebee colony fitness under field conditions. *BMC Evolutionary Biology,* 9, 152

Whitehorn, P.R., Tinsley, M.C., Brown, M.J.F., Darvill, B. & Goulson, D. (2011) Genetic diversity, parasite prevalence and immunity in wild bumblebees. *Proceedings of the Royal Society of London Series B - Biological Sciences,* 278, 1195-1202.

Whitehorn, P.R., O'Connor, S., Wackers, F.L., Goulson, D. (2012) Neonicotinoid Pesticide Reduces Bumble Bee Colony Growth and Queen Production. *Science* 336, 351-352

Widmer, A., Schmid-Hempel, P., Estoup, A. & Scholl, A. (1998) Population genetic structure and colonization history of *Bombus terrestris* s.l. (Hymenoptera: Apidae) from the Canary Islands and Madeira. *Heredity* 81, 563-572

Widmer, A. & Schmid-Hempel, P. (1999) The population genetic structure of a large temperate pollinator species, *Bombus pascuorum* (Scopoli)(Hymenoptera: Apidae). *Molecular Ecology* 8, 387-398

Wilcove, D.S., Rothstein, D., Dubow, J., Phillips, A. & Losos, E. (1998) Quantifying threats to imperiled species in the United States. *BioScience,* 48(8), 607-615.

Williams, P.H. (1982) The distribution and decline of British bumble bees (*Bombus* Latr.). *Journal of Apicultural Research* 21(4) 236-245

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

Williams, P.H. & Osborne, J.L. (2009) Bumblebee vulnerability and conservation worldwide. *Apidologie* 367-387

Williams, P.H., An, J., Brown, M.J.F., Carolan, J.C., Goulson, D., Huang, J. & Ito, M. (2012) Cryptic bumblebee species: consequences for conservation and the trade in greenhouse pollinators. *PloS One,* 7(3), e32992. doi:10.1371/journal.pone.0032992

Williamson, M. (1996) *Biological Invasions*. Chapman & Hall, London

Wilson, A.J. & Mellor, P.S. (2009) Bluetongue in Europe: past, present and future. *Philosophical Transactions of the Royal Society of London Series B - Biological Sciences* 364, 2669-2681

Yoneda, M., Furuta, H., Kanbe, Y., Tsuchida, K., Okabe, K. & Goka, K. (2008) Reproduction and transmission within a colony of bumblebee tracheal mite *Locustacarus buchneri* (Acari: Podapolipidae) in *Bombus terrestris* (Hymenoptera: Apidae). *Applied Entomology and Zoology* 43(3) 391-395

Experimental Appendices

Introduction

The experimental appendices contain the method, the results and a brief discussion of two experimental infections attempted in 2010 and 2011. In 2010, I experimentally infected a small number of wild caught *Bombus* summer queens with *S. bombi* larvae from queens collected in England and Ireland. In 2011, I experimentally infected *B. hypnorum* and *B. terrestris* queens reared in the laboratory from wild caught spring queens collected in spring 2011 with *S. bombi* larvae from queens collected in England.

Methods

Bee collection

In 2010 *Bombus* summer queens and males were collected in The Savill Garden, in Windsor Great Park. The single Swiss gyne reared in the laboratory in 2010 (see Chapter 2) was also included in this experiment.

In 2011 emerging spring *B. hypnorum* and *B. terrestris* queens were collected in Valley Gardens, in Windsor Great Park (see Chapter 3). The gynes and males produced by these queens were used in this project.

Bee Husbandry

In 2010, summer queens were kept in daylight in the laboratory, in individual plastic boxes with a tissue paper and paper base. Queens were fed with loose pollen in a small petri dish and sugar-water from a 15ml falcon tube, with holes drilled in the tip and a small petri dish to catch any leakage (as described in Chapter 2). The males were kept in the same way but with up to six males in a plastic box.

In 2011, laboratory reared *B. hypnorum* and *B. terrestris* gynes were kept in daylight either in individual queen rearing boxes or in in groups of up to 15 gynes from the same colony and fed pollen and sugar-water. The date the gynes were removed from their natal colony and the colony number was recorded. Up to 20 *B. hypnorum* or *B. terrestris* males were kept in the bee room (i.e. in the dark) in a wooden box and fed with loose pollen and sugar-water. The males were removed from their natal colonies every day for up to seven days, each colony was assigned a wooden

box for that seven day period and then assigned a new box for the subsequent seven day period. Thus I was able to identify the approximate age of the gynes and males, and ensure that gynes and males from different colonies were mated.

Bee mating

Bee mating took place in a laboratory, with naturally light around midday, to provide optimal conditions for mating. Males and gynes from differing colonies were mated either in a flight cage, if there multiple gynes from the same colony available, or in plastic boxes. I placed males that were at least five days old (Kelly 2009, Amin et al 2011) in a flight cage (500mm x 500mm x 600mm) for five minutes. Kelly (2009) suggested using a maximum of 30 gynes and 60 males in the flight cage, but my samples were much smaller, therefore I used a maximum of 25 males and six gynes. I then added the gynes to flight cage. The process for using a plastic box was similar although, only two or three males followed by one gyne (after five minutes) were placed in each plastic box. I observed their interactions for at least 30 minutes and transferred mating pairs from flight cage and to a separate plastic box. For the mating in the plastic box, the males that were not involved in the mating were removed. After 30 minutes, or when copulation was completed, the males were returned to their wooden box housing. Mated queens were housed in individual plastic boxes or queen rearing boxes and unmated gynes returned to their boxes. If the summer queens, collected in 2010 did not mate after three attempts, I assumed that they had mated prior to collection.

In 2011, the mating process was repeated until all unmated gynes had completed multiple (ca. 6) mating attempts. The mated queens were housed in the bee-room with *ad-libitum* sugar-water and pollen for about four days prior to hibernation (Kelly, 2009). After the mating process was completed, all the *B. hypnorum* and *B. terrestris* males were released at RHUL, close to the collection site.

Collection of parasite larvae from sand boxes

In 2010 spring queens collected in England, Switzerland and Ireland (see Chapter 2) were kept in plastic boxes on a layer of sand. The sand from the plastic boxes of all queens was checked for nematode larvae and any nematode larvae were transferred into plastic boxes of clean damp sand, ‘worm farms’, to mature and mate.

In 2011 spring queens were collected in England were kept in queen rearing boxes, unless they deposited *S. bombi* larvae in their faeces at the initial faeces checks when collected or at the 2nd faeces check approximately three weeks after collection, in which case they were kept in plastic boxes on sand. The sand from these boxes was also checked for nematode larvae and, if present, they were transferred to 'worm farms'.

To collect nematode larvae from worm farms, I poured sufficient water to cover the sand into the plastic box (worm farm) and to release it from the container (Kelly 2009, pers. comm. Joe Colgan). I poured the contents (sand and water) into a large conical flask (1.5l) and refilled the plastic box with water and repeated this process at least twice to remove any sand from the container. I half-filled the flask with running tap water and swirled the flask as it filled, to encourage the nematode larvae to separate from the sand. I then allowed the sand to settle in the base of the flask and poured the water through a 38um sieve, retaining the sand in the flask. The contents of the sieve were transferred to a clean plastic box by gently spraying water on the mesh and pouring the residue into the container. I half-filled the flask again and repeated the process at least twice. After the 3rd wash, I checked the liquid that had been poured off, under the dissecting microscope (x10-60) for nematode larvae. If none were seen I check for larvae in the 1st and 2nd wash, and transferred any larvae into a 50ml falcon tube. If larvae were seen in the third wash, the process was repeated until no nematode larvae were seen. The water containing the nematode larvae in the 50ml falcon tube was my nematode infested solution (NIS).

Preparation of NIS for experimental infections

To count the nematode larvae in the NIS, I set up a rack containing the 50ml falcon tubes of NIS, then swirled the first tube to get nematodes into suspension. I removed the lid, and using a Pasteur pipette, I transferred 2ml of the NIS to a nematode counting slide. As 2ml completely fills the surface of the slide and the 1ml rests on the counting grids, just the NIS below the counting grid is used for counting. The counting slide, contained the NIS, was placed under the dissecting microscope and the nematodes within the counting grids were counted 3 times, and an average was used to estimate the number of nematode larvae in the NIS. This was tested as the optimal number by calculating the average of three, six and nine counts without improving the variance of the average. The NIS from at least three infected queens was combined to create a homogenised NIS.

To prepare the dose for infecting the bumble bee queens, I calculated the concentration of nematode larvae in each tube, to produce a NIS mix of 200 nematodes per ml of solution (Kelly 2009). If necessary the NIS was concentrated, and the nematode larvae recounted.

Bee hibernation with NIS

To experimentally infect the bumble bee queens, they were hibernated in 50ml falcon tubes with three holes (that are large enough to allow air into the tube, but small enough to prevent the bee from crawling out), drilled in the lid with 5ml of damp sand infested with approximately 200 fertilised adult females. The tubes were filled to the 5ml marked with dry sand (that has been washed and autoclaved sand to remove contaminants). I added 1ml of water to the sand and 1ml of NIS (containing *ca.* 200 nematode larvae). This was sufficient moisture to dampen the sand, but not make it so wet that it was sticky. A queen was carefully placed in each tube and the lid screwed on.

The 50ml falcon tubes containing the bees were placed on Styrofoam racks with extra tubes filled with water, to ensure that the moisture levels are maintained (Figures 1 and 2). The rack is placed inside a black plastic bin liner, which is placed in a cardboard box. A cardboard lid is placed on the box and it is stored in a cold room at 4^{o}C for six weeks to simulate hibernation.

In 2010, 25 queens were hibernated, 13 with *S. bombi* parasites collected from English *Bombus* queens and 12 with *S. bombi* parasites collected from Irish *Bombus* queens. For the queens hibernated Windsor parasites, I used an estimated 2,690 nematode larvae in 26ml of NIS (i.e. approximately 200 nematode larvae per queen) and for the queens hibernated Dublin parasites, I used an estimated 2,487 nematode larvae in 24ml of NIS.

1	*B. hypnorum*	Water		3	*B. lucorum*
4	*B. lucorum*			7	*B. lucorum*
2	*B. terrestris*	Water		5	*B. terrestris*
		11	*B. terrestris*		
8	*B. terrestris*	Water		6	*B. terrestris*
12	*B. terrestris*	Water		9	*B. terrestris*
10	*B. terrestris*			13	*B. terrestris*

Figure 1: Windsor summer queens hibernated with *S. bombi* larvae Windsor

2	*B. lucorum*	Water	1	Swiss *B. terrestris*
5	*B. lucorum*		7	*B. lucorum*
4	*B. terrestris*	Water	3	*B. terrestris*
6	*B. terrestris*		8	*B. terrestris*
12	*B. terrestris*	Water	10	*B. terrestris*
9	*B. terrestris*		11	*B. terrestris*

Figure 2: A Swiss *B. terrestris* and Windsor summer queens hibernated with *S. bombi* larvae from Dublin

In 2011, a total of 75 *Bombus* queens were hibernated with nematode larvae: 33 *B. hypnorum* queens and 42 *B. terrestris* queens between 9th June and 30th September.

Table 1: Hibernation dates of *B. hypnorum* and *B. terrestris* queens experimentally infected with *S. bombi* larvae in 2011

Hibernation		Number of queens	
Date in	Date out	*B. hypnorum*	*B. terrestris*
9th July	21st July	3	0
17th July	29th July	4	0
24th July	5th Aug	2	0
1st July	12th Aug	1	0
11th July	22nd Aug	3	0
15th July	26th Aug	1	6
22nd July	2nd Sep	1	0
1st Aug	12th Sep	0	2
8th Aug	19th Sep	4	13
12th Aug	23rd Sep	12	13
19th Aug	30th Sep	2	12
	Total	33	42

Post-hibernation

The cardboard containing the hibernating bees was transferred from the cold room to bee-room. I removed the queens from 50ml tubes into individually labelled sandwich boxes supplied with sugar water and pollen. Queens were checked weekly for nematode larvae in their faeces for 6 weeks, and then sacrificed. All queens were dissected to check for both everted nematode uteri and nematode worms to ascertain parasite status.

Results

Infection success

My experimental infection success rates were low: only *ca.* 8% (2/25) in 2010 and ca. 8% (5/60) in 2011.

In 2010, only 2 of the 25 queens were successfully experimentally infected: 1 *B. lucorum* queen infected with parasites from Ireland contained 1 *S. bombi* uterus and 1 *B. hypnorum* queen infected with parasites from England contained 7 *S. bombi* uteri. None of the queens contained *S. bombi* nematode worms (except those attached to the everted uteri).

In 2011, 18 of the 25 *Bombus* queens died during hibernation. Only 73 of the 75 queens were dissected, and 13 of these were too decayed to record parasite status. Therefore parasite status from just 60 individuals was recorded. A total of 5 queens contained *S. bombi* uteri (2 *B. terrestris* queens and 3 *B. hypnorum* queens) and all of these contained just one everted nematode uterus. As in 2010, none of the queens contained *S. bombi* nematode worms (except those attached to the everted uteri).

Discussion

I achieved very limited success experimentally infecting hibernating queens with parasite larvae from queens collected in both England and Ireland. This may have been due to the dose of nematode larvae used (200 nematode larvae per queen) although Kelly (2009) found that this was sufficient to infect Irish queens with Irish parasites. Although the infection success was very low, this still suggests that the *S. bombi* harvesting and husbandry could work, but that both these, and the infection protocols, required enhancement.

From the initial dissections some of the Bombus queens that died during hibernation had (died and) decayed but none had dried out. This suggested that the hibernation conditions were suitably humid and that I used appropriate hibernation protocols.

The mortality rates of queens during hibernation in 2011 were high. This may have been due to the disturbance of the hibernating queens by the regular arrival and removal of queens and separate cardboard boxes in the cold room. It may also

have been due to the failure of the cold room during their hibernation, which may have been beyond my control, but it could have been identified more rapidly had I checked the temperature on a more regular basis.

In 2012, two experimentally infected *Bombus* queens (one *B. hypnorum* and one *B. terrestris*) were not dissected. I believed that these two samples were frozen, for dissection at a later stage then misplaced among my other samples in the freezer. This re-enforced the importance of labelling samples and the boxes they are stored in clearly, and ideally dissecting samples to obtain and record data as early as possible.

Overall, I succeeded in experimentally infecting one *B. lucorum* queen and one *B. hypnorum* queen in 2010 and two *B. terrestris* queens and three *B. hypnorum* queens in 2011. The infection intensity in the *B. hypnorum* queen was greater than in the *B. lucorum* queen in 2010 (seven and one nematode uteri per queen respectively). Furthermore, the number of *B. hypnorum* queens infected was greater than the number of *B. terrestris* queens infected in 2011 (three and two respectively). Although the sample size is very small, this suggests that the non-native *B. hypnorum* may be more susceptible to infection by S. bombi than native *Bombus* species. This may have consequences for the native Bombus populations in the UK, and for the parasite, due to the lack of competence of this non-native host seen in Chapter 4.