The impact of inbreeding and parasitism on bumblebees

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SUMMARY ABSTRACT

Many bumblebee species are suffering from the effects of habitat fragmentation and population isolation. In some cases, populations have lost genetic diversity due to genetic drift and it is possible they are now at heightened risk of extinction. Inbreeding may be particularly costly to bumblebees because, as Hymenoptera, their complementary sex determination system can lead to the production of sterile or inviable diploid males. However, little is known about the effect that diploid male production has on bumblebee colony fitness. Here, the consequences of brother-sister mating in the bumblebee *Bombus terrestris* are investigated, and the production of diploid males was found to exert considerable costs at the colony level by reducing productivity and survival. Diploid males may therefore act as indicators of the genetic health of populations, and their detection could be used as an informative tool in hymenopteran conservation. Due to the costs associated with inbreeding, selection may have favoured the evolution of kin recognition systems in bumblebees. Data are presented that suggest that *B. terrestris* can discriminate between kin and non-kin as gynes were less willing to mate with siblings compared to non-relatives.

Theory predicts that inbreeding may impose further costs on bumblebees through increased levels of parasitism, but empirical data are scarce. The relationship between population genetic diversity and parasite prevalence is assessed using Hebridean island populations of *Bombus muscorum* and *Bombus jonellus*. In the more outbred *B. jonellus*, there was no relationship between parasite prevalence and population heterozygosity. But prevalence of the gut parasite *Crithidia bombi* and the tracheal mite *Locustacarus buchneri* were found to be higher in populations of *B. muscorum* that had lower genetic

diversity. In addition to assessing infection status, the activity of the immune system was assessed in each individual bee. However, there was no relationship between population heterozygosity and these immune parameters. This suggests that, in some Hymenopteran species, as populations lose genetic diversity the impact of parasitism will increase, potentially pushing threatened populations closer to extinction. Therefore, preventing population fragmentation by the creation of suitable habitats and by ensuring connectivity between habitat patches are important aspects of hymenopteran conservation.

Finally, this thesis investigates the potential threat of pathogen spread from commercially reared bumblebees used for crop pollination to wild bumblebees. Although no direct evidence for parasite spillover is found, the prevalence of *C. bombi* was significantly higher in *B. terrestris* by the end of the season on farms that used commercial bumblebees compared to farms that did not. This high prevalence does suggest that pathogen spillover is a potential threat and it would be preferable to reduce the usage of commercial bumblebees where possible. For example, sowing wild flower mixes could boost natural pollinator populations, which in turn would benefit soft fruit pollination. Overall, this thesis contributes to our knowledge of the consequences of inbreeding in bumblebees and the relationship between genetic diversity and parasite prevalence. It provides a greater understanding of the factors that might be pushing threatened pollinators towards extinction and as a whole provides important information that may inform conservation practitioners, whose aim is to protect the future of our hymenopteran pollinators.

DECLARATION OF AUTHORSHIP

I, Penelope Ruth Whitehorn, declare that this thesis has been composed by myself and that it embodies the results of my own research. Where appropriate, I have acknowledged the nature and extent of work carried out in collaboration with others.

Signed …….

Date ……

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1.1 The global loss of biodiversity

1.1.1 Anthropogenic causes of species declines

Human intervention in natural ecosystems seriously threatens global biodiversity. Indeed, many ecologists believe that a global mass extinction may be occurring due to the rapid rate at which species are being lost (Diamond, 1989; McKinney & Lockwood, 1999). The destruction and degradation of natural habitats is the most important cause of such anthropogenic biodiversity decline (Tilman *et al.*, 1994). The loss of habitat also inevitably leads to its fragmentation, which causes formerly widespread species to become restricted to small patches where populations often become small and isolated and extinction risks are heightened (Fahrig, 2003). Human-mediated introduction of non-native species is also responsible for a large proportion of species declines. Such invasive alien species can detrimentally effect native species through introducing disease, competition, genetic hybridisation and habitat modification (Mack *et al.*, 2000) and are thought to be a leading cause of extinctions (Clavero & Garcia-Berthou, 2005).

1.1.2 The decline of bumblebees

Many bumblebee species have been subjected to habitat loss and invasive species, and have been suffering significant range contractions throughout the Northern Hemisphere over the last few decades (Kosior *et al*., 2007; Williams & Osbourne, 2009). These declines were initially documented in the UK due to the comprehensive historical information on the distribution and abundances of the native bumblebee species (Alford, 1980; Williams, 1982). It is now recognised that 3 of the 25 native species have

become extinct, 10 species have undergone severe range contractions and 7 species have been placed on the UK Biodiversity Action Plan (Goulson, 2010a). It has been more recently recognised that similar trends are occurring elsewhere in Europe. Fitzpatrick *et al.* (2007) compared distribution maps of Irish bumblebees pre- and post-1980 and found that similar species are declining in Ireland and Britain. Additionally, late-emerging species have suffered the most significant declines. A further study by Kosior *et al.* (2007) assessed the distribution and status of bumblebees in mainland Europe and found that approximately 30% of species were threatened throughout their range. The equivalent baseline data is not available in North America but there is emerging evidence that some bumblebee species have been suffering from dramatic declines in recent decades (Cameron *et al.*, 2011).

The primary cause of these losses is the intensification of agriculture, which has coincided with the period of most significant bumblebee declines in the latter half of the 20th century (Goulson *et al.*, 2008; Williams & Osborne, 2009). Such intensification has resulted in a loss of flower rich grasslands and other natural habitat, which has led to a decline in floral diversity. This leads to a loss of bumblebees as they are dependent on flowers for nectar and pollen, which almost exclusively comprise their diet. Indeed, a direct correlation has been found between the number of wild bee species and the floral diversity of an area (Hines & Hendrix, 2005). The intensification of agriculture has also lead to a loss of potential nesting sites for bumblebees, for example in hedgerows and unimproved grassland, and this is thought to have contributed to their decline (Goulson *et al.*, 2008).

Although it is recognised that agricultural intensification is partly responsible for bumblebee losses in North America (Grixti *et al.*, 2009), it is also thought that the spread of disease from commercial bumblebees is having a detrimental impact on bumblebee populations (Cameron *et al*., 2011). Studies have found higher prevalence of the bumblebee pathogens *Crithidia bombi* and *Nosema bombi* at sites near to glasshouses where commercial bumblebees are deployed and suggest this is evidence for pathogen spillover into the wild bumblebee population (Colla *et al.*, 2006; Otterstatter & Thomson, 2008). In the early 1990's bumblebee queens from North America were shipped to European rearing facilities, where it is thought they may have become infected with disease. The colonies were then shipped back to North America and released. Disease is thought by some to be responsible for the recent catastrophic declines in at least five native bumblebee species in North America since this time (Thorp & Shepherd, 2005; Winter *et al.*, 2006). However, it should be noted that there is no direct evidence for this at present (Brown, 2011).

1.2 Inbreeding and inbreeding depression

1.2.1 Overview

Genetic diversity is vital in maintaining the fitness of populations and is an important consideration in the conservation of species that have been undergoing population declines. Such diversity is required to withstand short-term environmental perturbations and is crucial in allowing populations to evolve and adapt to long-term environmental change (Frankham *et al.*, 2010). Large populations of naturally outbreeding species usually have extensive genetic diversity but small isolated populations are at risk from

losing their diversity. In smaller populations, random genetic drift can result in a steady, inexorable loss of genetic diversity, a process that may be greatly exacerbated during population bottlenecks. This process promotes homozygosity of certain alleles and causes rare alleles to be lost (Frankham *et al.*, 2010).

The loss of genetic diversity in small populations can also occur in the short term as a result of inbreeding (Keller & Waller, 2002). The term inbreeding is used in a number of different contexts but it invariably refers to situations where matings occur between relatives. Inbreeding due to such non-random mating refers to the degree of relatedness between mates, relative to two mates chosen at random from the population. Population subdivision can also cause non-random mating, which then leads to inbreeding (Keller & Waller, 2002). The consequence of all types of inbreeding is a loss of genetic diversity because it increases the frequency of individuals that are homozygous for alleles that are identical by descent (Keller & Waller, 2002). If this loss of genetic diversity leads to a loss of reproductive fitness it is termed inbreeding depression, which is predominantly caused by an increase in the frequency of homozygotes for deleterious recessives (Charlesworth & Charlesworth, 1999). It should be noted that if a species has always had a small effective population size, it may regularly inbreed and if deleterious recessives are frequently purged from populations, inbreeding depression may not occur. However, if a rare species was previously more common it is more likely that inbreeding depression will occur.

Inbreeding depression in diploid organisms significantly increases the risk of extinction (Frankham, 2005). This has been clearly demonstrated with stochastic computer models in a study by O'Grady *et al.* (2006). A meta-analysis of the literature was used to

estimate the impact of inbreeding depression on the fitness of species across a broad taxonomic range and this estimate was used to model its effect on extinction risk. It was found that inbreeding depression significantly reduced the time to extinction in all of the 30 species modelled. Further evidence of this relationship has been found under natural conditions. For example, small isolated populations of the Glanville fritillary butterfly *Melitaea cinxia* were found to have reduced heterozygosity and a resultant increase in extinction risk via effects on larval survival, adult longevity and egg-hatching rates (Saccheri *et al.*, 1998). Additionally, genetic factors have been found to be related to population dynamics and hence extinction risk in two species of wolf spider in the Genus *Rabidosa*. Smaller populations with lower genetic diversity were found to have reduced population growth rates, particularly under stressful environmental conditions and this increased their probability of extinction (Reed *et al.*, 2007).

1.2.2 Inbreeding in haplodiploids

Haplodiploid organisms have often been assumed to suffer less inbreeding depression as recessive deleterious and lethal mutations were thought to be purged through the haploid males (Werren, 1993). But some authors have challenged this assumption, primarily because purging will not be effective against female sex-limited traits, such as hibernation survival and fecundity (Henter, 2003). Indeed, a meta-analysis by this author concluded that when inbreeding is experimentally imposed on haplodiploid populations, substantial inbreeding depression does occur. For example, the haplodiploid wasp *Uscana semifumipennis* demonstrated significant inbreeding depression, with longevity and fecundity reduced 38% and 32% respectively (Henter, 2003).

Haplodiploids may suffer further genetic costs of inbreeding due to their single-locus complementary sex determination (sl-CSD) system, which is ancestral to the haplodiploid Hymenoptera. Under this system, individuals heterozygous at the polyallelic sex-determining locus develop into diploid females and hemizygotes develop into haploid males. When a diploid individual is homozygous at the sex locus a diploid male is produced. This rarely occurs in large outbreeding populations because many CSD alleles can be maintained by negative frequency-dependent selection. However, genetic drift in small populations increases diploid male production (DMP) by reducing CSD allelic richness (Cook & Crozier, 1995). Inbreeding also increases DMP as there is a higher probability that a matched mating will occur, where a female mates with a haploid male that carries a sex allele identical to one of her own and produces a colony where on average 50% of the offspring are diploid males (Duchateau *et al.*, 1994).

Diploid males represent significant fitness costs, primarily through their inviability or sterility. For example, in the parasitoid wasp *Brecon hebetor*, very few diploid males mature beyond the embryo stage (Petters & Mettus, 1980). In a few species, such as the sawfly *Athalia rosae ruficornis* and the wasp *Diadromus pulchellus*, diploid males can produce diploid sperm and mate, but this results in sterile or inviable triploid progeny so the costs are merely deferred by a generation (Naito & Suzuki, 1991; Elagoze *et al.*, 1994). It should be noted that in some hymenopteran species diploid males are viable, for example in the parasitoid wasp *Cotesia glomerata*, diploid males have been found to successfully reproduce (Elias *et al.*, 2009). It is thought that diploid male fertility has been selected for over time in this species as the occurrence of inbreeding is relatively frequent (Elias *et al.*, 2010). In social insects, however, diploid males do exert

substantial fitness costs as they effectively replace 50% of the female workforce and do not contribute to colony productivity and this can be viewed as 50% worker mortality (Duchateau *et al.*, 1994; Packer & Owen, 2001). In honey bees and ants this cost is reduced as the larvae are consumed by the workers, but in bumblebees they are reared to adulthood (Duchateau *et al.*, 1994). The production of diploid males has been shown to slow the rate of colony growth in *Bombus atratus* under laboratory conditions (Plowright & Pallett, 1979) and result in higher mortality of founding queens in the fire ant *Solenopsis invicta* (Ross & Fletcher, 1986). Modelling has demonstrated that DMP can initiate a rapid extinction vortex and suggests that haplodiploids are more prone to extinction due to genetic reasons than previously supposed (Zayed & Packer, 2005).

1.2.3 Inbreeding in bumblebees

The study of genetic diversity and inbreeding in bumblebees is particularly relevant because of the population declines and range contractions they have been experiencing. Due to the loss of habitat, populations of the rare species have become fragmented and genetically isolated and are therefore susceptible to inbreeding depression, which has serious implications for their persistence (Darvill *et al.*, 2006; Ellis *et al.*, 2006; Takahashi *et al.*, 2008).

The negative genetic consequences of population fragmentation and isolation are exacerbated in bumblebees as there are a number of factors that predispose them to inbreeding and a low level of heterozygosity. Firstly, as haplodiploids, in any one generation there are only 75% as many gene copies compared to diplodiploid organisms. The effective population size of a haplodiploid is, therefore, smaller than for

an equivalent diplodiploid (Packer & Owen, 2001). Secondly, the effective population size of bumblebees is further reduced by their social nature as it is determined by the number of successful nests in an area and not by the number of sterile workers, which are considerably more abundant (Goulson, 2010a). Finally, the majority of bumblebee species are monoandrous (Estoup *et al.*, 1995; Schmid-Hempel & Schmid-Hempel, 2000). This increases their susceptibility to inbreeding compared to polyandrous species, which effectively have more breeding individuals per generation (Page & Metcalf, 1982). Additionally, polyandrous species such as the field cricket *Gryllus bimaculatus* are able to avoid the costs of genetic incompatibility through postcopulatory selective fertilisation (Tregenza & Wedell, 2002).

Only a small number of studies have directly investigated inbreeding in bumblebees and these focus on the common and widespread species, *Bombus terrestris*. Duchateau *et al*. (1994) found that the growth rate of inbred colonies producing diploid males was only slightly affected. Gerloff *et al.* (2003) mimicked inbreeding through one generation of brother-sister mating in *B. terrestris* and studied its effects on two measures of fitness; immune defence and body size. Contrary to expectation, inbreeding did not significantly affect immune response or body size in either workers or haploid males and the variation in these response variables was largely explained by the maternal family and the colony of origin. Gerloff & Schmid-Hempel (2005) then extended this study to investigate the effects of inbreeding on hibernation survival, colony foundation success, colony size and the quantity and quality of reproductive output in the next generation. Colony size was negatively affected by inbreeding but variation in the other life history traits were again predominantly explained by maternal genotype. In contrast, an earlier study by Beekman *et al.* (1999) did find some evidence for inbreeding depression in *B.*

terrestris: inbreeding had a slight negative impact on the fecundity of the queens and on the size of colonies.

The apparent lack of severe inbreeding depression in *B. terrestris* partly explains why this species can be invasive and has been extremely successful in colonizing new areas from small founder populations. For example, *B. terrestris* is spreading rapidly across Tasmania after its introduction in the early 1990's, despite a severe genetic bottleneck and ensuing low genetic diversity (Schmid-Hempel *et al.*, 2007). However, this success is likely to have been aided by a favourable climate, lack of inter-specific competition and few parasites (Gerloff & Schmid-Hempel, 2005).

Rather than directly study inbreeding, a number of studies have investigated the population and genetic structure of various bumblebee species. The findings show that more common species, for example *B. terrestris* and *Bombus pascuorum*, exhibit little spatial genetic differentiation between populations (Estoup *et al.*, 1996; Widmer *et al.*, 1998; Pirounakis *et al.*, 1998; Widmer & Schmid-Hempel, 1999). However, when rare and common species are compared, the rare species with fragmented populations, such as *Bombus sylvarum* and *Bombus humilis*, have a much lower genetic diversity than common, widespread species such as *B. terrestris* and *B. pascuorum* (Ellis *et al.*, 2006). Similar results have been found in North America, where populations of declining species of bumblebees have lower levels of genetic diversity than co-occurring populations of species that are not suffering from declines (Cameron *et al.*, 2011). Additionally, extremely low effective population sizes have been found in two species of threatened bumblebees in the UK: in *B. sylvarum* effective population size ranged

from 21 to 72 (Ellis *et al.*, 2006) and in *Bombus distinguendus* the size ranged from only seven to 42 (Charman *et al.*, 2010).

Darvill *et al*. (2006) investigated inbreeding and population structure in *B. muscorum*, yet another rare and declining species now predominantly found in the Western Isles of Scotland. Isolated island populations were found to be genetically differentiated to those closer to the mainland and had substantially reduced genetic diversity. In addition, genetic diversity was lower than in the closely related but more common species, *B. pascuorum*. Subsequent work has shown that *B. muscorum* shows markedly higher population structuring and isolation by distance than the coexisting *Bombus jonellus* ($\theta = 0.13$ compared to $\theta = 0.034$). This indicates that *B. muscorum* has a lower dispersal ability (estimates of the maximum dispersal range being only 8 km, compared to 50 km for *B. jonellus*) and hence is more susceptible to population isolation due to habitat fragmentation (Darvill *et al.*, 2010). Diploid males have also been recorded in rare bumblebee species (Darvill *et al.*, 2006; Takahashi *et al.*, 2008), demonstrating that the loss of genetic variation has an associated fitness cost. It seems probable that inbreeding and loss of genetic diversity in isolated bumblebee populations reduces their fitness but it remains to be established if this is driving them to extinction.

1.2.4 Inbreeding and parasite susceptibility

One mechanism by which genetically impoverished populations may become extinct is through parasitism. Inbreeding and increased homozygosity can increase either the prevalence of parasites at the population level or susceptibility to parasites at the individual level. At the population level, a loss of genetic diversity due to inbreeding

reduces the capacity of the population to respond to novel virulent pathogen genotypes. The more genetically diverse a population is, the more likely it is that some individuals can resist a pathogen and this limits epidemic spread and facilitates evolution. In large populations, selection maintains this diversity but in small populations, alleles will be lost by genetic drift, and this will increase the probability that a pathogen that can kill one individual can kill many or all individuals (Frankham *et al.*, 2010). Studies in vertebrates have supported this, for example the genetic diversity of populations of the frog *Rana latastei* is negatively correlated with susceptibility to an emergent pathogen (Pearman & Garner, 2005). Similar relationships have been found in other taxa for example the endangered fish *Poeciliopsis o. occidentalis* (Hedrick *et al.*, 2001) and the rodent *Peromyscus maniculatus* (Meagher, 1999).

At the individual level, correlations between heterozygosity and variations in fitness related traits, for example parasite susceptibility, are collectively known as heterozygosity-fitness correlations (HFC). HFCs have been reported where loss of heterozygosity in individuals leads to higher rates of infection and disease, for example in sheep, (Coltman *et al.*, 1999), sparrows (MacDougall-Shackleton *et al.*, 2005), sea lions (Acevedo-Whitehouse *et al.*, 2006) and cooperative crows (Townsend *et al.*, 2009). However, this is certainly not a universal trait as a number of studies have recently emerged that show no such relationship (Pujolar *et al.*, 2009; Cote *et al.*, 2005). Indeed, Chapman *et al.* (2009) has used multivariate techniques to conduct a powerful meta-analysis of HFC studies and concluded that there was only weak evidence for heterozygosity-fitness correlations across many traits. Additionally, there is ongoing debate about the extent to which the measures of heterozygosity used in HFCs

(predominantly microsatellites) actually reflect the true inbreeding co-efficient of individuals (for example, David, 1998; Slate *et al.*, 2004; Ljungqvist *et al.*, 2010).

The majority of studies incorporated in the meta-analysis by Chapman *et al.* (2009) were vertebrates and the studies that have addressed the effects of inbreeding on immunity and/or parasitism in invertebrates have demonstrated that the relationship is complex. For example, Stevens *et al.* (1997) found that the effect of inbreeding on the susceptibility of the flour beetle *Tribolium castaneum* to parasitic nematodes was not consistent and depended on host lineage. The effect of inbreeding can also depend on host sex, for example inbred females of the autumnal moth *Epirrita autumnata* have a significantly reduced immune response, but there is no such effect in males (Rantala & Roff, 2007). It has also been shown that the effect of inbreeding on infection can depend on the parasite species, with inbred *Daphnia magna* hosts becoming more susceptible to one parasite species but not to another (Haag *et al.*, 2003). A recent study by Drayton & Jennions (2011) found that inbreeding in the cricket *Teleogryllus commodus* had no negative affect on immunity as measured by lysozyme-like activity and hemocyte counts. The effect of inbreeding on infection can also depend on the genetic diversity in the parasite population. Experiments have shown that genetically diverse populations of *Daphnia magna* only had lower infection rates compared to homogenous populations when exposed to a number of different parasite strains. No such effect of host heterogeneity was found in host populations exposed to single parasite strains (Ganz & Ebert, 2010).

There is, however, evidence to suggest that inbreeding can decrease the immunity of invertebrates at the individual level. Spielman *et al.* (2004) found that inbred

populations of *Drosophila melanogaster* had a significantly reduced resistance to the insecticidal toxin, thuringiensin, and live *Serratia marcescens* bacteria and this was shown to result from the loss of specific resistance alleles. A further laboratory study with *Drosophila* has also found evidence for inbreeding depression, which then led to increased susceptibility to parasites. Experimentally inbred lines of *Drosophila nigrospiracula* were found to have a reduced capacity to sustain defensive behaviours against the ectoparasitic mite *Macrocheles subbadius* (Luong *et al.*, 2007). Similarly, inbred termites suffer from increased disease susceptibility, which is due to a decrease in the efficacy of group level disease resistance rather than a loss of individual immunity per se (Calleri *et al.*, 2006). Fewer studies have investigated the relationship between heterozygosity and parasite prevalence at a population level but two studies have found no correlation in subpopulations of snails and earthworms (Trouve *et al.*, 2003; Field *et al.*, 2007). However, another study on a freshwater snail did find a negative correlation between population heterozygosity and probability of infection (Puurtinen *et al.*, 2004). Similarly, in *D. magna*, the transmission of a virulent parasite was found to be higher in inbred host populations (Ebert *et al.*, 2007).

In bumblebees, circumstantial evidence does exist supporting the hypothesis that inbred populations are more susceptible to parasitic infection. Firstly, the invasive *Bombus terrestris* in Tasmania is highly inbred due to small numbers of founding queens and individuals have been found to support very high loads of ectoparasitic mites (Schmid-Hempel *et al.*, 2007; Allen *et al.*, 2007). Secondly, an interdisciplinary study has looked at the population genetics and levels of pathogen infection in bumblebee populations across North America. The populations that were found to be declining had lower levels

of genetic diversity and significantly higher prevalence of the pathogen *Nosema bombi* compared to the stable bumblebee populations (Cameron *et al.*, 2011).

1.2.5 The parasite hypothesis

Despite the lack of consistent experimental evidence about the effect that inbreeding in bumblebees may have on immunity, there is reason to believe that parasite load will be greater in inbred populations that have a lower heterozygosity. This is due to the parasite hypothesis, which states that genetically diverse colonies of social insects have a selective advantage as they are more resistant to parasitism (Sherman *et al.*, 1988; van Baalen & Beekman, 2006). Fundamental to this hypothesis is the assumption that different host genotypes have a varying susceptibility to different parasite strains, which would mean that a parasitic infection is not likely to spread as rapidly and as far through a genetically heterogeneous colony (Sherman *et al.*, 1988; Schmid-Hempel, 1998). Experimental support for the hypothesis has been provided by a number of studies using bumblebees and their parasites as a model system and these are summarised below.

Shykoff & Schmid-Hempel (1991a) used the host-parasite system of *B. terrestris* and the intestinal trypanosome *C. bombi* to show that within species variation in susceptibility to the parasite does exist and infections spread more slowly between unrelated workers than among related workers. Strong effects of colony genotype on the probability of infection and transmission of *C. bombi* were also reported by Schmid-Hempel & Schmid-Hempel (1993). Further evidence for a genetic component in the patterns of infection has been provided by Wilfert *et al.* (2007) through studying quantitative trait loci (QTL) related to *C. bombi* infection in *B. terrestris*. Investigations

under field conditions have provided additional support for the parasite hypothesis. Liersch & Schmid-Hempel (1998) created genetically homogeneous and heterogeneous colonies of *B. terrestris* and placed them in the field where they were naturally exposed to parasitism. It was found that the genetically heterogeneous colonies had significantly lower prevalence, load and richness of a range of parasites, including protozoa, nematodes, mites and parasitoids. This work was taken further by Baer & Schmid-Hempel (2001) who artificially inseminated queens with sperm from one to four males, to represent different levels of polyandry. They found that the intensity and prevalence of *C. bombi* decreased with increasing levels of colony heterogeneity resulting from multiple inseminations.

The evidence outlined above does suggest that heterogeneous colonies of bumblebees suffer less damage from parasitic infections than homogeneous colonies but modelling has shown that this advantage may be smaller than expected. van Baalen and Beekman (2006) modelled the effect of colony heterogeneity on the fitness cost inflicted by parasites and diseases and their results supported the parasite hypothesis to an extent. However, in heterogeneous colonies more genotypes are present that are susceptible to different parasites and so they may actually suffer an increased frequency of infection at the colony level, despite there being less per-infection damage.

The parasite hypothesis is used to contribute to the explanation for the evolution of multiple mating in eusocial insects (Sherman *et al.*, 1988). Because polyandry reduces the average relatedness between colony members it was seen as a paradox for the original kin-selection explanations for the evolution of eusociality in hymenopterans (Hamilton, 1964). It is now understood that multiple mating can have selective

advantages by increasing the genetic heterogeneity within a colony, as seen in the case of parasites. However, bumblebees are predominantly monogamous (Estoup *et al.*, 1995) so theories concerning multiple mating are not generally applicable. But the parasite hypothesis provides a useful framework against which to predict what effect bumblebee genetic diversity might have on parasite susceptibility.

1.2.6 Inbreeding avoidance

Due to the potential fitness costs associated with inbreeding, one might predict that selection would have favoured the evolution of kin recognition and inbreeding avoidance behaviours in bumblebees. The mating behaviour of bumblebees has been well studied in the laboratory (for example, Sauter & Brown, 2001) but little is known about inbreeding avoidance behaviours. One study found that queens of *Bombus frigidus* and possibly *Bombus bifarius* preferentially mated with unrelated males when given a choice (Foster, 1992). Males of these two species exhibit the pre-mating behaviour known as 'patrolling', where males mark objects with a pheromone and visit them sequentially to encounter potential mates attracted by the scent (Alford, 1975; Williams & Zervos, 1991). In the natural situation it is unlikely that reproductives will encounter both siblings and non-siblings at the same time. Therefore, choice experiments such as Foster's (1992) perhaps do not represent the natural situation and clearly more research is need into the potential inbreeding avoidance behaviour of bumblebees.

1.3 Bumblebee parasites and immunity

1.3.1 The parasite of bumblebees

Bumblebees have long been known to harbour a great number and diversity of parasites (Alford, 1975; Schmid-Hempel, 1998) (see table 1.1). For the majority of the parasite species listed in Table 1, little is known about their biology, epidemiology or how the host-parasite interaction impacts bumblebee population biology. However, the most common parasites have been quite intensively studied and the following paragraphs introduce the parasite species that are encountered in the subsequent chapters.

Table 1.1 A summary of the parasites reported for bumblebees (taken & adapted from Schmid-Hempel, 2001).

Group	**Parasite**	**Remark**
Virus	Acute Bee Paralysis	Uncertain status in nature
	Entomopox virus	
Bacteria	*Spiroplasma*	In haemolymph
	Aerobacter cloaca and	
	other unidentified bacteria	
Fungi	*Acrostalagmus* sp.	Short hibernation in diseased queens.
	Beauveria bassiana	
	Candida sp.	
	Hirsutella sp.	
	Paecilomyces farinosus	Pathogenic
	Verticilium lecanii	
Protozoa	*Nosema bombi*	See section 1.3.1.2
	Crithidia bombi	See section 1.3.1.1
	Apicystis bombi	See section 1.3.1.3
	Neogregarina sp.	
Nematodes	*Sphaerularia bombi*	Infects overwintering queens, causing a behavioural change; queens do not found nests but seek further hibernating sites.
Hymenopteran	*Syntretus splendidus*	Probably exclusively attacks spring queens

parasitoids	*Melittobia acasta; M. chalybii*	
	Monodontomerus montivagus	
	Pediobius williamsoni	
Dipteran parasitoids	*Apocephalus borealis*	Feeds on thoracic muscles
	Boettcharia litorosa	
	Helicobia morionella	
	Brachioma devia; B. sarcophagina; B. setosa	Can be extremely destructive
	Conops algirus; C. argentifacies; C. elegans; C. flavipes; C. quadrifasciatus; C. vesicularis	Investigated particularly in *B. terrestris*
	Melaloncha sp.	
	Physocephala brugessi; P. dimidiatipennis; P. dorsalis; P. nigra; P. obscura; P. rufipes; P. sagittaria; P. tibialis; P. vittata	Pupa can be hyper-parasitised by pteromalid wasps
	Senotainia tricuspis	
	Sicus ferrugineus	Investigated particularly in *B. terrestris*
	Zodion sp.	
Lepidoptera	*Ephestia kühniella*	Feeds on colony provisions
Acari (mites)	A large number of species	Unclear status as parasites.
	Locustacarus buchneri	See section 1.3.1.4

1.3.1.1 Crithidia bombi

The intestinal parasite *C. bombi* (Trypanosomatidae, Zoomastigophorea, (Lipa & Triggiani, 1988)) is a single-celled flagellate. Recent molecular work has discovered that this species has two very distinct lineages and so it is now classified as two separate species: *C. bombi* & *Crithidia expoeki* (Schmid-Hempel & Tognazzo, 2010). But due to the recent nature of this discovery, this thesis only refers to *C. bombi*. The cells of the parasite attach to the walls of the mid- and hindgut in infected bumblebees and multiply rapidly. New parasite cells are then released from two to five days after the initial infection and pass out in the faeces, increasing in numbers for 8-13 days, after which the faecal pathogen load levels out but continues to fluctuate (Schmid-Hempel & Schmid-Hempel, 1993; Logan *et al.*, 2005). Horizontal transmission of the parasite occurs between workers within a colony by the ingestion of infective cells on nest materials.

Horizontal transmission between colonies occurs when foraging workers from uninfected colonies ingest cells left on flowers by workers from infected colonies (Durrer & Schmid-Hempel, 1994). A study by Imhoof & Schmid-Hempel (1999) showed that the rate of horizontal transmission by *C. bombi* is notably high, with all workers from lab-reared colonies of *B. terrestris* showing signs of infection after only 10 days of exposure in the field. In addition, the rate of horizontal transmission increases as the season progresses, due to the larger number of foraging workers. The vertical transmission of the parasite to the next generation depends on the parasite infecting daughter queens that survive to establish their own nests the following spring (Ulrich *et al.*, 2011).

The prevalence of *C. bombi* among bumblebees is typically high but varies among host species, locality and the time of year, usually falling within the range of 10% to 35% of bees infected (Shykoff & Schmid-Hempel, 1991b; Korner & Schmid-Hempel, 2005). Colonies infected with *C. bombi* are able to survive and reproduce, suggesting that the virulence of the parasite is low (Imhoof & Schmid-Hempel, 1999). However, *C. bombi* is pathogenic and can cause a slower colony development early in the season and a reduction in ovary size in queens as well as workers (Shykoff & Schmid-Hempel, 1991c). Infections may also affect the build-up of the queen's fat body for hibernation (Schmid-Hempel, 2001). In addition, the parasite can result in higher worker mortality under adverse environmental conditions. For example, starvation causes the mortality rate from the parasite to increase by more than 50% (Brown *et al.*, 2000). As such starvation can occur in natural situations, for example when rain or cold weather interrupts foraging, it is possible that *C. bombi* has significant adverse effects on the growth and survival of bumblebee colonies in the wild (Brown *et al.*, 2000). The

virulence of *C. bombi* has also shown to be context-dependent and can cause substantial loss of fitness for *B. terrestris* queens under stressful hibernation and colony founding (Brown *et al.* 2003b).

The nutritional status of the bumblebee host also affects the population dynamics of the parasite itself, as was demonstrated in a study by Logan *et al.*, (2005). It was found that pollen-starved bees maintained a significantly lower *C. bombi* population that developed later after infection. This has implications for the horizontal transmission of the parasite between colonies as there would be a decreased parasite population in the faeces of pollen starved bees, which would decrease inter-colony transmission. This could in turn decrease the overall parasite population and so *C. bombi* may be less prevalent in food stressed host populations than would be expected when only considering host susceptibility.

Infection by *C. bombi* also impairs cognitive processes and diminishes a bumblebees' ability to utilize floral information and make economic foraging decisions (Gegear *et al.*, 2006). Although this subtle behavioural change may not significantly reduce the fitness of the individual bee, the cost to the colony may be much more severe as the reproductive success of a colony is directly related to the foraging success of workers (Schmid-Hempel & Schmid-Hempel, 1998). *C. bombi* has been found to have further effects on the foraging behaviour of bumblebees with the number of flowers visited per minute and the flower handling time varying with infection intensity. Bees with more intense infections (i.e. more infectious bees) visited fewer flowers per minute and this may subsequently influence the probability of transmission but further work is needed to fully understand this complex system (Otterstatter & Thomson, 2006).

The interaction between *C. bombi* and its host (*B. terrestris* in all experiments) is strongly influenced by the genotypes of both. Transmission experiments have shown there is genotypic variation in the expression of host susceptibility and parasite infectivity (Schmid-Hempel & Schmid-Hempel, 1993; Schmid-Hempel *et al.*, 1999). It has also been shown that there is extremely high diversity of parasite genotypes, which again indicates that there are strong genotypic host-parasite interactions in this species (Schmid-Hempel & Funk, 2004). Such genotypic interactions suggest that *C. bombi* exerts considerable selection pressure on its host and this is supported by studies that have demonstrated the high virulence of this parasite, even if this virulence is condition or context dependent (Brown *et al.*, 2000; Brown *et al.*, 2003b).

1.3.1.2 Nosema bombi

Nosema bombi (Microsporidia, Nosematidae, Fantham & Porter, 1914) is another single-celled intestinal parasite and the only microsporidian known to infect bumblebees. The spores germinate in the gut lumen of infected bumblebees and primarily invade the mid-gut cells and the malpighian tubules but infections have also been found in fat tissue, nerve tissue, tracheae and reproductive organs (Larsson, 2007). After replication, the parasite releases mature spores back into the gut lumen, which begin to be passed out in the faeces from five days to as much as 21 days after the initial infection (McIvor & Malone, 1995). *N. bombi* is particularly infective of larvae and young bees (Rutrecht *et al.*, 2007), although it can also infect mature adults (Schmid-Hempel & Loosli, 1998). A higher infectivity of young bees favours the successful transmission of *N. bombi* because it can take up to 21 days before infective spores are passed out in the faeces. As the life expectancy of a bumblebee worker in the field is

roughly 20-30 days (Rodd *et al.*, 1980), young bees are much more likely to survive to the time post-infection when spores can be transmitted.

The transmission dynamics of *N. bombi* are less well understood than those of *C. bombi*. One study found a positive relationship between spore dose and infection success and that a transmittable infection can only be established if a bee ingests a minimum dose of 100,000 spores (Rutrecht *et al.*, 2007). This suggests that the nest is the major arena for infection, as spores will accumulate due to the concentration of bees and their faeces (particularly as infected colonies are dirtier, possibly due to the diarrhoea caused by the parasite and/or decreased cleaning behaviour of the workers) and these spores will be protected from destructive UV rays. This is further supported by the fact that *N. bombi* preferentially infects larva and young bees, both of which remain within the nest (Rutrecht & Brown, 2008). However, horizontal transmission between colonies must also occur as this one species of parasite infects many phylogenetically distant bumblebee species (Tay *et al.*, 2005). If no horizontal transmission takes place, genetic differentiation would have occurred in the parasite as it has done in its host, resulting in greater genetic diversity between *N. bombi* lineages.

The effect of *N. bombi* on its host appears to be variable, demonstrated by the contrasting results of different studies. A number of studies have found infection by *N. bombi* to be unrelated to the number of reproductives produced or the size of the colony, which suggests the parasite has few detrimental fitness effects (Fisher & Pomeroy, 1989; Imhoof & Schmid-Hempel, 1999; Whittington & Winston, 2003). However, these studies were simply correlative and did not experimentally infect bumblebees to investigate causal relationships. To further understand this parasite, Otti & Schmid-

Hempel (2007) investigated the effects of *N. bombi* in *B. terrestris* under standardised laboratory conditions. They found that the mortality rate of infected workers was five times higher than that of uninfected workers. Additionally, infected males had a lower survival and significantly less sperm and some gynes had extended abdomens, crippled wings and were unwilling to mate. Such severe infection effects in the sexuals are likely to either substantially reduce or completely eliminate their reproductive success. This high virulence may result from the unlimited food resources in the laboratory enabling the bees to support more intense infections than they would in the field. A field experiment was then conducted to investigate the effect of *N. bombi* under natural conditions (Otti & Schmid-Hempel, 2008). Infected queens produced significantly smaller colonies than uninfected queens and they also produced no sexual offspring, whereas a number of the uninfected colonies produced males. If *N. bombi* had similarly high virulence in all of its bumblebee host species, its transmission to further generations would be impeded. Rutrecht & Brown (2009) investigated this apparent paradox by conducting controlled laboratory infections in *Bombus lucorum*, a bumblebee species that occurs sympatrically with *B. terrestris*. Although *B. lucorum* was negatively affected by infection with *N. bombi*, the virulence did not appear to be as high as in *B. terrestris*, as colonies were still able to produce reproductives that were capable of mating, thus enabling the successful vertical transmission of this parasite.

The prevalence of *N. bombi* varies spatially, temporally and across species. This has been demonstrated in a study by Paxton (2005), which recorded the incidence of *N. bombi* in 21 bumble bee species from 7 European countries in 2003 and 2004. A total of 2846 bees were examined and microsporidia were detected in 9 of the species. Interestingly, *N. bombi* was found in all species where more than 60 individuals were

examined, which suggests the parasite is ubiquitous even if it exists at low levels in some species. The incidence of infection varied between countries, year and species. For example, 46% of *B. terrestris/lucorum* were infected in Ireland in 2003, whilst only 3% were infected in Sweden. And, overall, an average of 19.3% of *B. terrestris/lucorum* were infected, compared to only 3.7% of *B. pascuorum*. The factors that drive these high levels of variation are unknown.

1.3.1.3 Apicystis bombi

Comparatively little is known about the third gut microparasite to infect bumblebees. *A. bombi* (Neogregarinida, Lipotrophidae: Lipa & Triggiani, 1996) infects adult bumblebees through the ingestion of spores. These release sporozoites that penetrate through the gut wall infecting the fat body cells where they develop and multiply. Spores are then excreted in the faeces, to be transmitted to other individuals (Macfarlane *et al.*, 1995).

Apicystis bombi is known to have quite serious detrimental effects on its host; infected workers have a disintegrated fat body (Durrer & Schmid-Hempel, 1995) and infected colonies have a much decreased chance of growth and reproduction (Schmid-Hempel, 1998). In addition, *A. bombi* causes the premature death of queens after emergence (Macfarlane *et al.*, 1995; Rutrecht & Brown, 2008). Very little is known about the distribution of this parasite, although it is less common than both *C. bombi* and *N. bombi*.

1.3.1.4 Locustacarus buchneri

The endoparasite *L. buchneri* Stammer (Acari: Podapolipidae) infects the trachea of bumblebees. Gravid female mites overwinter inside hibernating bumblebee queens and when the queens become active in the spring, the mites pierce the tracheal wall with their mouthparts and feed on the haemolymph. Several mites may infest a single host and females deposit up to 50 eggs, which hatch into the mobile larviform females and males. After mating the females can migrate to other hosts within the nest via the host's spiracles (Alford, 1975) and will predominantly move from adult bees to 3rd or 4th instar bee larvae, when the wax-pollen larval surround opens to allow feeding (Yoneda *et al.*, 2008).

As with most parasite species, the prevalence of *L. buchneri* is highly variable. It is typically found in less than 10% of field caught bumblebees (Macfarlane *et al.*, 1995) but prevalence of up to 50% have been found in some species, although it is unclear why certain bumblebee species appear to be preferentially parasitised (Otterstatter & Whidden, 2004). Little experimental work has been carried out on this parasite but observations suggest that this mite can have negative fitness effects on its host. For example, Husband & Shinha (1970) reported that bees infected with large numbers of *L. buchneri* were suffering from diarrhoea and had a decreased foraging ability. Additionally, a *B. terrestris* queen that was observed to be weak and cease in its nest building activity, was found to contain a large number of *L. buchneri* and was almost entirely wasted away internally (Skou *et al.*, 1963). However, one laboratory study found that bumblebees infected with *L. buchneri* were no less efficient at foraging than uninfected bees (Otterstatter *et al.*, 2005), although the sample size was small.

1.3.2 The bumblebee immune system

The immune system of bumblebees, like that of all invertebrates, provides protection against the vast array of parasites and pathogens that may be encountered throughout their life history. The system is activated when an attacker breaks through the external barriers of the outer body wall or endothelia and is recognised as non-self. This recognition is achieved by pattern recognition receptors that identify pathogen associated molecular patterns (PAMPs) on the surface of the intruding microbe (Medzhitov & Janeway, 2000). The identification of PAMPs initiates a series of events that eventually leads to the appropriate defence response. Invertebrate defence responses are often classified into either the constitutive or inducible branches of immunity.

Constitutive immunity includes the production of haemocytes and the enzyme phenoloxidase (PO) from its inactive precursor prophenoloxidase (pro-PO). These immune responses are referred to as constitutive as they are present even without contact with a pathogen and as a result they can be activated rapidly and are effective against a broad range of parasites (Schmid-Hempel, 2005). PO catalyses the oxidation of phenols into quinones, which then polymerize into melanin (Soderhall & Cerenius, 1998). Melanin is deposited round the parasite, isolating and externalising it; such melanisation is a common defence mechanism in a wide range of invertebrates (e.g. Allander & Schmid-Hempel, 2000). Haemocyte-mediated immune responses include phagocytosis, nodulation and encapsulation. Phagocytosis occurs when a haemocyte encounters a pathogen smaller than itself and engulfs it. When multiple haemocytes bind to and smother larger pathogens, it is known as nodulation. Encapsulation is

similar to nodule formation but occurs on a larger, more organised scale in response to macro-parasite infection (Lavine & Strand, 2002).

The inducible element of the invertebrate immune system is slower to activate and may be tailored to particular pathogen classes. It involves the production of anti-microbial peptides, which are manufactured in the fat body, epithelium and hemocytes. A large number of antimicrobial peptides have been described, which are effective against a variety of pathogens (Hetru *et al.*, 1998). Recent work has shown that the bumblebee *B. terrestris* strongly upregulates three known antimicrobial peptides (abaecin, defensin 1 and hymenoptaecin) after wounding and bacterial infection (Erler *et al.*, 2011). Evidence has also shown that the invertebrate immune system is more sophisticated that previously supposed as it exhibits a form of immune memory and specificity. The offspring of immune challenged bumblebee queens showed significantly higher antibacterial activity than the offspring of control queens (Sadd *et al.*, 2005). This trans-generational immune priming has been demonstrated to be mediated by factors inside the egg (Sadd & Schmid-Hempel, 2007).

Immunocompetence (IC) can be defined as the ability of an organism to mount an immune defence against a parasite through either cellular, humoral or behavioural pathways (König & Schmid-Hempel, 1995; Schmid-Hempel & Schmid-Hempel, 1998; Adamo, 2004; Rantala & Roff, 2005; Wilson-Rich *et al.*, 2008). It is possible to empirically estimate IC by mimicking the challenge posed by a real parasite using synthetic material, which creates a standardised challenge against which different responses can be compared. The most universal method involves implanting a nylon monofilament into the abdomen of an insect, where it is exposed to the circulating

haemolymph. This triggers the encapsulation response, which can be measured as the degree of melanisation (König & Schmid-Hempel, 1995; Schmid-Hempel & Schmid-Hempel, 1998). This measure is a good illustration of the non-specific constitutive immune response as melanisation is used by invertebrates to respond to a wide and indiscriminate range of pathogens (Gupta, 1986).

In bumblebees, implanting a nylon monofilament mimics the action of conopid flies, which are widespread parasites of bumblebees that oviposit into the abdomen of worker bees (Schmid-Hempel *et al.*, 1990). The encapsulation response has helped elucidate many aspects of the bumblebee immune system (e.g. Schmid-Hempel & Schmid-Hempel, 1998; Allander & Schmid-Hempel, 2000). Additionally, the encapsulation response has been found to positively correlate with overall size of a bumblebee colony and the number of reproductives it produces, so it is a good correlate of fitness (Baer & Schmid-Hempel, 2003).

The level of phenoloxidase in an insect can be measured photospectrometrically and is also used to estimate an individual's IC. The role of PO in insect immunity is undoubtedly very complex and some studies have suggested its role is not essential as pathogens can sometimes be overcome in its absence (for example Leclerc *et al.*, 2006). However, more recent data have provided convincing evidence of the importance of the role of PO (summarised in Cerenius *et al.*, 2008). For example, the bacterial pathogen of the moth *Manduca sexta* can inhibit host PO but when this bacterium has a mutation that prevents the PO inhibitor from working it loses its virulence and the moth suffers no ill effects (Eleftherianos *et al.*, 2007). A genetic correlation has also been found

between PO levels in the haemolymph and ability to encapsulate artificial implants in larvae of the moth *Spodoptera littoralis* (Cotter & Wilson, 2002).

However, despite the essential role of PO in immunity, its measurement often does not explain resistance to certain parasites and correlations between levels of PO and resistance to real pathogens are not normally found. For example, Cornet *et al.* (2009) found no relationship between PO activity in *Gammarus pulex* and resistance to the bacteria *E. coli*, suggesting that other pathways are important in the defence against this infection. Similar results were found in *D. magna*, where variation in PO activity was not predicted by the differences in parasite resistance within and between populations (Mucklow *et al.*, 2004). Additionally PO activity did not predict resistance to two different pathogens in yellow dung flies *Scathophaga stercoraria* (Schwarzenbach & Ward, 2007). These studies do not question the importance of PO, but demonstrate the short falls of measuring a single immune parameter to explain the overall immunocompetence of an individual.

1.4 The commercialisation of bumblebees

1.4.1 Bumblebees as valuable pollinators

Bumblebees are hardy and efficient pollinators and provide essential reproductive services for numerous wild and cultivated flowering plants. Due to their relatively large body size and dense pile, bumblebees are able to forage in much lower temperatures than many other insects, including honeybees (Heinrich, 1979). Their dense hair allows

bumblebees to efficiently transport pollen and they are also able to sonicate (buzz pollinate) those flowers that shed pollen through apical pores (King, 1993). For these reasons, bumblebees provide a superior pollination service for a wide variety of plants, including many crops and as a consequence have been reared commercially for use in agriculture since the 1980s (Velthuis & van Doorn, 2006). The majority of commercially reared bumblebees are used for greenhouse tomatoes, but large numbers are also used for the pollination of various cucurbits, soft fruits, field beans, apples and almonds (Free & Williams, 1976; Stanghellini *et al.*, 1997; Stubbs & Drummond, 2001; Thomson & Goodell, 2001). Studies have since shown that bumblebees do indeed provide economic benefits to farmers through an increased crop yield (Serrano & Guerra-Sanz, 2006; Lye *et al.*, 2011).

1.4.2 The threat of commercial bumblebees

Despite the economic benefits that commercial bumblebees can provide, they do pose a threat to native bumblebee fauna and this can be through the spread of parasites and pathogens (Goulson, 2010b). The threat arises as the commercial bumblebees are not contained on the crop they are there to pollinate and frequently forage on surrounding wildflowers, where diseases can be transmitted (Morandin *et al.*, 2001; Whittington *et al.*, 2004). In North America, the accidental introduction of the gut parasite *N. bombi* with commercial bumblebees is thought by some to be responsible for the dramatic decline of seven species of native bumblebees since the 1990s (Winter *et al.*, 2006) although direct evidence is lacking (Brown, 2011). Additionally, studies have shown that pathogen spillover from commercial to native bumblebees has occurred in Canada. The prevalence of parasites in wild bumblebee populations was compared between sites

close to glasshouses using commercial bumblebees and sites over 50km from any commercial greenhouse. It was found that *C. bombi* was present at significantly higher prevalence at the sites near glasshouses. Additionally, bees foraging closest to the greenhouse had more intense infections (Colla *et al.*, 2006; Otterstatter & Thomson, 2008). Such pathogen spillover can occur even if the commercial bees are initially free of disease as the high densities of bumblebees in glasshouses provide suitable conditions for rapid spread of any pathogen with which they come into contact.

Another threat associated with the use of commercial bumblebees is competition for resources with native species. There are concerns for such competition occurring in Japan and South America as the non-native *B. terrestris* has become naturalised as a result of escapees from glasshouses (Matsumura *et al.*, 2004; Montalva *et al.*, 2011). *B. terrestris* has been shown to have four times the reproductive output of native Japanese bumblebees and they also overlap with native species in their preferred forage (Matsumura *et al.*, 2004; Inari *et al.*, 2005). There are also concerns for resource competition occurring in Chile due to considerable distributional overlap in native and non-native bumblebee species (Montalva *et al.*, 2011). In the UK, the commercial bees imported are predominantly the subspecies *B. terrestris dalmatinus* and *B. t. terrestris* but the native endemic subspecies is *B. t. audax*. The commercial subspecies have been found to have a greater foraging efficiency and reproductive rate compared to *B. t. audax* and so there are concerns that commercial bees established in the wild would have a competitive advantage (Ings *et al.*, 2006). There is also the risk of losing the native subspecies entirely through introgression as *B. t. dalmatinus* and *B. t. audax* readily interbreed (Ings *et al.*, 2005).

1.5 The bumblebee life cycle

Bumblebees are annual, primitively eusocial Hymenoptera that inhabit mainly the temperate regions of the world. Their annual life cycle is described in detail by Sladen (1912) and Alford (1975). Queens emerge from hibernation in the spring and individually found colonies. Once the queen has produced the first batch of offspring, the workers take over the tasks of foraging, brood care and nest maintenance. Sexuals (young queens and males) are produced towards the end of the colony cycle, usually in the late summer, and leave the nest to find a mate. Once mated the queens forage and find a hibernation site and the old founding queen, the workers and the males die. The following spring the queens that have survived hibernation give rise to the next generation (Alford, 1975).

1.6 Aims and objectives

Many bumblebee species are suffering from the effects of habitat fragmentation and population isolation. In some cases, populations have lost genetic diversity due to inbreeding and it is possible they are now at heightened risk of extinction. As bumblebees are keystone species and have a valuable role as pollinators, it is vital to understand what factors might be threatening these populations. Inbreeding may be particularly costly to bumblebees because, as Hymenoptera, their complementary sex determination system can lead to the production of sterile or inviable diploid males. Furthermore, inbreeding may cause increased levels of parasitism and this could play a role in driving the decline of isolated populations.

The overall aim of this thesis is to investigate the effect of inbreeding on bumblebees and establish whether the production of diploid males and a potentially increased susceptibility to parasites is contributing to their decline. A further aim is to investigate the potential threat of pathogen spillover from commercial bumblebees. An understanding of these effects is vital to establish what factors might be pushing threatened pollinators towards extinction and may assist with the development of appropriate conservation strategies.

The specific aims of each chapter are:

1. To assess the cost of inbreeding, in terms of diploid male production, in the bumblebee *Bombus terrestris*.

2. To investigate whether bumblebees attempt to avoid inbreeding through a kin recognition system.

3. To establish whether there is a relationship between levels of inbreeding and parasite prevalence in two bumblebee species, *Bombus muscorum* and *Bombus jonellus*, in the Western Isles of Scotland.

4. To further investigate the relationship between population genetic diversity, parasite prevalence and individual immunity in *B. muscorum*.

5. To assess whether bumblebees are at further risk due to the spread of pathogens from commercial bumblebees imported for soft fruit pollination.

Chapter 2 - Impacts of inbreeding on bumblebee colony fitness under field conditions

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2.1 Abstract

Inbreeding and the loss of genetic diversity are known to be significant threats to small, isolated populations. Hymenoptera represent a special case regarding the impact of inbreeding. Haplodiploidy may permit purging of deleterious recessive alleles in haploid males, meaning inbreeding depression is reduced relative to diploid species. In contrast, the impact of inbreeding may be exacerbated in Hymenopteran species that have a single-locus complementary sex determination system, due to the production of sterile or inviable diploid males. We investigated the costs of brother-sister mating in the bumblebee *Bombus terrestris*. We compared inbred colonies that produced diploid males and inbred colonies that did not produce diploid males with outbred colonies. Mating, hibernation and colony founding took place in the laboratory. Once colonies had produced 15 offspring they were placed in the field and left to forage under natural conditions.

The diploid male colonies had a significantly reduced fitness compared to regular inbred and outbred colonies; they had slower growth rates in the laboratory, survived for a shorter time period under field conditions and produced significantly fewer offspring overall. No differences in success were found between non-diploid male inbred colonies and outbred colonies.

Our data illustrate that inbreeding exacts a considerable cost in *B. terrestris* through the production of diploid males. We suggest that diploid males may act as indicators of the

genetic health of populations, and that their detection could be used as an informative tool in hymenopteran conservation. We conclude that whilst haplodiploids may suffer less inbreeding depression than diploid species, they are still highly vulnerable to population fragmentation and reduced genetic diversity due to the extreme costs imposed by the production of diploid males.

2.2 Introduction

The genetic health of populations is increasingly viewed as one of the most important factors in maintaining fitness in an uncertain and changing environment (Frankham et al., 2004). It is well established that inbreeding depression in diploid organisms significantly increases the risk of extinction (Saccheri et al., 1998). By contrast, haplodiploid organisms have often been assumed to suffer less inbreeding depression as deleterious recessive mutations were thought to be purged through the haploid males (Werren, 1993). However, some authors have challenged this assumption, partly because purging may not be effective against female sex-limited traits, such as hibernation survival and fecundity (Henter, 2003).

Haplodiploids may suffer further genetic costs of inbreeding due to their single-locus complementary sex determination (sl-CSD) system, which is ancestral to the haplodiploid Hymenoptera. Under this system, individuals heterozygous at the polyallelic sex-determining locus develop into diploid females and hemizygotes develop into haploid males. When a diploid individual is homozygous at the sex locus a diploid male is produced. The frequency of diploid males depends on the number of CSD alleles and so they are rarely produced in large outbreeding populations because many

alleles are maintained by negative frequency-dependent selection (Duchateau et al., 1994; Page & Metcalf, 1982). However, genetic drift in small populations is expected to increase diploid male production (DMP) by reducing CSD allelic richness (Cook & Crozier, 1995).

Diploid males represent significant fitness costs, primarily through their inviability or sterility (Petters & Mettus, 1980; Elagoze et al., 1994). In a few species, diploid males can produce diploid sperm and mate, but this invariably results in sterile triploid progeny so the costs are merely deferred by a generation (Naito & Suzuki, 1991). In social insects further costs of diploid males are apparent, as they replace 50% of the female workers and do not contribute to colony productivity (Duchateau et al., 1994). This has been shown to slow the rate of colony growth in *Bombus atratus*, under laboratory conditions (Plowright & Pallett, 1979) and result in high mortality of founding queens in the fire ant *Solenopsis invicta* (Ross & Fletcher, 1986). Recent modelling has demonstrated that DMP can initiate a rapid extinction vortex and suggests that haplodiploids are more prone to extinction than previously supposed (Zayed & Packer, 2005).

The study of genetic diversity and inbreeding in bumblebees is currently of particular importance as many species have been suffering from significant population declines and range contractions (Thorp & Shepherd, 2005; Kosior et al., 2007). This has been attributed predominantly to the intensification of agriculture and the associated loss of flower rich meadows and other habitats on which bumblebees depend (Goulson, 2010a; Goulson et al., 2005; Goulson et al., 2008; Carvell et al., 2006). The remaining populations of rare species have become fragmented, genetically isolated and suffer

from a loss of genetic diversity. They are now susceptible to inbreeding depression, with serious implications for their persistence (Darvill et al., 2006; Ellis et al., 2006; Takahashi et al., 2008).

The genetic consequences of population fragmentation and isolation are exacerbated in bumblebees as a number of factors predispose them to low levels of heterozygosity and hence inbreeding. Firstly, as haplodiploids, there are only 75% as many gene copies in any one generation compared to diplodiploid organisms, hence reducing the effective population size (Packer & Owen, 2001). Secondly, the effective population size of bumblebees is reduced still further by their social nature, as it is determined by the number of successful nests in an area and not by the number of more abundant sterile workers (Pamilo & Crozier, 1997). Finally, the majority of bumblebee species are monoandrous (Estoup et al., 1995; Schmid-Hempel & Schmid-Hempel, 2000). This increases their susceptibility to inbreeding compared to polyandrous species, which effectively have more breeding individuals per generation (Page & Metcalf, 1982) and which in some instances can avoid the costs of negative genetic incompatibility through postcopulatory selective fertilization (Tregenza & Wedell, 2002). Whilst small effective population size in haplodiploids may not result in inbreeding depression *per se* it will decrease CSD allelic richness, which in turn will increase diploid male production.

Investigations into the effects of inbreeding in bumblebees have had varying outcomes. Under laboratory conditions one generation of brother-sister mating in *B. terrestris* had no effect on immune defence or body size (Gerloff et al., 2003). However, a similar experiment found that inbreeding did have a significant negative effect on colony size, whereas the impact of inbreeding on other fitness traits was highly variable across

maternal genotypes (Gerloff & Schmid-Hempel, 2005). Additionally, when *B. terrestris* queens were sib-mated for several generations, a negative effect on queen fecundity and colony size was observed (Beekman et al., 1999). The cost of diploid male production is unclear: Duchateau *et al.* (1994) found that the growth rate of laboratory diploid male colonies of *B. terrestris* was not significantly affected, yet Plowright & Pallett (1979) found diploid male colonies of *B. atratus* had a significantly slower growth rate, albeit with a very small sample size. Diploid males have been observed in rare and threatened bumblebee species in the wild (Darvill et al., 2006; Takahashi et al., 2008), so the true costs of their production are important to ascertain.

This study aimed to determine the costs of brother-sister mating in the bumblebee *B. terrestris*, specifically focusing on survival and growth in field conditions and the fitness of diploid male colonies. Young *B. terrestris* gynes were mated in the laboratory with either their brothers or with un-related males. Their survival during hibernation was recorded and those queens that established colonies in the laboratory generated three experimental treatments:

1) Sib-mated queens not producing diploid male offspring (Inbred treatment)
2) Sib-mated queens producing diploid male offspring (Diploid male treatment)
3) Outbred queen colonies (Outbred treatment)

The rate of growth of these colonies was measured and once they had produced 15 offspring they were placed in the field. The development and survival of these colonies were followed throughout a summer season to demonstrate the costs of inbreeding and DMP in a natural setting.

2.3 Methods

2.3.1 Experimental protocol

10 laboratory colonies of *B. terrestris*, purchased from Koppert Biological Systems, The Netherlands, in February 2008, provided young queens and males. When these sexuals emerged they were removed from the maternal colony and housed in single sex sibling groups before being mated in large mesh sided flight cages between 1st April and 16th April 2008.

To generate the outbred treatment, maternal colonies were paired randomly and daughter queens were mated with the unrelated males from their paired colony. To generate inbred colonies, daughter queens were mated with their brothers. It was expected that approximately half the inbred matings would result in diploid male colonies. Only sibling groups were used; all males in the mating cage at any one time were brothers, and all queens were sisters. Bees were mated in groups (n = 15 to 60), always in a 1:2 ratio of queens to males. During copulation mating pairs were removed from the flight cage, placed into clear plastic boxes, then left undisturbed until copulation ended.

A total of 210 queens were successfully mated (82 non-sibmated and 128 sibmated); an average of 21 ± 3.2 (mean ± SE) queens per maternal colony. After mating, males were removed and queens were kept in the box for 48 hours under natural lighting with sugar water and fresh pollen *ad libitum* (honey bee pollen stored at -20^0C). After this period queens were housed individually in match boxes and hibernated in an incubator at 6 ^{o}C for 47 days.

Queens that survived hibernation were placed in individual wooden boxes (10cm x 10cm x 10cm) and kept under standard rearing conditions (28^0C, 60% relative humidity and red light (Plowright & Jay, 1966)). Sugar water (50% Attracker solution in distilled water, Koppert Biological Systems, The Netherlands) was provided *ad libitum* and pollen balls (ground fresh pollen mixed with Attracker) were provided three times a week. When a queen had produced five offspring, the new colony was transferred to a larger plastic box (25cm x 22cm x 14cm) with a separate feeding chamber. Following Duchateau *et al.* (1994), diploid male colonies were identified as those producing workers and males in approximately equal numbers from the first brood. When fifteen eclosed offspring had been produced the nest box was insulated and placed in a waterproof outer box. The colony was then transferred to the field site where the workers could forage under natural conditions. Colonies of all treatments were placed out in the field at approximately the same time. The field site was situated on the edge of Stirling University campus, from where ornamental gardens, deciduous woodland and mixed farmland were available within 500m radius (a conservative estimate of foraging range for this species, see Darvill *et al.*, 2004; Knight *et al*., 2005).

After field placement, colonies were checked weekly; on each occasion 10% of the offspring was removed and stored at -80^{o}C for later dissection in a separate study on parasite resistance. No offspring were removed if fewer than 10 were present. When the queen died, each colony's inner brood chamber was collected and frozen for subsequent inspection.

2.3.2 Variables measured

Hibernation survival

Following Gerloff & Schmid-Hempel (Gerloff & Schmid-Hempel, 2005) queens were classified as having survived hibernation only if they survived for at least 72 hours post hibernation; those that did not were unlikely to have survived under natural conditions. A considerable proportion of queens (0.108, n = 23) fell into this category. All subsequent analyses remain qualitatively unchanged if these queens are included.

Colony foundation

Queens were considered to have founded a colony if they successfully reared at least one offspring to adulthood. The number of days from the end of hibernation to the emergence of the first worker was recorded. Queens that had not laid eggs 12 weeks after the end of hibernation were removed from the experiment.

Colony growth

The number of colonies successfully rearing ≥5 and ≥15 offspring was recorded. These colony sizes were specifically relevant as at 5 offspring the colony was transferred to a larger box where the workers had to travel a short distance to find food and at 15 offspring the colony was transferred outside to forage independently.

Survival under field conditions

The number of weeks between the field placement date and the queen's death was recorded.

Final colony size

The final colony size was recorded when the queen died. For the colonies which were placed outside this was assessed by counting the number of empty cells in the brood clump. This is a reliable indirect measure of fitness as the number of reproductives produced by a colony is highly correlated with colony size (Gerloff & Schmid-Hempel, 2005; Muller & Schmid-Hempel, 1992). For colonies that had not reached 15 offspring, the experiment was ended 120 days after the queen had emerged from hibernation and the final colony size was counted at this time.

2.3.3 Statistical analyses

Data were analysed in SPSS 15.0 for Windows (2007 Chicago: SPSS Inc.). Binary logistic regression was used to investigate determinants of hibernation survival and colony foundation. Inbreeding treatment and maternal colony were entered as fixed factors and hibernation end date was included as a co-variate to control for variation in experiment start dates. Colony growth was analysed with binary logistic regression to assess whether or not colonies from different inbreeding treatments crossed the 5 worker and 15 worker size thresholds. Similarly, General Linear Models (GLMs) were used to investigate whether inbreeding status influenced the number of days to the emergence of a colony's 1^{st}, 5^{th} and 15^{th} offspring, as well as the impact of inbreeding on colony field survival time and total number of offspring produced. In each case inbreeding treatment and maternal colony were entered as factors and hibernation end date as a covariate. All data sets used in GLMs were normally distributed (verified with Anderson-Darling tests). Variables not contributing significantly to models were removed in a step-wise fashion. Pairwise differences between factor means were

investigated using Tukey's post hoc tests. Means are recorded ± their standard errors throughout.

2.4 Results

2.4.1 Hibernation survival

In total 93 queens (43.7%) survived the hibernation period and subsequent 72 hours. The probability of surviving hibernation was significantly affected by the maternal family line ($\chi^2_{9,}$ = 31.84; P < 0.0001); survival ranged from 11.5% to 68.0% between maternal colonies (see figure 2.1). Mating date was also a significant predictor of hibernation survival; queens mated earlier were more likely to survive ($\chi^2_{1,}$ = 19.28, p < 0.0001). There was no difference in survival between queens mated to unrelated males and sib-mated queens (46.34%, n = 82 and 41.98%, n = 131 respectively. $\chi^2_{1,}$ = 1.67; P = 0.199).

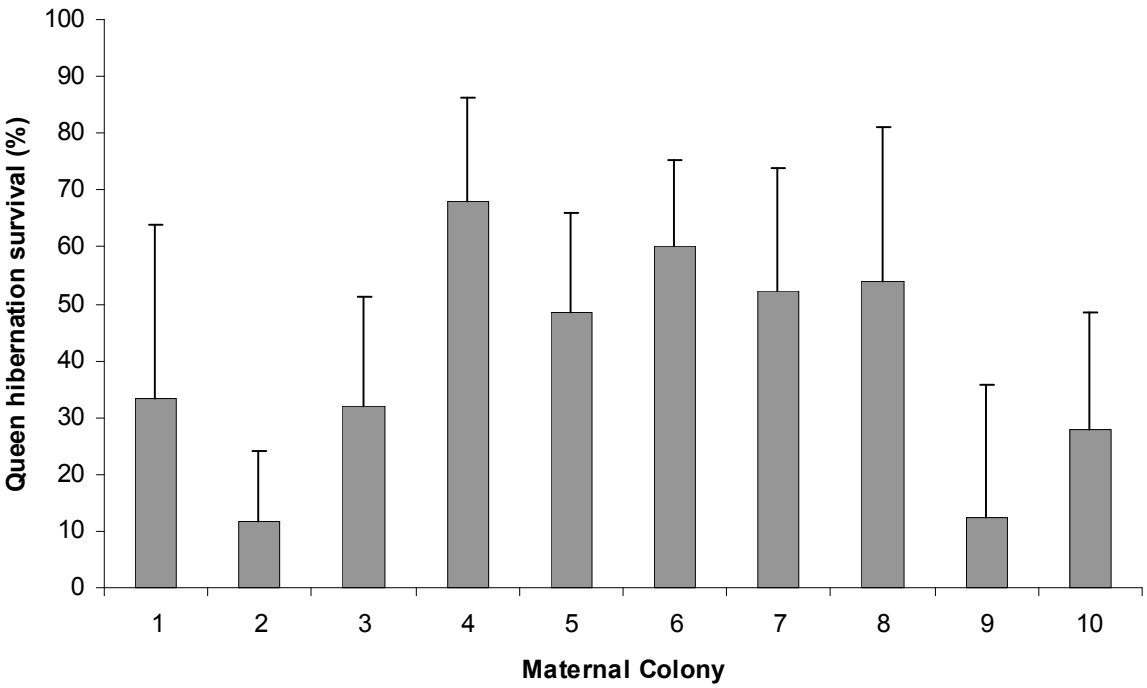

Figure 2.1 - Hibernation survival for experimental queens from each maternal colony.

The probability of surviving differed significantly between maternal colonies ($P < 0.0001$). Sample size within each maternal colony ranged from 8 to 40 and error bars show 95% shortest unbiased confidence limits.

2.4.2 Colony foundation

Out of the queens that survived hibernation, 47 produced at least one offspring and were considered to have successfully founded a colony. Of these 47 colonies, 20 were outbred, 17 were inbred and 10 were diploid male colonies. There was no difference between colony founding ability between queens mated to unrelated males and sib-mated queens (57.14%, n = 35 and 50.94%, n = 53 respectively. $\chi^2_{1,} = 0.326$, $p = 0.568$). Additionally, colony founding ability was not predicted by maternal colony ($\chi^2_{9,} = 14.25$, $P = 0.114$) or hibernation end date ($\chi^2_{1,} = 0.78$, $p = 0.378$).

2.4.3 Colony growth

The number of colonies reaching 5 and 15 offspring

The probability of a colony growing past the 5 and 15 offspring size thresholds was not influenced by inbreeding status ($\chi^2_{2,}$ = 0.36, P = 0.835; $\chi^2_{2,}$ = 1.70, P = 0.428 respectively) (figure 2.2). Maternal line did not significantly influence the number of colonies reaching 5 and 15 offspring ($\chi^2_{9,}$ = 11.42, P = 0.248; $\chi^2_{9,}$ = 11.76, P = 0.227 respectively) and neither did hibernation end date ($\chi^2_{1,}$ = 0.08, P = 0.779; $\chi^2_{9,}$ = 0.54, P = 0.461 respectively).

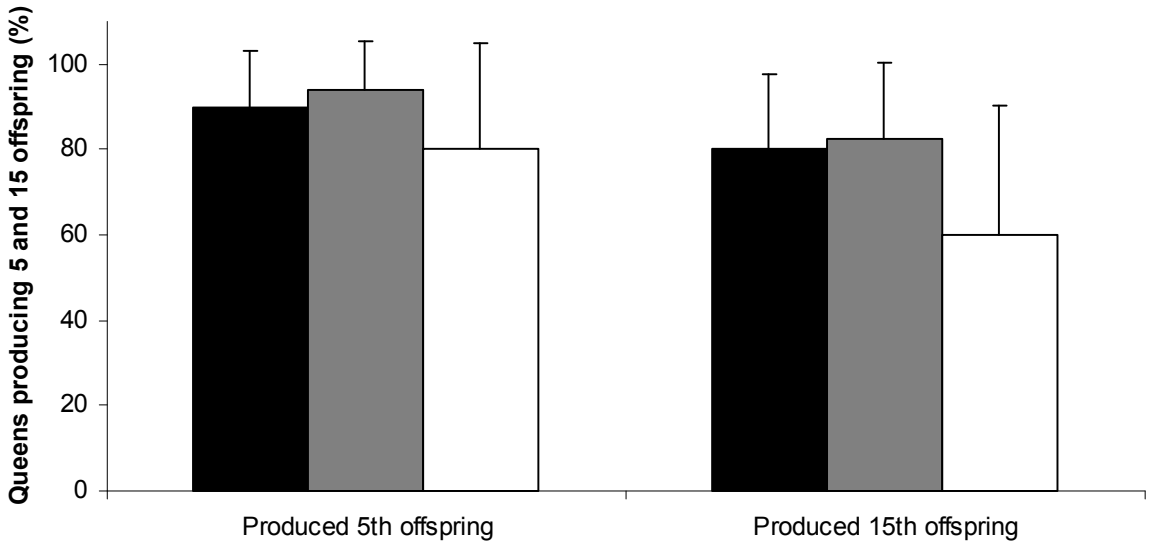

Figure 2.2 - The percentage of queens producing 5 and 15 offspring according to treatment.

Black bars represent the outbred treatment, grey bars the inbred treatment and white bars the diploid male treatment. No significant difference was found between these values (P = 0.835 for 5 offspring & P = 0.248 for 15 offspring). Sample size within each treatment ranged from 6 to 18 and error bars show 95% shortest unbiased confidence limits.

The rate of colony growth

We assessed the rate of growth of colonies after foundation by recording the time until they crossed three size thresholds: 1, 5 and 15 offspring. The inbreeding treatments did not significantly influence the time taken to reach these sizes, neither did maternal colony origin or hibernation end date (table 2.1). However, due to the earlier emergence of offspring in the DMP colonies, the mean interval between emergence of the 1st and 15th offspring was considerably longer for the diploid treatment (41.3 days ± 2.00, n = 6) than for either the inbred (26.51 days ± 1.18, n = 14) or outbred (23.6 days ± 1.21, n = 16) treatments (figure 2.3). This variation was highly significant ($F_{2, 25} = 35.13$; $P<0.001$). Post hoc tests confirmed that the diploid male treatment differed from both the others ($P<0.0001$), but that no difference existed between the inbred and outbred colonies ($P>0.246$). Maternal colony also influenced the number of days between the emergence of the 1st and 15th offspring ($F_{8, 25} = 6.35$, $p<0.001$).

Table 2.1 - Output of GLM for the rate of colony growth.

The rate of colony growth is represented by the number of days from the hibernation end date to the emergence of the 1st, 5th and 15th offspring and the number of days to the emergence of the 15th offspring from the 1st offspring, respectively, with respect to inbreeding treatment, maternal colony and the co-variate hibernation end date. Degrees of freedom are given in parentheses.

	Days to 1st offspring		Days to 5th offspring		Days to 15th offspring		Days from 1st to 15th offspring	
	F	P	F	P	F	P	F	P
Inbreeding Treatment	1.70 (2, 46)	0.194	0.75 (2, 39)	0.480	0.62 (2, 33)	0.545	35.13 (2, 25)	<0.001
Maternal Colony	1.68 (9, 34)	0.133	1.30 (8, 30)	0.281	1.70 (8, 24)	0.149	6.35 (8, 25)	<0.001
Hibernation end date	2.03 (1, 34)	0.164	1.37 (1, 30)	0.251	3.48 (1, 24)	0.074	2.09 (1, 24)	0.161

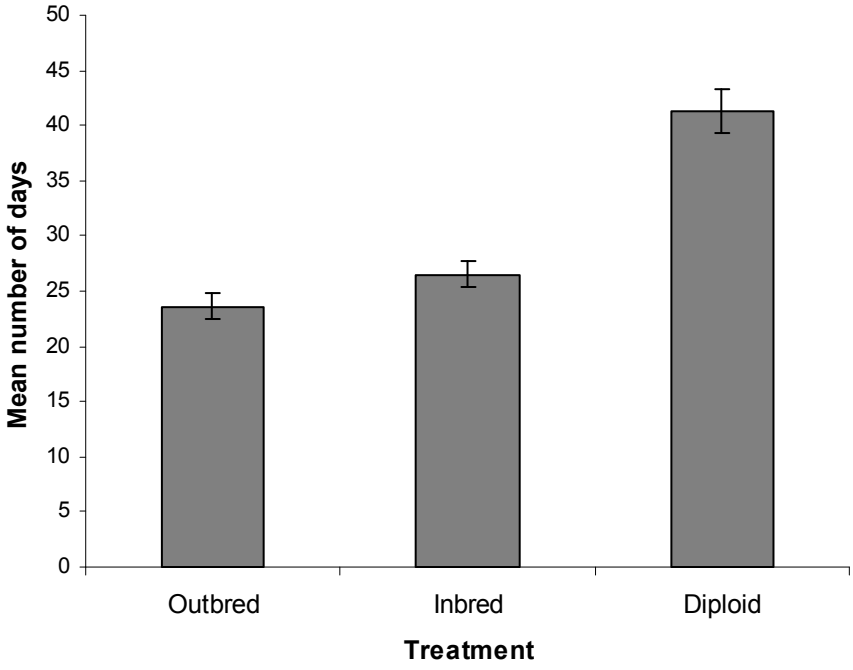

Figure 2.3 - Mean time from colony foundation to 15th offspring, according to treatment.

Bars show the mean duration of the period between the emergence of the 1st and 15th offspring. Means and their standard errors were predicted from the GLM. This measure of colony growth is significantly slower for the diploid male treatment than either outbred or inbred colonies ($P < 0.001$, see text).

2.4.4 Survival and growth under field conditions

Survival in the field

The diploid male colonies survived for a shorter time period under field conditions compared to the outbred and regular inbred colonies; a mean of only 1.5 (± 0.86) weeks, compared to means of 4.5 (± 0.54) and 3.4 (± 0.56) weeks respectively ($F_{2,32} = 4.33$, $p < 0.05$) (figure 2.4). Post hoc tests revealed the outbred and diploid male treatments were significantly different ($p < 0.02$); no significant difference existed for other pairwise comparisons (inbred-outbred $P= 0.388$, inbred-diploid male $P = 0.159$). Maternal line

and field placement date did not cause significant variation in field survival duration ($F_{7, 24} = 1.19$, $p = 0.345$ and $F_{1, 31} = 3.49$, $p = 0.071$ respectively).

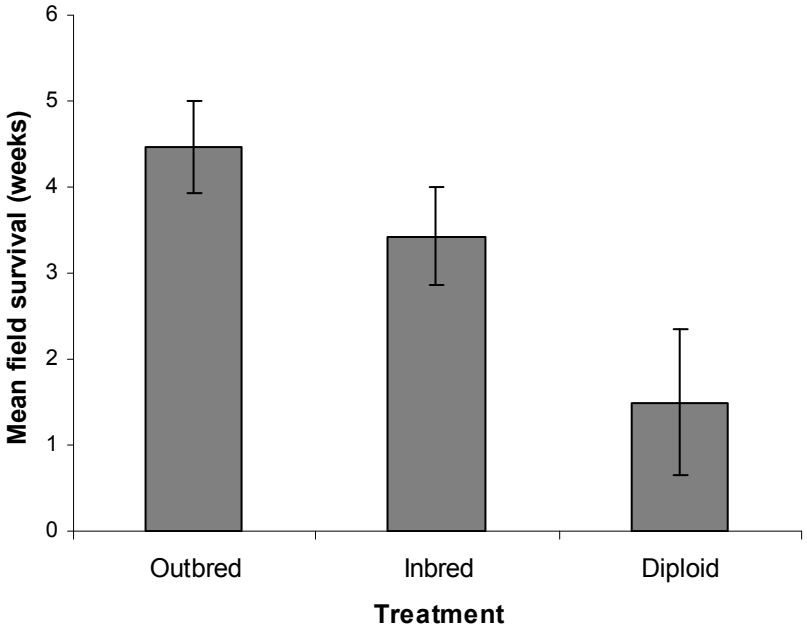

Figure 2.4 - The mean number of weeks colonies survived under field conditions according to treatment.

Bars represent the least square means and their standard errors as predicted by the GLM. Diploid male colonies survived significantly fewer weeks than the outbred colonies ($P < 0.05$, see text).

Colony growth in the field

The number of offspring a colony produces is a major determinant of colony fitness. All colonies had 15 offspring when placed into the field. Outbred and inbred colonies continued to grow under field conditions, producing total means of 30.9 (± 2.42) and 29.7 (± 2.50) offspring each. However, diploid male colonies produced very few additional offspring in the field, reaching a mean of only 15.8 (± 3.82) offspring. This striking variation between inbreeding treatments was significant ($F_{2, 32} = 6.03$, $p < 0.01$) (Figure 5). Post hoc tests confirmed that diploid male colonies differed significantly from both the outbred and inbred treatments ($p = 0.006$ & $p = 0.013$ respectively); the difference in mean size between outbred and inbred colonies was not significant ($p =$

0.935). Maternal colony and field placement date did not significantly influence final colony size ($F_{7, 24} = 1.43$, $p = 0.241$; $F_{1, 31} = 1.39$, $p = 0.248$ respectively).

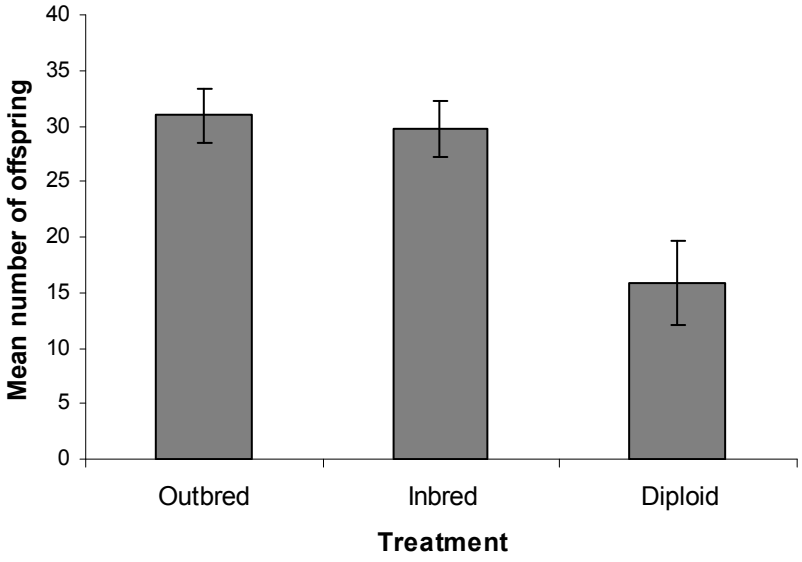

Figure 2.5 - The mean total number of offspring produced by colonies in the field according to treatment.

Bars represent least square means and their standard errors as predicted by the GLM. Diploid male colonies produced significantly fewer offspring than both the outbred and the regular inbred colonies ($P < 0.01$, see text).

2.5 Discussion

For the first time we demonstrate that brother-sister mating in *B. terrestris* exacts high costs under field conditions through the production of diploid males. A number of fitness parameters were negatively affected by diploid male production, including colony growth rate, total offspring production and colony survival, but no significant effects of inbreeding in the absence of diploid male production were detected.

The costs of diploid male production were first evident whilst colonies were growing in the laboratory, where the number of days between the emergence of the 1st and 15th offspring was considerably greater for the diploid male colonies. This slower growth rate presumably occurs because colony resources are diverted away from the production of industrious female workers; diploid males are idle within the colony and so the workforce is approximately halved, resulting in less brood care and slower growth. These findings augment the study by Plowright & Pallett (1979) who found that DMP colonies in *B. atratus* had a considerably slower rate of growth than all-worker-producing inbred colonies in laboratory conditions.

Overall colony fitness was gauged by the total number of offspring produced by the end of the experiment, as the number of reproductives reared by a colony is highly correlated with the number of workers (Muller & Schmid-Hempel, 1992; Gerloff & Schmid-Hempel, 2005). The mean number of offspring produced by the diploid male colonies was significantly lower than in the other treatments. In fact, the mean was only 15.8, which is barely greater than the colony size of 15 when nests were placed in the field. The low number of offspring in these colonies would result in fewer foraging workers and hence a lower food intake. This would have initially impeded growth and subsequently led to colony starvation. This is reflected in lower survival of DMP colonies; the queens survived approximately a third of the time of the outbred colonies, and died presumably due to starvation due to the lack of foraging workers. A similar outcome has been found in the fire ant *Solenopsis invicta*, where DMP colonies had lower brood weight, fewer adult workers and higher queen mortality compared to all-worker-producing colonies (Ross & Fletcher, 1986). This was explained by the queen

having to cope on her own for longer before there were sufficient workers to take over foraging duties.

As well as reducing colony survival, bumblebee diploid males impose a genetic load on populations as they yield no reproductive return for the resources invested in them. *B. terrestris* diploid males produce diploid (rather than haploid) sperm. They also have smaller testes and fewer spermatozoa than haploid males, and hence have reduced fertility (Duchateau & Marien, 1995). Although they develop normally in other respects and are capable of mating, Duchateau & Marien (1995) found that the queens mated to diploid males did not produce colonies. It has since been found that such queens are capable of producing a viable colony containing triploid offspring, but the triploid queens produced are inviable or infertile (Ayabe *et al.*, 2004). Therefore, as in other species such as the sawfly *Athalia rosae ruficornis*, the costs of diploid males are not all immediately apparent, but become so a generation later (Naito & Suzuki, 1991).

Diploid males have been found to be sensitive indicators of the loss of genetic diversity in Hymenoptera. For example, an apparently abundant species of orchid bee *Euglossa imperiali* was found to have large numbers of diploid males, ranging from 12% to 100% of the total population. This turned out to be the result of an extremely low effective population size (Zayed *et al.*, 2004). In a further study of more orchid bee species, the highest diploid male frequency and the lowest genetic variability was found in the rarest species (Lopez-Uribe *et al.*, 2007). Diploid males have also been found in rare and localised bumblebee species. In the Japanese bumblebee *Bombus florilegus*, diploid males were found in 28% of colonies produced in the laboratory from wild caught mated queens, a figure thought to be due to matched matings resulting from notably low

genetic diversity and small population size. Additionally, the frequency of triploid females was found to be 2.7% in natural populations (Takahashi *et al.*, 2008). Diploid males were detected at a frequency of 5% in the wild (with respect to haploid males) in the threatened bumblebee *Bombus muscorum*, again probably due to reduced genetic diversity brought about by population fragmentation and isolation (Darvill *et al.*, 2006). As diploid males are produced from the first brood, they will be found on the wing, even if the colony from which they have been produced dies prematurely, as our results suggest is highly likely. Because of the significant costs diploid males represent for bumblebee fitness, their frequency could potentially be used as an indicator of the genetic health of the population and hence its sustainability and conservation requirements (Zayed *et al.*, 2004). Where the production of diploid males is high, translocations from other populations might be considered as a means of increasing genetic diversity. However, given that DMP colonies are short-lived under field conditions, their apparent absence will not always indicate a genetically healthy population. A method of directly assessing CSD allele diversity would therefore be of great value.

In this experiment the only apparent cost of inbreeding was the production of diploid males, as the non-DMP inbred colonies did not differ significantly from the outbred colonies in all the variables measured. It should be noted, however, that this lack of difference could be due to the inbred colonies resulting from only one generation of brother-sister mating, which would not substantially decrease their level of heterozygosity relative to the outbred colonies. Indeed, one study has demonstrated decreased fecundity and colony size when *B. terrestris* queens are sibmated for several generations (Beekman *et al.*, 1999). Despite the fact that some evidence indicates that

Hymenoptera, including bumblebees (Gerloff & Schmid-Hempel, 2005; Beekman *et al.*, 1999), suffer from inbreeding depression, a meta-analysis has shown that the magnitude of fitness loss on inbreeding is less than that experienced by diploid insects (Henter, 2003). This supports ideas that deleterious recessive alleles are expressed and thus purged in haploid males (Werren, 1993). Our data show that the high costs of DMP following inbreeding far outweigh any apparently small effects of conventional inbreeding depression. Thus, whilst Hymenoptera may be spared some costs of inbreeding by virtue of their haplodiploidy, their sex determination system imposes unique costs through diploid male production. Due to these negative fitness effects, selection should act strongly on haplodiploids to avoid incestuous matings and the production of diploid males, a theory that has been supported by a recent study (Whitehorn *et al.*, 2009a). There is some evidence to suggest that this avoidance behaviour occurs through a kin recognition system (Foster, 1992).

Hibernation survival and colony growth in the laboratory were significantly influenced by maternal family line. This among-family variation has been found in a number of different fitness traits in bumblebees (Gerloff *et al.*, 2003; Gerloff & Schmid-Hempel, 2005) and is evidently an important aspect of their evolutionary ecology. The factors that maintain this variation in wild populations remain to be established. Mating date was another significant predictor of the variation in hibernation survival observed; queens that were mated first were more likely to survive than those mated at a later date, despite standardised hibernation duration and conditions. This substantiates the idea that individuals that are born and reproduce early in the season have a higher survival rate and fitness (Cushman *et al*., 1994; Gerloff & Schmid-Hempel, 2005).

We conclude that the diploid males produced following inbreeding impose large costs on bumblebees through their influence on a colony's survival and productivity. We suggest that they act as indicators of the genetic health of the population, and therefore their detection could be an indication of genetic problems in bumblebees and other social hymenopterans. Haplodiploidy may render the social Hymenoptera less susceptible to inbreeding depression compared to diploid species, due to purging. However, our data demonstrate that the magnitude of fitness costs from DMP following inbreeding may well be as extreme as those expected to result from conventional forms of inbreeding depression in diploid species.

2.6 Acknowledgements

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3.1

Chapter 3 - Kin recognition and inbreeding reluctance in bumblebees

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All authors commented on draft versions of this manuscript and the published version is presented here.

3.1 Abstract

Inbreeding frequently has a costly impact on fitness, thus selection has favoured the evolution of kin recognition and inbreeding avoidance behaviour in many species. As haplodiploid Hymenoptera, bumblebees are susceptible to additional costs of inbreeding due to their single-locus complementary sex determination (sl-CSD) system, which means that incest can result in the production of costly diploid males. Here we test whether *Bombus terrestris* reproductives are able to discriminate between kin and non-kin and whether their willingness to mate is adjusted accordingly. We found that *B. terrestris* reproductives took significantly longer to mate with siblings compared to non-relatives. This indicates that this species exhibits kin recognition and uses this information to determine mating behaviour.

3.2 Introduction

In species that suffer from inbreeding depression, mechanisms to avoid mating with close relatives are expected to be selected for (Pusey & Wolf, 1996). Kin recognition is one such mechanism and close relatives can be identified using either environmental (extrinsic) or genetic (intrinsic) clues (Holmes & Sherman, 1983). Extrinsic kin recognition is often context based: individuals learn environmental cues, such as the scent of their nest environment, then identify kin as those possessing the same environmental cues (Holmes & Sherman, 1982). Intrinsic kin recognition is independent of learning and is mediated by recognition alleles: individuals bearing the same alleles consider one another as kin (Keller & Ross, 1998). Increasingly, the definition of kin

recognition is restricted only to intrinsic mechanisms, although extrinsic mechanisms, such as nestmate recognition, can also lead to clear kin discrimination (Barnard & Aldhous, 1991; Todrank & Heth, 2003). Incest avoidance via kin discrimination has been reported for several insect species, including halictine bees (Smith & Ayasse, 1987), the field cricket *Gryllus bimaculatus* (Simmons, 1989), the ant *Iridomyrmex humilis* (Keller & Passera, 1993) the termite *Zootemopsis nevadensis* (Shellman-Reeve, 2001) and the cockroach *Blattella germanica* (Lihoreau *et al.*, 2007).

It might be expected that bumblebees have evolved methods of kin recognition as they are particularly susceptible to costs of inbreeding due to their single-locus complementary sex determination (sl-CSD) system (Zayed & Packer, 2005). The sex-determining locus is polyallelic; individuals that are heterozygous develop into diploid females, whereas hemizygotes become haploid males. However, if individuals are homozygous at the sex locus they develop as diploid males. This occurs rarely in large outbreeding populations because many CSD alleles can be maintained by negative frequency dependant selection (Duchateau *et al.*, 1994). However, genetic drift in small populations is expected to increase diploid male production (DMP) by reducing CSD allelic richness (Cook & Crozier, 1995).

Bumblebee diploid males yield no genetic return for the resources invested in them. *Bombus terrestris* diploid males have smaller testes and fewer spermatozoa than haploid males, and hence suffer reduced fertility (Duchateau & Marien, 1995). Queens that do mate with diploid males may produce a viable colony containing triploid offspring, but the triploid queens are infertile (Ayabe *et al.*, 2004). Additionally, as diploid males are produced from the first brood, the majority are on the wing too early in the season to

encounter virgin queens. The social nature of bumblebees predisposes them to further costs of DMP: diploid males are produced instead of 50% of the female workforce and do not contribute to colony productivity. This slows the rate of colony growth in *Bombus atratus* under laboratory conditions (Plowright & Pallett, 1979) and significantly reduces survival of *B. terrestris* colonies in the field (Whitehorn *et al.*, 2009b).

Diploid males occur in rare and localised bumblebee species in the wild. In the Japanese bumblebee *Bombus florilegus* 28% of sampled colonies contained diploid males; similarly, in the UK, 5% of *Bombus muscorum* males were found to be diploid. In both cases this is thought to result from low genetic diversity, small population size and fragmentation (Takahashi *et al*., 2008; Darvill *et al*., 2006). Recent modelling has demonstrated that DMP can initiate a rapid extinction vortex (Zayed & Packer, 2005), which has implications for the persistence of small genetically impoverished populations of bumblebees. In contrast, in large populations the risk of matings between bees with identical sex determination locus genotype is low, so long as siblings do not mate. However, bumblebee nests often produce large numbers of queens and males simultaneously, so encounters between siblings are likely and inbreeding avoidance behaviour is therefore beneficial.

The mating behaviour of bumblebees has been well studied in the laboratory (Djegham *et al.*, 1994; Tasei *et al.*, 1998; Sauter & Brown, 2001; Baer, 2003). By comparison, little is known about inbreeding avoidance behaviours. One study suggested that at least two bumblebee species recognise kin; when given a choice queens of *Bombus frigidus* and *Bombus bifarius* preferentially mated with unrelated males (Foster, 1992). Males of

these two species exhibit similar pre-mating behaviour known as 'patrolling', where males mark prominent objects with a pheromone and visit them sequentially to encounter potential mates attracted by the scent (Alford, 1975; Williams & Zervos, 1991). In such a situation it is unlikely that reproductives will encounter both siblings and non-siblings at the same time and so choice experiments such as Foster's (1992) perhaps do not represent the natural situation. Here we take an alternative approach to investigate kin recognition in *B. terrestris*, another species in which males exhibit patrolling behaviour in the wild.

Bombus terrestris is an annual, primitively eusocial bumblebee species. Under natural conditions, queens emerge from hibernation in spring and individually found colonies. Once the first batch of offspring has been produced, they take over the tasks of foraging, brood care and nest maintenance. Towards the end of the colony cycle, usually in the late summer, sexuals (young queens and males) are produced and leave the nest to find mates. The young queens mate only once and then enter hibernation; the old queen, the workers and the males then die. The following spring the queens that have survived hibernation give rise to the next generation (Alford 1975).

We present *B. terrestris* reproductives with either siblings or non-siblings and measure their propensity to mate. This may be a more realistic measure of inbreeding avoidance as a delayed propensity to mate in natural situations will reduce the chance of successful copulation.

3.3 Methods

3.3.1 Experimental Protocol

Eight laboratory colonies of *B. terrestris*, purchased from Koppert Biological Systems (The Netherlands) in February 2008, provided young queens and males. The colonies were checked each day and new sexuals that had emerged were removed and housed in single sex sibling groups. The sexuals were mated when between two and ten days old in mesh-sided flight cages (70cm x 70cm x 70cm) between 1st April and 16th April 2008. The matings took place in the laboratory, adjacent to large windows, between 10:00 and 15:00 so there were considerable quantities of natural light.

Young queens from each colony were either offered their brothers as mates or unrelated males from one other randomly chosen colony. Bees were mated in groups (n = 15 to 60), always in a 1:2 ratio of young queens to males. Only sibling groups were used, i.e. all males in the mating cage at any one time were brothers, and all queens were sisters. Mating pairs were removed from the flight cage during copulation. The mated queens then went into a separate study that we have published elsewhere, which required that we performed twice as many sibling matings as non-sibling matings. The willingness of queens to mate with their brothers compared to non-relatives was investigated by measuring the time between the release of bees into the flight cage and a copulation. All mating sessions were terminated after one hour. The proportions of mated and unmated queens were recorded for each mating batch where more than one mating occurred.

3.3.2 Statistical Analyses

Data were analysed in Minitab 15 (Minitab Inc., State College, PA, USA) with a General Linear Model. The response variable, time to mate, was box-cox transformed to fulfil the assumptions of normality. Mate identity (sibling vs. non-sibling), maternal colony, number of individuals in the cage and their interactions were included in the model. The model was sequentially simplified by the step-wise removal of non-significant terms. A further General Linear Model was used to analyse an additional response variable, proportion of queens mated within a batch. Mate identity, maternal colony and number of individuals in the cage were included in the model, which was again sequentially simplified. For bees originating from each of the eight colonies, individual t-tests were carried out to determine the significance of differences between the time to mate for sib and non-sib matings. Tests did not assume equal variance and were uncorrected for multiple comparisons. Means are recorded ± their standard errors throughout.

3.4 Results

The mating behaviour of 173 young queens from eight colonies was recorded; 70 with non-relatives, 103 with siblings. A mean of 10.8 minutes (± 0.94) passed before a sibling mating occurred, compared to a mean of only 4.5 minutes (± 1.15) for a mating between non-relatives. Pooling the data in this way revealed a highly significant difference between sibling and non-sibling matings ($F_{1,171} = 22.21$, $P < 0.001$). Bees originated from eight maternal colonies; for offspring from seven of these, sibling

matings were notably delayed relative to unrelated matings, in one case time to mate with siblings and non-relatives was similar (see figure 3.1). Two-sample t-tests showed that these differences were significant for three out of the eight colonies (P ranged from 0.018 to 0.024).

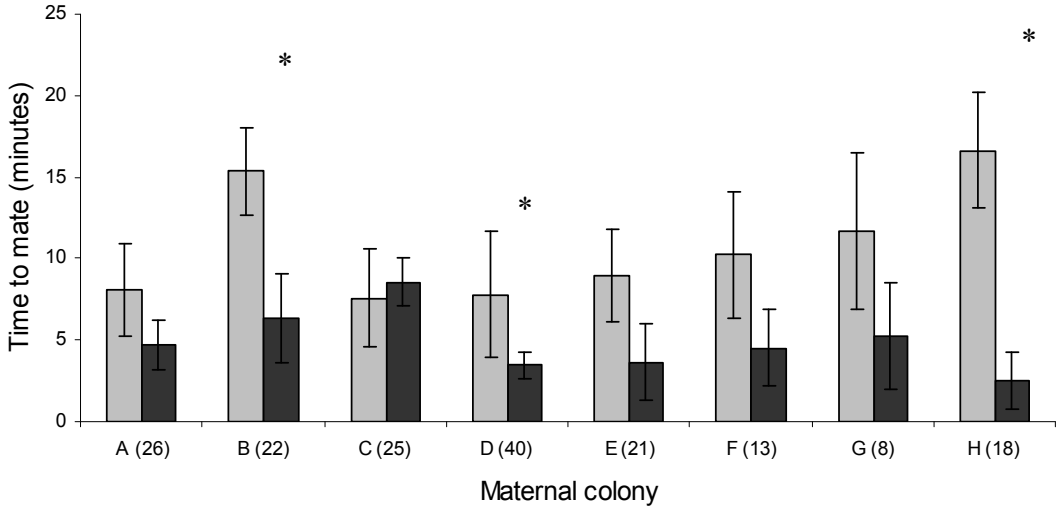

Figure 3.1: The mean time for reproductives from 8 colonies to copulate with siblings (light grey bars) and non-siblings (dark grey bars).

Bars represent the least squares means and their standard errors as predicted by a GLM. The GLM demonstrated that significantly more time elapsed before a queen mated with a sibling compared to a non-relative. Asterisks mark individual within-colony differences that were significant with 2-sample t-tests. The x-axis shows colony ID and sample size in parentheses.

Maternal colony, the number of bees in the mating cage and the interaction between the maternal colony and the identity of the mate did not significantly influence time to mate ($F_{7,152} = 0.7$, $P = 0.70$; $F_{5,152} = 0.73$, $P = 0.60$; $F_{7,152} = 0.97$, $P = 0.452$ respectively).

A mean proportion of 0.59 (± 0.06) queens mated within non sibling batches, compared to a mean proportion of 0.43 (± 0.05) queens within sibling batches. This difference was not significant ($F_{1,22} = 3.46$, $P = 0.076$). The proportion of mated queens was influenced by maternal colony ($F_{7,23} = 4.87$, $P = 0.002$) but not by the number of bees in the mating cage ($F_{6,16} = 0.94$, $P = 0.497$).

3.5 Discussion

For the first time, this study has demonstrated that *B. terrestris* reproductives are less willing to mate with their siblings than with unrelated individuals. Successful copulations between siblings took more than twice as long to initiate than matings between non-relatives. This suggests that *B. terrestris* has the ability to recognise kin and modulates its mating behaviour accordingly. Additionally, a greater proportion of queens mated with non-relatives, compared to siblings, but this difference was not significant. Variation in the reluctance to mate existed between maternal colonies but the trend for delayed sib-mating was evident in experiments on bees from seven of the eight maternal colonies tested. Among-family variation is common in bumblebees and has been found in a number of different fitness traits (Gerloff *et al.*, 2003; Gerloff & Schmid-Hempel, 2005).

A successful copulation is the result of a number of interacting factors, which can include male choice, female choice, male courtship behaviour, female response to this courtship and female reproductive status (Halliday, 1983). Several precopulatory behaviours occur in bumblebees. Conditions in the laboratory are too artificial for males to set up nuptial routes and exhibit their patrolling behaviour, but other behaviours that

occur once potential mates have encountered each other can be observed. Males approach females, inspect them with their antennae and then attempt to copulate. Females respond to males in three ways, either by remaining immobile, flying away, or exhibiting threat behaviour by raising their middle legs (Djegham *et al.*, 1994). However, it is not known how differences in these behaviours influence copulation success (Sauter & Brown, 2001).

Despite the uncertainty surrounding the role of precopulatory behaviours, it is likely that a successful copulation in bumblebees is ultimately the result of female choice for two main reasons. Firstly, the queen controls the onset of copulation as she must move her sting for the male to be able to insert his genitalia; queens are very choosy, often rejecting many males in the laboratory (Djegham *et al.*, 1994; Duvoisin *et al.*, 1999) as well as in the field (Kindl *et al.*, 1999). Secondly, because bumblebee queens mate only once (Estoup *et al.*, 1995; Schmid-Hempel & Schmid-Hempel, 2000) and males are capable of mating many times (Tasei *et al.*, 1998) selection acts more strongly on females to choose a mate that will maximise her fitness. This suggests that the different propensities to mate observed in our experiment were a result of variations in female behaviour and *B. terrestris* queens have the ability to recognise siblings.

Bombus terrestris colonies are almost invariably headed by a single, singly mated queen, ensuring high relatedness of all colony members. Queens may recognise siblings either because they are close kin or because they are nestmates; the former suggests they use intrinsic genetic cues, whereas the latter suggests extrinsic environmental cues are employed to determine mating reluctance. Because bumblebees are social insects it is possible that kin are recognised extrinsically through prior association as has been

found in other species (for example, Frommen *et al.*, 2007). Alternatively, an intrinsic mechanism may have developed. One such mechanism is known as phenotypic matching in which an individual recognises kin by assessing the similarities and differences between its own phenotype and that of unfamiliar conspecifics (Blaustein, 1983). This latter mechanism is thought to occur in the field cricket *Gryllus bimaculatus* where the females use their own cuticular compounds as a phenotypic template (Simmons, 1989).

It is beyond the scope of this paper to attempt to distinguish which method of discrimination *B. terrestris* may be employing. Nevertheless, whatever the underlying cognitive mechanisms, the behaviour observed in this study represents a clear example of kin discrimination (Tang-Martinez, 2001). These findings augment those of Foster (1992), who found that queens of *B. frigidus* and *B. bifarius* preferentially mated with unrelated males. In Foster's experiment the queens were given the choice between mating with a nestmate or a non-nestmate, which might suggest that queens compare males to see which are most different to themselves. However, in our experiment *B. terrestris* queens only ever encountered one type of male and still appeared to discriminate between kin and non-kin.

Such kin recognition and avoidance behaviour is expected to have been strongly selected for in bumblebees to avoid the costs of diploid male production that result from a mating between siblings. This is in accordance with the model of genetic complementarity, which assumes that females do not always choose a male with intrinsically superior genes. They may instead choose males with whom they have a higher genetic compatibility, i.e. the viability of offspring depends on the interaction

between the male and female genotypes (Tregenza & Wedell, 2002). Many studies of genetic complementarity have focused on polyandrous species where there is the potential for postcopulatory female choice (Birkhead & Pizzari, 2002; Colegrave *et al.*, 2002). However, the majority of bumblebee species are monoandrous (Estoup *et al.*, 1995; Schmid-Hempel & Schmid-Hempel, 2000) and in such species there must be some precopulatory indication of a male's relatedness. In the solitary wasp *Philanthus triangulum* this indication is through variation in the male's sex pheromone, which is more similar within than among families (Herzner *et al.*, 2006). In some social Hymenoptera, this indication has been shown to be mediated through the chemical composition of cuticular hydrocarbon recognition pheromones, for example in the bee *Lasioglossum zephyrum* (Smith & Wenzel, 1988), the wasp, *Polistes fuscatus* (Gamboa *et al.*, 1996) and the fire ant *Solenopsis invicta* (Keller & Ross, 1998).

The delayed propensity for *B. terrestris* to mate with siblings, demonstrated in this study, is likely to have been selected for as an inbreeding avoidance mechanism. This in turn decreases the production of costly diploid males. However, in small fragmented populations, mate choice is substantially reduced and sibling matings and diploid male production may become inevitable. This endorses the importance of habitat, and hence population connectivity when considering bumblebee conservation.

3.6 Acknowledgements

I would like to thank Steph O'Connor, Christiane Nitsch and Calum Brown for help with the laboratory work. I am also grateful to James Weir for technical support.

Chapter 4 - Parasite prevalence in *Bombus muscorum* and *Bombus jonellus*

4.1 Abstract

Many bumblebee species have been suffering from significant declines across their range in the Northern Hemisphere over the last few decades. The remaining populations of the rare species are becoming increasingly isolated due to habitat fragmentation and consequently have reduced levels of genetic diversity. The persistence of these populations may be threatened by inbreeding depression, which may result in a higher susceptibility to parasites. Here we investigate the relationship between genetic diversity and parasite prevalence in bumblebees, using the previously studied system of *Bombus muscorum* and *Bombus jonellus* in the Western Isles of Scotland. We recorded parasite prevalence in 17 populations of *B. muscorum* and 13 populations of *B. jonellus* and related the results to levels of heterozygosity. We found that prevalence of the tracheal mite *Locustacarus buchneri* was higher in populations of *B. muscorum* with lower genetic diversity but that there was no such relationship in the more genetically diverse *B. jonellus*. There was no relationship between the prevalence of the gut parasite *Crithidia bombi* and genetic diversity, but older bees were more likely to be infected. The prevalence of *Nosema bombi* and *Apicystis bombi* was too low to analyse. We also found that measures of individual heterozygosity were not as useful as the population level measures of genetic diversity at explaining variations in parasite incidence. This study provides important information on the parasite prevalence of relatively inbred and outbred bumblebee populations and species.

4.2 Introduction

The role bumblebees have as pollinators make them a vital component of ecosystems and also gives them great economic value. Over recent decades many bumblebee species have been declining across their range in the Northern hemisphere, predominantly due to the intensification of agriculture and the resultant loss of habitats (Williams & Osborne, 2009). These declines have been particularly severe in the UK where 3 of the 25 native species have become extinct, 10 species have undergone severe range contractions and 7 species have been placed on the UK Biodiversity Action Plan (Goulson, 2010a). The remaining populations of the rarer species have become isolated in habitat patches where there is still suitable forage and sites for nesting. There are instances of these populations going extinct, despite the continuing presence of good habitat. For example, Wicken Fen in central England supported 14 species of *Bombus* in the 1920s but by 1978 only six remained (Williams, 1986).

In order to implement the appropriate conservation strategies for bumblebees it is important to understand what is driving these remaining populations to extinction. Recent research has suggested that genetic factors might have a role; it has been found that rare species with fragmented populations, such as *B. sylvarum*, *B. humilis* and *B. muscorum* have a much lower genetic diversity than common, widespread species such as *B. terrestris* and *B. pascuorum* (Ellis *et al.*, 2006; Darvill *et al.*, 2006). Detailed study of the genetic diversity and population structure of *B. muscorum* has provided further information. *B. muscorum* has become rare across its range in the UK and is now predominantly found in the Western Isles of

Scotland. Darvill *et al.* (2006) found that the more isolated island populations of *B. muscorum* were genetically differentiated from those closer to the mainland and had substantially reduced genetic diversity. These studies suggest that habitat fragmentation and population isolation has led to inbreeding and a loss of genetic diversity in rare species of bumblebees. If the populations with reduced levels of genetic diversity also have lower fitness, inbreeding depression may be occurring. This might be the mechanism driving these populations towards extinction, as has been demonstrated in other invertebrate species (Saccheri *et al.*, 1998; Reed *et al.*, 2007).

One form of inbreeding depression, which may lead to an increased extinction risk, is higher levels of parasitism (de Castro & Bolker, 2005). Increased homozygosity can increase both the prevalence of parasites at the population level and susceptibility to parasites at the individual level (Frankham *et al.*, 2010). At the population level, the more genetic diversity present, the more likely it is that some individuals can resist a pathogen. If this genetic diversity is lost due to inbreeding, pathogen epidemics may spread more efficiently in the genetically homogenous population. Studies in a wide range of taxa have supported this by demonstrating that the genetic diversity of populations is negatively correlated with pathogen prevalence (for example, Puurtinen *et al.*, 2004; Pearman & Garner, 2005; Ebert *et al.*, 2007).

In order to establish whether a relationship between inbreeding and parasite susceptibility exists at the individual level, knowledge of the individual inbreeding co-efficient (*f*) is informative. This is calculated using detailed pedigree

information, but this is rarely available for wild populations (Marshall *et al.*, 2002). As an alternative, microsatellites have been increasingly used to provide a measure of multi-locus heterozygosity (MLH), which is then used to infer levels of relative inbreeding among individuals. However, there is often a poor correlation between heterozygosity at neutral markers and the true inbreeding co-efficient, particularly in large randomly mating populations (Pemberton, 2004; Slate *et al.*, 2004; Szulkin *et al.*, 2010). Despite this, there are many examples in the literature that present empirical observations of correlations between MLH and fitness related traits (including susceptibility to parasites) and these are known as heterozygosity-fitness correlations (HFCs) (reviewed in Chapman *et al.*, 2009). Whether inbreeding depression can be accurately quantified by measuring HFCs has been the subject of extensive and ongoing debate (for example, David, 1998; Balloux *et al.*, 2004; Slate *et al.*, 2004; Hansson & Westerberg, 2008; Chapman *et al.*, 2009; Ljungqvist *et al.*, 2010).

For HFCs to occur there has to be a correlation between the heterozygosity of functional loci, which have an effect on fitness, and the heterozygosity of neutral loci and such a correlation can result from either linkage disequilibrium (LD) or identity disequilibrium (ID). LD is the non-random association of alleles at two or more loci, which can occur particularly in recently bottle-necked populations. ID is the correlation of heterozygosity or homozygosity across loci and it can arise between any two loci if consanguineous matings occur. Under random mating, ID can result from genetic drift, bottlenecks and admixture (Szulkin *et al.*, 2010). ID can be measured using a sample of multilocus genotypes, by quantifying the excess of double heterozygotes at two loci relative to the expectation under random

association. This gives a parameter known as g2 and is an effective way to determine whether neutral markers do reflect true patterns of underlying inbreeding (David *et al.*, 2007; Szulkin *et al.*, 2010).

Circumstantial evidence does exist that supports the hypothesis that inbred populations of bumblebees are more susceptible to parasites. Firstly, the invasive and highly inbred *B. terrestris* in Tasmania has been found to support very high loads of ectoparasitic mites (Allen *et al.*, 2007). Secondly, in North America, declining bumblebee populations have lower levels of genetic diversity and a significantly higher prevalence of the pathogen *N. bombi* compared to stable bumblebee populations (Cameron *et al.*, 2011). Further support is provided by the theory that suggests genetically heterogeneous colonies of social insects are more resistant to parasitism (Sherman *et al.*, 1988; van Baalen & Beekman, 2006). This assumes that different host genotypes have varying susceptibility to different parasite strains, meaning that a parasitic infection is not likely to spread as rapidly or as far through a genetically heterogeneous colony (Sherman *et al.*, 1988; Schmid-Hempel, 1998). Investigations with bumblebees using lab-reared colonies placed under field conditions have provided support for this theory. Genetically heterogeneous bumblebee colonies had significantly lower prevalence, load and species richness of a range of parasites compared to genetically homogeneous colonies (Liersch & Schmid-Hempel, 1998; Baer & Schmid-Hempel, 2001).

This study aims to further investigate the relationship between genetic diversity and parasitism in bumblebees using the previously studied system of *B. muscorum* and *B. jonellus* in the Western Isles of Scotland. Darvill *et al.* (2010) collected samples

of these species and investigated their genetic diversity, population structure and dispersal ability using microsatellite markers. Both species are found throughout the island system and they make an interesting comparison. *Bombus muscorum* belongs to the subgenus *Thoracobombus* and is considered threatened. It has been placed on the UK Biodiversity Action Plan (UKBAP) along with three other species belonging to its genus. *Bombus jonellus* is a member of the subgenus *Pyrobombus* and has a widespread but local distribution and is not thought to be threatened (Benton, 2006). Darvill *et al.* (2010) found that the two species differed significantly in overall heterozygosity with *B. muscorum* exhibiting much lower genetic diversity. *B. muscorum* also shows markedly higher population structuring and isolation by distance *B. jonellus* ($\theta = 0.13$ compared to $\theta = 0.034$). *B. jonellus* has evidently retained genetic cohesion over greater distances and it was estimated that they are able to disperse >50km relatively frequently. In contrast, *B. muscorum* were estimated to disperse >8km only infrequently and the species also showed an increased frequency of population bottlenecks. These differences in dispersal distances suggest that *B. muscorum* is more susceptible to population isolation due to habitat fragmentation.

This study quantifies the prevalence of parasitic infection in a sub-sample of *B. muscorum* and *B. jonellus* collected by Darvill *et al.* (2010). This allows us to make within species comparison of infection susceptibility in inbred and outbred populations as well as comparisons between relatively inbred and outbred species. As each individual bee has been typed at up to 9 microsatellite loci it is possible to investigate whether any HFCs arise in these species and whether inbreeding is affecting parasite susceptibility on an individual level. Additionally, each

population has a measure of expected heterozygosity and so it is also possible to investigate whether genetic diversity at a population level affects parasite prevalence.

4.3 Methods

During the summers of 2003 to 2005, individuals of *B. muscorum* and *B.jonellus* were collected from islands in the Inner and Outer Hebrides and stored in 100% ethanol. In a previously published study (Darvill *et al.*, 2010), *B. muscorum* were genotyped at 8 microsatellite loci and *B. jonellus* were genotyped at 9 microsatellite loci. This gave each bee a measure of individual heterozygosity (the number of heterozygous loci divided by the number of genotyped loci). A measure of expected heterozygosity for each population was also calculated and these figures are published in Darvill *et al.* (2010) (table 4.1).

For the present study, the width of the thorax was measured using electronic digital calipers and the bee's age was estimated by assessing the extent of wing wear, using a four point scale (modified from Mueller & Wolfmueller, 1993). Before dissection and examination, each bee's abdomen was separated from its thorax and rehydrated by placing in 70% ethanol for approximately 15 hours and then in distilled water for a further 2 hours, before being drained and stored at -80^{o}C until subsequent dissection. Each bee was dissected in order to detect the presence of parasites Firstly, the abdomen was fixed dorsally to a wax tray with pins. Each bee had an individual wax tray to avoid contamination between samples. Micro-scissors were used to cut across the sternite, following the ventral curve and then either side

perpendicularly to the first incision. The ventral surface was then pinned back and insect ringer solution (9.1g NaCl; 0.52g KCl; 1.2g $CaCl_2.2H_2O$; 0.8g $MgCl_2.H_2O$; made to 1000ml distilled water) was pipetted over the abdomen to 'float' the contents. This was inspected under a dissecting microscope for the presence of the tracheal mite *L. buchneri* and any other macroparasites. A heterogeneous sample of fat body, extracted from the ventral & dorsal sides, and malpighian tubules was examined at 400x to determine the presence/absence of the microparasites *N. bombi* and *A. bombi*. The micro-scissors, forceps and pins were all sterilised with MicroSol 3+, followed by 100% Ethanol, between each bee dissection to remove DNA contamination.

The presence/absence of *C. bombi* was determined using diagnostic PCR primers. DNA was extracted using a method modified from Walsh *et al.*, (1991). Bee abdomens were homogenised in a buffer containing 500µl sterile distilled water, 0.025g Chelex-100 (Bio-rad), 17.5µl 1M dithiothreitol and 2.5µl proteinase K (20mg ml^{-1}). Samples were incubated at 56^{o}C for 90mins, then at 95^{o}C for 10mins before being centrifuged for 5mins at 10,000g. The presence of *C. bombi* was then determined by testing whether the parasite specific microsatellite primers Cri4F-Cri4R produced an amplification product (Schmid-Hempel & Funk, 2004). PCRs were run in 10µL volumes using HotStar Taq Plus PCR kits (Qiagen). Each reaction contained 5µl PCR Master Mix, 3µl dH_2O, 0.5µl of each forward and reverse primer (2µM) and 1µl template DNA. PCR amplification involved denaturing at 95 ^{o}C for 5 minutes, followed by 35 cycles of denaturing at 94 ^{o}C for 30 seconds, annealing at 50 ^{o}C for 1 minute and extension at 72 ^{o}C for 1 minute and then a final extension step at 72 ^{o}C for 10 minutes. Three pairs of microsatellite bumblebee primers (B10,

B11 & B96; Estoup *et al.*, 1995; Estoup *et al.*, 1996) were used to establish the presence of amplifiable template within each sample and any that did not yield product were discounted from further analysis of *C. bombi* prevalence. PCR reactions for the bumblebee microsatellite primers were run in 10µL volumes using Multiplex PCR kits (Qiagen). Each reaction contained 5µl PCR Master Mix, 3µl dH_2O, 0.2µM of each of the three primer pairs and 1µl template DNA. PCR amplification involved denaturing at 95 ^oC for 15 minutes, followed by 35 cycles of denaturing at 94 ^oC for 30 seconds, annealing at 50 ^oC for 90 seconds and extension at 72 ^oC for 90 seconds and then a final extension step at 72 ^oC for 10 minutes. All PCR reactions were run in a Peltier thermal cycler (DNA Engine Tetrad 2, Bio-Rad) with the appropriate positive and negative controls. PCR products were visualised on 1% agarose gels stained with 0.25mg ml^{-1} ethidium bromide. PCR was repeated on a subset of samples to ensure a consistent banding pattern.

It should be noted that, in a few cases, the 100% ethanol the bees were originally stored in had partially evaporated and the contents of the abdomen had blackened. However, it was still possible to detect macroparasites through dissection and PCR successfully detected a *C. bombi* infection in a blackened gut so these bees were left in the final analysis.

4.3.1 Statistical analyses

Population level analysis

All parasite data were analysed in R, version 2.12.0 (2010 The R Foundation for Statistical Computing). Binomial generalised linear mixed effect models were used

to investigate whether parasite prevalence (*C. bombi* and *L. buchneri* respectively) was influenced by the level of genetic diversity at the population level. *Bombus muscorum* and *B. jonellus* were analysed separately as island heterozygosity measures are different for the two species. Population-level heterozygosity, mean age, mean bee size (thorax width), mean sampling date, prevalence of the other species of parasite and finally island area as a proxy for bumblebee population size were entered as fixed effects. Sampling year was entered as a random factor.

Individual level analysis

Binomial generalised linear mixed effect models were also used to analyse determinants of parasitic infection on an individual level (presence or absence of *C. bombi* and *L. buchneri* respectively), with each bee as a replicate. Fixed effects included: bumblebee species, individual heterozygosity, age (entered as a co-variate with a four point scale), bee size (thorax width), sampling date (entered as a co-variate, numbered continuously from June 1st through to September) and infection status with the other species of parasite. Island and sampling year were entered as random factors.

Locustacarus buchneri abundance (the number of adult *L. buchneri* present in the abdomen, including uninfected bees) was analysed in a Bayesian framework using the MCMCglmm package in R (Hadfield, 2010). Generalised linear mixed models with a zero-inflated Poisson distribution were used and non-informative priors were set in all analyses. Prior sensitivity analysis was carried out and the final models are robust to variation in the values of priors. Model convergence was confirmed using Geweke's diagnostic (Geweke, 1992) and visual examination of the model output.

Fixed effects included: bumblebee species, individual heterozygosity, age, bee size, sampling day and infection with the other species of parasite. Island and sampling year were entered as random factors. Parameter estimates reported are means from the posterior distribution with 95% lower and upper credible intervals (CI). *L. buchneri* infection intensity (the number of adult *L. buchneri* present in the abdomen, excluding uninfected bees) was also analysed. In this case, generalised linear mixed models were used with penalised quasi-likelihood. The fixed effects and random factors were the same as for the MCMCglmm models.

All statistical tests were two-tailed and models were selected and simplified according to Aikaike's Information Criterion (AIC). All two-way interactions were investigated, not one of which was significant and so they are not presented here.

Computation of the estimate g_2

The microsatellite data were also used to calculate the parameter g_2 and its standard error for each of the island populations for each species. The parameter was computed in the software RMES, provided by David *et al.* (2007). This software also tests whether g_2 is significantly different from zero and thus indicates whether covariance in individual heterozygosity i.e. identity disequilibrium (ID), is occurring.

Figure 4.1 A map showing the location of the islands from which samples of B. muscorum and B. jonellus were taken (adapted from Darvill *et al.*, 2010)

4.4 Results

A total of 506 *B. muscorum* and 360 *B. jonellus* workers were dissected. The *B. muscorum* samples came from 17 island populations with a mean sample size of 29.8 (range: 20 to 41) from each island. The *B. jonellus* samples came from 13 island populations with a mean sample size of 27.7 (range: 18 to 30) from each island. The tracheal mite *L. buchneri* was the only macroparasite detected in these samples and had an overall frequency of 32%. The parasite was present in 15 out of

the 17 populations sampled for *B. muscorum* and it was present in all populations of *B. jonellus* (table 4.1).

Nosema bombi was detected at a very low overall prevalence (0.68%) and on only the following islands: Coll (1 infected *B. jonellus*), Mingulay (1 infected *B. muscorum*), Sandray (1 infected *B. muscorum*) and Tiree (3 infected *B. muscorum*). *Apicystis bombi* occurred at the same very low overall prevalence (0.68%) and was detected on the following islands: Barra (2 infected *B. muscorum*), Muck (2 infected *B. muscorum*), Muldoanich (1 infected *B. muscorum*) and South Uist (1 infected *B. jonellus*). Due to these low infection rates it was not possible to carry out any further analyses on these two parasite species.

It was possible to extract DNA and test for the presence of *C. bombi* from a total of 492 *B. muscorum* and 345 *B. jonellus*. The overall prevalence of *C. bombi* was 18.6% and it was present in 16 out of 17 *B. muscorum* populations and 10 out of the 13 *B. jonellus* populations (table 4.1).

Table 4.1 Population means for host genetic diversity and parasite prevalence. The figures in parentheses are the standard errors for genetic diversity and the 95% C.I. for parasite prevalence. Measures for heterozygosity are taken from Darvill et al., (2010).

		Bombus muscorum					***Bombus jonellus***				
	Island size	**He**	***Crithidia bombi* prevalence**		***Locustacarus buchneri* prevalence**		**He**	***Crithidia bombi* prevalence**		***Locustacarus buchneri* prevalence**	
	km^2		n	Mean	n	Mean		n	Mean	n	Mean
Barra	67	0.393 (0.113)	30	0.17 (0.06-0.35)	30	0.50 (0.32-0.68)	0.766 (0.048)	30	0.20 (0.08-0.39)	30	0.67 (0.47-0.82)
Canna	14	0.433 (0.086)	30	0.10 (0.03-0.28)	30	0.23 (0.11-0.43)	0.758 (0.070)	26	0.23 (0.10-0.44)	30	0.23 (0.11-0.43)
Coll	73	0.499 (0.091)	29	0.14 (0.05-0.33)	29	0.34 (0.19-0.54)	0.738 (0.073)	30	0.30 (0.15-0.50)	30	0.30 (0.15-0.50)
Colonsay	44	0.416 (0.086)	20	0.05 (0.003-0.27)	20	0.15 (0.04-0.39)	-	-	-		-
Eigg	30	0.533 (0.094)	24	0.17 (0.05-0.38)	30	0 (0-0.14)	0.757 (0.066)	21	0.24 (0.09-0.48)	30	0.37 (0.21-0.56)
Lunga	1	0.507 (0.108)	30	0.10 (0.03-0.28)	30	0 (0-0.14)	0.742 (0.076)	30	0.17 (0.06-0.35)	30	0.33 (0.18-0.53)
Mingulay	6	0.374 (0.115)	24	0 (0-0.17)	30	0.33 (0.18-0.53)	0.696 (0.048)	18	0 (0-0.22)	18	0.22 (0.07-0.48)
Monachs	4	0.305 (0.092)	29	0.13 (0.05-0.33)	30	0.13 (0.04-0.32)	-	-	-	-	-
Muck	6	0.425 (0.088)	26	0.19 (0.07-0.40)	30	0.20 (0.08-0.39)	0.751 (0.056)	24	0.25 (0.11-0.47)	30	0.20 (0.08-0.39)
Muldoanich	1	0.421 (0.103)	26	0.04 (0.002-0.22)	26	0.62 (0.41-0.79)	-	-	-	-	-
N. Uist	308	0.404 (0.113)	25	0.16 (0.05-0.37)	20	0.25 (0.10-0.49)	-	-	-	-	-
Pabbay	3	0.399 (0.118)	29	0.03 (0.002-0.20)	30	0.53 (0.35-0.71)	0.729 (0.046)	22	0 (0-0.19)	22	0.82 (0.59-0.94)
Rum	109	0.451 (0.077)	29	0.07 (0.01-0.24)	29	0.21 (0.09-0.40)	0.749 (0.079)	28	0.07 (0.01-0.25)	28	0.32 (0.17-0.52)
S. Uist	309	0.404 (0.113)	35	0.29 (0.15-0.47)	25	0.40 (0.22-0.61)	0.755 (0.054)	22	0.14 (0.04-0.36)	22	0.32 (0.15-0.55)
Sandray	4	0.367 (0.111)	30	0.10 (0.03-0.28)	30	0.57 (0.38-0.74)	0.763 (0.054)	29	0 (0-0.15)	30	0.47 (0.29-0.65)
Staffa	0.5	0.484 (0.091)	30	0.37 (0.21-0.56)	46	0.09 (0.03-0.22)	0.697 (0.082)	30	0.50 (0.32-0.68)	30	0.53 (0.35-0.71)
Tiree	75.25	0.499 (0.086)	41	0.46 (0.31-0.62)	41	0.27 (0.15-0.43)	0.715 (0.076)	30	0.63 (0.44-0.79)	30	0.27 (0.13-0.46)
Overall		**0.437 (0.015)**	**487**	**0.16 (0.13-0.20)**	**506**	**0.28 (0.24-0.32)**	**0.743 (0.005)**	**340**	**0.22 (0.18-0.27)**	**360**	**0.39 (0.33-0.43)**

4.4.1 Population level results

Bombus muscorum

The prevalence of *C. bombi* was not predicted by the overall heterozygosity of *B. muscorum* populations. (Z = -0.832, P = 0.406). Age, as measured by wing wear, and bee size both significantly influenced *C. bombi* prevalence, with populations with a lower mean age and smaller mean size more likely to be highly infected (Z = -2.86, P = 0.004 and Z = -5.14, P < 0.001 respectively). No other variable significantly affected the prevalence of *C. bombi* (table 4.2).

There was, however, a significant negative correlation between the prevalence of *L. buchneri* and *B. muscorum* population heterozygosity (Z = -3.51, P < 0.001, figure 4.2). There was also a significant positive correlation between island size and *L. buchneri* prevalence (Z = 3.02, P = 0.003). No other variable significantly affected the prevalence of *L. buchneri* (table 4.2).

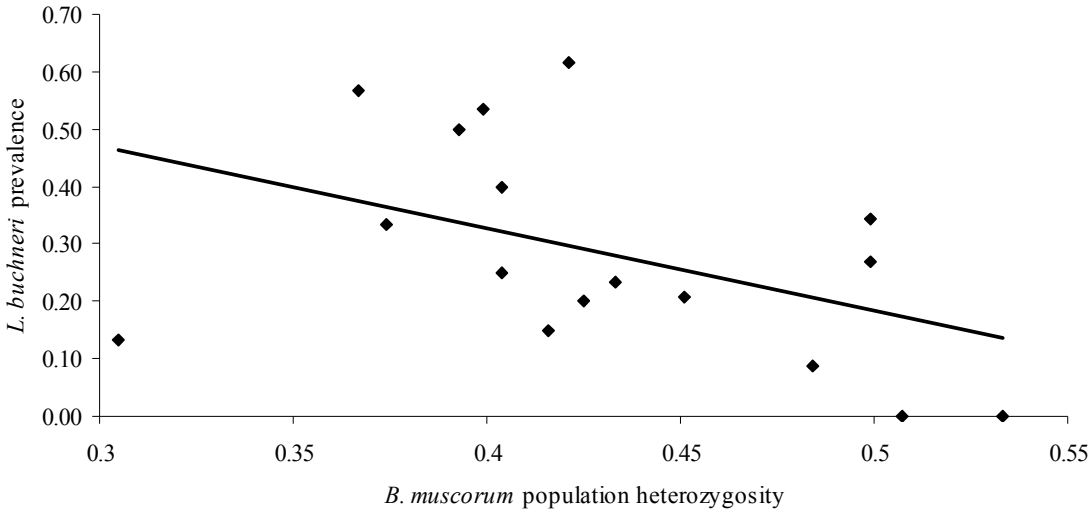

Figure 4.2 Relationship between *L. buchneri* prevalence and heterozygosity of host population.

Each point represents an island population. Islands with higher heterozygosity had significantly lower prevalence of *L. buchneri* ($P < 0.001$, table 4.2).

Table 4.2 Output of binomial generalised linear mixed effect models for the prevalence of *C. bombi* and *L. buchneri* in *B. muscorum* populations. Degrees of freedom are given in parentheses.

	Crithidia bombi				*Locustacarus bombi*			
	Co-efficient estimate	SE	Z	P	Co-efficient estimate	SE	Z	P
Heterozygosity of population	-3.096	3.722	-0.832 (1)	0.406	-9.169	2.61	-3.51 (1)	**<0.001**
Date	0.004	0.015	0.282 (1)	0.778	0.007	0.017	0.424 (1)	0.672
Age	-1.664	0.581	-2.864 (1)	**0.004**	-0.475	0.677	-0.702 (1)	0.483
Bee size	-3.859	0.751	-5.140 (1)	**<0.001**	-0.58	1.17	-0.494 (1)	0.672
Island Area	0.003	0.001	1.862 (1)	0.068	0.005	0.002	3.023 (1)	**0.003**
L. buchneri prevalence	-0.676	0.935	-0.723 (1)	0.469	-	-	-	-
C. bombi prevalence	-	-	-	-	-0.632	1.11	-0.571	0.568

Bombus jonellus

The prevalence of *C. bombi* was not predicted by the overall heterozygosity of *B. jonellus* populations. ($Z = -1.03$, $P = 0.301$). Age, as measured by wing wear, and bee size both significantly influenced *C. bombi* prevalence, but with the opposite trend to *B. muscorum*: populations with a higher mean age and greater mean size were more likely to be highly infected ($Z = 2.28$, $P = 0.022$ and $Z = 2.16$, $P = 0.031$ respectively). Populations that were on average sampled earlier in the season had a higher prevalence of *C. bombi* ($Z = -3.54$, $P < 0.001$). Populations that had a higher mean prevalence of *L. buchneri* also had a greater prevalence of *C. bombi* ($Z = 2.19$, $P =$

0.028). No variable significantly influenced the overall prevalence of *L. buchneri* in *B. jonellus* populations (table 4.3).

Table 4.3 Output of binomial generalised linear mixed effect models for the prevalence of *C. bombi* and *L. buchneri* in *B. jonellus* populations. Degrees of freedom are given in parentheses.

	Crithidia bombi				*Locustacarus bombi*			
	Co-efficient estimate	SE	Z	P	Co-efficient estimate	SE	Z	P
Heterozygosity of population	-10.364	10.023	-1.034 (1)	0.301	3.452	5.26	0.66 (1)	0.512
Date	-0.227	0.064	-3.535 (1)	**<0.001**	0.017	0.089	0.925 (1)	0.355
Age	2.859	1.253	2.283 (1)	**0.022**	-0.801	0.418	-1.915 (1)	0.056
Bee size	7.466	3.458	2.159 (1)	**0.031**	-0.118	1.701	-0.069 (1)	0.945
Island Area	-0.008	0.005	-1.639 (1)	0.101	-0.001	0.003	-0.289 (1)	0.772
L. buchneri prevalence	5.842	2.666	2.19 (1)	**0.028**	-	-	-	-
C. bombi prevalence	-	-	-	-	0.092	0.939	0.098 (1)	0.922

4.4.2 Individual level results

***Crithidia bombi* presence/absence**

Bombus jonellus were more frequently infected with *C. bombi* than *B. muscorum* (χ^2 = 4.77, df = 1, p = 0.029, figure 4.3) and the proportion of bees infected also increased

with age in both species (χ^2 = 7.68, df = 1, p = 0.006, figure 4.4). Neither the heterozygosity of individual bees, the size of the bee, whether it was infected with *L. buchneri* or the sampling date significantly affected the likelihood of *C. bombi* infection (table 4).

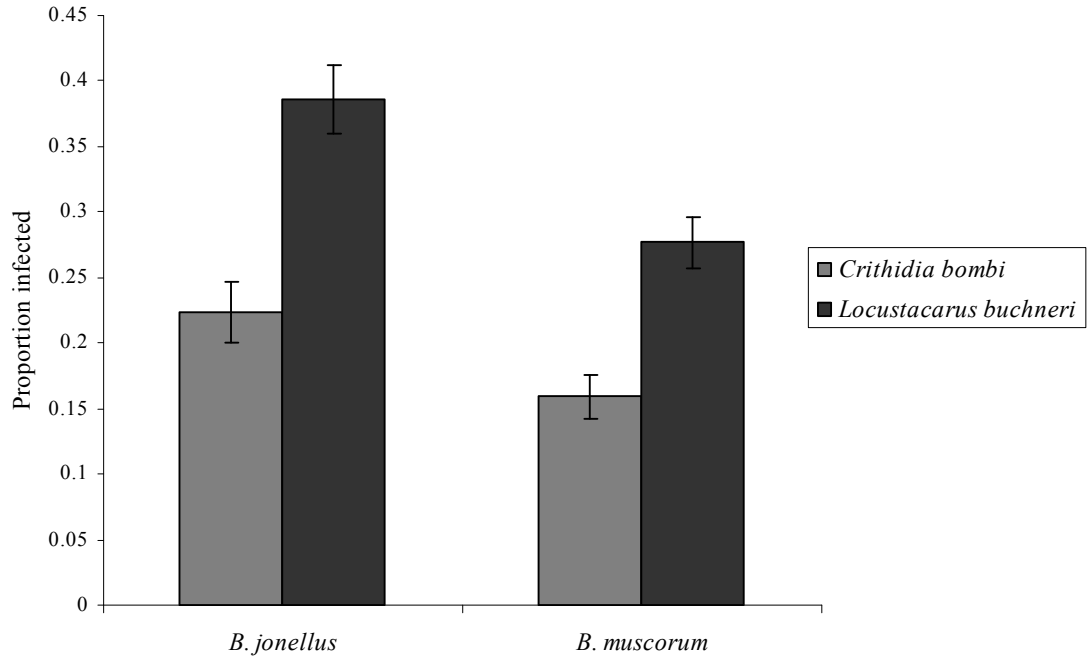

Figure 4.3 Proportion of *B. jonellus* and *B. muscorum* infected with *C. bombi* and *L.buchneri*.

A greater proportion of *B. jonellus* were infected with *C. bombi* and *L. buchneri* compared to *B. muscorum* (p = 0.029 and p = 0.001 respectively). Bars represent the mean prevalence and their standard errors.

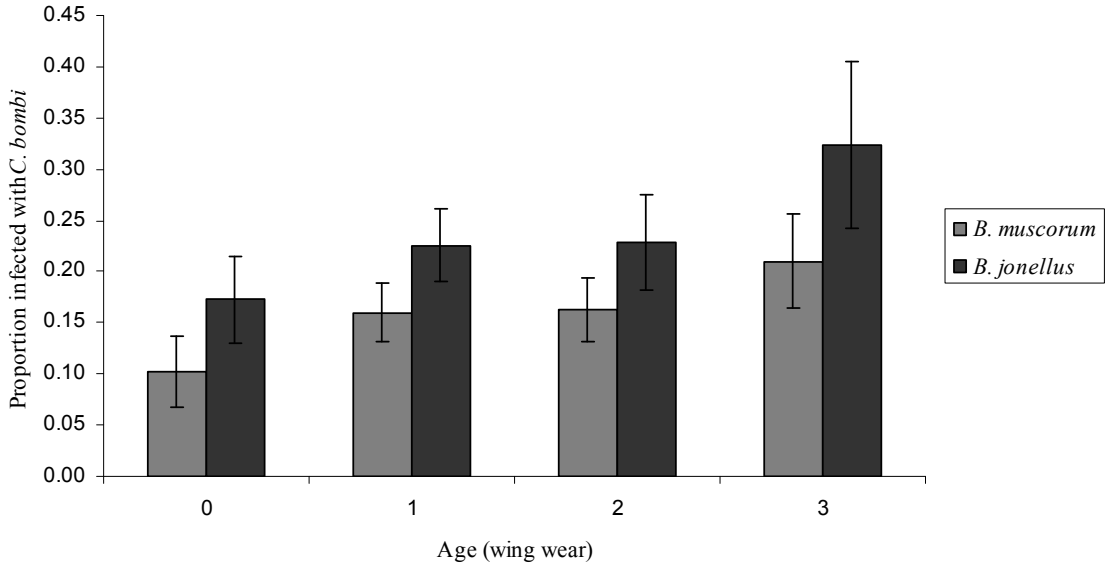

Figure 4.4 The prevalence *C. bombi* infection in different age groups, with wing wear as a proxy for age.

The likelihood of bees carrying *C. bombi* infections significantly increased with age ($p = 0.006$) when both host species were pooled.

Locustacarus buchneri presence/absence

Bombus jonellus were also more frequently infected with *L. buchneri than B. muscorum* ($\chi^2 = 10.12$, df = 1, $p = 0.001$, figure 4.3). Bees sampled later in the season were more likely to be infected ($\chi^2 = 4.79$, df = 1, $p = 0.029$) and infected bees were also significantly less likely to be infected with *C. bombi* ($\chi^2 = 42.82$, df = 1, $p < 0.001$). Individual heterozygosity, bee age and size did not significantly predict whether bees were infected with *L. buchneri* (table 4.4).

Table 4.4 Output of binomial generalised linear mixed effect models for the presence/absence of *C. bombi* and *L. buchneri* respectively. Degrees of freedom are given in parentheses. Log likelihood ratio tests provide χ2 and p values for each term.

	Crithidia bombi				*Locustacarus bombi*			
	Co-efficient estimate	SE	χ^2	P	Co-efficient estimate	SE	χ^2	P
B. muscorum compared to *B. jonellus*	-0.455	0.203	4.766 (1)	**0.029**	-0.529	0.166	10.12 (1)	**0.001**
Individual heterozygosity	0.144	0.701	0.042 (1)	0.839	-0.106	0.547	0.037 (1)	0.848
Age	0.294	0.106	7.679 (1)	**0.006**	0.141	0.085	2.741 (1)	0.098
Bee Size	-0.213	0.256	0.69 (1)	0.407	0.101	0.204	0.241 (1)	0.623
Sampling date	-0.006	0.009	0.417 (1)	0.518	0.024	0.009	4.791 (1)	**0.029**
L. buchneri presence	-0.006	0.024	0.073 (1)	0.788	-	-	-	-
C. bombi presence	-	-	-	-	-0.222	0.218	42.82 (1)	**<0.001**

Locustacarus buchneri abundance

Locustacarus buchneri abundance (the number of adult mites infecting a bee, including those bees that were uninfected) was significantly higher in *B. jonellus* compared to *B. muscorum* (p = 0.024, table 4.5). The abundance of *L. buchneri* also marginally increased with age (p = 0.050). Individual heterozygosity, the size of the bee, whether it was infected with *C. bombi* and the sampling date did not significantly affect likelihood of *L. buchneri* infection (table 4.5).

Table 4.5 MCMCglmm output for *L. buchneri* abundance

The parameter estimates shown here are with reference to *B. jonellus* and are on the log scale. The MCMC procedure for this model had a burn-in period of 5000, a total of 505,000 iterations and a thinning interval of 500. P-values ≤0.05 are written in bold.

	Parameter estimate	Lower 95% CI	Upper 95% CI	P value
Locustacarus buchneri abundance	2.111	-0.827	4.579	0.140
Species				
B. muscorum	-0.852	-1.652	-0.142	**0.024**
Heterozygosity	-0.901	-2.188	0.233	0.156
Age	0.195	0.005	0.388	**0.050**
Bee size	-0.065	-0.537	0.418	0.798
Sampling date	-0.005	-0.370	0.684	0.596
C. bombi presence	0.165	-0.028	0.018	0.657

However, when *L. buchneri* infection intensity was analysed (excluding all those bees that were uninfected) the results were different. No variable was found to significantly affect the load of *L. buchneri* within individual bees. This suggests that the difference in *L. buchneri* abundance between bumblebee species (table 4.5) is largely due to the higher number of uninfected *B. muscorum* compared to *B. jonellus* (table 4.6).

Table 4.6 The mean number of *L. buchneri* infecting the bumblebee hosts. Standard errors are given in parentheses.

	Including uninfected hosts		Excluding uninfected hosts	
	n	Mean	n	Mean
B. muscorum	506	1.75 (0.236)	139	6.35 (0.730)
B. jonellus	360	2.53 (0.278)	139	6.55 (0.573)

4.4.3 Relationship between microsatellite markers and levels of inbreeding

The parameter g_2 was computed for the 17 *B. muscorum* populations and the 13 *B. jonellus* populations to investigate the occurrence of identity disequilibrium. In each *B. muscorum* population at least one locus was not used in the calculation as there were no heterozygotes at those loci in the population. All 9 loci were used in the calculations of g_2 for *B. jonellus*. g_2 was only significantly different from zero in one population: *B. jonellus* on Mingulay (table 4.7 & 4.8).

Table 4.7 g_2 estimates for *B. muscorum* populations.

	Bombus muscorum					
	n	No. loci genotyped	No. loci used in g2 estimate	g2	SE	p
Barra	50	8	5	0.0001	0.0051	0.437
Canna	62	8	7	0.0305	0.0039	0.143
Coll	70	8	6	-0.0151	0.0026	0.722
Colonsay	67	8	7	-0.0104	0.0033	0.618
Eigg	64	8	7	0.0076	0.0017	0.225
Lunga	36	8	7	0.0285	0.0051	0.095
Mingulay	49	8	5	0.0833	0.0061	0.378
Monachs	44	8	6	0.0074	0.0055	0.355
Muck	52	8	7	0.0099	0.0037	0.371
Muldoanich	25	8	6	-0.0153	0.0136	0.515
North Uist	50	8	7	0.0192	0.0038	0.172
Pabbay	37	8	6	0.0337	0.0067	0.139
Rum	42	8	7	-0.0233	0.0029	0.856
South Uist	37	8	7	-0.0219	0.0055	0.695
Sandray	58	8	5	-0.0419	0.0045	0.887
Staffa	52	8	7	-0.0111	0.0025	0.693
Tiree	119	8	6	0.0113	0.0018	0.259

Table 4.8 g_2 estimates for *B. jonellus* populations.

	Bombus jonellus					
	n	No. loci genotyped	No. loci used in g2 estimate	g2	SE	p
Barra	82	9	9	-0.0074	0.0006	0.912
Canna	44	9	9	0.0036	0.0013	0.237
Coll	44	9	9	0.0047	0.0016	0.243
Colonsay	28	9	9	-0.0136	0.0017	0.920
Eigg	36	9	9	0.0012	0.0014	0.374
Lunga	38	9	9	0.0053	0.0019	0.261
Mingulay	32	9	9	0.0273	0.0043	0.018
Muck	47	9	9	-0.0005	0.0011	0.563
North Uist	50	9	9	-0.0139	0.0012	0.949
Pabbay	36	9	9	0.0180	0.0031	0.056
Rum	32	9	9	-0.0058	0.0012	0.811
South Uist	42	9	9	-0.0062	0.0011	0.769
Sandray	25	9	9	0.0003	0.0024	0.394
Staffa	46	9	9	-0.0063	0.0012	0.729
Tiree	56	9	9	0.0005	0.0012	0.425

4.5 Discussion

This study demonstrates that there is a relationship between the genetic diversity of bumblebee populations and the prevalence of at least one species of parasite. We found that *B. muscorum* populations with lower levels of heterozygosity had higher prevalence of the tracheal mite *L. buchneri*. This supports previous evidence that suggests low heterozygosity causes increased parasite prevalence in bumblebees. Firstly, high loads of ectoparasitic mites have been found on inbred bumblebees in Tasmania (Allen *et al.*, 2007) and secondly, a higher prevalence of *N. bombi* has been found in bumblebee populations that have reduced genetic diversity in the US (Cameron *et al.*, 2011). These findings are all in accordance with previous experimental work that has found genetic heterogeneity within colonies to be

negatively correlated with parasitic infections in social insects (Baer & Schmid-Hempel, 2001; Hughes & Boomsma, 2004; Seeley & Tarpy, 2007).

Relatively little research has been conducted on *L. buchneri* but limited data suggest that heavy infections might be associated with lethargy and reduced foraging (Husband & Sinha, 1970). In contrast, *Acarapis woodi*, the tracheal mite of honey bees *Apis mellifera*, has been studied in more detail. For example, experimental work has found that infection with *A. woodi* causes a reduction in the metabolic rate of individual bees and this may constrain activity, particularly in cool weather (Harrison *et al.*, 2001). Additionally, a review by McMullan & Brown (2009) acknowledges that honey bee colonies infected with tracheal mites have a greater mortality and this is again temperature dependent. It is certainly possible that *L. buchneri* inflicts similar costs on bumblebees. Parasitic infection may also have indirect effects on fitness simply by stimulating the immune system and *L. buchneri* infection can trigger a melanisation response in the host's trachea (pers. obs.). Colonies whose workers are immune challenged may have lower reproductive output, an effect that is exacerbated by harsh environmental conditions (Moret & Schmid-Hempel, 2001; Moret & Schmid-Hempel, 2004). Therefore, parasitism is likely to exert fitness costs on the hosts and as prevalence is higher in less genetically diverse populations, it may increase their risk of extinction as suggested by de Castro & Bolker (2005).

In contrast to the observations in *B. muscorum* there was no relationship between the prevalence of *L. buchneri* and the genetic diversity of *B. jonellus* populations. This may be a result of the appreciably lower range in the measures of population heterozygosity (only 0.019 compared to 0.228 for *B. muscorum*), which may mask

any influence that genetic diversity has on parasite prevalence. It could also result from the fact that *B. jonellus* is a more genetically diverse species than *B. muscorum* and has a greater dispersal ability, hence is more likely to avoid inbreeding depression (Darvill *et al.*, 2010). In contrast to *L. buchneri*, there was no relationship between the population heterozygosity in either bumblebee species and the prevalence of *C. bombi* (but see Chapter 5 and the General Discussion).

Although there was a population level relationship between genetic diversity in *B. muscorum* and prevalence of *L. buchneri*, there was no such relationship between individual heterozygosity and infection. In addition, there was no correlation between the individual heterozygosity in both bumblebee species and infection with *C. bombi*. This could be because inbreeding is not affecting susceptibility to parasites at an individual level. Another explanation is that heterozygosity at the neutral markers genotyped is not a good indicator of underlying inbreeding in these species. This is confirmed by the g_2 estimates, which suggest that in all but one population there is no identity disequilibrium in the microsatellites and so HFCs are unlikely to arise (Ljungqvist *et al.*, 2010; Szulkin *et al.*, 2010). This is possibly due to the relatively small number of loci genotyped, particularly in *B. muscorum* where not all the loci were used in computation of the g_2 estimates. A study by Slate & Pemberton (2002) concluded that in order to reliably detect HFCs a panel of ten or more microsatellite markers were needed.

The results from this study suggest that correlations between population genetic diversity and fitness can indicate the occurrence of inbreeding depression but that genetic diversity at neutral markers does not necessarily reflect individual levels of

inbreeding. These findings are in agreement with those found by Vali *et al.* (2008), who investigated the extent to which microsatellites (genotyped at 10-27 loci) explained nucleotide genetic diversity in populations of four different mammal species. They found that nucleotide diversity was correlated with population genetic diversity but there was a very weak association between microsatellite diversity and nucleotide diversity at the individual level. Other studies have also found that MLH is an unreliable predictor of individual genetic diversity (for example, Hedrick, 2001; Pemberton, 2004; Slate *et al.*, 2004).

The prevalence of the four parasite species observed in this study are comparable to those found in studies conducted elsewhere in Europe (Korner & Schmid-Hempel, 2005; Paxton, 2005). In the analyses of *C. bombi* and *L. buchneri*, *B. jonellus* had consistently higher infection rates compared to *B. muscorum*. This could reflect the inability of the more inbred *B. muscorum* to survive high levels of infection meaning that high parasite prevalence was not observed. However, it is perhaps more likely that this observation is simply due to an inter-specific difference in the parasitism rates of these two species, as such differences are commonly found in bumblebees (for example, Shykoff & Schmid-Hempel, 1991b; Korner & Schmid-Hempel, 2005). The reasons behind these differences remain unknown but are likely to relate to inter-specific variation in host genetics and parasite defence, environmental factors or parasite virulence.

On an individual level, infection with *C. bombi* is positively correlated with the age of bees in both species, as estimated by wing wear. This relationship is expected as the older bees are more likely to have been exposed to *C. bombi* during the course of their

lives and are therefore more likely to be infected. The trend could also relate to senescence of the immune system, which is known to occur under laboratory conditions where two key immune processes have been shown to decline with age (Doums *et al.*, 2002; Moret & Schmid-Hempel, 2009). On a population level, the relationship appears different for *B. muscorum* as populations with a lower mean age have a higher prevalence of *C. bombi*. This pattern could be expected if *C. bombi* is exerting a fitness cost on its hosts, which is likely as this parasite is known to increase the mortality rate of food-stressed worker bees by up to 50% (Brown *et al.*, 2000). Therefore, in populations of *B. muscorum* that have a higher overall prevalence of the parasite, it is mainly the younger bees that are left alive. The opposite trend is observed in *B. jonellus*: higher prevalence of *C. bombi* was observed in populations that have a greater mean age. This bumblebee species has a higher genetic diversity than *B. muscorum* and therefore may have an overall greater fitness and resistance to parasites. Consequently, bees may be able to survive for longer when infected with *C. bombi* and then it would be expected that the relationship between age and infection would be the same as that observed for the individual bees.

Bee size was another significant predictor of *C. bombi* prevalence in both bumblebee species and had the same relationship as age. In *B. muscorum*, a higher prevalence of *C. bombi* was observed in populations that had a smaller mean size and the opposite was true for *B. jonellus*. There is no evidence in the literature to suggest that the size of bumblebees influences their susceptibility to *C. bombi*, although this does remain a possibility. However, this relationship is more likely to have been due to an underlying relationship between age and size. Indeed, a Pearson's correlation between bee age and size with both species pooled revealed a significant positive relationship.

However, when each species was analysed separately there was no such relationship, which is why both variables were left in the analyses. It is likely that older bees are more abundant towards the end of the sampling period and previous studies have found that in some bumblebee species, forager size also increases throughout the season due to colonies producing larger workers (for example, Knee & Meddler, 1965; Plowright & Jay, 1968). Goulson *et al.* (2002) argue that larger foragers might survive for longer. Therefore, it is possible that, on average, older bumblebees are also larger, which would explain why age and size both predict *C. bombi* prevalence.

In conclusion, this study has demonstrated that low genetic diversity in *B. muscorum* populations is associated with a higher prevalence of the tracheal mite *L. buchneri*. This supports theories that suggest some parasite species can spread to higher prevalence in populations that are more genetically homogeneous. No such relationship was observed in *B. jonellus,* possibly due to the greater and less variable genetic diversity of this species. We also find that the number of microsatellites genotyped is not sufficient to detect any heterozygosity-fitness correlations at the individual level. However, the main finding does support the hypothesis that the persistence of small, isolated populations of bumblebees may be threatened due to inbreeding and the associated effects on levels of parasitic infection.

4.6 Acknowledgements

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Chapter 5 - Genetic diversity, parasite prevalence and immunity in wild bumblebees

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5.1 Abstract

Inbreeding and a consequent loss of genetic diversity threaten small, isolated populations. One mechanism by which genetically impoverished populations may become extinct is through decreased immune competence and higher susceptibility to parasites. Here we investigate the relationship between immunity and inbreeding in bumblebees, using Hebridean island populations of *Bombus muscorum*. We sampled nine populations and recorded parasite prevalence and measured two aspects of immunity: the encapsulation response and levels of phenoloxidase. We found that prevalence of the gut parasite *Crithidia bombi* was higher in populations with lower genetic diversity. Neither measure of immune activity was correlated with genetic diversity. However, levels of phenoloxidase declined with age and were also negatively correlated with parasite abundance. Our results suggest that as insect populations lose heterozygosity, the impact of parasitism will increase, pushing threatened populations closer to extinction.

5.2 Introduction

Genetic diversity is crucial in maintaining the fitness of populations by allowing them to withstand short-term environmental perturbations and evolve in response to long-term environmental change (Frankham *et al*., 2010). Small, isolated populations are at risk from losing their genetic diversity, either over the long term, predominantly as a result of genetic drift (Frankham *et al.*, 2010) or over the short term as a result of inbreeding (Keller & Waller, 2002). This can lead to inbreeding depression if there is

a loss of reproductive fitness, usually due to the increase in frequency of individuals homozygous for deleterious recessive alleles (Charlesworth & Charlesworth, 1999). In turn this may significantly increase the risk of local population extinctions (Frankham, 2005; O'Grady, 2006; Reed *et al.*, 2007).

One driver of extinction in genetically impoverished populations may be parasitism (de Castro & Bolker, 2005). Inbreeding and increased homozygosity can increase both the prevalence of parasites at the population level and susceptibility to parasites at the individual level (Frankham *et al.*, 2010). At the population level, a loss of genetic diversity due to inbreeding reduces the capacity of the population to evolve in response to novel virulent parasite genotypes. The more genetically diverse a population is, the more likely it is that some individuals can resist a pathogen. Studies in vertebrates have supported this, showing the genetic diversity of populations from a wide range of taxa is negatively correlated with pathogen prevalence (for example, Pearman & Garner, 2005; Hedrick *et al.*, 2001). At the individual level, lower heterozygosity may be associated with higher infection frequency and greater infection morbidity (Coltman *et al.*, 1999; Acevedo-Whitehouse *et al.*, 2006).

The studies that have addressed the effects of inbreeding on immunity and parasitism in invertebrates have demonstrated that the relationship is complex and can depend on host sex and genotype, as well as parasite species (Stevens *et al.*, 1997; Rantala & Roff, 2007; Haag *et al.*, 2003). However, inbreeding can decrease invertebrate pathogen resistance at the individual level, either through the loss of specific resistance alleles (Spielman *et al.*, 2004), reduced defensive behaviour (Luong *et al.*, 2007) or a lower efficacy of group level disease resistance (Calleri *et al.*, 2006). At

the population level, parasite transmission and the probability of infection is higher in inbred populations of some species (Puurtinen *et al.*, 2004; Ebert *et al.*, 2007). However, correlations between heterozygosity and parasite prevalence are not universal (Trouve *et al.*, 2003).

It is important to understand the relationship between genetic diversity and parasitism in bumblebees as many species have suffered from significant population declines across their range, predominantly due to the loss of habitats on which they depend (Williams & Osborne, 2009). The remaining populations of the rare species have become fragmented and genetically isolated. While common bumblebee species exhibit little genetic differentiation between populations (Estoup *et al.*, 1996; Widmer & Schmid-Hempel, 1999), rare species appear to have much lower genetic diversity and considerable population subdivision (Ellis *et al.*, 2006). One example is *B. muscorum*, which is a rare and declining bumblebee species in the UK, now predominantly found in the Western Isles of Scotland. Isolated island populations have substantially reduced genetic diversity (Darvill *et al.*, 2006). *B. muscorum* shows markedly higher population structuring and isolation by distance than the coexisting *Bombus jonellus*, possibly due to its poor dispersal ability (Darvill *et al.*, 2010). Hence it is more susceptible to population isolation and inbreeding.

To date, only one study has tested how inbreeding in bumblebees influences immunity at the individual level. Gerloff *et al.* (2003) found one generation of sib-mating had no negative impact on the encapsulation response. Nevertheless, parasite load may be greater in locations where inbreeding is most acute as theory suggests that genetically diverse colonies of social insects have a selective advantage due to higher parasite

resistance (Sherman *et al.*, 1988; van Baalen & Beekman, 2006). This assumes that different host genotypes have varying susceptibility to different parasite strains, meaning that a parasitic infection is not likely to spread as rapidly or as far through a genetically heterogeneous colony (Sherman *et al.*, 1988; Schmid-Hempel, 1998). Investigations with bumblebees using lab-reared colonies placed under field conditions have provided support for this theory. Genetically heterogeneous bumblebee colonies had significantly lower prevalence, load and species richness of a range of parasites compared to genetically homogeneous colonies (Liersch & Schmid-Hempel, 1998; Baer & Schmid-Hempel, 2001).

Here we aim to test the hypothesis that wild bumblebee populations that have a lower genetic diversity have a concomitant decrease in immunocompetence and an increase in parasite prevalence. We use the previously studied island populations of *B. muscorum* in the Western Isles of Scotland and measures of population genetic diversity are taken from Darvill *et al.* (2006). Immune competence was estimated by measuring two aspects of constitutive immunity; the encapsulation response and levels of the enzyme phenoloxidase (PO). The encapsulation response assay is a well established method of measuring an insect's ability to respond to a foreign body and the synthetic implant provides a standardised challenge against which individual responses can be compared (König & Schmid-Hempel, 1995). PO is a key component of the invertebrate immune system; it is stored as the inactive precursor pro-PO and activated when infection is detected (Soderhall & Cerenius, 1998). Parasite prevalence was measured by dissecting bees and recording any parasitic infections present. These investigations allowed us to assess the impact of inbreeding on parasitism and immune parameters in wild insect populations.

5.3 Methods

Nine Hebridean islands off the west coast of Scotland were visited between 4th August and 20th August 2009 (Barra, Coll, Iona, Mingulay, North Uist, South Uist, Sandray, Staffa, and Tiree). A total of 246 *B. muscorum* workers were collected, with a mean of 27.3 (range: 23 to 30) from each island. As samples were taken in the peak season for bumblebees, the numbers taken were unlikely to negatively impact the fitness of colonies. Collected bees were stored in hair curlers (Superdrug, UK) with access to sugar water (50% Attracker solution in distilled water, Koppert Biological Systems, The Netherlands).

On the day of capture, bees were subjected to an encapsulation assay using an abiotic implant, following the methods of König & Schmid-Hempel (1995). Each bee was anaesthetised with CO_2, placed under a dissecting microscope and secured with pins so that its ventral side was exposed. A fine sterile pin was used to make an incision in the inter-segmental membrane between the second and the third sternite. A nylon implant (diameter 0.16mm, mean length 1.44 ± 0.012 mm) was inserted through this incision, where it would be exposed to the circulating haemolymph. Four hours later the bee was freeze-killed in liquid nitrogen, before being stored in a -80^0C freezer for later examination. A temperature data logger (Tinytag, Gemini data loggers, UK) recorded the ambient temperature during each assay and a mean was calculated for each four hour implant period.

Before dissection and examination each bee's abdomen was separated from its thorax and defrosted on ice. The implant was dissected out and mounted onto a slide using

Eukitt® (Electron Microscopy Sciences, USA). The degree of encapsulation was then measured by viewing the implant on a light table, with constant background illumination. A picture of the implant was taken and the mean grey value calculated using Image J software (U. S. National Institutes of Health, USA). This value was then subtracted from a control value (the mean grey value for an implant that had not been placed in a bee) to give a value for the encapsulation response (König & Schmid-Hempel, 1995).

The abdomen was inspected for the presence of the tracheal mite *L. buchneri* and any other macroparasites. The gut (excluding the honey sac) was then removed and homogenised in 200µl of insect ringer solution (9.1g NaCl; 0.52g KCl; 1.2g $CaCl_2.2H_2O$; 0.8g $MgCl_2.H_2O$; made to 1000ml with distilled water). 10µl of homogenate was examined at x400 magnification to determine the presence/absence of the microparasites *C. bombi, N. bombi* and *A. bombi*. If present, a further sample was examined on a haemocytometer and the number of cells in the two 0.1µl grids was counted. The intensity of infection was recorded as the mean number of cells in 0.1µl of gut homogenate.

The width of the thorax was measured using electronic digital calipers and the bee's age was estimated by assessing the extent of wing wear, using a four point scale (modified from Mueller & Wolfmueller, 1993). The phenoloxidase (PO) activity assay was adapted from Brown *et al*., (2003a). The thoraces were homogenised in 300µl phosphate buffer saline (PBS: 8.74g NaCl; 1.78g $Na_2HPO_4.2H_2O$; 1000ml distilled water; pH 6.5) before being centrifuged at 15.7 G (4^oC for 10mins). The supernatant was used to measure the concentration of the active PO as well as the total

PO (proPO plus the active PO). Reaction mixtures for the active PO measurements contained 20µl of the thorax supernatant, 140µl distilled water, 20µl PBS and 20µl L-DOPA solution (4mg ml^{-1} distilled water). For the total PO measurements, reaction mixtures contained 20µl of the thorax supernatant, 120µl distilled water, 20µl PBS, 20µl L-DOPA solution and 20µl bovine α-chymotrypsin solution (Sigma, C4129; 2.1mg ml^{-1} distilled water) and were incubated for five minutes at room temperature. The reaction was allowed to proceed at 30 ^{o}C for 40 minutes in a microplate reader (Versamax, Molecular Devices, USA). Absorbance readings were taken every 10 seconds at 480nm and analysed using SOFTmaxPRO 4.0 software (Molecular Devices, USA). Enzyme activity was measured as the slope (V_{max} value) of the reaction curve during the linear phase of the reaction. Eight replicate assays were performed on each bee, four of total PO (including chymotrypsin) and four of active PO (without chymotrypsin). All measures of PO were corrected for bee size as approximated by the thorax width cubed.

5.3.1 Statistical analyses

Data were analysed in R, version 2.7.2 (2008 The R Foundation for Statistical Computing). Conservative population-level analyses were first carried out using each population as a replicate and employing Pearson's correlations to investigate relationships between measures of genetic diversity, effective population size and parasite prevalence. The measures of genetic diversity (heterozygosity and allelic richness) were taken from Darvill *et al.*, (2006) and were based on 9 microsatellite loci, of which one was monomorphic and a second almost so. Effective population size estimates were computed using Colony version 2.0 (Wang, 2009; Jones & Wang, 2010). In-depth individual based analyses followed and as causal relationships

between variables are unknown a series of models were used, exchanging the dependent variable to explore all relationships. Population-level heterozygosity was used in individual analyses as a number of studies have shown it is a more accurate predictor of heterozygosity than using individual measures based on a relatively small number of loci (Pemberton, 2004).

Binomial generalised linear models were used to investigate determinants of parasite prevalence (*C. bombi* and *L. buchneri* respectively). Due to overdispersion in the *L. buchneri* prevalence data, a quasibinomial model was used. Zero-inflated negative binomial models (ZINB) were used to investigate the variables influencing parasite abundance (both the number of *C. bombi* cells per 0.1µl gut homogenate and the number of adult *L. buchneri* present in the abdomen). General linear models were used to investigate the variables influencing both the levels of phenoloxidase and the encapsulation response. Both these response variables were Box-Cox transformed to fulfil the assumptions of normality. In all models, island heterozygosity, population size, individual thorax width, wing wear, phenoloxidase (total PO divided by thorax width cubed) and the load of the other parasite were entered as co-variates. All statistical tests were two-tailed and all two-way interactions were investigated. Models were selected and simplified according to Aikaike Information Criterion (AIC). Only significant interactions are presented here. Means are recorded ± their standard errors throughout.

5.4 Results

Three species of parasite were detected; the gut trypanosome *C. bombi*, the tracheal mite *L. buchneri* and a conopid fly *Physocephala* sp.. *C. bombi* was detected at very high frequency in all island populations: prevalence ranged from 77% to 100%. *L. buchneri* was also detected in all populations, with a prevalence ranging from 3% to 53% (table 5.1). Larvae of *Physocephala* sp. were detected at very low prevalence (3%) and on only two islands (Staffa = 2 infected bees, Iona = 6 infected bees) and no further analysis was carried out for this parasite. No *Nosema bombi* or *Apicystis bombi* were detected.

Table 5.1 Population means for host genetic diversity, parasite prevalence and immunocompetence measures.

The figures in parentheses are the standard errors for genetic diversity, PO and encapsulation and the 95% C.I. for parasite prevalence. Measures for heterozygosity and allelic richness are taken from Darvill *et al.*, (2006).

		Bombus muscorum genetic diversity		Effective	*Crithidia*	*Locustacarus*			
Island	N	Heterozygosity	Allelic richness	population size	*bombi* prevalence	*bombi* prevalence	Total PO (Vmax)	Active PO (Vmax)	Encapsulation (grey value)
Barra	30	0.393 (0.11)	3.10 (0.66)	59	0.90 (0.78-1.02)	0.30 (0.14-0.46)	7.92 (1.10)	2.33 (0.47)	36.29 (3.17)
Coll	28	0.499 (0.09)	3.46 (0.69)	66	0.93 (0.83-1.03)	0.54 (0.36-0.72)	2.54 (0.45)	0.89 (0.18)	19.00 (1.84)
Iona	29	0.554 (0.09)	4.22 (0.91)	68	0.79 (0.63-0.95)	0.03 (-0.03-0.09)	4.05 (0.57)	1.25 (0.22)	25.49 (4.18)
Mingulay	27	0.374 (0.12)	2.99 (0.64)	46	0.93 (0.83-1.03)	0.15 (0.01-0.29)	7.19 (1.12)	2.55 (0.36)	27.71 (3.55)
N. Uist	23	0.404 (0.11)	3.32 (0.66)	81	1.00	0.30 (0.12-0.48)	4.99 (0.65)	1.48 (0.25)	22.95 (2.90)
S. Uist	23	0.404 (0.11)	3.32 (0.66)	81	0.91 (0.79-1.03)	0.39 (0.19-0.59)	5.49 (0.83)	1.62 (0.28)	30.53 (4.14)
Sandray	27	0.367 (0.11)	3.05 (0.63)	39	1.00	0.44 (0.26-0.62)	5.79 (0.65)	2.39 (0.49)	16.05 (1.35)
Staffa	30	0.484 (0.09)	3.33 (0.51)	52	0.77 (0.59-0.95)	0.23 (0.07-0.39)	3.76 (0.48)	0.87 (0.14)	22.29 (2.59)
Tiree	29	0.499 (0.09)	3.27 (0.55)	79	0.89 (0.77-1.01)	0.27 (0.11-0.43)	5.46 (0.61)	1.53 (0.18)	26.42 (2.46)

5.4.1 Population level results

Pearson correlations revealed general relationships between measures of host genetic diversity and parasite prevalence. There was a negative relationship between heterozygosity and the prevalence of both parasites; this correlation was only significant in the case of *C. bombi* (*L. buchneri*: r = -0.338, p = 0.374; *C. bombi*: r = -0.67, p = 0.048, figure 5.1). Heterozygosity and allelic richness were tightly correlated (r = 0.815, p = 0.007) and heterozygosity had better explanatory power in the analyses so was used in subsequent tests as the measure of genetic diversity. As effective population size had no correlation with parasite prevalence it was excluded from the subsequent in-depth analysis of parasite prevalence.

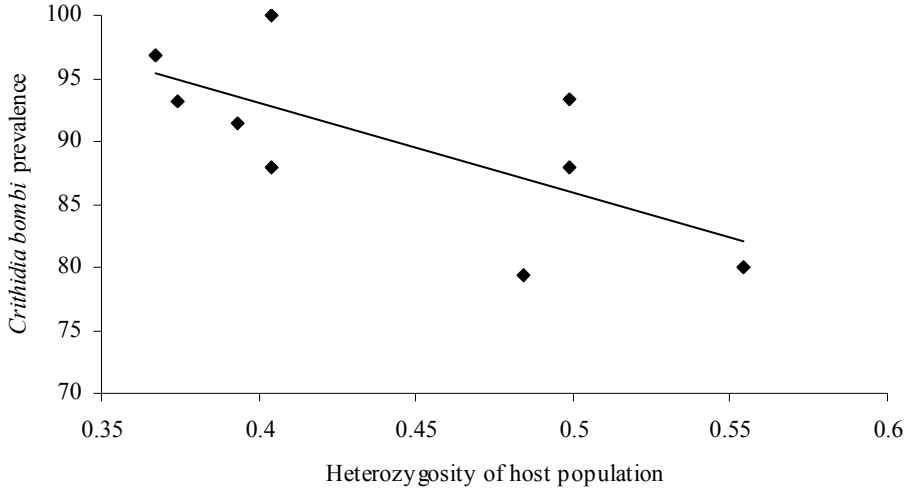

Figure 5.1 Relationship between *C. bombi* prevalence and heterozygosity of host population.

Each point represents an island population. Islands with higher heterozygosity had significantly lower prevalence of *C. bombi* (P = 0.003, table 5.2).

The negative correlation between *C. bombi* prevalence and host population heterozygosity remained significant in the detailed analysis that took into account other explanatory variables (Z = -2.99, P = 0.003). Age, as measured by wing wear, also significantly influenced *C. bombi* prevalence, with populations with a greater mean age more likely to be highly infected (Z = 2.02, P = 0.043). No other variable significantly affected the prevalence of *C. bombi* (table 5.2). No variable significantly affected the prevalence of *L. bombi* (table 5.2).

Table 5.2 Output of binomial GLM and quasibinomial GLM for prevalence of *C. bombi* and *L. buchneri* respectively. Degrees of freedom are given in parentheses.

	Crithidia bombi				*Locustacarus buchneri*			
	Co-efficient estimate	SE	Z	P	Co-efficient estimate	SE	T	P
Heterozygosity of population	-12.65	4.22	-2.99 (1)	**0.003**	-9.01	5.17	-1.74 (1)	0.132
Date	0.048	0.052	0.815 (1)	0.415	0.053	0.051	1.04 (1)	0.348
Age	1.37	0.679	2.02 (1)	**0.043**	-0.156	1.83	-0.085 (1)	0.940
Bee size	-2.81	2.90	-0.697 (1)	0.334	-2.36	2.08	-1.13 (1)	0.320
Encapsulation	-0.089	0.052	-1.72 (1)	0.086	0.014	0.081	0.171 (1)	0.875
Phenoloxidase	-20.49	261.56	-0.078 (1)	0.938	-48.67	29.67	-1.64 (1)	0.152
L. buchneri prevalence	-6.91	4.73	-1.46 (1)	0.145	-	-	-	-
C. bombi prevalence	-	-	-	-	0.587	27.86	0.021 (1)	0.987

5.4.2 Trends across individuals in parasite abundance

Here we use the parameter abundance to represent the number of parasites infecting a bee, including those bees that were uninfected. *Crithidia bombi* abundance was significantly positively correlated with both heterozygosity in the local host population and host population size, while it was negatively correlated with phenoloxidase levels (table 5.3, figure 5.2). *C. bombi* abundance was positively correlated with *L. buchneri* abundance and this effect was amplified in bees that also had high levels of PO (there was a significant positive interaction between the two factors). There was also a significant interaction between the effects of *L. buchneri* abundance and bee size: large bees with a high *L. buchneri* load had lower *C. bombi* loads than expected. Age and size also interacted with *C. bombi* abundance, with older, larger bees having lower parasite loads (table 5.3). *Locustacarus buchneri* abundance, in contrast, was only significantly predicted by levels of PO (table 5.3), with higher infection intensities observed in bees with lower levels of PO.

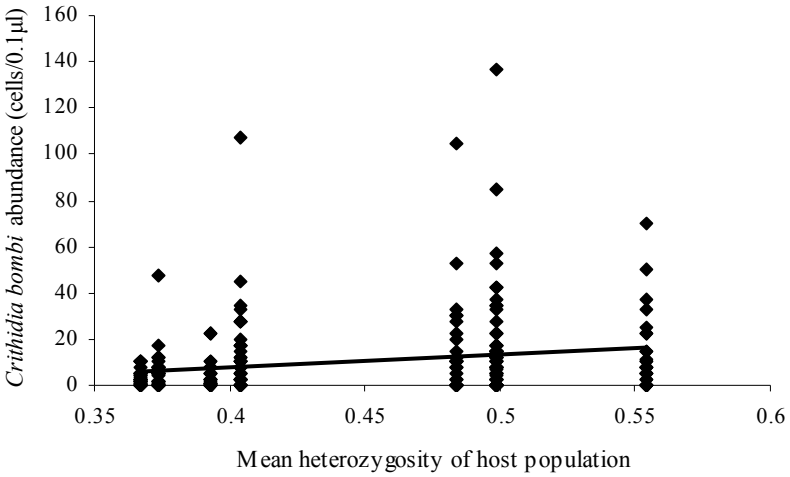

Figure 5.2 The relationship between *C. bombi* abundance and heterozygosity of host population.

Bees from populations with higher heterozygosity had higher parasite abundance (P = 0.001, table 5.3).

Table 5.3 Factors affecting *C. bombi* and *L. buchneri* abundance.

The response variables are the number of *C. bombi* cells per 0.1 µl and the number adult *L. buchneri* per bee respectively. Co-efficient estimates and SE are taken from the zero-inflated negative binomial model output. Log likelihood ratio tests provide χ^2 and p values for each term.

Crithidia bombi abundance					*Locustacarus buchneri* abundance			
Source	Co-efficient estimate	SE	χ^2	P	Co-efficient estimate	SE	χ^2	P
Heterozygosity of population	4.67	1.33	10.12	**0.001**	-2.96	2.71	1.16	0.282
Population size	0.021	0.006	12.96	**<0.001**	0.010	0.013	0.560	0.454
Date	-0.02	0.029	0.467	0.494	-0.049	0.029	2.79	0.095
PO	-59.49	22.26	6.74	**0.009**	-18.94	6.36	8.55	**0.003**
L. bombi load	0.881	0.391	5.60	**0.018**	-0.008	0.01	1.70	0.192
Bee size	0.216	0.609	0.126	0.722	0.391	0.235	2.75	0.097
Age	0.601	0.608	0.968	0.325	-0.089	0.141	0.402	0.526
Encapsulation	0.004	0.049	0.008	0.931	0.006	0.009	0.433	0.510
Age*Size	-0.051	0.016	9.43	**0.002**	-	-	-	-
L. bombi load*size	-0.193	0.077	6.98	**0.008**	-	-	-	-
L. bombi load*PO	4.36	1.83	6.54	**0.011**	-	-	-	-

5.4.3 Trends across individuals in immune parameters

A total of 233 nylon implants were successfully dissected out of the bees. The encapsulation response showed large variation and the degree of greyness on the implants ranged from 0.73 units to 83.73 units, with a mean of 25.50 ± 1.05 units. This melanisation did not show any correlation with the length of the implant ($r = 0.032$, $p = 0.616$) nor with the ambient temperature during the four hour assay ($r = 0.066$, $p = 0.298$). The encapsulation response had no relationship with population level heterozygosity but was significantly predicted by levels of phenoloxidase; bees with higher levels of PO showed greater encapsulation responses. The population size and date also significantly influenced the encapsulation response; smaller populations had lower responses and the response also declined over the sampling period (table 5.4).

Table 5.4 Output for General Linear Models for the encapsulation response and levels of total phenoloxidase. Degrees of freedom are given in parentheses.

	Encapsulation		Phenoloxidase	
	F	P	F	P
Heterozygosity of population	1.41 (1, 226)	0.236	28.07 (1, 222)	**< 0.001**
Population size	5.23 (1, 226)	**0.023**	0.25 (1, 221)	0.62
Date	10.35 (1, 226)	**0.001**	4.07 (1, 222)	**0.045**
Age	2.66 (1, 226)	0.104	5.03 (1, 222)	**0.002**
Bee size	0.234 (1, 225)	0.629	11.26 (1, 222)	**< 0.001**
C. bombi load	0.475 (1, 225)	0.491	6.14 (1, 222)	**0.014**
L. bombi load	0.009 (1, 224)	0.922	32.38 (1, 222)	**< 0.001**
Encapsulation	-	-	4.17 (1, 222)	**0.042**
Phenoloxidase	4.23 (1, 226)	**0.041**	-	-

Total and active phenoloxidase showed a strong positive correlation ($r = 0.816$, $p < 0.0001$) so only total PO was used in all analyses. Levels of total phenoloxidase, in contrast to the encapsulation response, were significantly predicted by a large number of variables. Lower levels of PO were found in bees from populations with higher heterozygosity (table 5.4). Levels of PO were also found to decline with age (figure 5.3). Additionally, larger bees were found to have higher levels of volume-corrected PO. As expected from previous analyses, bees with higher infection intensities of *C. bombi* and *L. buchneri* had lower levels of PO (figures 5.4 & 5.5).

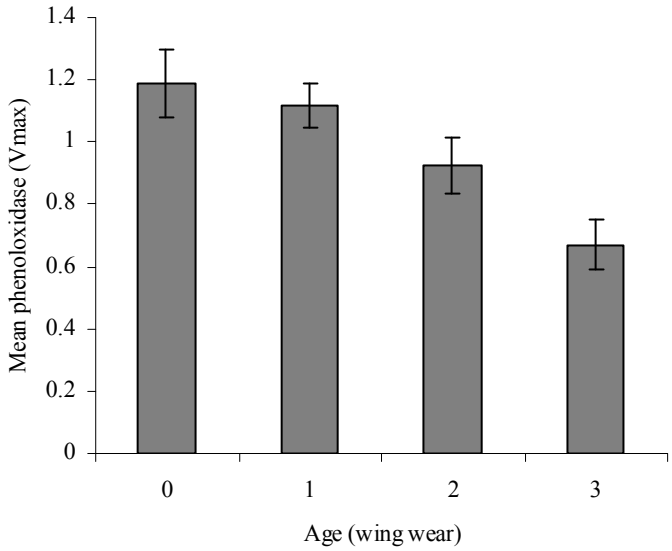

Figure 5.3 Mean levels of PO for each age group, with wing wear as a proxy for age.

Bars represent the least square means and their standard errors as predicted from the GLM. PO was found to significantly decline with age (p = 0.002, table 5.4).

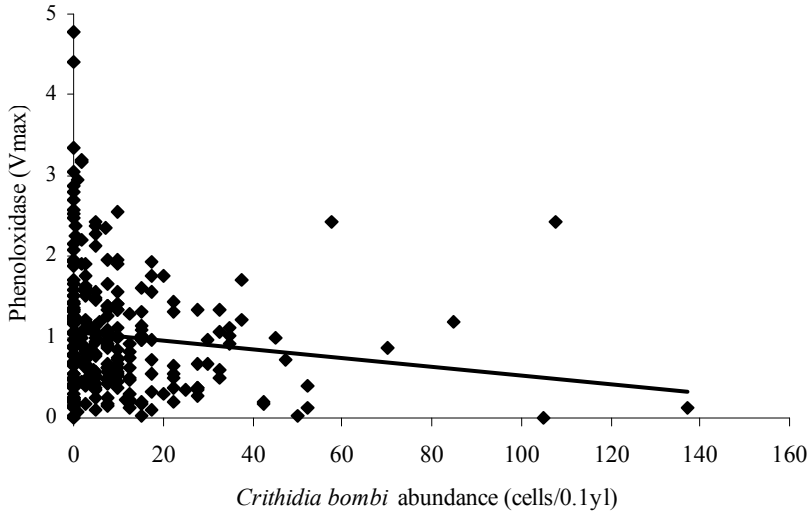

Figure 5.4 The relationship between phenoloxidase and *C. bombi* abundance.

Levels of phenoloxidase significantly declined with increasing *C. bombi* abundance (P = 0.014, table 5.4).

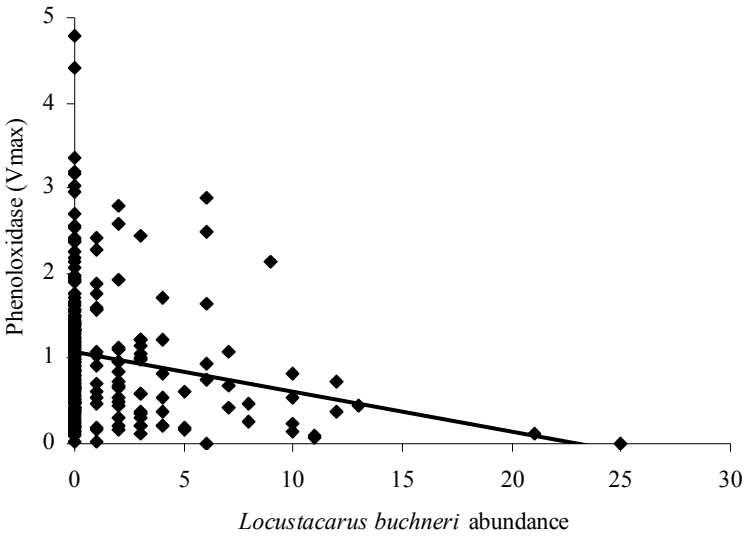

Figure 5.5 The relationship between phenoloxidase and *L. buchneri* abundance.

Levels of phenoloxidase significantly declined with increasing *L. buchneri* abundance ($P < 0.001$, table 5.4).

5.5 Discussion

This study is the first to demonstrate a relationship between the genetic diversity of natural bee populations and the prevalence of parasites. *Bombus muscorum* populations with lower levels of heterozygosity had higher prevalence of the gut parasite *C. bombi*. This field based study using wild bumblebee populations supports previous laboratory and experimental work that found genetic heterogeneity within colonies to be negatively correlated with parasitic infections in social insects (Baer & Schmid-Hempel, 2001; Hughes & Boomsma, 2004; Seeley & Tarpy, 2007). Additionally, high loads of the ectoparasitic mite have been found on the invasive *B. terrestris* in Tasmania, which is inbred due to small numbers of founding queens (Allen *et al.*, 2007).

There are two mechanisms that might result in low heterozygosity causing increased parasite prevalence. Inbred individuals may have low immune competence, resulting in greater susceptibility to infection and, secondly, parasite infections may be able to spread faster through populations with lower genetic diversity. We measured two immune system parameters, phenoloxidase levels and the encapsulation response. We found no evidence to suggest that bees from less heterozygous populations had inferior immune activity. However, it is possible that other immune system components may suffer negatively from inbreeding. Our results are consistent with the theory that population genetic homogeneity leads to higher parasite prevalence (Sherman *et al*., 1988; Schmid-Hempel, 1998). The theory assumes that host genotypes differ in their ability to resist different parasite strains, which is certainly true for *C. bombi* as several studies have demonstrated a strong genetic component to the susceptibility of bumblebees to this parasite using cross infection experiments, genetic analysis and QTL mapping (Imhoof & Schmid-Hempel, 1998; Schmid-Hempel *et al.*, 1999; Wilfert *et al.*, 2007).

Higher parasite prevalence in more genetically depauperate populations has been found in a number of other species, particularly vertebrates, for example Whiteman *et al.* (2006). This relationship has not been so commonly studied in invertebrates but experimental work has shown that a lower genetic diversity increases the probability of parasitic infection in *Daphnia magna* and the freshwater snail *Lymnaea stagnalis* (Puurtinen *et al.*, 2004; Ebert *et al.*, 2007). Thus parasitism may be a mechanism that increases the risk of extinction in small, isolated and inbred populations (de Castro & Bolker, 2005). This is particularly relevant in the case of bumblebees due to their recent population declines in Europe, North America, Japan and China (Williams & Osborne,

2009). Populations of rare species are becoming fragmented and isolated, which has led to a decline in their genetic diversity (Ellis *et al.*, 2006).

It is likely that higher prevalence of bumblebee parasites reduces fitness and increases mortality in inbred populations. *Crithidia bombi* increases the mortality rate of food-stressed worker bees by up to 50% (Brown *et al.*, 2000) and reduces worker foraging efficiency (Otterstatter *et al.*, 2005). Infection also reduces the fitness of colony-founding queens by 40% (Brown *et al.*, 2003b; Yourth *et al.*, 2008), which could have a severe impact on declining populations. Relatively little research has been conducted on *L. buchneri* but limited data suggest that heavy infections might be associated with lethargy and reduced foraging (Husband & Sinha, 1970). Parasitic infection may also have indirect effects on fitness simply by stimulating the immune system; *C. bombi* infection elicits PO production (Brown *et al.*, 2003a) and *L. buchneri* infection triggers a melanisation response by the host (pers. obs.). Colonies whose workers are immune challenged may have lower reproductive output, an effect exacerbated by harsh environmental conditions (Moret & Schmid-Hempel, 2001; Moret & Schmid-Hempel, 2004).

Interestingly, whilst bees in inbred populations were more likely to be infected with *C. bombi*, these populations also had lower mean parasite abundance. This could reflect the inability of inbred bees to survive high levels of infection meaning that high spores loads were not observed. A small but significant positive correlation was observed between the parasite load of *C. bombi* and *L. buchneri*. This may suggest that some individual bees are more generally susceptible to parasitic infection or that the two parasite species act synergistically.

Levels of PO were shown to decline with increasing bee age. A decline with age in both the encapsulation response and PO has been reported for bumblebees under laboratory conditions (Doums *et al.*, 2002; Moret & Schmid-Hempel, 2009) but this is the first study to suggest such immune senescence occurs in the wild. Levels of PO were also negatively correlated with the load of both parasite species. This is in agreement with a study by Siva-Jothy *et al.* (2001), who found that PO levels became negatively correlated with gut parasite burden in a damselfly, after an acute immune challenge with a nylon implant. This suggests a similar trade-off may be occurring in bumblebees; those infected with *C. bombi* may be unable to upregulate PO when subjected to the nylon implant we inserted. An alternative explanation for the negative correlation between PO and parasite load would be that more intense infections are able to establish in bees with lower immune capacity (Nigam *et al.*, 1997). A negative correlation between heterozygosity and PO was also observed, but it seems likely this is due to the tight correlation between PO and *C. bombi* abundance. Levels of PO were positively correlated with the encapsulation response. As the enzyme was measured after the implant had been inserted, the correlation reflects the involvement of PO in the immune cascade that results in encapsulation (Soderhall & Cerenius, 1998).

In conclusion, this study has demonstrated that low genetic diversity in *B. muscorum* populations is associated with a higher prevalence of parasites, although we detected no associated loss of immune competence. This supports theories that suggest population genetic homogeneity enables parasites to spread to higher prevalence. Inbreeding negatively affects a range of fitness traits in insects; our current data suggest that elevated parasitism may pose an additional threat to isolated populations.

5.6 Acknowledgements

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6.1

Chapter 6 - Investigating the impact of deploying commercial *Bombus terrestris* for crop pollination on pathogen dynamics in wild bumblebees

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All authors commented on draft versions of this manuscript.

6.1 Abstract

The use of commercial bumblebees for crop pollination has been implicated in the decline of wild bumblebees through the spread of pathogens. This study investigates whether diseases from commercial bumblebees threaten native species in the UK. We sampled bumblebees from ten soft fruit farms: five that deploy commercial *Bombus terrestris* and five that do not. Each farm was visited monthly throughout the summer and workers of *B. terrestris, Bombus pratorum, Bombus pascuorum* and *Bombus lapidarius* were captured. The faeces of these bees were inspected for the gut microparasites *Crithidia bombi, Nosema bombi* and *Apicystis bombi*. The prevalence of *A. bombi* and *N. bombi* was too low to analyse. The prevalence and abundance of *C. bombi* was significantly different among bumblebee species. Overall, the prevalence of *C. bombi* was lower on farms deploying commercial bumblebees. However, *C. bombi* prevalence in *B. terrestris* rose sharply on commercial farms at the end of the season, suggesting that the high density of commercial bees increases parasite transmission. However, we found no evidence of pathogen spillover to wild species. This study provides an important insight into interactions between native and commercial bumblebees and their parasites in Europe.

6.2 Introduction

The commercial use of bumblebees as pollinators for agricultural crops has been common practise since the 1980s when techniques for mass rearing bumblebees were developed (Velthuis & van Doorn, 2006). The majority are used for greenhouse

tomatoes, but large numbers are also used for the pollination of various cucurbits and soft fruits (Velthuis & van Doorn, 2006; Stanghellini *et al*., 1997; Stubbs & Drummond, 2001). As bumblebees are highly efficient pollinators, they can provide economic benefits to fruit growers through increased yield (Serrano & Guerra-Sanz, 2006; Lye *et al*., 2011). However, their use does not come without risk. Commercially produced bumblebees pose three main threats to native bumblebee fauna; competition for resources (Ings *et al.*, 2006; Inoue *et al.*, 2008; Inoue *et al.*, 2010); hybridisation with native subspecies (Kondo *et al.*, 2009) and finally, the spread of parasites (Colla *et al.*, 2006). It is vital to understand the relevance of these threats to bumblebees as populations of many species have been declining over recent decades (Williams & Osborne, 2009; Cameron *et al.*, 2011). These declines have been predominantly attributed to the intensification of agriculture and the associated loss of habitats on which bumblebees depend (Goulson *et al*., 2008; Williams & Osborne, 2009).

Recent work from North America suggests that diseases from commercial bumblebees may pose a significant additional threat to native species (Winter *et al*., 2006; Colla *et al*., 2006; Cameron *et al.*, 2011) and this threat can take two forms. Firstly, the use of commercial bumblebees, frequently imported from foreign countries, could introduce a novel pathogen or pathogen genotype, which is virulent in wild populations (Goka *et al.*, 2000; Goka *et al.*, 2006). Secondly, if the unusually high densities of bumblebees associated with commercial use elevate disease prevalence, pathogens may spill over to cause increased infection rates in wild bumblebee populations (Otterstatter & Thomson, 2008). Such pathogen spillover can occur even if the commercial bees arrive uninfected if they contract and amplify local pathogens. The potential exists for both processes to occur when commercial bumblebees are deployed as they regularly forage on wild

flowers adjacent to the crop (Morandin *et al*., 2001; Whittington *et al*., 2004). Transmission of intestinal parasites can then occur when infected and uninfected individuals forage on the same flower (Durrer & Schmid-Hempel, 1994). Infection with intestinal parasites such as *Crithidia bombi* and *Nosema bombi* can substantially reduce the fitness of individual bumblebees and the reproductive output of colonies (Brown *et al.*, 2003; Otti & Schmid-Hempel, 2008).

The introduction of novel pathogens can potentially have severe consequences. In North America, the accidental introduction of the gut parasite *N. bombi* with commercial bumblebees is thought by many to be responsible for the dramatic decline of seven species of native bumblebees since the 1990s (Winter *et al*., 2006; Cameron *et al.*, 2011), although direct evidence is lacking (Brown, 2011). Within Europe, this may be considered less of a threat as the source and destination locations of commercial bees contain the same parasite species. However, the introduction of novel pathogen strains remains a risk. For example, the gut trypanosome *C. bombi* is known to consist of a large number of different strains (Schmid-Hempel & Funk, 2004). Higher mortality has been found when bumblebees are infected with a *C. bombi* strain from a distant location compared to infection from a local source (Imhoof & Schmid-Hempel, 1998). Thus, the importation of bumblebees from abroad could potentially introduce novel parasite strains to which the local populations are more susceptible.

Pathogen spillover occurs when a heavily infested host reservoir population transmits a pathogen to a nearby susceptible population (Daszak *et al*., 2000). In the case of the commercial use of bumblebees, the reservoir population consists of the imported colonies and the susceptible population is the local natural bumblebee fauna. The

pathogen may already exist within the susceptible population but spillover occurs if the commercial bees maintain higher parasite loads, which is likely due to the unnaturally high densities of commercial colonies within greenhouses or polytunnels. Pathogen spillover from commercial to wild bees has been shown to occur in Canada. The prevalence of parasites was compared between sites close to glasshouses using commercial bumblebees and sites over 50km from any commercial greenhouse. It was found that *C. bombi* was present at significantly higher prevalence at the sites near glasshouses. Additionally, bees foraging closest to the greenhouse had more intense infections (Colla *et al*., 2006; Otterstatter & Thomson, 2008). It should be noted that pathogen spillover can occur even if the commercial bees are free of disease in the factory; high densities of bumblebees in glasshouses provide suitable conditions for rapid spread of any pathogen with which they come into contact.

Despite studies in North America, no comparable research into the potential threat of pathogens and parasites from commercial bumblebees has been published in Europe and this paper aims to investigate whether such a threat exists. We focus on the use of commercial bumblebees for the pollination of soft fruit where nest boxes are placed in open ended polytunnels and open field situations. The spread of pathogens to wild bumblebees is of particular concern in such situations as there is no containment of the commercial bees. We investigate this using soft fruit farms in the UK as a study system, where there is undoubtedly the potential for commercial bumblebees to pose a threat as approximately 60,000 *B. terrestris* nests are currently imported from mainland Europe each year (Goulson, 2010b). We compare the prevalence and abundance of pathogens in bumblebees on farms that do deploy commercial bumblebees and on farms that do not. If commercial bumblebees are acting as a source of infection, we would assume there to

be an elevated prevalence of infection among foraging bumblebees on the farms where they are deployed.

6.3 Methods

Ten soft fruit farms in East and Central Scotland were selected for this study (see table 6.1). Five farms deployed commercially reared *B. terrestris* to aid pollination (hereafter referred to as "commercial farms" and five did not ("wild farms"). Wild farms were located at least 4 km from a farm that used commercial bumblebees to minimise the presence of any foraging commercial bees. The foraging range of bumblebees is difficult to measure and estimates vary, but most agree that *B. terrestris* rarely forage more than 1.5 km from their nest (Darvill *et al.*, 2004; Knight *et al.*, 2005; Osborne *et al.*, 2008; Wolf & Moritz, 2008). Sampling took place at each farm for one day in May, June, July and August. Worker bumblebees of the species *B. terrestris, B. pascuorum, B. pratorum* and *B. lapidarius* were collected using sweep nets. Bees were collected either directly from the raspberry or strawberry crop or from wildflowers growing within 10 metres of the crop. No attempt was made to distinguish between the morphologically similar *B. terrestris, B. lucorum, B. magnus* and *B. cryptarum* and this species group is referred to as simply *B. terrestris*. On the commercial farms this group includes the commercial bumblebees. Commercial bumblebees were also sampled directly from their colonies on one day in May and June; at this time the nestboxes had been open and the bees foraging for varying periods of time. Bees were held individually in clear sampling tubes with ventilation holes in the lids and were left until they had defecated. The faeces were collected into microcapillary tubes, which were then sealed at each end and stored in a chilled box. Bees were released at the end of the

sampling period unharmed. The faeces were later inspected at x400 magnification to detect the presence of *C. bombi, N. bombi* and *A. bombi*. No attempt was made to distinguish between *C. bombi* and the newly discovered *C. expoeki* (Schmid-Hempel & Tognazzo, 2010). If present, the intensity of infection was recorded using a haemocytometer: the number of cells in a 0.1µl grid was counted.

Table 6.1 Information on the ten farms from which samples were collected.

Farm name	Longitude	Latitude	Farm type	Ha soft fruit	No. nestboxes imported per year
Allanhill	2° 46.8' W	56° 19.2' N	Commercial	45	300
Blacketyside	2° 59.2' W	56° 12.7' N	Commercial	40	200
Broadslap	3° 36.5' W	56° 19.7' N	Commercial	8	6
SCRI	3° 04.2' W	56° 27.4' N	Commercial	18.5	6
Seaton	2° 33.1' W	56° 34.2' N	Commercial	40	350
Briarlands	4° 02.6' W	56° 10.1' N	Wild	0.5	-
Kincreich	2° 55.2' W	56° 35.3' N	Wild	6	-
Mill of Montague	3° 19.2' W	56° 26.2' N	Wild	6	-
Milton of Ruthven	3° 09.6' W	56° 38.5' N	Wild	40	-
Newmills	3° 18.0' W	56° 30.4' N	Wild	6	-

6.3.1 Statistical analyses

Data were analysed in R, version 2.12.0 (2010 The R Foundation for Statistical Computing). Chi-squared tests established whether differences existed between the proportion of infected bees in different species. Binomial generalised linear mixed effect models were used to analyse determinants of *C. bombi* prevalence and each bumblebee species was analysed separately. The residuals were tested for autocorrelation using the Durbin-Watson statistic but this was not detected. *Crithidia bombi* abundance (the number of *C. bombi* cells per 0.1µl faeces, including uninfected bees) was analysed in a Bayesian framework using the MCMCglmm package in R

(Hadfield, 2010). Generalised linear mixed models with a zero-inflated poisson distribution were used and non-informative priors were set in all analyses. Prior sensitivity analysis was carried out and the final models are robust to variation in the values of priors. Model convergence was confirmed using Geweke's diagnostic (Geweke, 1992) and visual examination of the model output. Parameter estimates reported are means from the posterior distribution with 95% lower and upper credible intervals (CI). A binomial generalised linear mixed effect model was used to investigate the difference in prevalence of *N. bombi* between the treatments. Prevalence of infection was too low to allow bumblebee species to be analysed separately for this parasite. In all the mixed effect models, sampling month (entered as a covariate 1, 2, 3 or 4), treatment (presence or absence of commercial bumblebees) and bumblebee species were entered as fixed effects and the individual farms were entered as a random effect. Means are recorded ± their standard errors throughout.

6.4 Results

A total of 946 worker bumblebees was collected from the ten farms and screened for pathogens over the four month sampling period. Additionally, 103 commercial bumblebee workers were collected directly from their nestboxes in May and June. All three parasite species were detected and the overall prevalence in the bees collected foraging was: *C. bombi* 39.22%; *N. bombi* 2.01% and *A. bombi* 0.74%. The number of bees infected with *A. bombi* was too small to allow further analyses on this parasite.

6.4.1 Crithidia bombi prevalence

The proportion of bees infected differed significantly across the different species, being highest in *B. pratorum* and lowest in *B. pascuorum* ($\chi^2 = 53.09$, df = 3, $p < 0.001$, figure 6.1, table 6.2). The prevalence of *C. bombi* infection in commercial bumblebees collected directly from their nestbox in May and June was 35.92 ± 4.75%, which is similar to the prevalence in *B. terrestris* collected from commercial farms (28.57 ± 5.66%; $\chi^2 = 1.09$, df = 1, p = 0.297) and wild farms (47.73 ± 5.36%; $\chi^2 = 2.73$, df = 1, p = 0.099) in May and June.

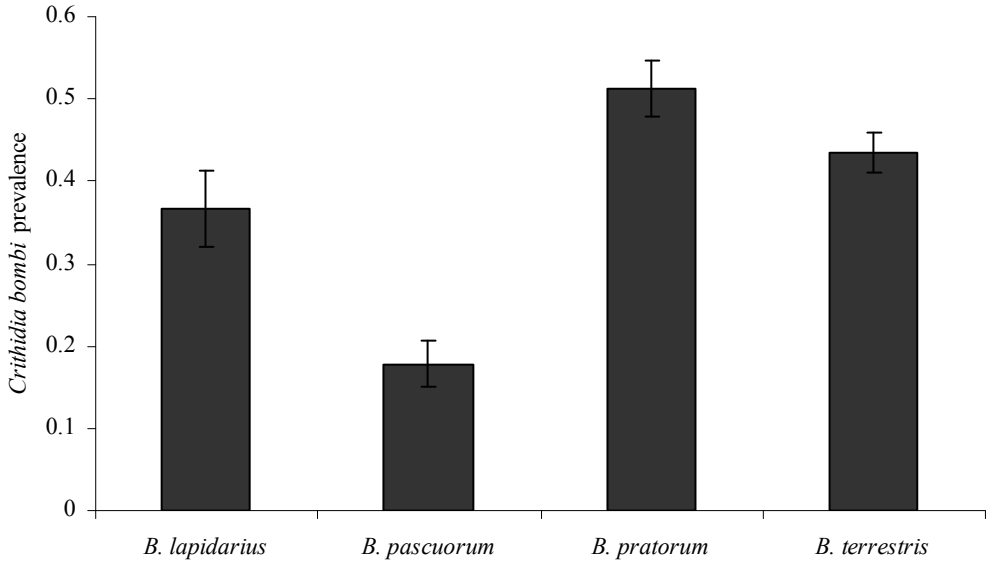

Figure 6.1 Prevalence of *C.bombi* in the four bumblebee species.

The proportion of bees infected was significantly different among the species ($\chi^2 = 53.09$, df = 3, $p < 0.001$). Bars represent the mean prevalence and their standard errors.

Table 6.2 Mean prevalence of *C. bombi* infection for the four bumblebee species in the two farms types across the sampling period.

	B. terrestris						***B. pascuorum***						***B. pratorum***						***B. lapidarius***					
	Commercial			**Wild**			**Commercial**			**Wild**			**Commercial**			**Wild**			**Commercial**			**Wild**		
	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	N	Mean	SE	n	Mean	SE	n	Mean	SE	n
May	0.250	0.25	4	0.500	0.29	4	0.000	-	1	0.400	0.25	5	0.417	0.15	12	0.345	0.09	29	0.333	0.33	3	1.000	-	1
June	0.283	0.06	60	0.476	0.05	84	0.069	0.05	29	0.300	0.15	10	0.257	0.08	35	0.486	0.09	35	0.423	0.10	26	0.191	0.09	21
July	0.307	0.05	75	0.417	0.06	72	0.154	0.06	39	0.143	0.07	28	0.400	0.08	40	0.824	0.07	34	0.316	0.11	19	0.400	0.25	5
August	0.712	0.06	66	0.406	0.06	64	0.243	0.07	37	0.191	0.06	42	0.792	0.08	24	0.875	0.13	8	0.412	0.12	17	0.471	0.13	17

Bombus terrestris

Averaging across the whole season, the proportion of *B. terrestris* infected with *C. bombi* was significantly higher on the wild farms compared to the commercial farms (χ^2 = 17.95, df = 1, $p < 0.001$). There was also a significant interaction between the treatment and the sampling month (χ^2 = 19.07, df = 1, $p < 0.001$): month significantly predicted *C. bombi* prevalence on commercial farms due to the marked increase in August, whilst prevalence on wild farms did not change significantly over time (figure 6.2).

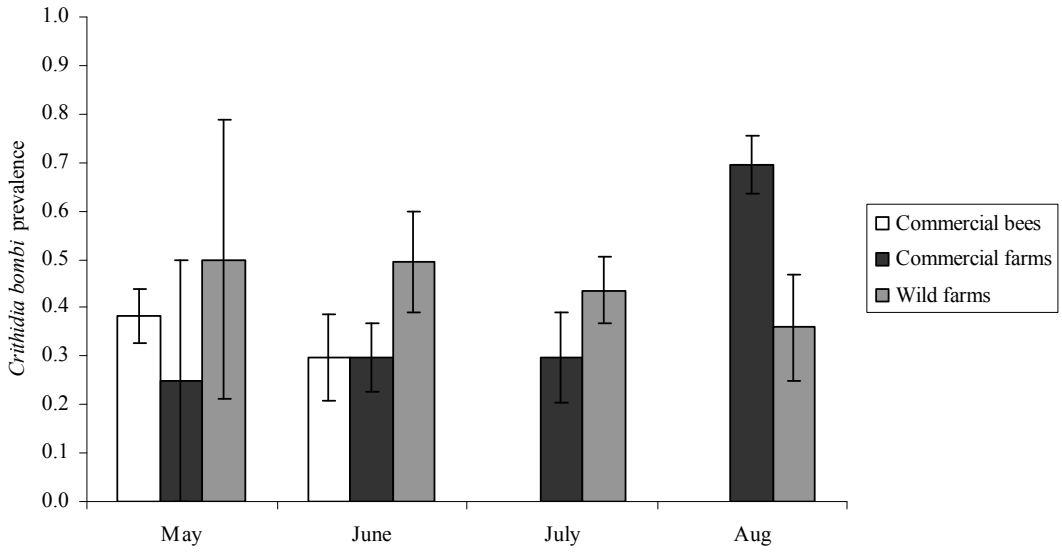

Figure 6.2 Prevalence of *C. bombi* in *B. terrestris* over the sampling period in the two farm types.

Prevalence on commercial farms was significantly affected by month due the marked increase in August ($Z = 4.75$, $p < 0.001$). No significant change in prevalence occurred on wild farms ($Z = 0.976$, $p = 0.329$). There was no difference in the prevalence of *C. bombi* in commercial bees collected from nest boxes and in foraging *B. terrestris* collected on commercial farms (χ^2 = 1.09, df = 1, $p = 0.297$). Bars represent the mean prevalence and their standard errors.

Bombus pratorum

The prevalence of *C. bombi* was significantly higher on wild farms than on commercial farms ($\chi^2 = 6.33$, df = 1, p = 0.012, figure 6.3) and also significantly increased over the sampling period ($\chi^2 = 30.27$, df = 1, $p < 0.001$). There was no interaction between the treatment and month ($\chi^2 = 0.887$, df = 1, p = 0.346), indicating that this increase occurred at a similar rate on both farm types.

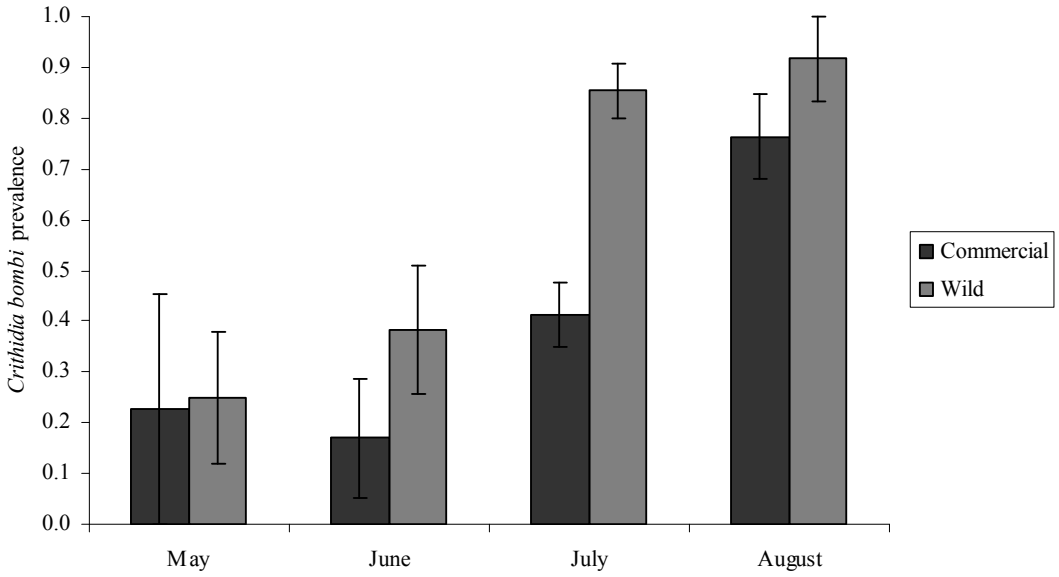

Figure 6.3 Prevalence of *C. bombi* in *B. pratorum* over the sampling period in the two farm types.
Prevalence was higher in wild farms (p = 0.012) and significantly increased over time ($p < 0.001$). Bars represent the mean prevalence and their standard errors.

Bombus pascuorum and *Bombus lapidarius*

Similar results were obtained for both species and as so few workers were collected in May, this month was excluded from the analysis of both. The prevalence of *C. bombi* in *B. pascuorum* and *B. lapidarius* was not significantly different in each farm type ($\chi^2 = 0.038$, df = 1, p = 0.847 and $\chi^2 = 0.473$, df = 1, p = 0.492 respectively) and did not

significantly change over time (χ^2 = 1.52, df = 1, p = 0.217 and χ^2 = 1.26, df = 1, p = 0.262 respectively). Temporal patterns were similar on commercial and wild farms: there was no significant interaction between month and treatment (χ^2 = 2.46, df = 1, p = 0.117 and χ^2 = 2.20, df = 1, p = 0.138).

6.4.2 Crithidia bombi abundance

Considering the load of infection in each individual bee, *C. bombi* abundance for all bumblebee species did not differ significantly between the two treatments and did not change significantly over time (table 6.3). There was also no significant interaction between these two variables. The abundance was, however, significantly different between the four bumblebee species (figure 6.4).

Table 6.3 MCMCglmm output for *C. bombi* abundance.

The parameter estimates shown here are with reference to *B. terrestris* and the commercial treatment and are on the log scale. The MCMC procedure for this model has a burn-in period of 5000, a total of 50,5000 iterations and a thinning interval of 500. P-values <0.05 are written in bold.

	Parameter estimate	Lower 95% CI	Upper 95% CI	P value
Crithidia bombi abundance	4.770	3.001	6.562	**<0.001**
Species				
B. lapidarius	1.094	0.121	1.903	**0.022**
B. pascuorum	-3.025	-4.321	-1.741	**<0.001**
B. pratorum	-1.040	-1.629	-0.416	**<0.001**
Month	-0.005	-0.487	0.445	0.980
Treatment	1.697	-0.459	4.129	0.110
Month*Treatment	-0.609	-1.258	0.025	0.064

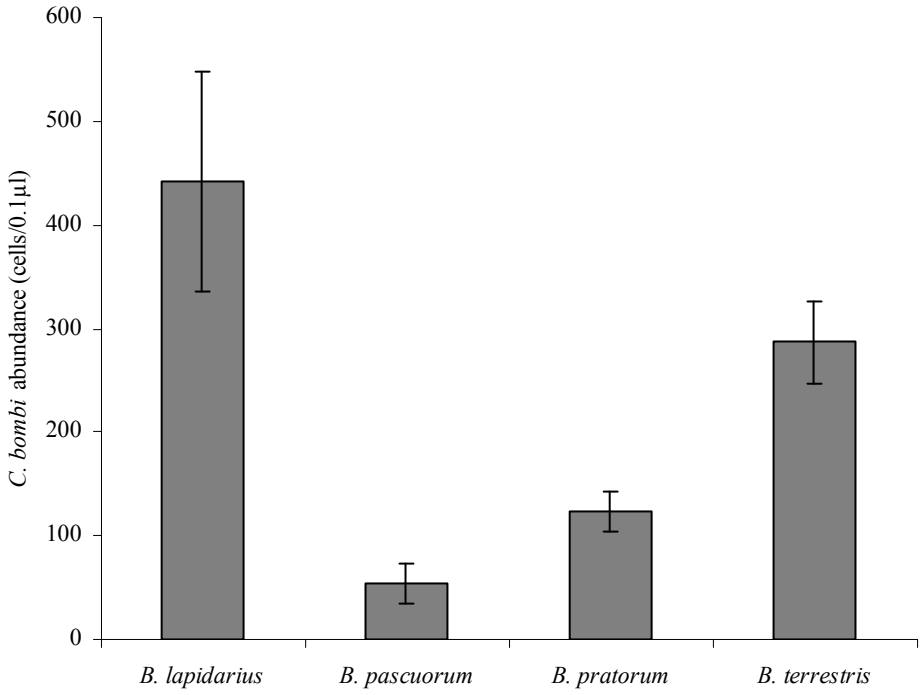

Figure 6.4 Mean *C. bombi* abundance for the four bumblebee species.

All species comparisons were significant: *B. lapidarius* had a significantly greater mean load than all the other species (*B. pascuorum*: $p < 0.001$; *B. pratorum*: $p < 0.001$; *B. terrestris*: $p = 0.022$). *B. terrestris* had a significantly greater mean load than *B. pascuorum* and *B. pratorum* ($p < 0.001$ and $p < 0.001$ respectively). *B. pratorum* had a significantly higher mean load than *B. pascuorum* ($p = 0.004$). Bars represent the mean abundance and their standard errors

6.4.3 Nosema bombi

When all bumblebee species were pooled, a greater proportion was infected with *N. bombi* on commercial farms (2.95 ± 0.97%) compared to wild farms (1.15 ± 0.54%). However, this difference was not significant ($\chi^2 = 3.09$, df = 1, $p = 0.079$). Due to the small number of bees infected (n = 19) it was not possible to analyse species separately. However, this comparison could be confounded by an uneven distribution of species infected in the two treatments: only *B. terrestris* were found to be infected on wild

farms whilst a few individuals of all four bumblebee species were infected on commercial farms. Additionally, two of 103 commercial bumblebees collected directly from their nestboxes were infected with *N. bombi*.

6.5 Discussion

The decline of insect pollinators is of universal concern due to the ecological and economic benefits they provide. The global trade in commercial bumblebees may have contributed to this decline, partially through the spread of pathogens and parasites (Colla *et al*., 2006; Brown *et al.*, 2011; Cameron *et al.*, 2011). However, the impact of commercial pollination practises is likely to differ depending on location and ecological circumstances. This paper offers the first insight into the potential impacts of commercial bumblebees on parasite dynamics in European bumblebee populations.

No evidence for the spread of pathogens from commercial bees to other bumblebee species was found: parasitic infection in wild bumblebee species was no higher at commercial farms compared to wild farms (and was lower in one wild bumblebee species). This contrasts markedly with the situation in Canada, where commercial bumblebees used in glasshouses acted as a source of infection to wild bumblebees in the surrounding area (Colla *et al*., 2006; Otterstatter & Thomson, 2008). Overall, we found a lower prevalence of *C. bombi* in *B. terrestris* on commercial farms compared to wild farms, particularly early in the season. This could be a dilution effect caused by the new arrival of large numbers of uninfected commercial bumblebees. Our study did not investigate whether parasites were present in commercial nest boxes when they arrived from the suppliers; hence we cannot discern whether the infections observed in

commercial bees were contracted largely or exclusively whilst bees were foraging on farms following deployment. However, previous studies have found commercial bees to arrive from the supplier infected with parasites (Goka *et al.*, 2000; Colla *et al.*, 2006 and references therein).

Interestingly, the prevalence of *C. bombi* increased through the season in *B. terrestris* on commercial farms, whilst it remained approximately constant on wild farms. This was driven by a marked increase in infection rate at the end of the season in August. Although both wild and commercial *B. terrestris* (and also *B. lucorum, B. magnus* and *B. cryptarum*) would have been sampled on commercial farms, the majority are likely to have been commercial bees due to the close proximity of their nest boxes. One possible explanation for this pattern is an increased rate of density dependent transmission of *C. bombi* due to the elevated bumblebee population on commercial farms. Greater transmission rates would then amplify the prevalence of this parasite. Alternatively, commercial bumblebees may have higher susceptibility to *C. bombi* than local *B. terrestris*. Genetic variation exists in *B. terrestris* for *C. bombi* susceptibility (Wilfert *et al.*, 2007), thus it is possible that commercial *B. terrestris* could be poorly adapted to defend against local *C. bombi* genotypes. This effect may be intensified as commercial *B. terrestris* have undergone selection in a factory environment for several generations, which might have altered immune investment. The significantly higher prevalence of *C. bombi* on commercial farms by the end of the season does suggest that pathogen spillover is a threat as there is a possibility that wild bumblebees, including newly emerged queens, may become infected by contact with commercial bees. Such infection of queens would cause fitness losses as *C. bombi* is known to substantially reduce their colony founding success (Brown *et al*., 2003b). However, recent research suggests that

queens may be more resistant to *C. bombi* than workers, which would lessen the impact of any epidemic (Ulrich *et al.*, 2011). Further research into the rates of interspecific transmission by the strains of *C. bombi* infecting wild and commercial bumblebees would be required to assess the risks of these late-season epidemics spreading to other species in the surrounding areas.

The overall mean prevalence of *C. bombi* was similar to that in central Europe and was also significantly different among bumblebee species (Shykoff & Schmid-Hempel, 1991b). *Bombus pratorum* suffered from the highest rate of infection, particularly at the end of the sampling period. This species emerges early from hibernation in the spring throughout the UK and nests can produce reproductives as early as April (Goulson, 20010a). Therefore, individuals still on the wing by the end of the summer are highly likely to be infected as they would have had a long period of exposure to *C. bombi*. The intensity of infection with *C. bombi* also varied significantly across bumblebee species but interestingly shows a different pattern to the prevalence of infection. *B. lapidarius* was found to suffer from considerably higher parasite loads than all three other bumblebee species and *B. terrestris* had significantly higher loads than *B. pascuorum* and *B. pratorum*. The reasons behind these differences remain unknown but it may relate to inter-specific differences in host genetics and parasite defence, environmental factors or parasite virulence.

The proportion of bees infected with *N. bombi* was too low in this study to allow an in-depth analysis. To obtain a good picture of the infection dynamics of this parasite species, results from more than one season would be required as the prevalence of *N. bombi* is known to vary spatially, temporally and across species by substantial amounts

(Paxton, 2005; Larsson, 2007). *N. bombi* appears to be a rare pathogen in this habitat and consequently may only have a small impact on the bumblebee populations in the area. Our dataset is too small to make any conclusions but it is interesting to note that the prevalence of this parasite was higher on the commercial farms, although this difference was not significant. Previous authors have thought that the presence of commercial bumblebees can possibly amplify the prevalence of *N. bombi* (Colla *et al.*, 2006; Cameron *et al.*, 2011). This is potentially concerning as bumblebees infected with *N. bombi* have substantially reduced fitness (Otti & Schmid-Hempel, 2008; Rutrecht & Brown, 2009).

This study assesses one aspect of the dangers associated with the use of commercial bumblebees for pollination services. We have shown that the presence of commercial *B. terrestris* amplifies the prevalence of *C. bombi* by the end of the season, which represents a potential threat in terms of pathogen spillover. But we find no evidence that this threat is being realised in terms of transmission to wild bumblebees; our data suggest that this late-season epidemic may remain within the commercial bees. However, more research over a larger temporal and spatial scale is needed before any generalisations on the disease risks posed by commercial bees can be made. Indeed, much is still to be understood about bumblebee diseases, particularly viruses. For example, deformed wing virus (DWV) is a honey bee pathogen that has been found to infect bumblebees and has a seriously detrimental effect on fitness (Genersch *et al*., 2006). A potential transmission route for this virus could be through commercial bumblebees as honeybee pollen is used in the rearing process (Velthuis & van Doorn, 2006). Further research is also needed into the other detrimental ecological consequences associated with commercial bumblebees, such as hybridisation with

native subspecies and competition for resources. Due to the uncertainties surrounding these potential costs, it would be preferable to develop viable alternatives where possible and thus reduce the need for commercial bumblebees. For example, sowing wild flower mixes can boost natural pollinator populations (Carvell *et al.*, 2007), which in turn may benefit soft fruit pollination.

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Chapter 7 - General Discussion

Habitat fragmentation and invasive species threaten global biodiversity and have been responsible for the decline and extinction of many populations and species. Bumblebees are one example of a taxon subject to such threats and, as valuable pollinators, there is a pressing need to conserve them. Although advances are continually being made towards this end (for example, Carvell *et al*., 2007; Goulson *et al*., 2011), there is still much to be learnt about their evolutionary ecology that would help inform conservation decisions. Specifically, it is known that rare species and isolated populations of bumblebees are losing their genetic diversity (Darvill *et al.,* 2006; Ellis *et al.*, 2006), but it is not known whether they are suffering from inbreeding depression. Additionally, little is understood about the effects of parasites and pathogens on inbred populations and whether they might increase the risk of extinction. This thesis contributes to our knowledge of these particular issues and provides a greater understanding of the factors that might be pushing threatened pollinators towards extinction.

As haplodiploid Hymenoptera, it has been previously assumed that bumblebees may suffer less inbreeding depression than diplodiploid organisms, as deleterious recessive mutations were thought to be purged through haploid males (Werren, 1993). However, their haplodiploidy may in fact exacerbate the costs of inbreeding as their single-locus complementary sex determination system (sl-CSD) can result in the production of sterile or inviable diploid males (Cook & Crozier, 1995). This diploid male production (DMP) has been found to occur in wild populations of threatened bumblebees (Darvill *et al*., 2006; Takahashi *et al*., 2008) and so it is important to establish its costs. Previous studies have only considered the costs associated with diploid males under laboratory conditions and have produced contrasting results (Duchateau *et al*., 1994; Plowright &

Pallett, 1979). The study presented in Chapter 2 of this thesis is the first to investigate the cost of DMP to bumblebee colonies under field conditions.

We demonstrated that DMP in *B. terrestris* does impose severe costs through its influence on colony productivity and survival. Diploid male colonies had a significantly slower growth rate in the laboratory, augmenting the results of Plowright & Pallet (1979). This slower growth results from an effectively smaller workforce as diploid males are idle within the colony and are produced instead of industrious female workers. Additionally, we found that diploid male colonies produced significantly fewer offspring overall, which represents a substantial fitness cost as the number of reproductives reared by a colony is highly correlated with the number of workers (Gerloff & Schmid-Hempel, 2005; Muller & Schmid-Hempel, 1992). Finally, colony survival in the field was severely impeded by DMP: queens survived only one third of the time of those in outbred colonies, presumably dying of starvation due to the lack of foraging workers.

As well as the costs identified in Chapter 2, it is known that diploid males impose a genetic load on populations as they yield little or no reproductive return for the resources invested in them. Although bumblebee diploid males can sometimes mate and triploid individuals have been observed in the wild (Takahashi *et al*., 2008; Darvill, 2007), diploid males do have reduced fertility (Duchateau & Marien, 1995) and triploids are invariably infertile (Ayabe *et al*., 2004). Because diploid males represent such substantial fitness costs for bumblebees, their frequency of occurrence would make a good indicator of the genetic health of wild populations, as suggested by Zayed *et al.* (2004). A high frequency would suggest that the population is suffering from a loss of

genetic diversity and may even be at risk of extinction (Zayed & Packer, 2005). Such information is essential when deciding where to focus and prioritise conservation effort.

There would be large fitness benefits for bumblebees in avoiding the production of diploid males where possible. Such avoidance could be achieved if bumblebees had evolved some method of kin recognition. Even in large populations, where matings between individuals with identical sex determination genotypes is unlikely, a mechanism of kin recognition would be useful. This is because bumblebee nests often produce large numbers of males and new queens simultaneously, which makes encounters between siblings highly probable. Data presented in this thesis suggests that *B. terrestris* reproductives do indeed discriminate between kin and non-kin, being less willing to mate with their siblings than with non relatives (Chapter 3). Indeed, matings between siblings took more than twice as long to initiate under laboratory conditions than between non-relatives; under natural conditions this delay would reduce the chance of a successful copulation. Prior to this study, very little was known about inbreeding avoidance behaviour in bumblebees. However, research by Foster (1992) on two American bumblebee species, *Bombus frigidus* and *Bombus bifaricus*, found that queens preferred to mate with unrelated males in choice experiments. Our study supports this finding and additionally demonstrates that queens appear to discriminate between kin and non-kin even when not given a choice and only encountering one type of male.

Unfortunately we were unable to establish what cues bumblebees use for kin discrimination. As bumblebees are social insects they may simply use extrinsic cues, such as scent of the nest environment, which would only enable them to discriminate between their siblings and other individuals. However, it has been found that some

insects are able to employ more sophisticated intrinsic methods of kin recognition. For example, kin discrimination in some social Hymenoptera is mediated through the chemical composition of cuticular hydrocarbon recognition pheromones (e.g. Gamboa *et al*., 1996; Keller & Ross, 1998). It is thought that in at least one species of wasp, *Polistes fuscatus*, this enables them to recognise non-nestmate kin, which are more distantly related than siblings (Gamboa, 2004). Further experimentation to find out whether bumblebees can recognise kin that have not been reared in the same nest environment would be interesting, and would demonstrate whether or not they are capable of intrinsic kin recognition. In small, inbred populations, mating with relatives becomes inevitable, but it would still be an advantage to recognise more closely related kin. This would reduce the chance of a matched mating, and therefore also reduce the chance of producing costly diploid males.

The study detailed in Chapter 2 investigated the effects of only one generation of sib-mating on aspects of colony fitness. This may explain why no difference in the fitness of outbred colonies and inbred colonies that did not produce diploid males was found. Just one study has investigated the fitness of bumblebees over successive generations of inbreeding (Beekman *et al.* 1999), and found that such continuous inbreeding resulted in a decline in queen fertility and colony size under laboratory conditions. If logistics allowed, a useful extension to the work presented in this thesis would be to continue the inbreeding for a greater number of generations and investigate all aspects of fitness, including parasite susceptibility. As it stands, one generation of sib-mating does not accurately represent natural situations, where small population size and limited gene flow inevitably results in consanguineous matings over a number of generations. However, previous genetic studies of the bumblebee populations in the Western Isles of

Scotland have shown that they provide an interesting study system for the investigation of the effects of inbreeding in natural, unmanipulated populations.

Darvill *et al.* (2006 & 2010) investigated the genetic diversity, population structure and dispersal ability of *B. muscorum* and *B. jonellus* from a number of Hebridean islands. The studies showed that more isolated populations of *B. muscorum* had significantly lower levels of heterozygosity, and that this species as a whole was substantially more inbred and had lower dispersal abilities than *B. jonellus*. Quantifying the prevalence of parasitic infection in these two species has allowed us to make comparisons of infection incidence in relatively inbred and outbred populations as well as inbred and outbred species (Chapter 4). The results of this investigation revealed a relationship between heterozygosity and parasitism: the more inbred populations of *B. muscorum* had higher prevalence of the tracheal mite *L. buchneri*. However, there was no apparent relationship between population genetic diversity of *B. muscorum* and *C. bombi* prevalence. This latter parasite species is known to vary in prevalence substantially across host species, localities and times of year (Shykoff & Schmid-Hempel, 1991b; Korner & Schmid-Hempel, 2005). The bees analysed in Chapter 4 had been collected by Darvill *et al.* (2006 & 2010) over multiple years and throughout the summer season from June to September. Although these variables were accounted for in the statistical analyses, they could have masked any effect of genetic diversity on *C. bombi* prevalence.

There was also no apparent relationship between genetic diversity and parasite prevalence in the more outbred bumblebee species, *B. jonellus*. This may have been because the measures of population heterozygosity fell within a considerably smaller

range than for *B. muscorum* (a range of only 0.019 compared to 0.228), potentially masking any influence that genetic diversity has on parasite prevalence. This could also result from the fact that *B. jonellus* is a more genetically diverse species than *B. muscorum* and has a greater dispersal ability (Darvill *et al.*, 2010). This may enable it to avoid inbreeding depression and hence the associated costs such as increased parasite prevalence. Interestingly, *B. jonellus* had higher infection rates of both *C. bombi* and *L. buchneri* than *B. muscorum*. It is possible that the more inbred *B. muscorum* individuals have a lower fitness and are therefore unable to survive high levels of infection, meaning that high parasite prevalence were not observed in this species. However, it is more likely that this observation is unrelated to genetic diversity and instead due to an inter-specific difference in the parasitism rates of these two species. This could result from variation in host genetics and parasite defence, environmental factors or parasite virulence. Such differences are commonly found across bumblebee species (e.g. Shykoff & Schmid-Hempel, 1991b; Korner & Schmid-Hempel, 2005), although the reasons for them remain unknown. There are clear opportunities for further research in this area; for example, it would be interesting to investigate the susceptibility of *B. muscorum* and *B. jonellus* workers to *C. bombi* under standardised laboratory conditions.

The striking relationship between population genetic diversity and *L. buchneri* prevalence in *B. muscorum* prompted a more detailed investigation into this bumblebee species (Chapter 5). This study focused on nine island populations and sampled bees over a period of 16 days in August 2009. The short time frame limited variation in parasite prevalence that might occur due to time of year. The study found that *C. bombi* prevalence was higher in populations with lower genetic diversity, therefore suggesting

that more inbred populations do have higher parasite prevalence; confirmation of the conclusions of Chapter 4. There was also a negative relationship between heterozygosity and prevalence of *L. buchneri* but, unlike the results in Chapter 4, this was not significant. This could have been because fewer island populations were sampled, and covered a smaller range in measures of population heterozygosity (a range of 0.187 compared to a range of 0.228 in Chapter 4).

The variation in parasite prevalence among islands could conceivably result from factors other than the genetic diversity of the bumblebee populations. The most obvious are environmental factors, but unfortunately it was beyond the scope of this study to measure these. It is possible that a less favourable environment, for example one with higher levels of rainfall, could impact the health and immunity of bumblebees and render them more susceptible to parasites. However, it is unlikely that such environmental factors are sufficiently variable across the Hebridean islands to produce the marked differences observed in parasite prevalence. Alternatively, flower abundance may have differed among the islands and, as at least one species of bumblebee parasite is transmitted on flowers (Durrer & Schmid-Hempel, 1994), this in turn may have affected parasite abundance. It would be constructive to conduct a follow-up study to investigate whether a positive relationship exists between parasite abundance and flower abundance on these Hebridean islands.

One striking difference between the two parts of this thesis that investigate parasitism in *B. muscorum* in the Hebrides (Chapters 4 & 5), is the difference between the prevalence of *C. bombi*. In Chapter 4 the overall prevalence observed was 15.9% but in Chapter 5 it was 89.8%. Although inter-annual differences in rates of parasitism in bumblebees are

frequently found (for example, Paxton, 2005), a difference of this magnitude is rare. Different detection techniques were used in the two investigations: In Chapter 5, *C. bombi* was detected through microscopic examination of the hind gut, while in Chapter 4 diagnostic PCR was used. However, it is unlikely that these techniques would have resulted in the observed prevalence differences. PCR is a more sensitive method (Schmid-Hempel & Funk, 2004), and it would perhaps be expected to detect a higher prevalence than dissection, but in fact the opposite was observed. Another possibility is that the condition of the bee specimens affected the reliability of the detection rates of *C. bombi*. The bee specimens dissected in Chapter 4 had been collected between 2003 and 2005 and stored in 100% ethanol. Despite this, in some cases, the ethanol had evaporated and the contents of the bee abdomens had blackened. Although tracheal mites were still in evidence, it is possible that in a small proportion of bees the gut had degraded to a degree where PCR was no longer able to detect *C. bombi* DNA. By contrast, the bees sampled for Chapter 5 were fresh frozen shortly after being caught and then only defrosted for dissection. It is unlikely any *C. bombi* cells in the hindgut would have degraded in this time, ensuring a high detection rate. Ideally, identical sampling and specimen storing methods would have been used for both studies.

Despite the limitations to the investigations in Chapters 4 and 5, the results support the theory that population genetic homogeneity leads to higher parasite prevalence (Sherman et al., 1988; Schmid-Hempel, 1998). The results are also supported by other circumstantial evidence in bumblebees (Allen *et al*., 2007; Cameron *et al*., 2011) as well as in many other species, particularly vertebrates (for example, Whiteman *et al*., 2006). This thesis investigates how inbreeding impacts parasitism in real island populations and provides a proxy to understand the impacts of inbreeding in fragmented habitat

islands on the mainland. The results suggest that if bumblebee populations become sufficiently isolated, causing a lack of gene flow and a subsequent loss of genetic diversity, they may suffer from higher levels of parasitism. Such a cost associated with inbreeding may push threatened populations closer to extinction. However, much is still to be understood, for example it is not known how large or connected habitat fragments need to be to maintain genetic diversity and only poor estimates of the actual sizes of most isolated populations exist. Such information is crucial to conservation efforts and research into these issues would be invaluable.

The isolation of populations is a contemporary problem globally, caused by the fragmentation of habitats and changing climatic conditions. The ranges of many different species are now subdivided into habitat islands between which there is limited migration. This has been shown to result in inbreeding in a wide range of taxa other than bumblebees - for example termites in tropical rainforests (Dupont *et al.*, 2009) and European tree frogs (Andersen *et al.*, 2004). As such population fragmentation and isolation increases the risk of species extinctions (Fahrig, 2003), restoring habitat connectivity is key to slowing the loss of biodiversity. Conservation management is increasingly addressing this through the creation of habitat corridors and connections between protected areas (Worboys *et al.*, 2009; Gilbert-Norton *et al.*, 2010). For bumblebees and other insect pollinators, agri-environment schemes and urban gardens are going some way to achieving this and improving habitat availability (Carvell *et al*., 2007; Osborne *et al*., 2008).

The final part of this thesis investigated the potential threat of pathogen spread from commercial to wild bumblebees (Chapter 6). This is an important and timely area of

research as recent work in North America has suggested that diseases from commercial bumblebees may pose a significant threat to native species (Winter *et al*., 2006; Colla *et al*., 2006; Cameron *et al*., 2011). No comparable work has been undertaken in Europe, despite the widespread usage of commercial bumblebees. The results presented in this thesis contrast with those of the North American studies as we found that parasitic infection in wild bumblebee species was no higher at farms that deployed commercial bumblebees than at farms that did not. This suggests that pathogen spillover may not be a substantial threat to wild bumblebees species in the UK. However, care should be taken when interpreting these results as they are based on pathogen prevalence from just one year and on a relatively small spatial scale. As bumblebee parasite prevalence is known to vary spatially and temporally (Shykoff & Schmid-Hempel, 1991b; Paxton, 2005), the experiment would ideally be repeated over a longer time-scale and over a larger area to account for such limitations.

Although no direct evidence for pathogen spillover was found, the prevalence of one species of parasite, *C. bombi*, increased through the season in *B. terrestris* on commercial farms, whilst it remained approximately constant on wild farms. This was driven by a marked increase in infection rate at the end of the season in August, which could have resulted from an increased rate of density-dependent transmission of *C. bombi* on commercial farms. This high prevalence does suggest that pathogen spillover is a potential threat, as there is a possibility that wild bumblebees - including newly emerged queens - may become infected by contact with commercial bees. Further study, including quantification of the infection rates of queens, is needed before any conclusions can be drawn. Additional investigation into the genetic diversity of commercial bumblebees and their resistance to parasites would also be valuable in

gaining a greater understanding of this issue. Despite the lack of direct evidence for pathogen spillover, the precautionary principle suggests that we should not interpret these results as an indication that the impact of commercial bumblebees is lower here than in other countries. Regulations should be imposed that ensure the commercial nest boxes imported to the UK are adequately screened for parasites. Techniques that would facilitate such screening include a recently developed multiplex PCR for the molecular detection of *C. bombi* and *A. bombi* simultaneously (Meeus *et al*., 2010a) and a multiplex PCR with broad range primers to detect honeybee viruses in bumblebees (Meeus *et al*., 2010b).

In conclusion, the work presented in this thesis demonstrates that inbreeding in bumblebees is particularly costly due to the production of diploid males but that bumblebees may be able to avoid their production through kin discrimination. We suggest that the detection of diploid males could be an informative tool in hymenopteran conservation as they can act as indicators of the genetic health of populations. If bumblebee populations do become inbred and lose genetic diversity, we have found that they may be pushed further towards extinction through increased parasitism. The potential exists for this pressure to be exacerbated through the use of commercial bumblebees and an associated higher parasite prevalence. Therefore, preventing population fragmentation and isolation is key for the conservation of bumblebees. This can be achieved through the creation of suitable flower rich habitats and by ensuring connectivity between habitat patches.

Chapter 8 - References

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