UNIVERSITY OF SOUTHAMPTON

Faculty of Medicine, Health and Biological Sciences
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The conservation genetics of the bumblebees *Bombus muscorum* and *Bombus jonellus* in a model island system

by

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ABSTRACT

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Prior to commencing this thesis, it was not known whether fragmented populations of rare bumblebees suffered fitness costs as a consequence of inbreeding or a loss of genetic diversity. Theory predicted that they could be particularly susceptible to local extinction due to small effective population sizes and the production of diploid males, but empirical data were lacking. Almost nothing was known about the dispersal capabilities of even common species of bumblebee, and it was not known whether interspecific differences might increase the sensitivity of some species to habitat fragmentation. This thesis compares the population genetics of two bumblebee species (*B. muscorum* and *B. jonellus*) in a model island system (Hebrides, UK). Both species show significant levels of population stucturing (*B. muscorum,* $\theta = 0.13$ and *B. jonellus,* $\theta = 0.034$) and isolation by distance. Pairwise comparisons between populations suggest that *B. jonellus* disperse > 50 km relatively frequently. By contrast, *B. muscorum* populations are spatially structured on a smaller scale, and are estimated to disperse > 8 km only infrequently. Populations of both species show signs of recent population bottlenecks under some microsatellite mutation models, and under all models bottlenecks were more frequent for *B. muscorum.* Diploid males were found at low frequency in *B. muscorum* but were not detected for *B. jonellus.* However, we find triploid individuals in both species, indirectly confirming that diploid males were present in the previous generation. This is the first time that triploids have been detected in any wild bee populations. For both species, triploid frequencies were negatively correlated with population size, and those restricted to less than $13km^2$ of suitable habitat were particularly at risk. Estimated total triploid frequencies peaked at 20% with respect to normal diploid workers, and were higher in *B. muscorum* than in *B. jonellus*, perhaps due to the greater dispersal range of the latter species. These results indicate that closely-related species exhibit cryptic but fundamental differences in aspects of their ecology which influence their susceptibility to habitat fragmentation. Observed differences may in part explain differential declines of mainland populations of bumblebees and will greatly inform future conservation strategies.

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Chapter 1 - General Introduction

1.1 Habitat destruction and species declines

1.1.1 An overview

The destruction and degradation of natural habitats as a result of human activity is a serious threat to global biodiversity. Formerly widespread species become restricted to remaining fragments of suitable habitat, and the resulting populations are frequently small and isolated. Within these small fragments, inbreeding depression, combined with disruptions to social structure, mating behaviour, feeding patterns and seasonal dispersal may reduce the fecundity of remnant populations. In addition, population growth may be reduced as a result of the Allee effect (Stephens & Sutherland 1999; Stephens *et al.* 1999). In recent years, the problems of habitat loss and fragmentation of populations have been highlighted by a number of high profile cases, including the preservation of avian diversity in Brazil (Marini & Garcia 2005), and the conservation of the red panda (*Ailurus fulgens*; Li *et al.* 2005), the South China tiger (*Panther tigris amoyensis*; Xu *et al.* 2007), the giant panda (*Ailuropoda melanoleuca*; Zhan *et al.* 2007) and Mexican wolves (*Canis lupus baileyi*; Fredrickson *et al.* 2007). Owing to a number of anthropogenic processes, populations and indeed entire species are being lost at such an alarming rate, across numerous taxa, that many authors suggest that the planet is entering the 6th recorded period of mass extinctions (Leakey & Lewin 1995; Thomas *et al.* 2004)

1.1.2 The decline of bumblebees

Many bumblebee species are thought to have declined dramatically in recent decades, both in Europe and North America (reviewed in Goulson 2003a). The distribution and conservation status of *Bombus* species worldwide are poorly documented. The best studied populations occur in the UK where, of the 19 native species of true bumblebee, only 6 remain common and widespread (*B. terrestris, B. lucorum, B. hortorum, B. pascuroum, B. pratorum* and *B. lapidarius*). Three species are now nationally extinct, although *B. pomorum* was only ever known from a few specimens and may never have been a genuine resident. *B. cullumanus* was a chalk-grassland species which declined due to habitat destruction, and was last recorded in about 1941 (BMNH collection). Finally, *B. subterraneus*, which was once widespread across southern England, declined dramatically in the years after World War II. It was last recorded at Dungeness in 1988. All of the 10 remaining extant species have, to varying degrees,

undergone comparable range declines and reductions in abundance (*B. ruderatus, B. distinguendus, B. muscorum, B. humilis, B. soroeensis, B. ruderarius, B. monticola, B. sylvarum, B. jonellus, B. magnus*). The available evidence suggests that bumblebee diversity in the UK is severely threatened, and worldwide trends may mirror those seen here, given that the primary cause (agricultural intensification) is steadily affecting all temperate regions of the northern hemisphere.

Agricultural intensification is thought by most to be the primary cause of these declines (Williams 1986; Osborne & Corbet 1994), through extensive habitat destruction associated with the 1947 Agriculture Act, and later, the Common Agricultural Policy (Goulson 2003). Vast tracts of previously suitable land have been lost to intensive agriculture since World War Two (Fuller 1987). Coupled with this drive toward increased productivity came a number of changes to farming methods that had additional consequences for biodiversity (reviewed in Goulson 2003). Many traditional practices were abandoned in favour of more intensive methods. Species rich hay meadows were replaced by monocultures of grass cut for silage. Rotations involving nitrogen-fixing leguminous crops such as clovers (*Trifolium* spp.) were no longer necessary once cheap fertilisers were commonplace. Increased pesticide use, the grubbing out of hedgerows, and ploughing right up to those that remain all reduced the diversity of wildflowers found within farmland. It is this loss of floral diversity that is believed to be the major cause of declining bee diversity in agricultural areas (Banaszak 1983, 1992; Gathmann *et al.* 1994; O'Toole 1994). These same changes are likely to have reduced the availability of nest and hibernation sites, and increased exposure to insecticides, both of which may have contributed to declines (Goulson 2003).

Although the reasons for bumblebee declines are well accepted and understood, it remains less clear why some species have faired so much worse than others. It is thought that in some cases climatic factors may be important (Williams 1986, 1988; Williams 2005), although these are unable to explain rarity in every case. Diet breath is also thought to be a factor, with rarer species tending to be more specialised (Goulson & Darvill 2004). Recent work has highlighted potential interactions between tongue-length and emergence times (Goulson *et al.* 2005; Fitzpatrick *et al.* 2007). Additional factors have been mooted, including differences in foraging range (Walther-Hellwig & Frankl 2000; Darvill *et al.* 2004). No single hypothesis seems able to provide a universal explanation for the differential declines experienced by our bumblebee fauna. As important pollinators of both crops and wildflowers (Corbet *et al.* 1991),

they are of great commercial and ecological significance, and addressing their declines should be considered a conservation priority. Changes in the composition of the pollinator community are thought likely to cause widespread changes in plant communities (Corbet *et al.* 1991), with knock-on effects for higher trophic levels.

One of the major consequences of habitat destruction is that remaining populations of rare bumblebees have become fragmented, and are now separated from one another by considerable distances (Edwards & Broad 2005). The consequences of this fragmentation for the long-term viability of remaining populations remain largely unknown, and their deduction is the primary aim of this thesis.

1.2 The effect of habitat fragmentation

1.2.1 The consequences of small population size

Small populations are inherently more vulnerable to local extinctions due to environmental and demographic stochasticity (Frankham *et al.* 2002). If these populations form part of a broader metapopulation then regional extinctions can be balanced by subsequent recolonisation, and dispersal between populations will ensure genetic cohesion (Frankham *et al.* 2002). However, if habitat fragmentation results in the isolation of small populations, then they may face an additional extinction threat through inbreeding and the loss of genetic diversity (Keller & Waller 2002; Reed & Frankham 2003; Spielman *et al.* 2004). If fragmentation is severe then extinct patches may never be repopulated, regardless of the suitability of the habitat.

A simple but significant consequence of small population size is that genetic diversity will be lower relative to large populations (Frankham 1996). However, when discussing the effects of population size it is crucial to make the distinction between census population size (N_c) and effective population size (N_e). N_e relates to the number of individuals that reproduce successfully in each generation (Beebee & Rowe 2004), and is often an order of magnitude lower than N_c due to the presence of non-breeding individuals, along with other factors including sterility, reduced fecundity, sex-ratio effects and mortality (Frankham 1995). The most extreme reductions in N_e occur during population bottlenecks (where N_e falls and remains

low for a number of generations), and founder events (when previously uninhabited patches are colonised by a small number of individuals). Clearly, fewer mutations per generation will occur in small populations, given a constant mutation rate per individual. In addition, the process of genetic drift results in rare alleles being lost more quickly in small populations, due to demographic stochasticity (Frankham *et al.* 2002). It is the harmonic mean of N_e over a number of generations that determines the expected genetic variation (Packer & Owen 2001). Therefore, occasional decreases to small population sizes will have a disproportionately large effect on average N_e and therefore genetic diversity (Barrett & Charlesworth 1991; Leberg 1992; Hartl & Pucek 1994). This process is not confined to neutral genetic markers. When N_e drops substantially below a previous long-term average, chance dominates selection with regard to the alleles that persist, even at non-neutral loci (Beebee & Rowe 2004). It follows that heterozygosity (a common measure of genetic diversity) is lower in small populations, and this has been demonstrated empirically (Frankham 1996).

1.2.2 What genetic threats exist?

Small populations with low genetic variation (and therefore low phenotypic variation) are less able to withstand short-term environmental perturbation (Allendorf & Leary 1986; Keller *et al.* 1994), and have a reduced evolutionary potential (Fisher 1930; Soule 1980; Lande 1995; Frankham 1999). In addition, within these populations deleterious mutations will tend to accumulate, since selection is less effective in small populations (Lynch *et al.* 1995). These processes could reduce the viability of populations in the long-term. By contrast, *inbreeding* may rapidly threaten populations in the short-term (Keller & Waller 2002). By increasing the frequency of individuals that are homozygous for alleles that are identical by descent, inbreeding increases the frequency with which deleterious recessive alleles are expressed. This is termed inbreeding depression, and can lead to rapid population extinction through an extinction vortex (Gilpin & Soulé 1986; Lacy 1993). There are related fitness costs associated with reduced frequencies of heterozygotes. For certain traits, the fittest phenotype results from heterozygosity at the coding locus. This is variously termed heterozygote advantage, overdominance, or heterosis (Beebee & Rowe 2004). Considerable confusion arises through the use of the term inbreeding, as it is used in a number of different contexts (Jacquard 1975). Keller and Waller (2002) provide an excellent review of the terminology, which is summarised below.

1.2.3 Inbreeding terminology

The term 'inbreeding' is used in a number of contexts, all of which describe situations in which matings occur among relatives, resulting in an increase in homozygosity (Jacquard 1975). However, inbreeding is always a relative measure, never an absolute one (Wright 1969), so the differences reflect the reference population to which they refer.

Pedigree inbreeding

Inbreeding occurs under this definition when two parents share common ancestors (Figure 1.1). The extent of inbreeding is determined by the amount of ancestry that is shared by the parents of an inbred individual. F is therefore defined as the probability of two homologous genes in an individual being identical by descent. Note that it is possible for an individual to be homozygous at a locus without the two alleles being identical by descent, due to size homoplasmy (Section 1.4). Pedigree F only measures the inbreeding contributed by a particular pedigree, and as such it is always *relative* to a particular ancestral generation. In practice pedigree information is only available for a limited number of generations. Averaged over all individuals within a population, pedigree inbreeding is equivalent to Wright's F_{IT} (Wright 1978a).

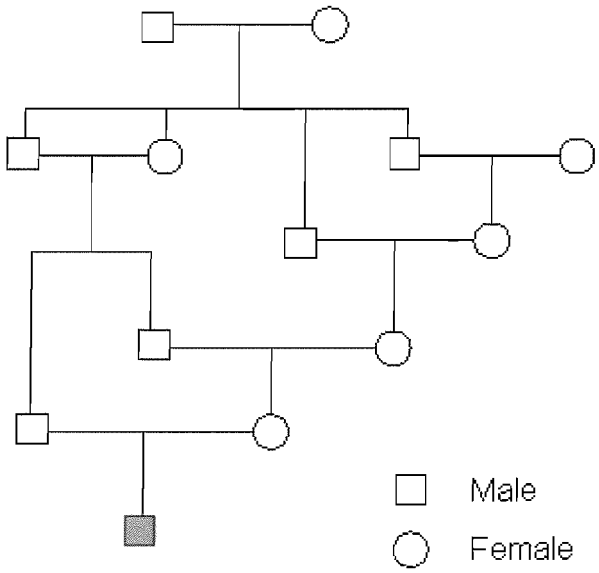


Figure 1.1 Pedigree inbreeding. In this instance all of the individuals are the descendents of the initial pair. Furthermore the shaded individual's father's uncle is also his mother's grandfather. As a result, the shaded individual's pedigree inbreeding coefficient, *F*, is 0.305. (Adapted from Keller and Waller, 2002).

Inbreeding due to non-random mating

In this context the term refers to the degree of relatedness between mates *relative* to two mates chosen at random from the population. The degree of inbreeding within a non-randomly mating population is measured relative to a randomly mating population of the same size. This type of inbreeding is sometimes determined through detailed pedigrees (Keller & Arcese 1998), but is more usually inferred by calculating the parameter F_{IS}. This is the difference between the observed heterozygosity, and that expected under random mating ($F_{IS} = 1 - H_o/H_e$). When $F_{IS} > 0$ it suggests that inbreeding is occurring more frequently than would be expected under random mating. An $F_{IS} < 0$ indicates that less inbreeding than expected is occurring, suggesting the presence of inbreeding avoidance mechanisms. In small randomly mating populations, mating between relatives will result in high levels of pedigree F (F_{IT}), but yet F_{IS} will be zero.

Inbreeding due to population subdivision

When populations are divided into several essentially isolated sub-populations, inbreeding will also occur, purely because N_e is restricted and genetic drift occurs within population fragments. This occurs irrespective of whether random mating occurs within these fragments. This final definition of inbreeding corresponds to Wright's F_{st}, and measures inbreeding across the whole population, relative to that expected under random mating in the total population. In the absence of non-random mating within populations, significant values of F_{ST} therefore demonstrate that the population as a whole is spatially structured. Very few migrants per generation (N_m) are needed to prevent genetic structuring between populations (Beebee & Rowe 2004). Theoretically an $N_m > 1$ is all that is required to prevent subpopulations diverging from one another as a result of drift. In practice, however, a greater number of migrants will often be required in order to prevent inbreeding in natural populations, as many migrants will not successfully reproduce (Vucetich & Waite 2000).

The total inbreeding within a collection of sub-populations (F_{it}) is a function of both within and between population inbreeding. The three parameters are linked by the following formula:

$$1 - F_{IT} = (1 - F_{IS})(1 - F_{ST})$$

1.2.4 Measuring inbreeding - the relationship between F and H_E

For many species it is far from straightforward to follow individuals closely enough to allow pedigrees to be reconstructed. However, the relationship between individual inbreeding coefficients and various fitness characters is still of great interest. As a result, a number of publications have examined the heterozygosity of individuals, averaged across a small number of loci, and reported significant correlations with fitness-related traits (reviewed in Coltman & Slate 2003). The suggestion that such a correlation exists is appealing to population geneticists. However, both theoretical (Balloux *et al.* 2004) and empirical (Slate *et al.* 2004) studies have concluded that the correlation between individual inbreeding coefficients (F) and heterozygosity, where present, is very weak. A recent review concluded that heterozygosity-fitness correlations should be treated with caution, suggesting that in these cases the microsatellites used may be linked to other loci that affect fitness (Pemberton 2004).

Heterozygosity nevertheless remains an important parameter when comparing populations, as it accurately reflects differences in genetic diversity (Beebee & Rowe 2004).

1.2.5 Inbreeding in wild populations

In the past, a number of authors have questioned the importance of genetic factors in the long-term viability of wild populations, arguing that effects will be small relative to demographic and environmental stochasticity (Caro & Laurenson 1994; Caughley 1994). There are also a number of mechanisms by which inbreeding can be avoided, including long range dispersal, kin recognition, or polyandry (Pusey & Wolf 1996). However, a recent meta-analysis concluded that most species are *not* driven to extinction before they are affected by genetic factors (Spielman *et al.* 2004). Indeed there is now a wealth of evidence suggesting that inbreeding reduces both individual and population performance. Numerous effects have been documented, including a reduction in larval survival, adult longevity, egg-hatching rates and resistance to disease and environmental stress (reviewed in Keller & Waller 2002). However, rather than having a direct effect, inbreeding is thought to make individuals more susceptible to environmentally inflicted mortality. For example, inbred individuals from a population of song sparrows (*Melospiza melodia*) died at a much higher rate during a severe storm than did outbred birds (Keller 1998).

It is important to note that genetic processes will not necessarily condemn a species to extinction, regardless of the state of its environment. The Mauritius kestrel was, by 1974, reduced to a single breeding pair (Groombridge *et al.* 2000) after spending many generations with population size below 50, which is often regarded as the minimum for viability (Frankham *et al.* 2002). However, an intensive program of conservation and habitat management has restored numbers to over 200 pairs (Safford & Jones 1997). Whether the reduced genetic variation within this population limits its ability to withstand environmental perturbations, or constrains future evolutionary potential remains to be seen.

Most studies of the effects of inbreeding in wild populations have focused on vertebrates, with few studies of populations of invertebrates (Keller & Waller 2002). Of note is the oft cited case of the Glanville fritillary (*Melitaea cinxia*) on the Åland islands in Finland. Saccheri *et al.* (1998) found that populations with low heterozygosity had a higher extinction risk, due to

decreased larval survival, adult longevity and egg hatching rates. Similarly, Schmitt and Hewitt (2004) found that in regions of low genetic diversity, butterfly populations decline more rapidly, although in this case reduced adaptability rather than inbreeding depression was implicated.

1.3 The conservation genetics of social Hymenoptera

1.3.1 An overview of sociality

Eusociality, where certain related individuals never attempt to reproduce themselves, but cooperate in helping others to reproduce, is very rare in nature (Goulson 2003). The naked mole rat (*Heterocephalus glaber*) and a few other arthropod groups (including termites) are the sole examples outside the Hymenoptera. However, eusociality is common in the Hymenoptera, and is thought to have evolved independently on several occasions (Wilson & Holldobler 2005). Why should this be?

Social Hymenopterans are haplodiploid, whereby males are haploid (are produced from unfertilised eggs) and females are diploid. Bumblebee colonies are founded by a single queen who has mated only once (Estoup *et al.* 1995b; Schmid-Hempel & Schmid-Hempel 2000). The worker caste, which are produced from fertilised eggs, are not sterile, and are able to lay haploid eggs. Haplodiploidy has some interesting consequences for intra-colony relatedness (Figure 1.2). Most importantly, full-sisters are on average 75% related to one another, but only 50% related to their own offspring. This predisposes Hymenopterans to cooperate, as kin-selection theory (Hamilton 1964) predicts that an individual should help her mother produce new queens (sisters) rather than lay her own male eggs (Goulson 2003).

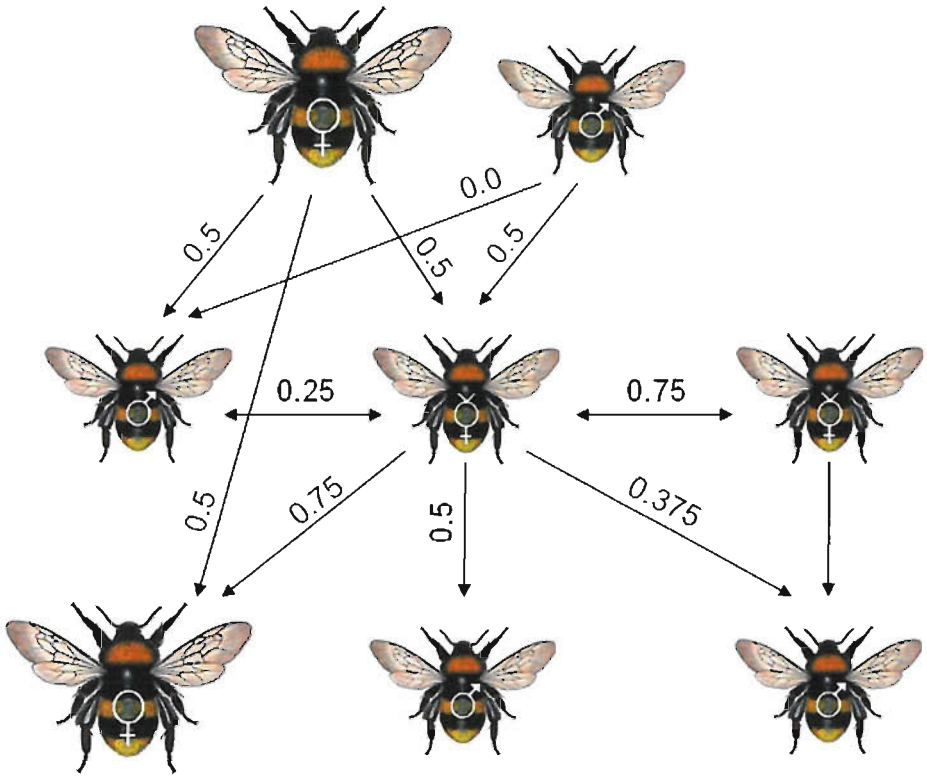


Figure 1.2 The relatedness amongst individuals from a bumblebee colony. Note that workers are more related to their sisters (including new queens) than they are to their own offspring. However, workers are more closely related to their sons and nephews then they are to their own brothers.

1.3.2 The influence of sociality on conservation genetics

One of the major consequences of habitat destruction is that remaining populations become fragmented, and are separated from one another by considerable distances. A healthy population of butterflies can persist on quite a small area of land for many decades. For example, a south-facing downland of just a few hectares near Folkestone in south-east England has supported a population of many thousands of Adonis blue butterflies (*Lysandra bellargus*) for at least 100 years, even though this population is very isolated (Harper *et al.* 2003). The last bumblebee species to disappear from the United Kingdom (*B. subterraneus*), was once widespread across southern England, but declined dramatically in the years after World War II, and was last recorded at Dungeness in 1988. Its decline followed a characteristic pattern, with populations becoming increasingly small and isolated, and subsequently disappearing, despite the apparent suitability of the remaining habitat (in contrast with the Adonis blue butterfly). Why might this be so? Understanding the consequences of the

fragmentation and isolation of remnant populations of bumblebees is of great importance to conservationists, given the current distributions of many rare species. Of course, bumblebees are not the only group to have declined in the last century, but it seems that they may have been particularly hard hit.

Social Hymenoptera are particularly susceptible to a loss of genetic diversity as it is the effective population size N_e, rather than the census population size N_c which determines the rate of genetic drift. N_e may therefore be lower than the number of individuals by several orders of magnitude (Pamilo & Crozier 1997; Wilson & Holldobler 2005), and dependent simply on the number of colonies which successfully reproduce. In many social insects, including bumblebees, large numbers of reproductively inactive individuals live within the colony, and a large area is needed to support a single nest, resulting in a low N_e within an area (Goulson 2003). The N_e contributed by a single colony depends on the number of queens in a colony (N_f) and the number of males they have mated with (N_m), according to Wright's (1933) formula:

$$N_e = \frac{9N_m N_f}{(2N_f + 4N_m)}$$

The effective population size of a given species can therefore be increased as a result of polygyny (more than one queen per nest) and polyandry (where queens mate with more than one male) (Chapman & Bourke 2001). Many species of bees, wasps, and ants are both polygynous and polyandrous to varying degrees. However, in bumblebees, all colonies are founded by a single queen, and the vast majority of species are monoandrous (Estoup *et al.* 1995b; Schmid-Hempel & Schmid-Hempel 2000). It follows that the effective population size is equal to the number of successful nests x 1.5, not x 2 as would be the case for a diplo-diploid organism (Wright 1933; Crozier 1976). It seems therefore that population sizes of bumblebees may be low, even relative to other social insects, making them particularly susceptible to the loss of genetic diversity.

An additional and highly significant cost may be imposed on inbred populations of haplodiploid Hymenoptera as a result of their sex determination mechanism (Cook & Crozier 1995). The system (parthenogenetic arrhenotoky) ordinarily results in diploid individuals developing into females, and haploid individuals into males. However, the mechanism

underlying this process, known as the Single Locus Complimentary Sex Determination mechanism (sl-CSD) has important consequences for small populations (Cook & Crozier 1995). The mechanism centres around a polyallelic sex-determining locus. Individuals heterozygous at this locus develop into females, and homozygous (or hemizygous) individuals develop into males. It is of course possible for a diploid individual to be homozygous at this locus, but in normal outbred populations, allelic diversity is such that this occurs very infrequently (e.g. Duchateau *et al.* 1994). However, as populations diminish in size, genetic drift will lead to a reduction in the number of sex alleles in the population, increasing the probability of a 'matched mating' (Owen & Packer 1994). A queen that mates with a male who shares one of her sex determining alleles will produce a colony in which 50% of her workforce are diploid males. In honeybees and ants, diploid male larvae are consumed by the workers, which minimises their cost, but in bumblebees they are reared to adulthood (Plowright & Pallett 1979; Duchateau *et al.* 1994). Bumblebee diploid males are viable but effectively sterile, and therefore represent a considerable cost to the colony best viewed as 50% worker mortality (Packer & Owen 2001). The colony-level impact of diploid male production is reduced in polyandrous and polygynous hymenopteran species, and some authors have suggested that these strategies may have originally arisen in response to DMP (Cook & Crozier 1995; van Wilgenburg *et al.* 2006b). Recent modelling work has shown that DMP, where present, can initiate a rapid extinction vortex. Zayed and Packer (2005) conclude that this renders haplodiploids more (rather than less) prone to extinction as a result of reduced genetic diversity then diplodiploid taxa. Fascinatingly, not all diploid males are truly sterile, and in 5% of cases, matings result in colonies which produce triploid females, and in some cases triploid males (Ayabe *et al.* 2004). These colonies are extremely small and weak however, and do not survive long (Ayabe *et al.* 2004).

1.3.3 Evidence of inbreeding depression in Hymenoptera

Low effective population sizes, and an associated loss of genetic diversity will not necessarily lead to reductions in population fitness. Inbreeding depression through the expression of deleterious alleles will only occur if a population harbours an appreciable genetic load (Keller & Waller 2002). It is thought that the haploid males in haplodiploid Hymenoptera provide a mechanism for the purging of recessive deleterious alleles, by exposing them to selection in each generation (Bruckner 1978; Crozier 1985; Werren 1993; Antolin 1999; Packer & Owen

2001). Indeed it has been shown theoretically that they carry a reduced genetic load relative to diploid species across a wide range of mutation rates, selection intensities, and dominance levels (Werren 1993). As such, Werren (1993) has suggested that haplodiploids are more likely to survive the transition from outbreeding to inbreeding than are diploids.

However, it is clear that purging will not be effective against female sex-limited traits, such as fecundity or hibernation survival (Gerloff *et al.* 2003; Henter 2003; Gerloff & Schmid-Hempel 2005). Few studies have attempted to determine the consequences of inbreeding in bumblebees. Gerloff and Schmid-Hempel (2005) found significant reductions in colony foundation success and hibernation success (reductions of 23% and 6% respectively) in response to brother-sister mating. In this case little evidence of inbreeding depression was found for reproductive output or cumulative fitness, as was the case for Duchateau *et al.* (1994), but in both instances nests were reared in the lab and fed *ad libitum.* However, Beekman *et al.* (1999) found that inbred queens laid fewer eggs, suggesting that colony growth might be slower. Gerloff *et al.* (2003) found no evidence for a reduced encapsulation (immune) response following the insertion of small plastic beads. A recent meta-analysis concluded that, although haplo-diploid insects suffer less from inbreeding than diploid insects, substantial inbreeding depression does occur, which in one case resulted in a 38% decrease in longevity and a 32% reduction in fecundity (Henter 2003).

Diploid males represent a clear example of inbreeding depression, and have been detected in numerous wild populations of Hymenopterans (Plowright & Pallett 1979; Kukuk & May 1990; Pamilo *et al.* 1994; Roubik *et al.* 1996; Zayed & Packer 2001; Stahlhut & Cowan 2004a, b). Their frequency has been proposed as an indicator of population fitness (Zayed *et al.* 2004). Diploid male production (DMP) had not been detected in wild outbred populations of bumblebees prior to the present study, and its absence has been used to infer that *B. terrestris* possesses at least 46 sex-determining alleles (Duchateau *et al.* 1994). However, sib-mating in the lab has demonstrated that the sl-CSD operates in bumblebees, and indeed the descendents of a small population introduced to Tasmania were found to produce diploid males with high frequency (Buttermore *et al.* 1998). The relationship between genetic diversity and DMP remains far from clear, with a number of studies reporting low frequencies of DMP despite high levels of inbreeding (Paxton *et al.* 2000; Stahlhut & Cowan 2004a).

1.3.4 Studies of the population genetics of hymenopteran species

There have been remarkably few studies of the population genetics of fragmented populations of Hymenopterans. Previous studies have largely focused on ant species and report mixed findings, generally related to the colony structure present. For example, fragmented populations of the wood ant, *Formica lugubris*, showed no detectable inbreeding and had high genetic variability, perhaps as a consequence of polygyny maintaining a high effective population size (Gyllenstrand & Seppa 2003). Similarly, Maki-Petays *et al* (2005) found little evidence for reduced genetic diversity in two polygynous ant species in response to habitat fragmentation. A significant amount of genetic structuring was found between subpopulations, but social structure, rather than limited dispersal led to restricted gene flow. Studies of the monogynous ant *Formica exsecta* provide some evidence of inbreeding in social insects, with population sub-structuring at a very local scale, significant inbreeding coefficients (F_{IS}) and consequently a high degree of queen–male relatedness (Pamilo & Rosengren 1984; Pamilo 1991; Sundstrom *et al.* 2003). Similarly, breeding between relatives has been shown in ant species where very few colonies participate in nuptial flights at any one time (Hasegawa & Yamaguchi 1995). Finally, there is some evidence of inbreeding in the lek-mating species *Pogonomyrmex occidentalis* (Cole & Wiernasz 1997).

Given the potentially serious consequences of inbreeding in bumblebees, it is essential that we understand its prevalence within wild bumblebee populations. Populations that are geographically isolated from one another will begin to diverge (via genetic drift) only if gene flow between them is negligible. Neutral genetic markers can be used to estimate population structuring, relative levels of genetic diversity and dispersal range. Initial studies of genetic diversity were carried out during the 1970's and 1980's, and used allozymes; these are polymorphic enzymes that can be separated using gel electrophoresis. However, it seems that *Bombus* exhibit unusually little variation in their allozymes, rendering this approach of little value (Pamilo *et al.* 1984; Scholl *et al.* 1990; Owen *et al.* 1992; Scholl *et al.* 1992). The development of several highly variable microsatellite markers (see section 1.4) for bumblebees (Estoup *et al.* 1993), along with advances in DNA sequencing facilitated the direct assessment of variation in DNA sequences between both individuals and populations.

Initial studies focused largely on two common and widespread European species, *B. terrestris* and *B. pascuorum*. In *B. terrestris,* there appears to be little population substructuring within mainland Europe, suggesting that dispersal is frequent and that there are no substantial isolating barriers between populations (Estoup *et al.* 1996). However, populations on various Mediterranean islands and Tenerife (Canary Islands) were distinct, indicating that substantial bodies of water do provide a more-or-less complete barrier to movement (Estoup *et al.* 1996; Widmer *et al.* 1998). In *B. pascuorum*, populations throughout most of mainland Europe are similar, but differ markedly from those found south of the Alps in Italy, suggesting that substantial mountain ranges can also act as barriers to dispersal (Pirounakis *et al.* 1998; Widmer & Schmid-Hempel 1999). There were also small differences between populations in Scandinavia and those in the body of mainland Europe. Widmer and Schmid-Hempel (1999) conclude that *B. pascuorum* probably invaded Europe from two refugia following the last ice-age, with one population coming to occupy most of Europe from Spain to Sweden, and the other remaining trapped in Italy. More recently, Shao *et al.* (2004) compared seven mainland and island populations of *B. ignitus* in Asia and similarly found that mainland populations were genetically similar, but distant offshore populations had significantly differentiated, perhaps as a consequence of founder effects and drift. Additional insights into the dispersal of bumblebees come from monitoring their spread after artificial introduction into new areas. In New Zealand, they spread by up to 140 km per year (Hopkins 1914) and colonised islands up to 30 km off shore (Macfarlane & Griffin 1990), although we cannot be certain that their dispersal was not artificially aided. Indeed they are absent from islands at distances ranging from 16-55 km from the mainland (Macfarlane & Gurr 1995). In Tasmania, to which *B. terrestris* was introduced in 1992, spread has been much slower, at about 10 km per year (Stout & Goulson 2000).

In many cases it seems that genetic structuring is observed when populations are separated by appreciable barriers, like mountain ranges or stretches of water. It is particularly interesting to note that the population of *B. terrestris* on an island just 3km offshore was significantly differentiated from the mainland (Estoup *et al.* 1996). Whilst it is conceivable that bumblebees have a decreased propensity for dispersal over water, this suggests that under some circumstances even generally common and widespread species may be susceptible to localised population structuring. Many rare species of bumblebees have very fragmented distributions, existing in 'islands' of suitable habitat separated by 'oceans' of arable farmland. It is clearly of

great importance that we ascertain the population structure for these species, and quantify the genetic diversity within habitat remnants.

1.4 Microsatellites and their use in conservation genetics

Microsatellites are neutral markers found widely dispersed throughout the genomes of most eukaryotes (Hamada *et al.* 1982). They consist of tandem repeats of short-sequence motifs, usually of 2, 3 or 4 base pairs, repeated many times. This regular pattern makes them prone to occasional replication errors, and over time a great diversity of different alleles may accrue. Microsatellites are numerous, highly variable and easy to score, making them excellent genetic markers (Queller *et al.* 1993). By comparing the frequencies of different alleles between different populations, usually using several different microsatellite loci, much can be learnt about population genetics. In addition, they are inherited in a simple Mendelian fashion, making them suitable for studies of relatedness and inbreeding (Queller *et al.* 1993; Frankham *et al.* 2002).

Microsatellites are identified by a standard procedure which involves making and screening a library of short DNA sequences (Rassmann *et al.* 1991; Hughes & Queller 1993). Fragments in the region 300-600bp are cut from the genome using restriction enzymes and ligated into plasmid vectors. These vectors are transformed into *E. coli*, and colonies are cultured before being screened with a number of labelled candidate repeat sequences (CT_{10}, GC_{10} etc). Colonies which hybridise with these probes are then directly sequenced, enabling primers to be designed from the flanking regions either side of the microsatellites (see Queller *et al.* 1993). Using these primers, microsatellites can be amplified by PCR (Saiki *et al.* 1985), separated according to size by electrophoresis, and finally scored by comparison with standards of known size (Queller *et al.* 1993).

Estoup *et al.* (1993) were the first to characterise microsatellites in bees. They identified 26 in *B. terrestris* and an additional 75 in *Apis mellifera*. Of these 26, a subset of particularly variable markers has been used in a number of subsequent studies of bumblebee population genetics (see previous section). A further set of bumblebee microsatellites have recently been developed, which will doubtless prove useful in future studies (Funk *et al.* 2006).

Microsatellites, through their ability to infer relatedness, have also been used to study a number of other aspects of bumblebee ecology. The number of different alleles in worker offspring was used by Estoup *et al.* (1995b) to determine whether queens of five bumblebee species (*B. terrestris, B. lucorum, B. lapidarius, B. pratorum* and *B. hypnorum*) mated more than once. Schmid-Hempel & Schmid-Hempel (2000) extended this work to include an additional three species (*B. pascuorum, B. sicheli* and *B. hortorum*). In all species, with the exception of *B. hypnorum,* queens were found to mate only once (monoandry). That most species are monandrous makes the process of identifying sisters on the basis of shared alleles considerably more straightforward. Sisters from the same colony are on average 75% related to one another (see section 1.3) which allows them to be distinguished from non-sisters with a high degree of certainty (Queller *et al.* 1993). This has enabled a number of studies to estimate bumblebee foraging range and nest density, by detecting sisters from distant sample sites, and assessing their frequency relative to non-sisters (Chapman *et al.* 2003; Darvill *et al.* 2004; Knight *et al.* 2005).

Microsatellites have been favoured by population geneticists for several years due to their variability, ease of use and relatively low cost (Queller *et al.* 1993). However, in recent years two issues have been identified which warrant consideration. Firstly, a number of standard measures, such as heterozygosity (H_E) and the inbreeding co-efficient (F) assume that two alleles that are identical in size are the same. However, it is becoming increasingly evident that size homoplasy is commonplace in bumblebee microsatellites (Estoup *et al.* 1995c; Viard *et al.* 1998; Yokoyama *et al.* 2004). Size homoplasy (SH) refers to the situation where different alleles are present that are identical in length, but not identical by descent. If SH is common within populations then it may reduce the accuracy with which relatedness can be calculated, and overestimates of the extent of within-population inbreeding (F_{IS}) may be observed. If however, most SH occurs between rather than within populations, as was found by Viard *et al.* (1998), genetic differentiation between populations (F_{ST}) might be underestimated. However, a recent review concluded that SH is only likely to present a significant problem for population geneticists when mutation rates are high, population sizes are large, and strong allele size constraints exist (Estoup *et al.* 2002). In general the review concluded that in most cases the large amount of variability at microsatellite loci compensates for any homoplasmious evolution.

The second issue relating to the suitability of microsatellites for use in conservation genetics centres on current uncertainties over the mechanism of mutation. Size homoplasmies arise from mutations (insertions, deletions or substitutions) that either do not change the length of the allele, or change it to the length of another allele already present in the population. This is related to, but distinct from the normal mutational processes that give rise to variation at microsatellite loci. A number of different models exist, and empirical work suggests that no one model is applicable to all loci (Estoup *et al.* 2002). The predominant mutational model is of great relevance in population genetics, as it affects the rate at which SHs arise (Estoup *et al.* 2002), and the methodology used to detect recent population bottlenecks (Piry *et al.* 1999). First proposed was the infinite allele model (IAM: Kimura & Crow 1964), in which any number of tandem repeats may be gained or lost, always resulting in an allele not already present within a population. As such, it is the only model under which SH cannot occur (Estoup *et al.* 2002). The K-allele model (KAM: Crow & Kimura 1970) describes a less extreme version of the IAM, where there are a finite number of allelic states (K), and any allele has a constant probability of mutating into one of the other (K-1) alleles. In contrast, the stepwise mutation model (SMM: Kimura & Ohta 1978) allows for the loss or gain of just a single tandem repeat in each mutation event. SHs accumulate under this model more rapidly than under any other (Estoup *et al.* 2002). Finally, the two-phase model (TPM: Dirienzo *et al.* 1994) describes mutation patterns that involve a majority of one-step changes, with a smaller proportion of multi-step changes. In practice, perfect microsatellites [e.g. $(CT)_n$] seem to deviate less from the SMM than do interrupted [e.g. $(CT)_nAT(CT)_nTT(CT)_n$] or compound microsatellites [e.g. $(CT)_n(GT)_n$], both of which often seem to follow the TPM (Estoup *et al.* 2002). Research in this area is ongoing, and the findings of this thesis may have to be viewed in the light of future studies.

1.5 The bumblebee lifecycle

A very detailed account of bumblebee lifecycle and be found in Alford (1975), which is summarised by Goulson (2003). In short, bumblebees have an annual lifecycle (Figure 1.3). Queens and males typically emerge from mature nests in late-summer and autumn, and mate. Males are able to mate several times, but available evidence suggests that in the majority of species females only mate once (are monoandrous) (Estoup *et al.* 1995b; Schmid-Hempel & Schmid-Hempel 2000). After mating, queens spend several weeks foraging and build up substantial fat reserves, which they survive on whilst hibernating throughout the winter in a north-facing bank. They emerge from hibernation in spring and immediately begin foraging on nectar. The emergence time varies between species and may be related to dietary specialisation (Goulson & Darvill 2004). Nectar supplies may be in short supply at this time of year, and the availability of spring forage probably has a significant, but as yet unknown, effect on the distribution and abundance of certain species (Goulson 2003). Nesting sites vary between species, and frequently include areas of tussocky grass or disused rodent burrows. The queen incubates an initial brood, laid in a lump of pollen which is covered in wax exuded from her abdomen. Once these have hatched (after about 4 days) she begins foraging for both nectar and pollen which she feeds to the growing larvae. The total development time for this first brood is about 4-5 weeks and during this time the colony may be particularly susceptible to prolonged periods of inclement weather. After emerging, the workers take over the foraging duties and help the queen tend the nest and produce subsequent broods. The nest grows rapidly from this point onwards, until at some point between April and August (depending on the species) it switches to producing new males and queens.

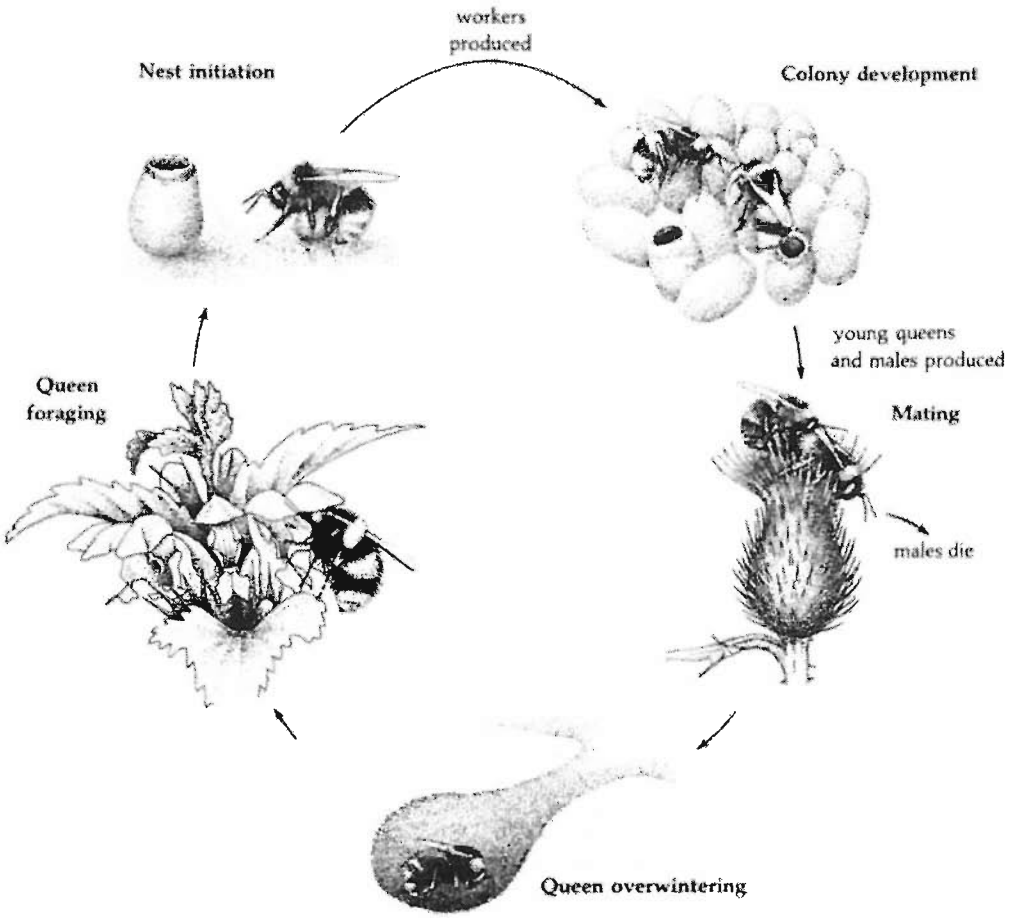


Figure 1.3 The life cycle of a true bumblebee (after Prys-Jones & Corbet 1991).

1.6 Aims and objectives

Habitat loss has led to the fragmentation and isolation of many bumblebee populations throughout the UK, and elsewhere. Concerningly, some of these populations have subsequently disappeared, despite the (apparent) suitability of remaining habitat fragments. It is possible that inbreeding is accelerating local extinctions, or that dispersal is insufficient to maintain a metapopulation on the scale imposed by habitat loss. If we are to conserve our declining bumblebee species, we urgently need to determine a number of facts concerning their ecology. Specifically, it would be desirable to determine:

a) The minimum population size needed to avoid a high rate of stochastic local extinctions, and to maintain sufficient genetic diversity to avoid inbreeding depression
b) The area of habitat needed to support such a population
c) The dispersal range of bumblebees. This is an important parameter, as dispersal allows the recolonisation of locally extinct populations and counters the loss of genetic diversity that may otherwise occur in small populations

This thesis aimed to answer these questions for two bumblebee species; an ambitious undertaking given that these ecological attributes are not easy to measure. In order to discern the effects of population size and isolation it is necessary to know the precise distributions of the studied species. Unfortunately, accurate distribution maps with sufficient resolution are not available for much of the UK. Without exhaustive ground surveys the confounding effects of undetected stepping-stone populations could never be ruled out. To overcome this problem, populations on oceanic islands were studied, where the extent of suitable habitat and degree of isolation could be easily quantified. The model island system (Hebrides, UK) contains islands of a range of sizes, which are isolated to varying degrees. The drawback of this approach is that dispersing individuals are unable to stop and forage *en route*, so the scale of dispersal estimates may need to be adjusted before they are applicable to mainland populations.

The two species chosen for study (*B. muscorum* and *B. jonellus*) were both widespread within the island system and frequently occurred on the same islands, facilitating a comparison between the two. In addition, they represent an interesting contrast on two levels. *B. muscorum* is considered threatened, and has not coped well with the habitat fragmentation, particularly in

England. *B. jonellus* by contrast is not thought to be threatened, and persists with a widespread but local distribution, where suitable habitat allows. *B. muscorum* is a member of the subgenus *Thoracobombus*, and is one of four (of 5 in total) members of this subgenus to have been placed on the UK Biodiversity Action Plan. *B. jonellus* belongs to the subgenus *Pyrobombus*, none of which are considered threatened in the UK, and several of which have been involved in well documented colonisations in recent decades.

Over the course of the following chapters I aim to:

1. Study the population genetics of *B. muscorum* in an attempt to understand its dispersal capabilities and the minimum area needed to support a viable population.
2. Compare parameter estimates for *B. muscorum* with those of *B. jonellus* to determine whether interspecific differences exist.
3. Quantify diploid male and triploid frequencies with the aim of determining the minimum population size required to avoid reductions in population fitness.

Peripheral studies also examine:

4. Whether aggregated males outside a mature *B. muscorum* nest are the offspring of the colony, or whether they have been attracted from other colonies in the vicinity and are attempting to mate with emerging gynes
5. Patterns of variation in the size of foraging workers, both within and between islands.

Chapter 2 - An introduction to the research system

This short chapter sets the scene for later research chapters by providing background information about the island system (Hebrides, UK) and the study species. I give a brief overview of current knowledge about the ecology of the study species, modified largely from Benton (2006). To this I add data on the foraging ecology of the study species in the Hebrides collected during the course of field work for later chapters, as this information is unlikely to be published elsewhere. These data do not represent a controlled study of habitat and forage use, but I believe they represent a reasonable approximation.

2.1 The Hebrides

The Hebrides are a diverse archipelago comprising over 120 named islands (80 of them inhabited) off the west coast of Scotland (Figure 2.1a). The islands are composed of some of the oldest rocks in the British Isles (up to 3,000 million years old), and range in size from small offshore skerries just a few hundred metres across to large islands of many thousands of hectares (Angus 2001).

Several different habitat types occur, some of which have been largely unaffected by man's long presence on the islands, while others are heavily dependent on his intervention. The most notable example of the latter is machair, a globally scarce habitat found only in parts of Scotland and western Ireland. This habitat is found on several Hebridean islands, and results from the deposition of shell-based sand by strong prevailing winds, forming low lying calcareous plains (Angus 2001). In the broad sense the 'machair system' actually stretches from the fore dunes through grassland, machair lochs, fens and salt marshes to the blackland where the sand based soil merges into peat rich moorland (Angus 2001). From the perspective of bumblebees, the most important area is the floristically rich machair grassland. This botanically unique habitat relies on low intensity crofting practices including small scale cultivation and grazing (Owen *et al.* 2001; Gaynor 2006). Other habitats of importance for bumblebees include rotationally grazed pasture, hay meadows, domestic gardens, heather moorland and bog.

To date, much of the Hebrides have escaped the agricultural intensification which is thought to be responsible for declines in bumblebee species richness observed elsewhere (Williams 1986; Osborne & Corbet 1994). Low intensity farming, coupled with adjacent natural habitats,

creates a patchwork of habitats which support a diverse assemblage of bumblebees. Species richness peaks on Coll and Tiree, where 10 species can be found (*B. lucorum, B. cryptarum, B. magnus, B. jonellus, B. hortorum, B. lapidarius, B. ruderarius, B. pascuorum, B. muscorum,* and *B. distinguendus*). Elsewhere, species richness is lower, limited presumably by a combination of habitat diversity and the dispersal abilities of individual species. Most widespread are *B. muscorum* and *B. jonellus*, both of which occur on most of the islands in the archipelago, and it is these two species which this thesis focuses on.

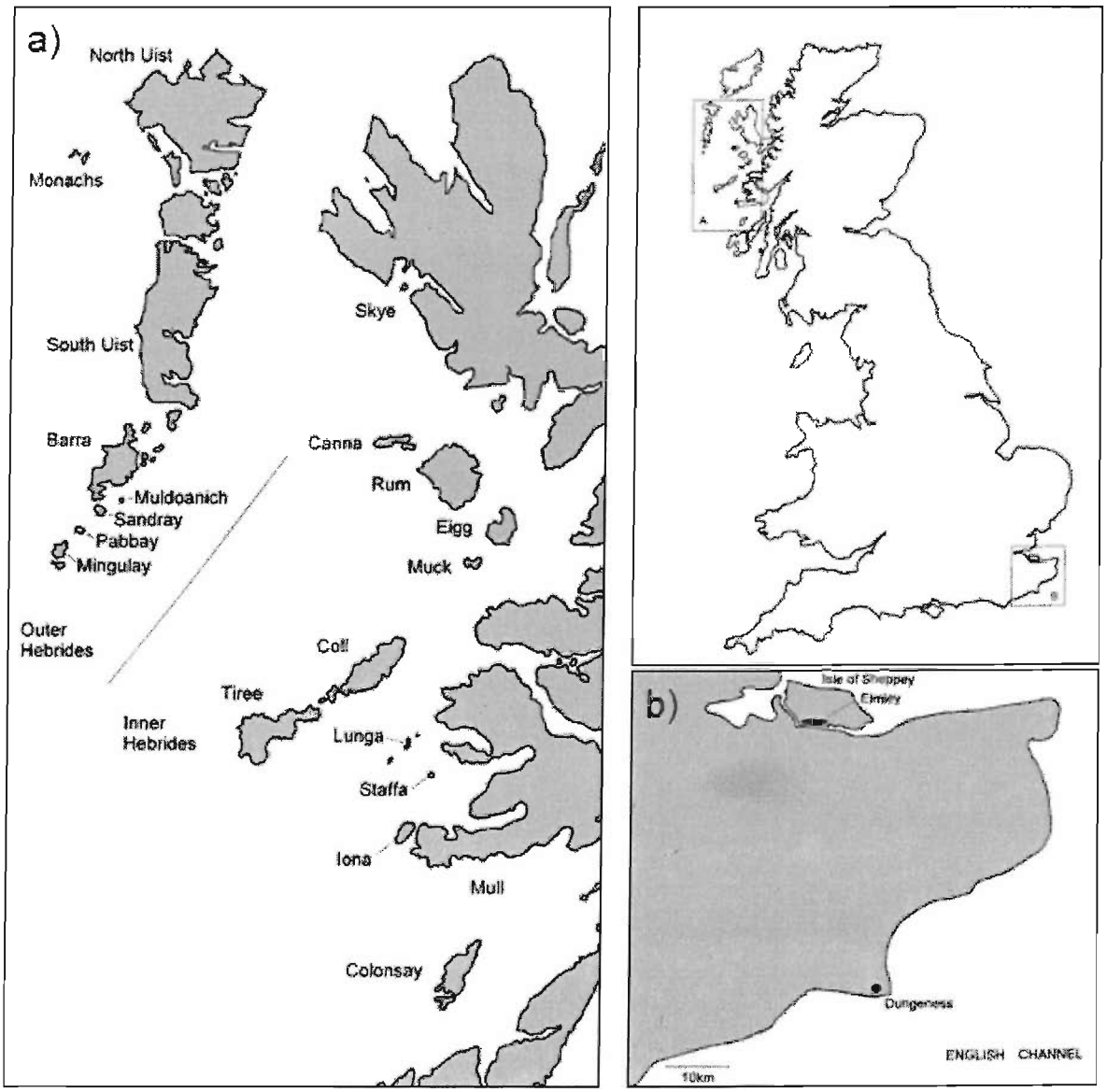


Figure 2.1 Maps of the areas studied, showing; a) the visited islands in the Hebrides (those named) and, b) the two sites in southern England from which *B. muscorum* were collected (see Chapter 3).

2.2 The study species

2.2.1 *Bombus muscorum* (Linnaeus)

a) Description

This species belongs to the sub-genus *Thoracobombus*, a group of mainly long-tongued bees, which includes the common and widespread species *B. pascuorum* (Alford 1975). In general appearance *B. muscorum* is similar to *B. pascuorum.* Its thorax and abdomen are ginger, although the thorax is generally a little darker, giving a two-tone appearance (Prys-Jones & Corbet 1991). There are several named subspecies in the UK, differentiated on the basis of coat colours. The most distinctive of these (*agricolae* Baker, previously called *agricolae*) occurs on the Outer Hebrides, and on some of the islands in the Inner Hebrides. The face, ventral surfaces and legs are all black, and the thorax is a deep chestnut brown, giving it a very distinctive appearance (Prys-Jones & Corbet 1991). Elsewhere in the Inner Hebrides and on the adjacent mainland one finds the subspecies *pallidus* Evans. This has pale hairs on ventral surfaces and legs, and the red-brown shade to the thorax is intermediate between *agricolae* and the sub-species found in the south of the UK, *sladeni* Vogt (Alford 1975).

b) Distribution

Sladen (1912) describes this species as widespread throughout Britain, but not abundant, and states that it was more common in Scotland and Ireland than in England. It had a localised distribution in central southern England and in the Midlands. Following declines in recent decades, this species in now sparsely distributed in England and Wales, and occurs primarily along the coast (Figure 2.2). It is more widespread (but local) in Scotland, occurring inland, along the coast, and on many of the Scottish islands.

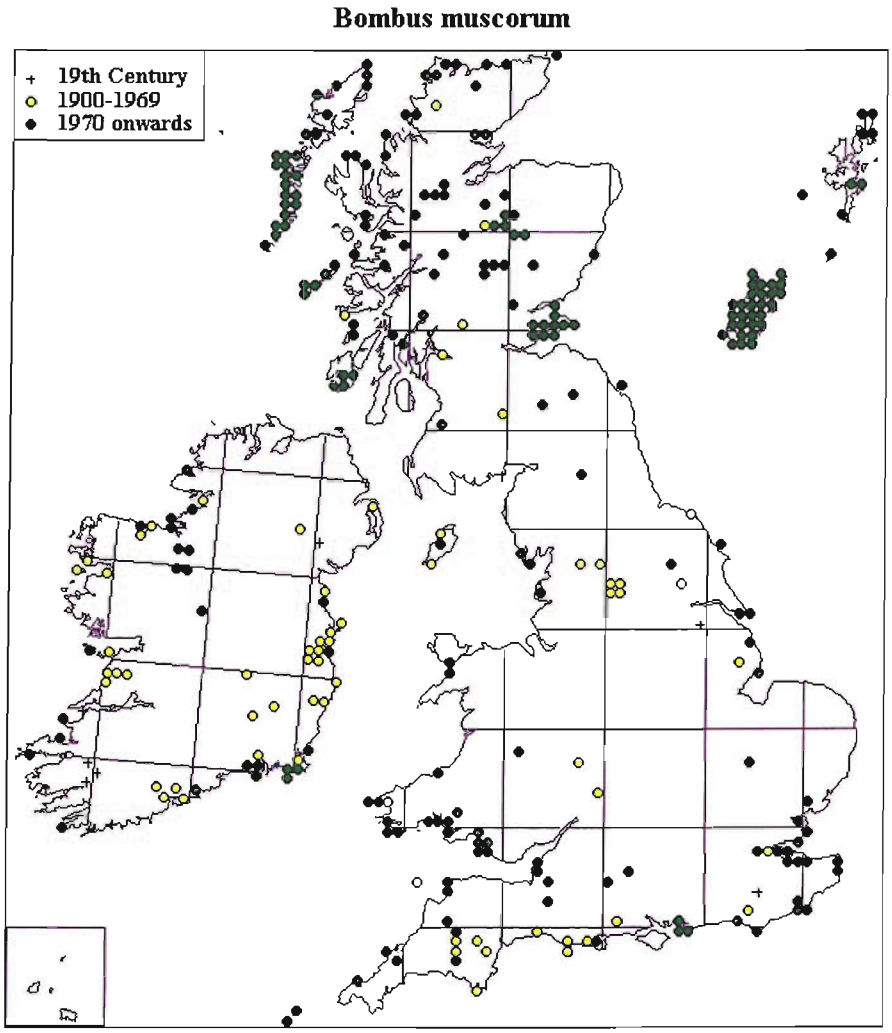


Figure 2.2 The distribution of *B. muscorum* in the British Isles on a 10 km x 10km grid. Green dots represent post-1970 records and yellow dots pre-1970. Reproduced with permission from the Bees Wasps and Ants Recording Society (BWARS).

c) Ecology

In southern England, queens are relatively late emerging, with the first appearing during May (Benton 2006). In the Hebrides, where all species emerge later than in southern England, they are one of the earlier species, with queens in flight from late May (pers. obs.). They are thought to nest primarily on the ground surface (like other carder bumblebees), using dead grasses or moss to cover the surface of the nest (Alford 1975). Workers begin to appear in late May/early June and peak in numbers during July (from late June in the Hebrides, peaking in August). Benton (2006) states that a mature nest contains relatively few workers, estimated at fewer than 100.

B. muscorum has a mainly coastal distribution in England and Wales, where it survives along sea walls and flood defences. It is said to prefer cool, damp, open grasslands, with moss or leaf litter for nesting (Alford 1975). It has been recorded foraging on a range plant species, including *Lamium purpureum*, *Trifolium repens*, *Trifolium pratense*, *Vicia cracca*, *Lathyrus pratensis, Vicia sativa, Cirsium vulgare* and *Lotus* spp. (for a full list, see Benton 2006). Ellis (2005) found that 78% of all visits were to the Fabaceae, primarily *Trifolium repens*, *Trifolium pratense*, *Lotus* spp. and *Vicia cracca*. In mainland Scotland, *B. muscorum* primarily uses *Erica tetralix* on wet moorland and heath (M. Macdonald, pers. comm.). In the Hebrides, they use two distinct habitat types; machair-type areas and heath land. Of the total worker sample, 49% were foraging on machair or flower-rich meadow, and 48% on heathland. There was no machair on many of the islands, in which case all of the workers were caught from heath land. Within machair type habitats, 39% of visits were to *Trifolium pratense*, 14.7% were to *Cirsum vulgare*, 13.4% to *Centaurea nigra*, 10.9% to *Succisa pratensis* and 8.5% to *Trifolium repens*. On heathland, 63.7% of visits were to *Erica cinera*, 16% were to *Calluna vulgaris*, 7.9% to *Potentilla erecta* and 7.1% to *Erica tetralix*.

d) Conservation status

Formerly widespread throughout Britain, but not abundant (Sladen 1912), *B. muscorum* is now considered an increasingly scarce species, with a scattered but mainly coastal distribution (Benton 2006). It has, for some time now, been the subject of an English Nature (now Natural England) Species Recovery Project. It has recently been added to the UK Biodiversity Action Plan (UKBAP), where it joins three other UK representatives of this sub-genus (*B. sylvarum*, *B. humilis* and *B. ruderarius*).

2.2.2 *Bombus jonellus* (Kirby)

a) Description

This species is a member of the sub-genus *Pyrobombus*, a group of mainly short-tongued bees, which includes the common and widespread species *B. pratorum* (Alford 1975). Workers resemble *B. hortorum* in general appearance, a distinctive species found commonly in urban gardens throughout the UK. Its face and thorax have a black ground-colour, with yellow bands on both the collar and scutellum. The abdomen is also black, with a yellow band on the first tergite and a white tail (tergites 5 & 6). Males are similar, but have more extensive (and

brighter) yellow areas. The subspecies *hebridensis* occurs on the Outer Hebrides, and has a pinkish tail, resembling the closely related species *B. pratorum*.

b) Distribution

This species is widespread but local throughout Britain, with the greatest density of populations in both the south and the north (Benton 2006). Within Scotland it is widespread in the north, and is present on many of the Scottish islands, including Orkney and Shetland (Figure 2.3b).

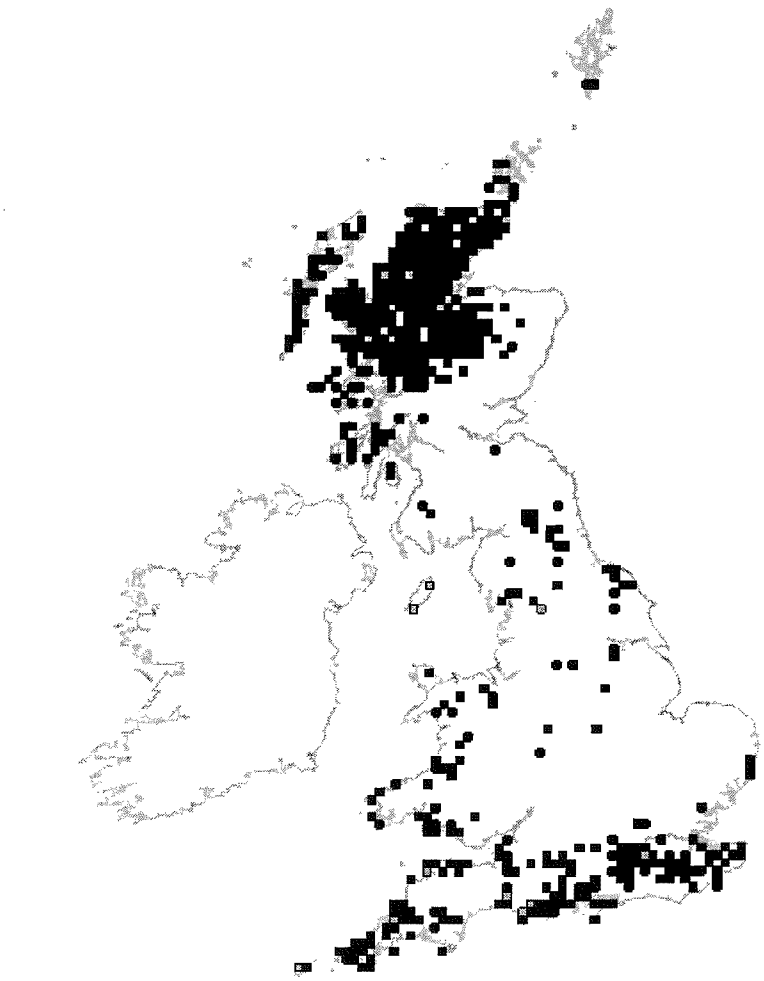


Figure 2.3 The distribution of *B. jonellus* in the British Isles on a 10 km x 10km grid. Black dots represent post-1970 records and grey dots those from pre-1970. Reproduced with permission from the Bees Wasps and Ants Recording Society (BWARS), accessed via the National Biodiversity Network (NBN) database.

c) Ecology

This is a relatively early emerging species in southern England, with queens appearing in late March or April (Benton 2006). They are thought to nest both below ground and on the surface. The colony cycle here is short, and the species frequently raises a second brood later in the season (Alford 1975). Workers begin to appear at the end of April in the south, and new queens from the first brood emerge in June or July. On the Scottish mainland, queens are abundant by mid-June, and workers can be seen from June onwards, reaching a peak by late September. *B. jonellus* is not though to raise a second brood in the north. In the Hebrides, queens can be seen from mid-June, but workers are scarce until mid-July, whereafter numbers rise rapidly (pers. obs.). Colonies are thought to contain 50-120 workers at maturity (Benton 2006).

B. jonellus is predominantly associated with heathland and moorland, but also occurs in some coastal habitats. In some areas it is also known to visit gardens. In Scotland, *B. jonellus* is said to use machair grassland in addition to heather moorland (Benton 2006). In the Hebrides, on most islands 100% of workers were sampled from heather moorland. None were found using machair on Coll or Tiree, despite the abundance of this habitat. Small numbers were observed using machair on South Uist. In the south of the UK, queens and workers have been recorded using a broad range of forage plants (see Benton 2006), but in the Hebrides and on the adjacent mainland they were much less polylectic; 72.2% of workers were found visiting *Calluna vulgaris*, 18.7% were on *Erica cinerea*, 3.2% on *Succisa pratensis*, 2.5% *Centaurea nigra* and 1.1% on *Erica tetralix*. However, Staffa and Lunga, the smallest islands visited, were the only islands on which *B. jonellus* was found using *Succisa pratensis* and *Centaurea nigra*. Patches of *Erica cinerea* and *Calluna vulgaris* on these islands were relatively small, and many foragers were also using *Centaurea nigra* (76.5% of Staffa workers) and *Succisa pratensis* (47.8% of Lunga records).

d) Conservation status

This species is not thought to have declined significantly in recent decades, although some populations have been lost from the central and southern lowlands of England. It remains locally abundant in heathland and moorland, and also occurs at low density on some grassland sites in southern England (for example, Salisbury Plain). It is not considered threatened, and is not part of the UK Biodiversity Action Plan.

Chapter 3 - Population structure and inbreeding in a rare and declining bumblebee, *Bombus muscorum* (Hymenoptera: Apidae).

This chapter has been published as:

Darvill B, Ellis JS, Lye GC, Goulson D (2006) Population structure and inbreeding in a rare and declining bumblebee, *Bombus muscorum* (Hymenoptera: Apidae). *Molecular Ecology* **15**, 601-611.

The *B. muscorum* from southern England were caught and genotyped by Jon Ellis. General laboratory assistance, including DNA extraction, was provided by Gillian Lye. All authors commented on draft versions of this manuscript, and the published version is presented here.

3.1 ABSTRACT

Owing to habitat loss populations of many organisms have declined and become fragmented. Vertebrate conservation strategies routinely consider genetic factors, but their importance in invertebrate populations is poorly understood. Bumblebees are important pollinators, and many species have undergone dramatic declines. As monoandrous social Hymenopterans they may be particularly susceptible to inbreeding due to low effective population sizes. We study fragmented populations of a bumblebee species, on a model island system, and on the mainland where it is rare and declining. We use microsatellites to study: population genetic structuring and gene flow; the relationships between genetic diversity, population size and isolation; frequencies of (sterile) diploid males - an indicator of inbreeding. We find significant genetic structuring ($\theta = 0.12$) and isolation by distance. Populations >10km apart are all significantly differentiated, both on oceanic islands and on the mainland. Genetic diversity is reduced relative to closely related common species, and isolated populations exhibit further reductions. Of 16 populations, 10 show recent bottlenecking, and 3 show diploid male production. These results suggest that surviving populations of this rare insect suffer from inbreeding as a result of geographic isolation. Implications for the conservation of social Hymenopterans are discussed.

3.2 INTRODUCTION

The destruction of natural habitats as a result of human activity is one of the most serious threats to survival faced by many species. Formerly widespread species become restricted to remaining fragments of suitable habitat, and the resulting populations are frequently small and isolated. Without frequent immigration these populations are prone to the loss of genetic diversity through bottlenecks and drift (Frankham *et al.* 2002; Keller & Waller 2002). The maintenance of genetic diversity is crucial for the long-term survival of many populations and species (Frankham *et al.* 2002; Hansson & Westerberg 2002; Keller & Waller 2002; Reed & Frankham 2003). Reduced genetic diversity lowers the capacity of a population to respond to environmental change, and may lead to inbreeding depression caused by the expression of deleterious alleles.

It has been argued that in wild populations the impact of inbreeding will be negligible relative to demographic and environmental stochasticity (Caro & Laurenson 1994; Caughley 1994), and avoided due to processes such as long-range dispersal, kin recognition and polyandry (Pusey & Wolf 1996). There is, however, now a wealth of evidence suggesting that inbreeding may reduce both individual and population performance. Numerous effects have been documented, including a reduction in larval survival, adult longevity, egg-hatching rates and resistance to disease and environmental stress (reviewed in Keller & Waller 2002). However, the vast majority of such studies have focused on vertebrate and plant species. The relatively large population sizes of invertebrates compared to vertebrates may explain why few signs of inbreeding have been detected (Van Dongen *et al.* 1998; Gyllenstrand & Seppa 2003; Henshaw & Crozier 2004; Keller *et al.* 2004; Molbo *et al.* 2004). In contrast, Saccheri *et al.* (1998) found reduced heterozygosity in small isolated populations of the butterfly *Melitaea cinxia* and a resultant increase in extinction risk. Given the vast number of invertebrate species, and the diversity of their life histories, it is not yet possible to generalize as to the relative importance of genetic processes in the decline of endangered populations.

As a result of their sociality, social insects exhibit characteristics which may increase their susceptibility to inbreeding. Effective population sizes may be small, despite an apparent abundance of (sterile) workers, and haplodiploidy may reduce genetic variation (Pamilo & Crozier 1997; Chapman & Bourke 2001). It has been argued that population fitness will not be

reduced by inbreeding in haplodiploid Hymenoptera, as haploid males provide a mechanism for the purging of recessive deleterious alleles (Sorati *et al.* 1996). However, the single-locus complementary sex determination (sl-CSD) system found in social Hymenopterans has an important consequence for the fitness of populations which begin to lose genetic diversity. Individuals homozygous at the sex locus develop into sterile diploid males, which do not benefit the colony, and therefore represent a cost (Cook & Crozier 1995). The overall fitness of the population is thus directly related to the number of different alleles at the sex locus, which may in turn be related to the size and isolation of the population.

Habitat fragmentation further increases the susceptibility of populations to a loss of genetic diversity, through drift and bottlenecks (Frankham *et al.* 2002; Keller & Waller 2002). Previous studies of social Hymenoptera have largely focussed on ant species and report mixed findings, generally related to the colony structure present. For example, fragmented populations of the wood ant, *Formica lugubris*, showed no detectable inbreeding and had high genetic variability, perhaps as a consequence of polygyny (more than one queen per nest) inflating the effective population size (Gyllenstrand & Seppa 2003). Similarly, Maki-Petays *et al.* (2005) found little evidence for reduced genetic diversity in two polygynous ant species in response to habitat fragmentation. A significant amount of genetic structuring was found between subpopulations, although a closer analysis revealed that social structure played a key role in restricting gene flow. Conversely, five studies of monogyne ants (only one queen per nest) provide some evidence of inbreeding in social insects. Sundstrom *et al.* (2003) report a high degree of population structure at a local scale in *Formica exsecta* along with sex-biased gene-flow, significant inbreeding coefficients (F_{IS}) and a high degree of queen-male relatedness. Two further studies of the same population of the same species also report evidence for inbreeding (Pamilo & Rosengren 1984; Pamilo 1991). Similarly, inbreeding has been shown in ant species where very few colonies participate in nuptial flights at any one time (Hasegawa & Yamaguchi 1995). Finally, there is some evidence of inbreeding in the lek-mating species *Pogonomyrmex occidentalis* (Cole & Wiernasz 1997).

Bumblebees are social hymenopterans which live in annual colonies founded by a single queen. The majority of species are monoandrous (Estoup *et al.* 1995b; Schmid-Hempel & Schmid-Hempel 2000; Sauter *et al.* 2001; Payne *et al.* 2003), which decreases the amount of genetic variation present in each colony, relative to that of polygynous or polyandrous species, and therefore increases their susceptibility to inbreeding (Chapman & Bourke 2001). In

addition, the effective population size is determined by the number of successful nests in a given area, rather than the number of (sterile) workers. Many bumblebee populations have declined dramatically in recent decades, both in Europe and North America, primarily as a result of agricultural intensification and associated habitat loss (reviewed in Goulson 2003). Of the United Kingdom's 25 native species, three are now extinct, and several remain only in small isolated populations (Goulson 2003). As bumblebees are important crop and wildflower pollinators (Corbet *et al.* 1991), their declines may have serious consequences for agriculture and for wildflower populations.

Previous studies of bumblebee population genetics have focussed on common and widespread species: *Bombus terrestris* (Estoup *et al.* 1996; Widmer *et al.* 1998), *B. pascuorum* (Pirounakis *et al.* 1998; Widmer & Schmid-Hempel 1999) and *B. ignitus* (Shao *et al.* 2004). Genetic differentiation between mainland sites separated by several hundreds of kilometres was low, and genetic variability was high. Comparisons between mainland sites and distant offshore islands found significant genetic differentiation, and reduced genetic diversity on some islands, possibly as a consequence of founder effect and drift (Estoup *et al.* 1996; Widmer *et al.* 1998; Shao *et al.* 2004).

Here, we study a rare and declining bumblebee species which exists in a series of small fragmented populations, in order to:

1) determine whether the populations exhibit genetic structuring, and estimate dispersal range;
2) attempt to establish the relationship between genetic diversity, population size and isolation;
3) detect the presence of diploid males as an indicator of inbreeding.

3.3 MATERIALS AND METHODS

3.3.1 Study species

B. muscorum (L.) is a rare bumblebee which has declined dramatically in recent years, particularly in the south of the UK where agricultural changes have been most pronounced. Once widespread on the mainland, it now survives only in a series of small fragmented populations, although it is still relatively abundant on some Scottish islands (Edwards & Broad 2005). *B. muscorum* occurs within the UK as a number of different subspecies, differentiated on the basis of coat colour. Three races occur within the study areas: *B. muscorum sladeni* (Vogt) is found in south and central England and Wales; *B. muscorum pallidus* (Evans) in northern England, mainland Scotland and some Inner Hebridean islands; and *B. muscorum agricolae* Baker on the Shetland Isles, the Outer Hebrides and some of the Inner Hebrides. Of these three races *B. muscorum sladeni* has declined the most, and is now very rare. *B. muscorum* occurs in a range of habitats (Goulson *et al.* 2006). In the south it occurs on coastal marshes, shingle and calcareous grasslands, and is strongly associated with Fabaceae (Goulson & Darvill 2004). In the north it is also frequently found on moorland and machair (Goulson *et al.* 2005).

3.3.2 Sample collection

During the summers (June – Sept) of 2003 and 2004, individuals of *B. muscorum* were collected from 14 islands in the Inner and Outer Hebrides (Scotland, UK), and from two southern UK sites (Figure 2.1). All known southern UK populations were visited, although at some, workers were insufficiently abundant for adequate samples to be collected. Previously recorded populations on the Scottish mainland were also visited, but here too workers were very scarce or absent, and samples were not collected. Non-lethal tarsal samples were taken following Holehouse *et al.* (2003). Workers were caught from numerous locations within each population (where possible >200m from one another) to minimise the probability of sampling individuals from the same colony. When encountered, male bumblebees were also caught in order to assess the frequency of diploid males, and were destructively sampled to conclusively assess their sex. Samples were preserved in pure ethanol and stored at ambient temperature.

3.3.3 Molecular methods

DNA was extracted using the HotShot protocol (Truett *et al.* 2000). Workers were genotyped at up to 9 microsatellite loci: B132, B131, B118, B100, B96, B10, B11, B124, B126 (Estoup *et al.* 1995b; Estoup *et al.* 1996). B100 was found to be monomorphic, as was B11 in all but one population. Microsatellites were amplified by polymerase chain reaction (PCR) in 10μL volumes using QIAGEN Multiplex PCR kits. Each reaction contained approximately 10ng template DNA, 1μL Q-solution, 5μL PCR Master Mix and 0.2μM of each primer. Samples were initially denatured at 95°C for 15 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 51°C for 90 s and extension at 72°C for 90s. A final extension step at 72°C for 10 min then followed. PCR products were visualised on an ABI PRISM™ 377 semi-automated sequencer using an internal size standard (GeneScan ROX 350, Applied Biosystems). Fragment sizes were scored using Genotyper (Applied Biosystems). Repeat PCRs were carried out on individuals believed to be diploid males, and on any samples that had failed to amplify or were uncertainly scored.

3.3.4 Statistical methods

The dataset was first checked for unexpected mutation steps, large gaps in the data or unusually sized alleles using MSA (Dieringer & Schlotterer 2003). A number of different software applications were used for subsequent analyses, some of which do not deal well with missing data. Where a full dataset was not available (for Elmley at B10, and several populations at B131) the locus or populations were excluded from the analysis. Tests for genotypic linkage disequilibrium and departure from Hardy-Weinberg equilibrium (HWE) were performed using GENEPOP version 3.4 (Raymond & Rousset 1995). Sequential Bonferroni corrections (Rice 1989) were applied to minimise Type I errors.

The genetic population structure was assessed with *F*-statistics (Wright 1951), using Weir & Cockerham's (1984) estimators (F, f and θ), as implemented in FSTAT version 2.9.3 (Goudet 2001). Global *F*-statistics were calculated for all populations, and pairwise θ for all pairs of populations. Means and standard errors of the estimates were obtained by jack-knifing over samples and loci. Significance levels were determined by permuting alleles (100,000 permutations) using MSA, applying Bonferroni corrections (Rice 1989).

An analysis of molecular variance (AMOVA) was performed using ARLEQUIN version 2.001 (Schneider *et al.* 2000), with the populations divided into 3 groups, corresponding to the three recognised subspecies, and also to their geographical locations. The significance of the results was estimated by performing 16,000 permutations.

Genetic isolation by distance (IBD) is expected to increase linearly with the logarithm of physical separation in two-dimensional space (Rousset 1997; Hardy & Vekemans 1999). Isolation by distance between the Hebridean populations was therefore examined by regression of pairwise estimates of genetic distance, as defined by $\theta/(1- \theta)$, against the corresponding logarithms of the geographic separation between populations (Rousset 1997). A Mantel test (Mantel 1967) was used to assess the significance of any correlation, performing 50,000 permutations in ISOLATION BY DISTANCE (Bohonak 2002).

A Neighbour-Joining (NJ) tree (Saitou & Nei 1987) relating the populations was constructed using Nei's chord-distance (Nei *et al.* 1983). To assess the stability of the tree nodes, 1000 bootstrap replications were performed using the PHYLIP package of programs (Felsenstein 2004). SEQBOOT, GENDIST and NEIGHBOUR were used to create replica datasets, calculate Nei's chord distance, and construct neighbour-joining trees, respectively. The final condensed consensus tree was produced using MEGA version 3 (Kumar *et al.* 2004), showing only nodes supported by more than 50% of bootstraps.

Possible loss of genetic variation through bottlenecking - a recent reduction in population size without substantial subsequent immigration - was tested for using BOTTLENECK version 1.2.02 (Piry *et al.* 1999). Three mutation models have been proposed for microsatellites: the stepwise mutation model (SMM); infinite allele model (IAM); and two-phase mutation model (TPM). Shao *et al.* (2004) followed the IAM, arguing that bumblebee microsatellites, which are interrupted-repeats, have been shown to not fit the SMM (Estoup *et al.* 1995a; Estoup *et al.* 1995c). Indeed, as dinucleotide repeats, multi-step changes may be frequent in these microsatellites (Huang *et al.* 2002). However, in support of the TPM, others have argued that mutation patterns involve a majority of one-step changes, with a smaller proportion of multi-step changes (Ellegren 2000; Schlotterer 2000). Since this debate is ongoing, tests were conducted using both the infinite-allele model (IAM), and the two-phased model of mutation

(TPM) allowing for varying multi-step changes between 5 and 10%. 100,000 iterations were performed in each case.

In order to ascertain whether the genetic diversity of the populations was related to either their isolation or to their area, a two-way analysis of variance (ANOVA) was performed using SPSS version 12. Genetic diversity was assessed by calculating Nei's unbiased heterozygosity (Nei 1987) and allelic richness using FSTAT. Allelic richness was calculated, because the average number of alleles per locus is sensitive to sample size (El Mousadik & Petit 1996). The habitat area utilised by each population was defined as the number of kilometre squares in which *B. muscorum* was found foraging. Isolation was measured as the distance to the nearest population, or (in the case of the Hebridean populations) the distance to the mainland, whichever was smaller. The area of land within 5, 10, 15 and 20 km of each Hebridean island was estimated using 1:50,000 Ordnance Survey maps, in order to take into account the size as well as the proximity of potential source populations. The allelic richness and H_E of Hebridean populations were compared to southern UK populations using Mann Whitney U-tests in SPSS version 12. In addition, the H_E of both Hebridean and southern UK populations of *B. muscorum* was compared to that of the closely related species, *B. pascuorum* (data from Widmer & Schmid-Hempel 1999). Data from a previous study of *B. terrestris* and *B. lucorum* are also included in Table 1 for comparison (Estoup *et al.* 1996).

3.4 RESULTS

3.4.1 Hardy-Weinberg and linkage disequilibrium

In total 854 females and 64 males were genotyped (Table 3.1). Neither global tests by population nor by locus detected any significant deviation from HWE. Significant linkage disequilibrium ($P < 0.05$) was found between two pairs of loci, B132-B131 and B96-B126, when testing each locus pair across all populations. Tests within each population found significant linkage disequilibrium in only one population (Staffa), for these locus pairs. A global test across all populations excluding Staffa found no significant linkage disequilibrium, so subsequent analyses were carried out with and without this population.

Table 3.1 The sample size, average (unbiased) heterozygosity (H_E) and allelic richness of each of the 16 populations (± S.E.), along with data for mainland continental populations of *B. pascuorum* (from Widmer & Schmid-Hempel 1999), *B. terrestris and B. lucorum* (from Estoup *et al.* 1996). Allelic richness and H_E were calculated using all loci except B131

Population	Sub-species	Sample size	Allelic Richness*	H_E
Barra	*agricolae*	50 ♀ 3 ♂	3.10 ± 0.66	0.393 ± 0.113
Mingulay	*agricolae*	49 ♀ 1 ♂	2.99 ± 0.64	0.374 ± 0.115
Muldoanich	*agricolae*	25 ♀ 6 ♂	3.63 ± 0.90	0.421 ± 0.103
Pabbay	*agricolae*	37 ♀ 16 ♂	3.33 ± 0.68	0.399 ± 0.118
Sandray	*agricolae*	58 ♀ 1 ♂	3.05 ± 0.63	0.367 ± 0.111
Outer Hebrides Average			**3.22 ± 0.12**	**0.391 ± 0.010**
Colonsay	*pallidus*	67 ♀ 0 ♂	3.21 ± 0.50	0.416 ± 0.086
Lunga	*pallidus*	36 ♀ 6 ♂	3.43 ± 0.56	0.507 ± 0.108
Staffa	*pallidus*	52 ♀ 0 ♂	3.33 ± 0.51	0.484 ± 0.091
Canna	*agricolae*	62 ♀ 3 ♂	3.11 ± 0.57	0.433 ± 0.086
Coll	*agricolae*	70 ♀ 0 ♂	3.46 ± 0.69	0.499 ± 0.091
Eigg	*agricolae*	64 ♀ 2 ♂	3.30 ± 0.51	0.533 ± 0.094
Muck	*agricolae*	52 ♀ 0 ♂	2.91 ± 0.42	0.425 ± 0.088
Rum	*agricolae*	42 ♀ 1 ♂	2.91 ± 0.48	0.451 ± 0.077
Tiree	*agricolae*	119 ♀ 2 ♂	3.27 ± 0.55	0.499 ± 0.086
Inner Hebrides Average			**3.21 ± 0.07**	**0.472 ± 0.014**
Overall Hebrides Average			**3.22 ± 0.06**	**0.443 ± 0.014**
Dungeness	*sladeni*	23♀ 6 ♂	3.95 ± 0.62	0.522 ± 0.088
Elmley †	*sladeni*	48♀ 17 ♂	4.06 ± 0.92	0.496 ± 0.125
Southern UK Average			**4.01 ± 0.06**	**0.509 ± 0.013**
B. pascuorum		an average of 22.7 per site	**5.49 ± 0.16**	**0.563 ± 0.009**
B. terrestris		an average of 37.5 per site	**5.96 ± 0.12**	**0.610 ± 0.009**
B. lucorum		40	**7.00 ± 2.00**	**0.598 ± 0.115**

* For *B. pascuorum, B. terrestris and B. lucorum* the average number of alleles per locus is given. However, when sample sizes are similar, the two measures are comparable.

† For Elmley, data for B10 was incomplete.

3.4.2 Population structure

Overall genetic structuring was high, with $\theta = 0.119 \pm 0.023$ S.E. ($P < 0.00001$) [excluding Staffa, $\theta = 0.120 \pm 0.025$ S.E. $P < 0.00001$]. Estimates of F_{IS} were small (mean $F_{IS} = 0.01 \pm 0.01$ S.E.). Pairwise θ values were highly significant ($P < 0.01$) for 109 of 120 comparisons, and were significant ($P < 0.05$) for 3 pairs of populations (Mingulay-Muldoanich, Mingulay-Sandray and Muldoanich-Sandray). The remaining 7 comparisons between the cluster of islands comprising Mingulay, Muldoanich, Pabbay, Sandray and Barra were not significant. Coll and Tiree also showed no significant differentiation from one another.

An analysis of molecular variance (AMOVA) found significant structuring within populations ($P < 0.000001$) and among populations within sub-species groups ($P < 0.000001$), but not between sub-species groups ($P = 0.58$), confirming that the observed structuring was due to genetic differentiation between populations rather than a sub-species effect (Table 3.2).

3.4.3 Gene flow between populations

Table 3.2 Analysis of molecular variance (AMOVA) examining the partitioning of genetic variation*

Source of variation	d.f.	Sum of squares	Variance	% total	*P*
Within populations	1595	2503.443	1.56956	87.00	< 0.000001
Among populations within subspecies groups	12	340.418	0.24297	13.47	< 0.000001
Among subspecies groups	2	37.336	−0.00841	−0.47	0.58
Total	1609	2881.197	1.80411		

*Prior to performing this AMOVA, population Elmley and locus B131 were removed as ARLEQUIN does not perform well when some populations are missing data for one or more loci.

There was a highly significant correlation between genetic distance (θ/1- θ) and the natural logarithm of physical separation ($P < 0.0001$, $R^2 = 0.514$), for the 14 Hebridean populations (Figure 3.1). Some populations separated by distances of between 3 and 10 kilometres were significantly genetically differentiated from one another, and all populations more than 10km apart were significantly differentiated. The two southern UK populations were 49 km apart, and were separated by a significant genetic distance of 0.040 ($P = 0.0072$).

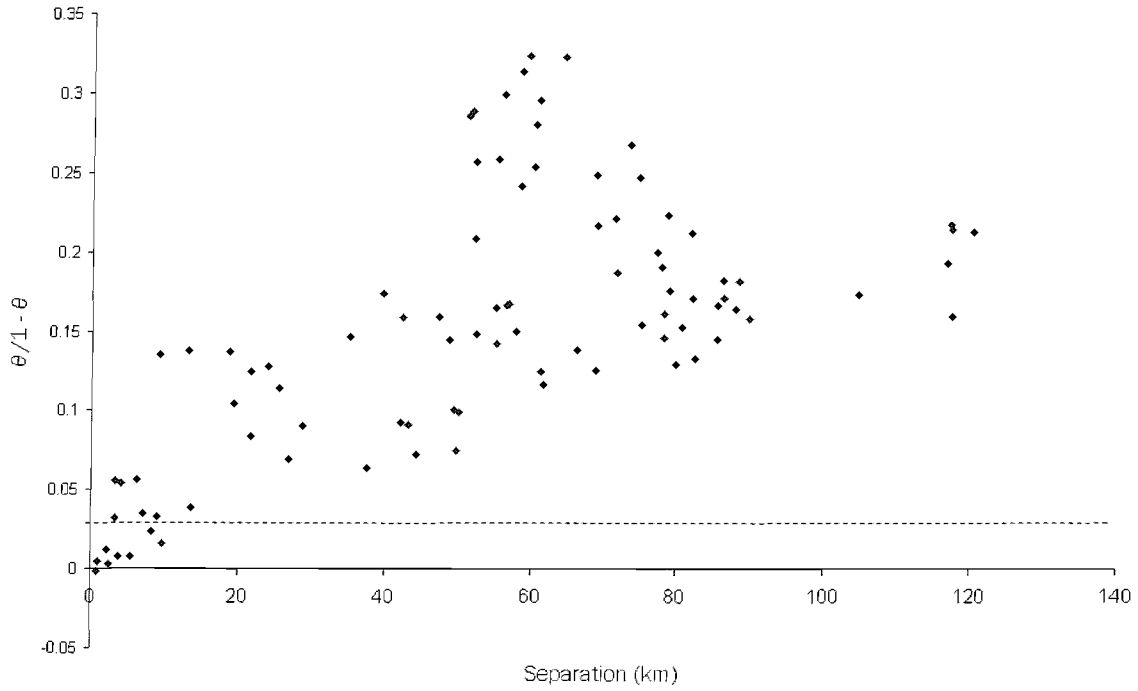


Figure 3.1 The (unlogged) physical separation of *B. muscorum* populations and the genetic differentiation between them. Mantel test, $P < 0.0001$. Population pairs above the dotted line are significantly differentiated from one another ($P < 0.05$).

The neighbour-joining tree grouped together populations which were geographically close to one another (Figure 3.2). This corresponds well with the results of the isolation by distance analysis. This tree is largely in accordance with the 3 sub-species of *B. muscorum* previously designated on morphological grounds. However, microsatellite data alone are not ideal for phylogenetic analyses (Frankham *et al.* 2002), and further studies using mitochondrial DNA are needed to clarify the status of these phenotypes.

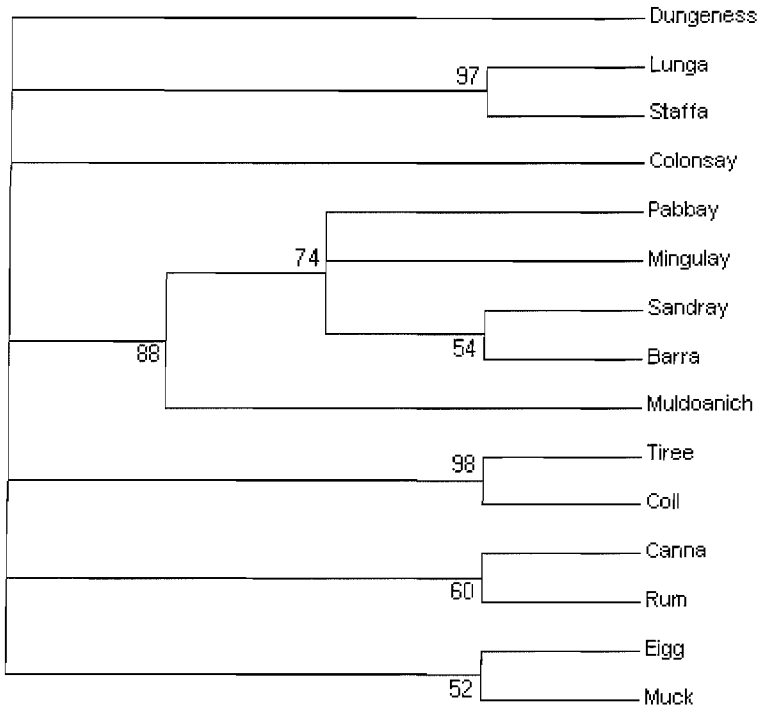


Figure 3.2 Condensed neighbour-joining tree (unrooted) relating 15 of the 16 *Bombus muscorum* populations (Elmley was excluded due to missing data at the locus B10). Numbers represent bootstrap support. Only nodes supported by more than 50% of bootstraps are shown.

3.4.4 Population bottlenecks

Under the infinite allele model (IAM), ten of the fourteen Hebridean populations (Barra, Coll, Canna, Eigg, Colonsay, Lunga, Muck, Rum, Staffa, Tiree) showed significant signs of recent bottlenecking (Wilcoxon Test, one-tailed for heterozygote excess, $P < 0.05$). The remaining islands (Mingulay, Muldoanich, Pabbay, Sandray) along with the two southern UK populations (Dungeness and Elmley) showed no sign of recent bottlenecking. Under both the TPM and SMM models, only Eigg showed significant signs of recent bottlenecking ($P = 0.004$).

3.4.5 Genetic diversity

The analysis of variance found no relationship between expected heterozygosity (H_E) and either isolation or habitat area ($F_{1,12}$ = 0.389, P = 0.545 and $F_{1,12}$ = 0.720, P = 0.414 respectively). The observed variation in H_E was, however, well explained by the location of the islands, with Outer Hebridean islands having significantly lower values on average ($F_{1,13}$ = 16.43, P = 0.002). No relationship was found between allelic richness and either isolation or habitat area ($F_{1,12}$ = 0.128, P = 0.727 and $F_{1,12}$ = 0.158, P = 0.699 respectively). In contrast to H_E, variation in allelic richness was not well explained by location, with the Inner and Outer Hebrides having a similar number of alleles per locus ($F_{1,13}$ = 0.006, P = 0.941). The more complex measures of isolation which took into account the size as well as the proximity of potential source populations did not better explain the observed variation in H_E or allelic richness.

Overall, allelic richness was significantly lower in the Hebridean populations than in southern UK sites (One-tailed Mann Whitney; $P < 0.01$). H_E was also lower in the Hebridean populations, although the difference was not significant (One-tailed Mann Whitney; P = 0.1). Both Hebridean and southern UK populations of *B. muscorum* showed significantly lower H_E than populations of the closely related species, *B. pascuorum* (One-tailed Mann Whitney; $P <$ 0.001 and P = 0.021, respectively).

3.4.6 Diploid males

In total 64 males were caught and genotyped, 41 from the Hebrides and 23 from southern UK sites. Of these, 3 were diploid (at 3 or more loci), representing an overall frequency of 5% with respect to haploid males. Two of these were caught in the Hebrides (on Pabbay and Tiree) and one in the south (Elmley), representing frequencies of 5% and 4% respectively. Few males were caught from most populations and the diploid males detected represent a considerable proportion of the males collected (50% of males caught on Tiree, 6.3% of Pabbay males, and 5.9% of Elmley males).

3.5 DISCUSSION

3.5.1 Hardy-Weinberg and linkage disequilibrium

Global tests by population and by locus found no deviation from HWE suggesting that null alleles are absent, or at very low frequencies, and that mating is random. Linkage disequilibrium was found between two pairs of loci in one population (Staffa). Physical linkage of these loci in just one of 16 populations is unlikely. Four other hypotheses could explain this result: (1) selection acting on certain genotypic combinations, (2) recent immigration from a genetically differentiated population, (3) a recent population bottleneck, (4) sampling bias due to the collection of groups of workers from the same colonies. Of these explanations the last two seem the most likely: under the TPM model, a recent bottleneck was detected on Staffa; Staffa is the smallest of all of the populations, with an area of just 0.5 km^2, so it is possible that a small number of workers from the same colony were collected in the course of sampling.

3.5.2 Population structure and gene flow

Genetic structuring over all populations was moderately high (θ = 0.119), and an AMOVA confirmed that the observed structuring was largely due to differences between individual populations, and not due to differences between the three sub-species that were sampled. Significant genetic differentiation was evident between populations as little as 3km apart, and all populations separated by 10km or more were significantly differentiated from one another. A clear pattern of isolation by distance was evident (Figure 3.1), with the populations demonstrating a regional equilibrium between gene flow and drift (see Hutchison & Templeton 1999). In support of this, the neighbour-joining tree grouped together populations which were geographically close (Figure 3.2). It is clear that dispersal in this species is limited, and that gene flow over distances greater than 10km is uncommon. The two southern UK populations, separated by a distance of 49km, were significantly differentiated from one another, but the genetic distance between them was lower than might be expected from comparison with populations in the Hebrides (0.040), possibly suggesting that long distance dispersal is slightly more common over land. It could be argued that large bodies of water represent a greater obstacle than large areas of unsuitable habitat, since the latter would

contain some suitable forage, enabling dispersing bees to top-up energy reserves en route. Over-land gene flow might therefore occur over much greater distances. However, it is perhaps more likely that this similarity occurs because in the recent past these populations were linked by stepping-stone populations that have since disappeared (Edwards & Broad 2005). Until recently, these populations were probably part of a much larger metapopulation extending around the coast of southern England and Wales.

Given that the bumblebee species previously studied are common and ubiquitous it is perhaps unsurprising that little genetic variation was detected over large distances (Estoup *et al.* 1996; Pirounakis *et al.* 1998; Widmer & Schmid-Hempel 1999). Even with limited dispersal, if all populations are contiguous, genetic cohesion will remain relatively high. However, their data reveals that susceptibility to genetic differentiation is not unique to *B. muscorum*. The population of *B. terrestris* on Samos Island, just 3km away from the mainland was found to be significantly differentiated from continental populations (Estoup *et al.* 1996). Furthermore, the island of Elba, less than 10km from the mainland was highly differentiated from continental populations ($\theta > 0.11$). Differentiation between island populations and the mainland was attributed to founder effects and genetic drift, suggesting that dispersal over these distances is infrequent.

3.5.3 Genetic diversity and bottlenecks

The majority of the Hebridean populations (10 of the 14) showed signs of recent bottlenecking under the IAM mutation model. Under the more conservative TPM model, a bottleneck was detectable in one population (Eigg). No clear relationship was evident between population size, isolation and genetic diversity, possibly as a result of the obscuring effect of these frequent bottlenecks. It was, however, clear that the more isolated Outer Hebridean islands had significantly reduced genetic diversity compared to the Inner Hebrides. The southern UK populations were more heterozygous than those in the Hebrides, and allelic richness was also significantly higher in the south. It is possible that the recent bottlenecks experienced by many Hebridean populations may account for these differences. Alternatively, this may reflect the relatively recent contraction in the range and effective population size of southern sites. In the absence of severe bottlenecks, reduced effective population size results in a gradual loss of genetic diversity, rather than a sudden drop (Frankham *et al.* 2002). Southern populations may

therefore not yet have reached mutation-drift equilibrium. Nevertheless, all populations of *B. muscorum* showed significantly reduced genetic variation compared to other *Bombus* species previously studied (Table 3.1). In particular, the closely related species *B. pascuorum* shows significantly higher genetic variation than *B. muscorum*. It seems probable that the low genetic diversity in remaining isolated populations is due to drift acting on small effective population sizes, coupled with periodic bottlenecks.

3.5.4 The cost of inbreeding

Theory predicts that, as monoandrous social Hymenopterans, fragmented populations of bumblebees are susceptible to a loss of genetic diversity (Chapman & Bourke 2001). Here, for the first time, we provide evidence from wild populations suggesting that this is indeed the case. A recent meta-analysis concluded that the relationship between genetic diversity and population fitness was highly significant, and that reduced heterozygosity has a deleterious effect (Reed & Frankham 2003), but there nevertheless remains a clear distinction between inbreeding and inbreeding depression. It has been suggested that haploid males in Hymenoptera provide a mechanism for the purging of recessive deleterious alleles, reducing their susceptibility to inbreeding depression (Sorati *et al.* 1996). Indeed, the only study of bumblebees to date found no evidence for reduced immune response or body size following inbreeding (Gerloff *et al.* 2003). [Note that, following submission of this chapter as a manuscript, Gerloff & Schmid-Hempel (2005) demonstrated that hibernation survival and colony foundation success were both significantly reduced in inbred lines, and that nest sizes were on average smaller]. However, males express genes relevant to only a fraction of the bumblebee lifecycle. Deleterious mutations affecting the development of female morphology, or traits such as hibernation survival and nest-foundation success would not be exposed to purging. Although it is true to say that many deleterious mutations will be purged by haploid males, in our view it is not true that bumblebees are invulnerable to inbreeding depression. Indeed, a recent meta-analysis concluded that, although haplodiploid insects suffer less from inbreeding than diploid insects, substantial inbreeding depression does occur, in one case resulting in a 38% decrease in longevity and a 32% reduction in fecundity (Henter 2003). The reduced genetic diversity of remaining populations of *B. muscorum* may therefore reduce their fitness directly, and will reduce their capacity to respond to environmental change.

The single-locus complementary sex determination (sl-CSD) system found in bumblebees presents an additional cost of inbreeding through the production of sterile diploid males (Cook & Crozier 1995). Their production in the wild has been proposed as an indicator of the vulnerability of bee populations (Zayed *et al.* 2004). For the first time in naturally occurring populations of bumblebees we find diploid males at detectable frequencies. Although few individuals were detected, males were infrequently encountered whilst sampling workers, and therefore the diploid males detected represent a considerable proportion of the males collected from within those populations. It is likely that continued erosions of effective population size and genetic diversity will result in higher levels of diploid male production, with concomitant consequences for population fitness (Zayed & Packer 2001).

3.5.5 Conservation

In the wake of agricultural intensification, populations of many rare Hymenopterans have declined and fragmented. The distributions of bumblebees are reasonably well known, and demonstrate that remaining populations frequently survive only in small, isolated pockets of suitable habitat. Despite the habitat within these areas remaining suitable, populations within them often become extinct. For example, *B. subterraneus* was widespread in England during the early 1900's, occurring in a range of flower-rich habitats (Alford 1975). By the 1960's it was restricted to a few isolated coastal sites in Essex and Kent, but these populations rapidly became extinct, despite the continued suitability of the habitat. The species was last recorded in Britain in 1988 at Dungeness. Seven once widespread UK species have undergone similarly dramatic declines in recent decades, trends which are mirrored throughout Europe and North America (reviewed in Goulson 2003). The distributions of other hymenopterans are less well known, but many are considered scare or threatened, and exist only in small isolated populations (Edwards & Broad 2005). To date it was not known whether genetic factors might be contributing to their continued declines. Here, for the first time, we demonstrate that populations of a rare and threatened hymenopteran exhibit reduced genetic diversity, and we show that long-range dispersal is uncommon. Additionally, we detect diploid males, an indicator of inbreeding. If, as seems likely, genetic factors are accelerating the declines of social hymenopterans, many of which are important crop and wildflower pollinators (Corbet *et al.* 1991), eco-systems engineers (Jones *et al.* 1994), and natural pest-predators (Van Mele & Cuc 2000), then steps must be taken to conserve what genetic diversity remains.

Chapter 4 - A comparison of the population genetics of *B. jonellus* and *B. muscorum*

4.1 ABSTRACT

Habitat loss has led to the population fragmentation of once widespread invertebrates. It is thought that many taxa withstand this to a degree through large population sizes or long-range dispersal. However, as monoandrous social Hymenopterans, bumblebees may be particularly sensitive to habitat fragmentation due to low effective population sizes. Furthermore, the production of sterile diploid males as a consequence of inbreeding is likely to lead to reduced fitness and increased local extinction risk in small populations. It has recently been recognised that dispersal in bumblebees is limited, and population structuring combined with a loss of genetic diversity can be evident on a local scale. However, it was not known whether dispersal, and therefore sensitivity to habitat fragmentation, might differ between species. Here we compare two bumblebee species (*Bombus muscorum* and *B. jonellus*) in a model island system. We use microsatellites to: compare the extent of population genetic structuring and estimate dispersal; investigate relationships between genetic diversity, population size and isolation; and quantify frequencies of (sterile) diploid males. Both species demonstrate population sub-structuring (*B. muscorum,* $\theta = 0.13$ and *B. jonellus,* $\theta = 0.034$) and isolation by distance. *B. jonellus* populations retain genetic cohesion over greater distances and are estimated to disperse > 50 km relatively frequently. By contrast, *B. muscorum* populations are locally fragmented, exhibit reductions in genetic diversity, show increased frequencies of population bottlenecks and are estimated to disperse > 8 km only infrequently. Diploid males were found at low frequency in *B. muscorum* but were not detected for *B. jonellus.* These results indicate that closely-related species may exhibit cryptic but fundamental differences in aspects of their ecology which influence their susceptibility to habitat fragmentation. Observed differences may in part explain differential declines of mainland populations of bumblebees and will greatly inform future conservation strategies.

4.2 INTRODUCTION

Over recent decades, many bumblebee species have declined dramatically, both in Europe and North America, primarily as a result of agricultural intensification and associated habitat loss (reviewed in Goulson 2003). As a consequence, populations of many species are now fragmented, and in several cases are thought to be threatened. In the United Kingdom, of 25 native species, three are now extinct and several remain only in small isolated populations (Benton 2006). The decline of bumblebees may have serious consequences for the pollination of agricultural crops and wildflowers (Corbet *et al.* 1991), so it is crucial that we fully understand the underlying causes. To date, proximate (and ultimate) explanations for differential declines of bumblebee species have included differences in: tongue length (forage availability and/or competition: Corbet *et al.* 1995); emergence time (nest-site and/or forage competition; see Benton 2006); niche breadth/resource specialisation (forage availability; Goulson *et al.* 2005) and climatic niche space (forage availability interacting with niche space: Williams 2005). Most authors agree that no single theory is capable of explaining observed declines, and it is most likely that several factors are important.

Whatever the reasons, declines in several species have led to the fragmentation of remaining populations. In some instances, local extinctions have continued, despite the apparent suitability of habitat fragments, and the population as a whole has continued to decline. Within a functioning metapopulation, dispersal is of key importance, as it ensures that stochastic local extinctions are followed by subsequent re-colonisation. However, with limited dispersal, not only will suitable patches remain unoccupied, but inbreeding may further accelerate declines. In the absence of occasional immigration, populations lose genetic diversity through bottlenecks and drift (Frankham *et al.* 2002; Keller & Waller 2002). Low genetic diversity reduces the long-term viability of populations via inbreeding depression (the expression of deleterious alleles) and a lowered capacity to respond to environmental change (Frankham *et al.* 2002; Hansson & Westerberg 2002; Keller & Waller 2002; Reed & Frankham 2003).

As a result of their sociality bumblebees exhibit characteristics which increase their susceptibility to inbreeding over and above other invertebrates (Pamilo & Crozier 1997; Chapman & Bourke 2001: see Introduction). This prompted recent work which attempted to determine population structure in a range of species. Initial studies of common and ubiquitous species (*B. terrestris* and *B. pascuorum*) found little or no genetic differentiation over large

distances (Estoup *et al.* 1996; Pirounakis *et al.* 1998; Widmer *et al.* 1998; Widmer & Schmid-Hempel 1999; but see Herrmann *et al.* 2007). More recently, Darvill *et al.* (2006) and Ellis *et al.* (2006) studied fragmented populations of rare and declining species (*B. muscorum* and *B. sylvarum* respectively). Significant genetic structuring was found in both species (θ = 0.08 - 0.12) and in *B. sylvarum* estimates of effective population size were very low (range 21-72). Genetic diversity was considerably lower than in closely related common species and several populations showed signs of recent population bottlenecks. Low frequencies of sterile diploid males (an indicator of inbreeding) were found in both species. However, there was no clear relationship between genetic diversity and either isolation or population size, perhaps because of the confounding effect of recent bottlenecks, or because populations had not yet reached mutation-drift equilibrium (Frankham *et al.* 2002).

Although we are beginning to understand the potential for (and importance of) population sub-structuring and inbreeding in bumblebees, we have little idea whether all species are equally at risk. Molecular markers have recently revealed significant differences in the nesting density and foraging range of a number of bumblebee species (Darvill *et al.* 2004; Knight *et al.* 2005). It is conceivable that species may also vary in their dispersal ability, which would greatly influence their ability to cope with habitat fragmentation.

Here we compare two bumblebee species, *Bombus muscorum* (L.) and *Bombus jonellus* (Kirby) in a model island system (Hebrides, UK). On the mainland, the distributions of both species are very fragmented, although *B. muscorum* is now rare and declining whereas *B. jonellus* is widespread and abundant where suitable habitat allows. Within the system both species are relatively abundant, and they are generally sympatric on the study islands, allowing a direct comparison between species.

For each species we quantify:

1. Population genetic structuring and estimates of dispersal range
2. The relationship between genetic diversity, population size and isolation
3. The frequency of diploid males – a possible indicator of inbreeding.

4.3 MATERIALS AND METHODS

4.3.1 Study species

B. muscorum has declined dramatically in recent years, particularly in the south of the UK where agricultural changes have been most pronounced. Although once scarce but widespread on the mainland, it now survives only in a series of small fragmented populations. However, in the Scottish Highlands and on some Scottish islands it remains locally abundant due to the continued suitability of the habitat (see Chapter 2) (Edwards & Broad 2005). In the Hebrides it is found in a variety of habitats, including heathland and machair (Goulson *et al.* 2005).

B. jonellus is strongly associated with heathland and is known to specialise on *Calluna vulgaris* (see Chapter 2), but it also occasionally occurs in gardens in the south of England. Its distribution on the mainland it also somewhat fragmented, due to its specialist habitat requirements, but where suitable habitat remains it can be quite abundant. Unlike *B. muscorum* it seems to cope well in a fragmented landscape, and occupies relatively small and isolated patches of remnant heathland.

4.3.2 Sample collection

During the summers (June – Sept) of 2003- 2005, individuals of *B. muscorum* and *B. jonellus* were collected from islands in the Inner and Outer Hebrides (Scotland, UK), aiming for a range of island sizes and varying levels of isolation. Previous published genotypic data for *B. muscorum* (Darvill *et al.* 2006) were supplemented by an additional 4 populations (181 new individuals) to strengthen the comparison between the two species. Efforts were made to collect samples from the west coast of mainland Scotland, but despite extensive searching of previously occupied areas, *B. muscorum* was very scarce or absent, and samples of this species were not collected. Non-lethal tarsal samples were taken following Holehouse *et al.* (2003). Workers were caught from numerous locations within each population (where possible >200m from one another) to minimise the probability of sampling individuals from the same colony. When encountered, males of both species were also caught in order to assess the frequency of diploid males, and were destructively sampled to conclusively assess their sex. Samples were

preserved in pure ethanol and stored at ambient temperature. In total 1061 *B. muscorum* (965 ♀ & 96 ♂) and 762 *B. jonellus* (758 ♀ & 4 ♂) were genotyped (Table 4.3).

4.3.3 Molecular methods

DNA was extracted using the HotShot protocol (Truett *et al.* 2000). Workers were genotyped at up to 9 microsatellite loci in each species: (*B. muscorum* = B132, B131, B118, B96, B10, B11, B124, B126; *B. jonellus* = B132, B100, B131, B96, B10, B11, B124, B126, B121) (Estoup *et al.* 1995b, 1996). Microsatellites were amplified by polymerase chain reaction (PCR) in 10μL volumes using QIAGEN Multiplex PCR kits. Each reaction contained approximately 10ng template DNA, 1μL Q-solution, 5μL PCR Master Mix and 0.2μM of each primer. Samples were initially denatured at 95°C for 15 min, followed by 35 cycles of denaturing at 94°C for 30 s, 90 s at the appropriate annealing temperature (see below) and extension at 72°C for 90s. A final extension step at 72°C for 10 min then followed. Annealing temperatures for *B. muscorum* were in the range 51-52°C, and for *B. jonellus* spanned 47-53°C PCR products were visualised on an ABI PRISM™ 377 semi-automated sequencer using an internal size standard (GeneScan ROX 350, Applied Biosystems). Fragment sizes were scored using Genotyper (Applied Biosystems). Repeat PCRs were carried out on individuals believed to be diploid males, and on any samples that had failed to amplify or were uncertainly scored.

4.3.4 Statistical methods

Both datasets were first checked for unexpected mutation steps, large gaps in the data or unusually sized alleles using MSA (Dieringer & Schlotterer 2003). *B. muscorum* samples collected in 2003 were not genotyped at 131, and where appropriate this locus was excluded from subsequent analyses. Tests for genotypic linkage disequilibrium and departure from Hardy-Weinberg equilibrium (HWE) were performed using GENEPOP version 3.4 (Raymond & Rousset 1995). Sequential Bonferroni corrections (Rice 1989) were applied to minimise Type I errors. Where deviations from HWE or apparent linkage disequilibrium suggested the presence of sisters within a population sample, KINSHIP v 1.3.1 (Goodnight & Queller 1999) was used to remove all but one representative from each nest, following the method of Darvill *et al.* (2004).

Genetic population structure was assessed with *F*-statistics (Wright 1951), using Weir & Cockerham's (1984) estimators (F, f and θ), as implemented in FSTAT version 2.9.3 (Goudet 2001). *F*-statistics were calculated for all populations, and pairwise θ for all pairs of populations. Means and standard errors were obtained by jack-knifing over samples and loci. Both global θ and R_{ST} (Rousset 1996; Goodman 1997) were calculated, as the latter (although strictly appropriate only to microsatellites mutating in a step-wise fashion) accounts for differences in the variance of loci and sample sizes. Significance levels of both global and pairwise θ values were determined by permuting alleles (100,000 permutations) using MSA, applying strict Bonferroni corrections (Rice 1989).

In a non-linear system genetic isolation by distance (IBD) is expected to increase with the logarithm of physical separation (Rousset 1997; Hardy & Vekemans 1999). Isolation by distance in each species was therefore examined by regression of pairwise estimates of genetic distance θ/(1 - θ) against the corresponding logarithms of geographic separation (Rousset 1997). A Mantel test (Mantel 1967) was used to assess the significance of any correlation, performing 50,000 permutations in ISOLATION BY DISTANCE (Bohonak 2002). To test for interspecific differences in the degree of isolation by distance, a Monte Carlo analysis was performed using POPTOOLS (version 2.6.9, CSIRO, www.cse.csiro.au/poptools). The observed average (interspecific) difference in inter-island genetic distances was compared to the equivalent value from data randomised by shuffling (without replacement). A *P*-value was estimated by calculating the number of times the real interspecific differences exceeded that of the randomised data in 10000 randomisations.

Possible loss of genetic variation through bottlenecking (or founder effects) was tested for using BOTTLENECK version 1.2.02 (Piry *et al.* 1999). Three mutation models have been proposed for microsatellites: the stepwise mutation model (SMM); infinite allele model (IAM); and two-phase mutation model (TPM). The microsatellites used here are predominantly interrupted-repeats (Estoup *et al.* 1995a; Estoup *et al.* 1995c), so may not fit the SMM, which led Shao *et al.* (2004) to follow the IAM. However, in support of the TPM, others have argued that the majority of mutations are one-step changes (Ellegren 2000; Schlotterer 2000), with a lower frequency of multi-step changes. Since this debate is ongoing, tests were conducted using all three models, allowing for either 70% or 90% stepwise mutations within the TPM. 100,000 iterations were performed in each case.

Population size, isolation, dispersal and associated variables might all be expected to explain variation in genetic diversity (expected heterozygosity (H_E) and allelic richness). A multi-factorial ANOVA with step-wise removal of non-significant terms was performed using SPSS version 15 to identify important factors. Island size and suitable habitat area per island were estimated using 1:50,000 Ordnance Survey maps, with the latter estimated from field observations of where each species was foraging. Population sizes were ranked from 1-5 based primarily on the area of suitable habitat (1-3, 3-10, 10-30, 30-60 and > 60 km^2), but populations in which foragers were found to be particularly abundant or scarce were moved up or down a single category accordingly. Isolation was defined as the distance to the nearest island, or the distance to the mainland, whichever was less. Additionally, historically important probable source populations were identified from distribution maps (Mull, Mainland and Skye) and the minimum distance to the nearest of these was determined. The area of land within 5, 10, 15 and 20 km of each island was estimated using 1:50,000 Ordnance Survey maps, in order to take into account both the size and proximity of potential migrant sources. The habitat area of the nearest neighbouring island was also included as a covariate in the analysis. Finally, island group (Inner or Outer Hebrides) was included in all analyses as a fixed factor. This analysis was repeated using the probability of a recent genetic bottleneck (*P*-value from Wilcoxon's one-tailed test for heterozygote excess, under IAM, SMM and TPM) as the dependent variable.

4.4 RESULTS

4.4.1 Hardy-Weinberg and linkage disequilibrium

a) *B. muscorum*

Due to the inherently low levels of genetic diversity present in these populations it was not possible to reliably test for the presence of sisters within the dataset due to low statistical power. Nevertheless, neither global tests per locus nor per population detected any significant deviation from HWE. Significant linkage disequilibrium ($P < 0.05$) was found between four pairs of loci, B132-B131, B96-B126, B131-B96 and B131-B118 when testing each locus pair

across all populations. Tests within each population found significant linkage disequilibrium in just two populations for these locus pairs (Staffa at B132-B131, B96-B126 and the Monachs at B131-B96 and B131-B118). A global test across all populations excluding both Staffa and the Monachs found no significant linkage disequilibrium, so subsequent analyses were carried out with and without these two populations.

b) *B. jonellus*

Global tests per locus detected highly significant deviations from HWE for locus B131. Indeed for eleven individuals (out of a total of 758), no alleles amplified at this locus, despite re-extraction and amplification. The likely explanation, the presence of null-alleles, makes this locus unsuitable for population genetics, so it was excluded from subsequent analyses. Global tests per locus and per population (excluding B131) again found significant deviations from HWE (at several loci in several populations), as did tests for linkage disequilibrium (at several locus pairs in several populations). Kinship was therefore used to remove sisters from each population, leaving only one representative per nest. Subsequent tests showed no significant deviation from HWE, nor evidence of linkage disequilibrium.

4.4.2 Population structure

a) *B. muscorum*

Overall population structuring was moderately high, with $\theta = 0.128 \pm 0.025$ S.E. ($P < 0.0001$) [excluding Staffa and Monachs, $\theta = 0.120 \pm 0.026$ S.E. $P < 0.0001$], and with $R_{ST} = 0.1024$ [excluding Staffa and Monachs, $R_{ST} = 0.1005$]. The global mean F_{IS} was close to zero (-0.009 ± 0.013) [excluding Staffa and Monachs, F_{IS} = -0.001 ± 0.014 S.E.], and no individual populations were found to have F_{IS} values that deviated significantly from zero (all $P > 0.05$, following 2880 randomisations in FSTAT).

Genetic differentiation between populations (pairwise θ) was highly significant ($P < 0.01$) for 132 of 153 comparisons, significant ($P < 0.05$) in 3 cases and non-significant for the remaining 18. With the exception of Coll and Tiree, pairwise comparisons between all Inner Hebridean islands and between all Inner and Outer Hebridean islands were significant. The

majority of Outer Hebridean islands were not significantly differentiated from one another (17 out of 28 comparisons; Table 4.1). The populations from North and South Uist were genetically indistinguishable ($\theta = 0.008$) and were pooled for subsequent analyses.

Table 4.1 Pairwise values of θ (top triangle) for the sampled populations of *B. muscorum*, and associated *P*-values. The values in the bottom triangle are adjusted *P*-values following strict Bonferroni correction.

	Barra	Canna	Coll	Colonsay	Eigg	Iona	Lunga	Mingulay	Monach	Muck	Muldoanich	N .Uist	Pabbay	Rum	Sandray	Staffa	S. Uist	Tiree
Barra	-	0.137	0.228	0.175	0.158	0.084	0.133	0.016	0.032	0.178	0.012	0.011	0.008	0.230	-0.002	0.141	0.002	0.205
Canna	0.002	-	0.148	0.148	0.077	0.057	0.111	0.122	0.235	0.113	0.129	0.099	0.104	0.052	0.142	0.111	0.127	0.141
Coll	0.002	0.002	-	0.130	0.065	0.070	0.121	0.239	0.277	0.094	0.195	0.217	0.219	0.083	0.244	0.111	0.237	0.005
Colonsay	0.002	0.002	0.002	-	0.117	0.083	0.137	0.177	0.219	0.166	0.138	0.163	0.162	0.136	0.178	0.128	0.188	0.143
Eigg	0.002	0.002	0.002	0.002	-	0.033	0.067	0.146	0.225	0.051	0.133	0.121	0.146	0.053	0.182	0.091	0.140	0.069
Iona	0.002	0.002	0.002	0.002	0.002	-	0.068	0.084	0.147	0.082	0.055	0.068	0.083	0.063	0.102	0.081	0.090	0.067
Lunga	0.002	0.002	0.002	0.002	0.002	0.002	-	0.127	0.189	0.060	0.138	0.114	0.114	0.126	0.149	0.034	0.105	0.120
Mingulay	n.s.	0.002	0.002	0.002	0.002	0.002	0.002	-	0.067	0.175	0.037	0.016	0.003	0.211	0.023	0.153	0.014	0.222
Monach	0.008	0.002	0.002	0.002	0.002	0.002	0.002	0.002	-	0.209	0.061	0.068	0.051	0.295	0.037	0.208	0.056	0.246
Muck	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	-	0.181	0.140	0.160	0.119	0.198	0.083	0.146	0.085
Muldoanich	n.s.	0.002	0.002	0.002	0.002	0.002	0.002	0.028	0.002	0.002	-	0.020	0.032	0.202	0.031	0.143	0.042	0.172
N. Uist	n.s.	0.002	0.002	0.002	0.002	0.002	0.002	n.s.	0.002	0.002	n.s.	-	0.014	0.172	0.022	0.150	0.008	0.199
Pabbay	n.s.	0.002	0.002	0.002	0.002	0.002	0.002	n.s.	0.002	0.002	n.s.	n.s.	-	0.199	0.008	0.127	0.012	0.204
Rum	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	-	0.244	0.125	0.201	0.090
Sandray	n.s.	0.002	0.002	0.002	0.002	0.002	0.002	0.034	0.002	0.002	n.s.	0.037	n.s.	0.002	-	0.154	0.012	0.224
Staffa	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	-	0.144	0.102
S. Uist	n.s.	0.002	0.002	0.002	0.002	0.002	0.002	n.s.	0.002	0.002	0.008	n.s.	n.s.	0.002	n.s.	0.002	-	0.218
Tiree	0.002	0.002	n.s.	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	-

b) *B. jonellus*

Significant population structuring was also found in this species, with global $\theta = 0.034 \pm 0.006$ S.E. ($P < 0.0001$) and $R_{ST} = 0.0392$. However, global values of both θ and R_{ST} were significantly lower in *B. jonellus* than those observed in *B. muscorum* (for θ, two-tailed Mann Whitney, $P = 0.002$ [excluding Monachs and Staffa, $P = 0.005$]; and for R_{ST}, two-tailed Mann Whitney, $P = 0.027$ [excluding Monachs and Staffa, $P = 0.074$]). Global F_{IS} values were close to zero (0.014 ± 0.005 S.E.), and no individual populations had F_{IS} values that deviated significantly from zero (all $P > 0.05$, following 2880 randomisations in Fstat,).

Pairwise θ values were non-significant ($P > 0.05$) for 54 of 153 comparisons, were significant ($P < 0.05$) in 5 cases and were highly significant ($P < 0.01$) for the remaining 94. Unlike in *B. muscorum*, comparisons between Inner Hebridean islands found no significant differentiation between populations in the majority of cases (40 out of 58). However, all comparisons between Inner and Outer Hebridean islands were significant. Within the Outer Hebridean island group, 8 out of 15 pairwise comparisons were non-significant (Table 4.2). As with *B. muscorum*, populations from North and South Uist were genetically indistinguishable ($\theta = -0.001$) and were pooled prior to subsequent analyses.

Table 4.2 Pairwise values of θ (top triangle) for the sampled populations of *B. jonellus* with associated *P*-values. The values in the bottom triangle are adjusted *P*-values following strict Bonferroni correction.

	Barra	Canna	Coll	Colonsay	Eigg	Lunga	Mainland	Mingulay	Muck	Mull	N. Uist	Pabbay	Rum	Sandray	Skye	Staffa	S. Uist	Tiree
Barra	-	0.041	0.038	0.040	0.031	0.036	0.037	0.028	0.041	0.042	0.017	0.008	0.041	0.003	0.038	0.069	0.010	0.059
Canna	0.002	-	0.004	0.009	0.005	0.006	0.011	0.074	0.014	0.001	0.057	0.068	-0.004	0.051	0.004	0.017	0.050	0.014
Coll	0.002	n.s.	-	0.006	0.003	0.003	0.008	0.072	0.014	0.002	0.053	0.065	0.004	0.049	0.002	0.007	0.048	0.008
Colonsay	0.002	n.s.	n.s.	-	0.004	0.007	0.004	0.067	0.007	0.002	0.054	0.064	0.011	0.053	0.003	0.018	0.045	0.025
Eigg	0.002	n.s.	n.s.	n.s.	-	0.005	0.008	0.056	0.005	0.004	0.039	0.055	0.002	0.044	0.001	0.020	0.035	0.022
Lunga	0.002	n.s.	n.s.	n.s.	n.s.	-	-0.002	0.051	0.006	0.007	0.055	0.053	0.005	0.050	0.000	0.017	0.041	0.018
Mainland	0.002	n.s.	n.s.	n.s.	n.s.	n.s.	-	0.058	0.009	0.011	0.057	0.056	0.013	0.054	0.000	0.018	0.041	0.021
Mingulay	0.002	0.002	0.002	0.002	0.002	0.002	0.002	-	0.061	0.071	0.058	0.016	0.071	0.037	0.063	0.096	0.044	0.098
Muck	0.002	0.017	0.015	n.s.	n.s.	n.s.	n.s.	0.002	-	0.012	0.065	0.067	0.014	0.052	0.007	0.029	0.053	0.034
Mull	0.002	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.002	n.s.	-	0.048	0.067	0.002	0.049	0.003	0.012	0.045	0.016
N. Uist	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	-	0.019	0.053	0.020	0.053	0.087	-0.001	0.070
Pabbay	n.s.	0.002	0.002	0.002	0.002	0.002	0.002	n.s.	0.002	0.002	0.005	-	0.065	0.013	0.063	0.101	0.014	0.082
Rum	0.002	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.002	n.s.	n.s.	0.002	0.002	-	0.053	0.001	0.019	0.047	0.014
Sandray	n.s.	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.024	n.s.	0.002	-	0.050	0.087	0.019	0.075
Skye	0.002	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.002	n.s.	n.s.	0.002	0.002	n.s.	0.002	-	0.015	0.044	0.018
Staffa	0.002	0.003	n.s.	0.028	0.003	0.005	0.002	0.002	0.002	n.s.	0.002	0.002	0.006	0.002	0.005	-	0.081	0.025
S. Uist	n.s.	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	n.s.	n.s.	0.002	n.s.	0.002	0.002	-	0.063
Tiree	0.002	0.002	n.s.	0.002	0.002	0.002	0.002	0.002	0.002	0.005	0.002	0.002	0.031	0.002	0.002	0.002	0.002	-

4.4.3 Gene flow between populations

For both species there was a highly significant relationship between genetic distance (θ/1- θ) and the natural logarithm of physical separation (Figure 2; $P < 0.00002$, $R^2 = 0.369$ and $P < 0.00002$, $R^2 = 0.298$ for *B. muscorum* and *B. jonellus* respectively). The extent of this relationship differed significantly between species, with *B. jonellus* populations retaining genetic cohesion over greater distances ($P < 0.0001$). Both species were not present on all of the islands sampled so, for clarity, only islands on which both were found are included in Figure 4.1.

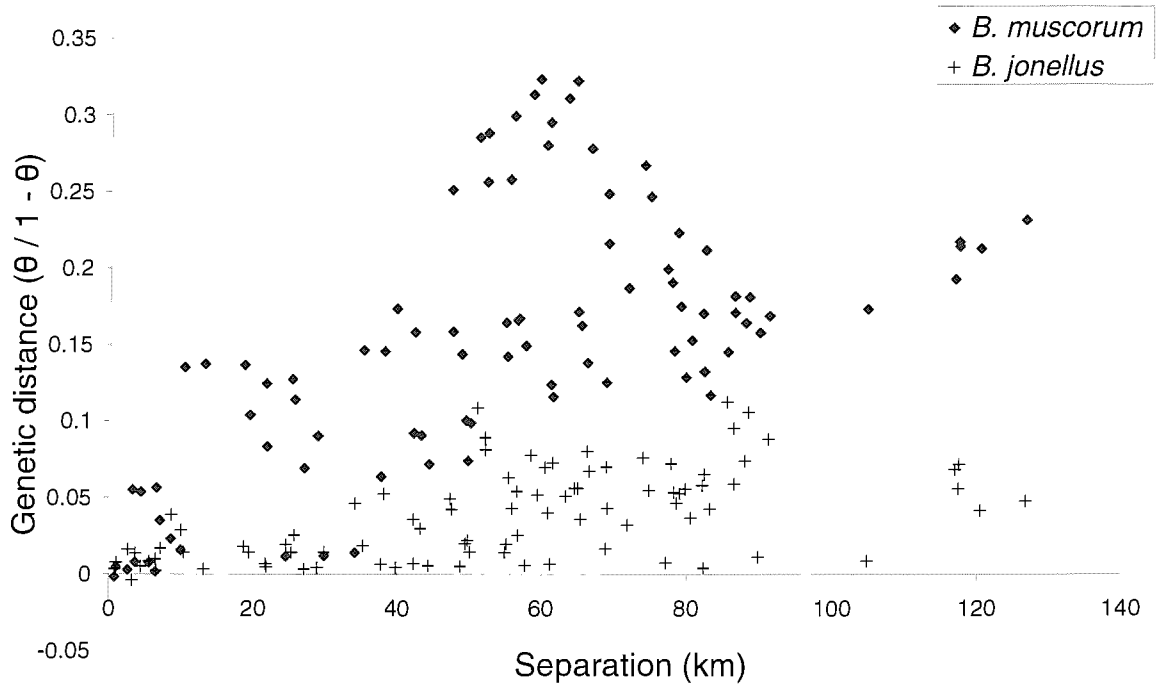


Figure 4.1 The physical separation of populations and the corresponding genetic distances between them, for both *B. muscorum* and *B. jonellus*. Populations for which only one species was sampled are excluded from this figure (Mull, Iona, Skye, Mainland, Monachs, Muldoanich).

Some *B. muscorum* populations as little as 3.2 km apart were significantly differentiated from one another, as were the majority of populations separated by more than 10 kilometres. All discrete populations more than 34km apart were significantly differentiated, irrespective of stepping-stone populations. By contrast, in *B. jonellus* no populations less than 7.1km apart were significantly differentiated and some populations separated by as much as 104km remained genetically undifferentiated.

4.4.4 Population bottlenecks

Under the infinite allele model (IAM) fourteen of the seventeen *B. muscorum* populations (all except Pabbay, Sandray and Muldoanich) showed significant signs of recent bottlenecking (Wilcoxon Test, one-tailed for heterozygote excess, $P < 0.05$). Using the TPM model (using the default settings in which 70% of mutations are stepwise) only six of the populations (Coll, Eigg, Muck, Rum, Iona, Monachs) showed bottlenecking. More stringent settings (90% stepwise mutations) showed only Eigg as significant ($P = 0.0039$), although Rum ($P = 0.055$) and the Monachs ($P = 0.078$) approached significance. Using the SMM, only Eigg showed significant signs of recent bottlenecking ($P = 0.004$). Populations of *B. muscorum* from the Inner Hebrides showed a greater probability of having passed through a recent bottleneck (Mann Whitney test, $P = 0.001$; $P = 0.013$ and $P = 0.026$ for the IAM, TPM and SMM respectively). No such trend was in evidence for *B. jonellus* (Mann Whitney tests, all $P > 0.05$), however nine of the seventeen *B. jonellus* populations showed evidence of a recent bottleneck under the IAM (all except Colonsay, Lunga, Mainland, Mingulay, Muck, Mull, Skye and Staffa). When following the TPM model (default settings, 70% stepwise mutations), only three populations (Coll, Barra and Sandray) showed significant signs, and no populations were significant when using more stringent settings in the TPM (90% stepwise) or when following the SMM.

4.4.5 Genetic diversity

The two species differed significantly in overall genetic diversity, both for expected heterozygosity (H_E) and allelic richness (Mann-Whitney tests; both $P < 0.001$), with *B. muscorum* the more genetically depauperate. Both island group (Inner or Outer Hebrides) and the distance to the nearest neighbouring population were important factors in determining the H_E of *B. muscorum* populations when included simultaneously in the GLM (Figure 4.2). Outer Hebridean islands demonstrated lower levels of H_E ($F_{1,14} = 35.595$, $P < 0.001$; Table 4.3) and more isolated of populations showed further reductions in H_E ($F_{1,14} = 6.885$, $P = 0.020$; Figure 4.3).

Table 4.3 The sample size, average (unbiased) heterozygosity (H_E) and allelic richness of each of the populations (± S.E.). For *B. muscorum*, allelic richness and H_E were calculated using all loci except B131. Allelic richness values are standardised to the smallest sample size (which for both species is 25).

	Bombus muscorum			*Bombus jonellus*		
Population	**Sample size**	**Allelic Richness**	H_E	**Sample size**	**Allelic Richness**	H_E
Barra	50 ♀ 3 ♂	3.10 ± 0.66	0.393 ± 0.113	82 ♀ 0 ♂	8.63 ± 1.70	0.766 ± 0.048
Mingulay	49 ♀ 1 ♂	2.99 ± 0.64	0.374 ± 0.115	32 ♀ 1 ♂	7.50 ± 1.31	0.696 ± 0.048
Muldoanich	25 ♀ 6 ♂	3.63 ± 0.90	0.421 ± 0.103	-	-	-
Pabbay	37 ♀ 16 ♂	3.33 ± 0.68	0.399 ± 0.118	36 ♀ 0 ♂	7.55 ± 1.33	0.729 ± 0.046
Sandray	58 ♀ 1 ♂	3.05 ± 0.63	0.367 ± 0.111	25 ♀ 0 ♂	9.00 ± 1.80	0.763 ± 0.054
Monachs	44 ♀ 49 ♂	2.22 ± 0.39	0.305 ± 0.092	-	-	-
Uists	87 ♀ 3 ♂	3.32 ± 0.66	0.404 ± 0.113	92 ♀ 0 ♂	9.30 ± 1.98	0.755 ± 0.054
Outer Hebrides Average		**3.09 ± 0.17**	**0.380 ± 0.014**		**8.40 ± 0.37**	**0.742 ± 0.013**
Colonsay	67 ♀ 0 ♂	3.21 ± 0.50	0.416 ± 0.086	28 ♀ 0 ♂	10.24 ± 2.31	0.756 ± 0.064
Lunga	36 ♀ 6 ♂	3.43 ± 0.56	0.507 ± 0.108	38 ♀ 0 ♂	9.90 ± 2.07	0.742 ± 0.076
Staffa	52 ♀ 0 ♂	3.33 ± 0.51	0.484 ± 0.091	46 ♀ 0 ♂	8.19 ± 1.70	0.697 ± 0.082
Iona	50 ♀ 5 ♂	4.22 ± 0.91	0.554 ± 0.087	-	-	-
Canna	62 ♀ 3 ♂	3.11 ± 0.57	0.433 ± 0.086	44 ♀ 0 ♂	9.58 ± 2.01	0.758 ± 0.070
Coll	70 ♀ 0 ♂	3.46 ± 0.69	0.499 ± 0.091	44 ♀ 1 ♂	9.05 ± 1.86	0.738 ± 0.073
Eigg	64 ♀ 2 ♂	3.30 ± 0.51	0.533 ± 0.094	36 ♀ 1 ♂	9.87 ± 2.09	0.757 ± 0.066
Muck	52 ♀ 0 ♂	2.91 ± 0.42	0.425 ± 0.088	47 ♀ 0 ♂	8.81 ± 1.79	0.751 ± 0.056
Rum	42 ♀ 1 ♂	2.91 ± 0.48	0.451 ± 0.077	32 ♀ 0 ♂	10.00 ± 2.10	0.749 ± 0.079
Tiree	119 ♀ 2 ♂	3.27 ± 0.55	0.499 ± 0.086	54 ♀ 1 ♂	8.18 ± 1.71	0.715 ± 0.076
Skye	-	-	-	43 ♀ 0 ♂	10.03 ± 2.10	0.745 ± 0.068
Mull	-	-	-	37 ♀ 0 ♂	10.68 ± 1.93	0.769 ± 0.057
Inner Hebrides Average		**3.32 ± 0.12**	**0.480 ± 0.015**		**9.50 ± 0.24**	**0.743 ± 0.006**
Overall Hebrides Average		**3.23 ± 0.09**	**0.437 ± 0.015**		**9.16 ± 0.23**	**0.743 ± 0.005**
Mainland UK †	71 ♀ 23 ♂	**4.01 ± 0.06**	**0.509 ± 0.013**	42 ♀ 0 ♂	**10.02 ± 1.98**	**0.755 ± 0.071**

† For *B. muscorum* these figures represent average values for the two Southern UK samples genotyped in chapter 3.

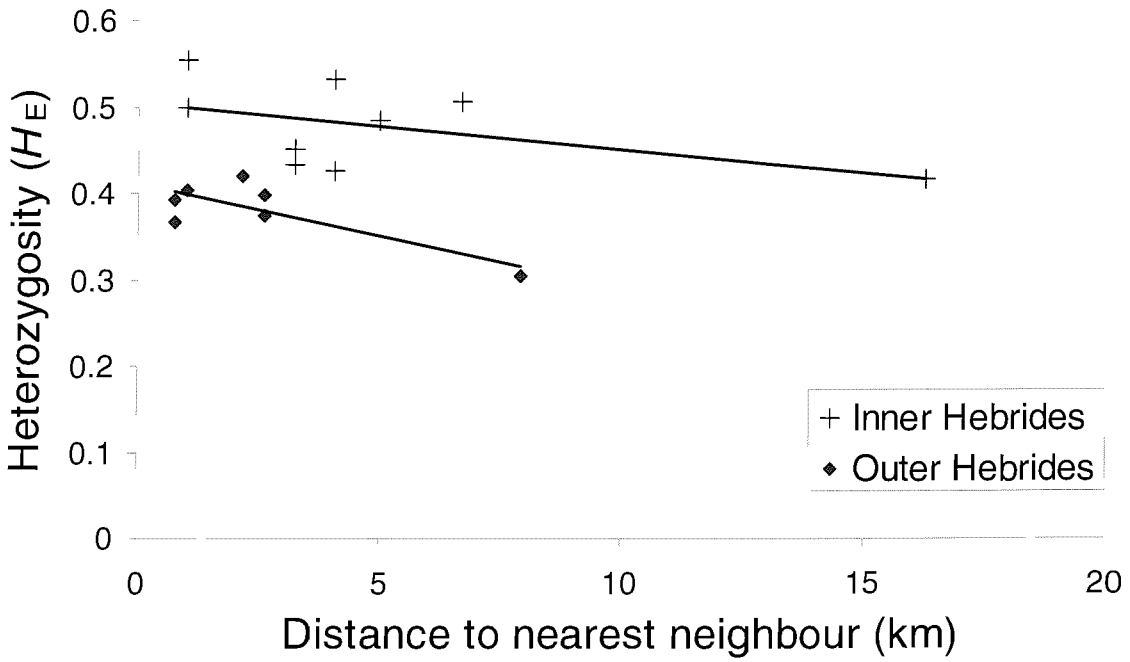


Figure 4.2 The effect of isolation on the genetic diversity (H_E) of *B. muscorum* populations. More isolated islands were less genetically diverse, as were populations from the Outer Hebrides.

The heterozygosity of populations was also well explained by the distance to historical source populations ($F_{1,15}$ = 14.830, P = 0.002), although this effect was insignificant relative to the importance of island group (Inner or Outer). However, when considering the Inner Hebrides alone, distance to historical source populations appeared to relate to H_E levels. The relationship was non-significant when considering all islands in the group ($F_{1,7}$ = 1.781, P = 0.219). However, Coll and Tiree are separated by just 1km, and may not represent truly independent units in this instance. Pooling these populations (dotted line) revealed a significant relationship ($F_{1,7}$ = 8.602, P = 0.022; Figure 4.3). The area of land (migrant sources) within 5km of a population did not have a significant effect on H_E ($F_{1,15}$ = 0.112, P = 0.742), and nor did any of the other measures of source area.

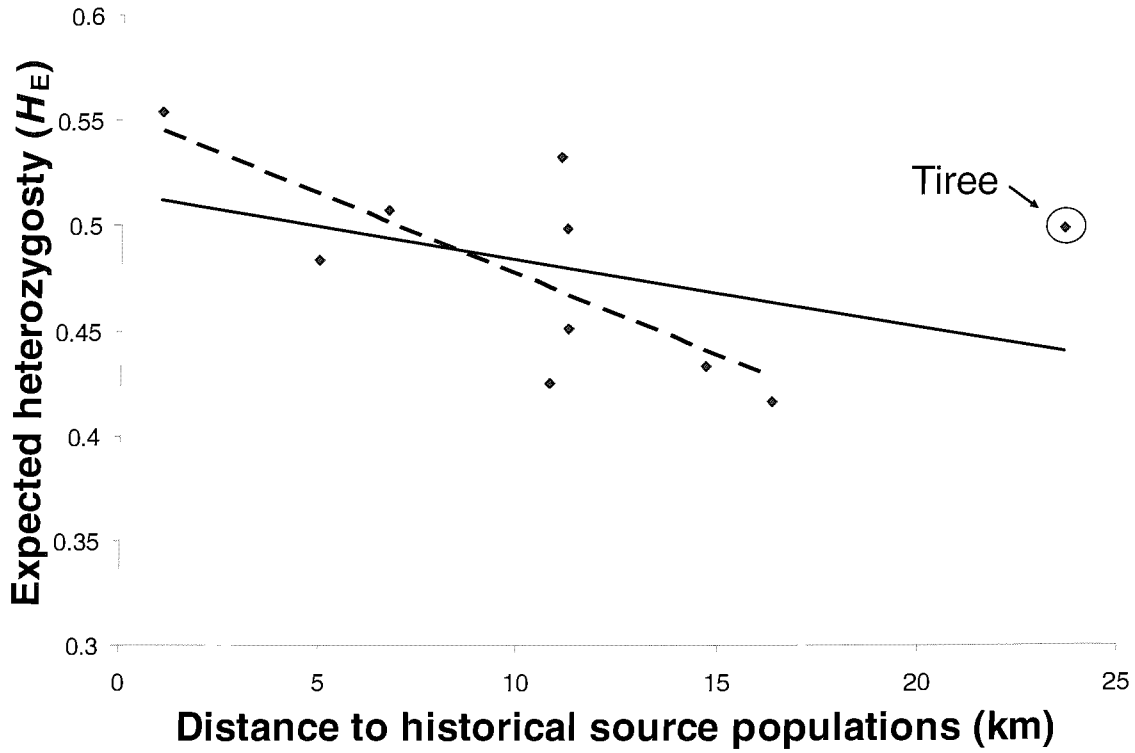


Figure 4.3 The relationship between isolation (from historically important source populations) and heterozygosity (H_E) for Inner Hebridean populations of *B. muscorum*. The outlying point is Tiree, which is just 1km from its neighbour Coll, and perhaps not truly independent.

The allelic richness of *B. muscorum* populations was not affected by the distance to their nearest neighbours, or by their distance from historical source populations. Allelic richness was however well explained by both the area of (source) habitat within 5km ($F_{1,14} = 18.740$, $P = 0.001$) and island group ($F_{1,14} = 9.357$, $P = 0.008$). However, the relationship was heavily skewed by unusual populations: Iona in the Inner Hebrides is adjacent to the historically important source population of Mull, and the Monachs in the Outer Hebrides are small and very isolated (Figure 4.4).

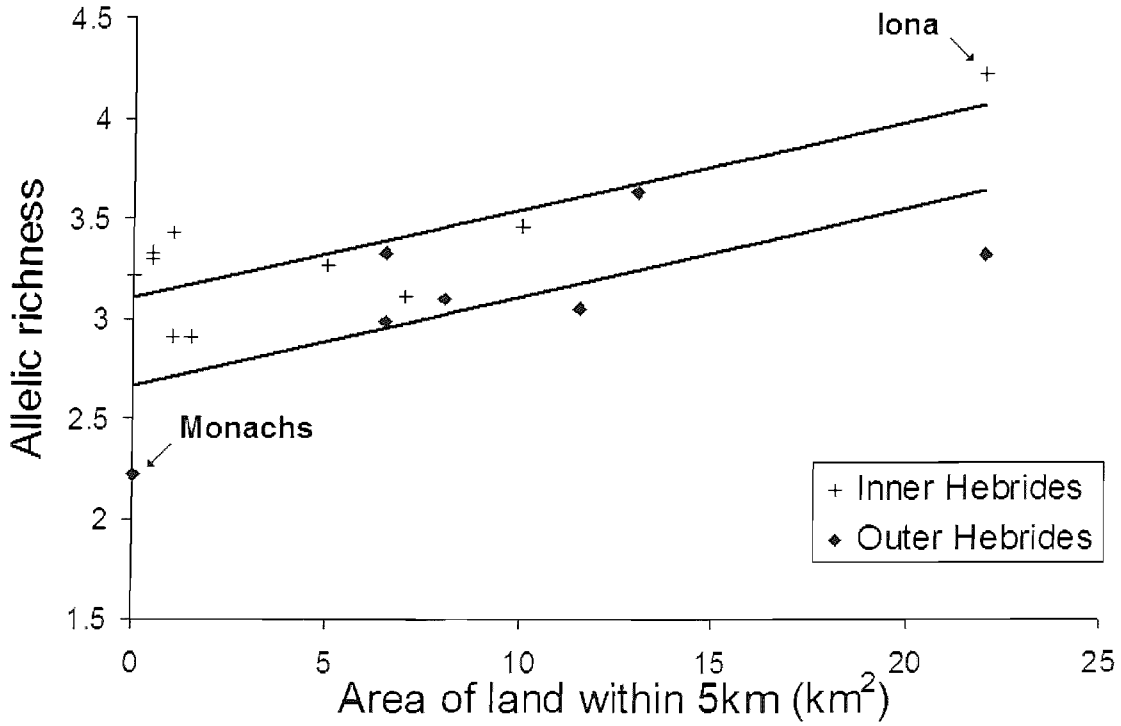


Figure 4.4 The observed relationship between the allelic richness of *B. muscorum* populations and the area of land within 5km of their shores. Inner Hebridean islands had, on average, higher levels of allelic richness. The apparent trends are heavily skewed by Iona and the Monachs.

Unlike in *B. muscorum*, the variation in the expected heterozygosity (H_E) of *B. jonellus* populations was not well explained by island group ($F_{1,15}$ = 0.048, P = 0.829) or isolation ($F_{1,15}$ = 0.002, P = 0.966). However, variation in H_E was well explained by population size, with large populations retaining high levels of heterozygosity (Jonckheere-Terpstra test, P = 0.046; Figure 4.5).

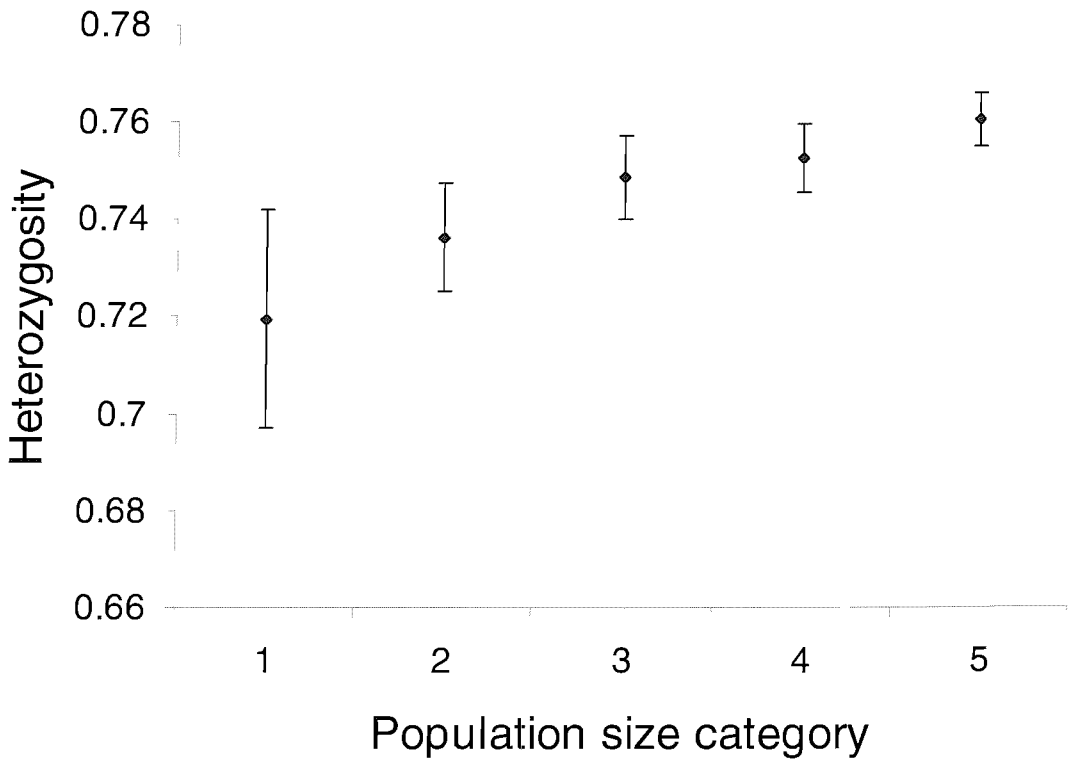


Figure 4.5 The expected heterozygosity (H_E ± S.E) of *B. jonellus* populations of varying size categories. Large populations, on average, retained higher levels of genetic diversity.

Allelic richness was not strongly affected by the area of potential source populations within 5km ($F_{1,15}$ = 2.002, P = 0.178), but was instead dependent on their distance from the historically (and/or present day) important source populations of Mull, Skye and the mainland ($F_{1,15}$ = 14.512, P = 0.002; Figure 4.6). The Outer Hebrides did show, on average, reduced levels of allelic richness, but it was the distance to source populations, rather than island groups which was the most important factor in describing allelic richness (with both in the model $F_{1,15}$ = 0.501, P = 0.490).

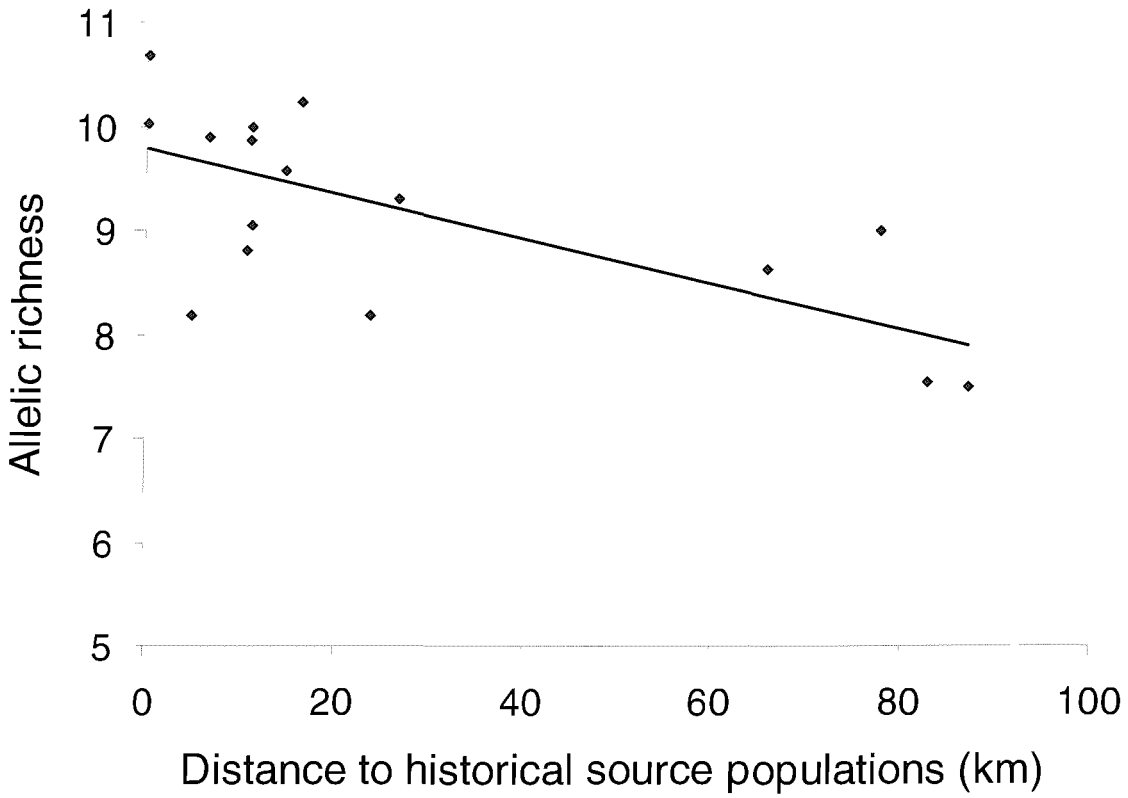


Figure 4.6 The relationship between the distance to historically important source populations and the allelic richness of *B. jonellus* populations. The lowest levels of allelic richness are found in the most isolated islands.

4.4.6 Diploid male production

In *B. muscorum,* a total of 98 males were caught and genotyped from the Hebrides, and of these 2 were diploid (at 3 or more loci), representing an overall frequency of 2% with respect to haploid males. The diploid individuals were caught on Pabbay and Tiree. In *B. jonellus* only 4 males were caught, and none were diploid.

4.5 DISCUSSION

4.5.1 Hardy-Weinberg and linkage disequilibrium

In general, the absence of deviations from HWE or patterns of linkage suggest that the chosen loci (except the discarded B131) were suitable for use in this study. Due to low levels of genetic diversity it was not possible to attempt the removal of sisters from the *B. muscorum* data set, but nevertheless problems were not broadly evident. This suggests low frequencies of sampled sisters in most populations. Sampling was spread over a broad area where possible, which was greatly facilitated by the range of habitats in which *B. muscorum* was found. *B. jonellus* by contrast specialised on *Calluna vulgaris* to such an extent that it was not possible to find them in many locations on each island (see chapter 2), which perhaps explains the relatively higher frequencies of sisters detected in this species. It is also possible that population sizes of *B. muscorum* were significantly larger, reducing the probability of sampling workers from the same colony twice. The linkage disequilibrium observed in *B. muscorum* populations on Staffa and the Monachs probably reflects the presence of sisters amongst the sampled workers, as these were the smallest (and among the most isolated) islands visited. However, other explanations are possible, notably: immigration from a genetically differentiated population; a recent population bottleneck; or severe inbreeding (Beebee & Rowe 2004). All are plausible, but the presence of sisters within the sample seems the most likely explanation.

4.5.2 Genetic diversity, population size and isolation

The two species differed significantly in overall heterozygosity (H_E) and allelic richness, which in itself does not necessarily reflect the relative genetic health of one species over and above the other. It does, however, add to a general consensus of low genetic diversity in scarce species relative to widespread congeners (Zayed *et al.* 2005, see also Chapter 8). Isolated populations of *B. muscorum* showed further reductions in H_E highlighting the importance of gene flow in gaining and retaining genetic diversity. The H_E of populations also seemed to be weakly related to the isolation of Inner Hebridean islands from historical source populations. This relationship was not evident with allelic richness, perhaps due to the confounding effects of recent population bottlenecks in this species. Instead, allelic richness was weakly (but

significantly) correlated with the area of (source) habitat within 5km although this relationship was largely an artefact of the consanguinity of Iona and Mull, and the isolation of the Monachs.

Unlike in *B. muscorum*, observed variation in the H_E of *B. jonellus* populations was correlated closely with population size, with large populations retaining high levels of heterozygosity. However, in the chain of islands comprising Barra, Sandray, Pabbay and Mingulay, increasingly isolated islands showed reduced H_E indicating the simultaneous importance of isolation. Allelic richness was strongly linked with distance to the likely source populations of Skye, Mull and the mainland, highlighting the significance of large populations as both pools and sources of genetic diversity. However, as with H_E, population size was an important component in the maintenance of genetic diversity, with small islands (e.g. Staffa and Mingulay) having low levels of allelic richness relative to more isolated islands with larger population sizes (e.g. Colonsay). It seems most likely that both isolation and population size are important in gaining and preserving genetic diversity for both species.

4.5.3 Population structure and gene flow

Genetic differentiation was evident in both species, but significantly was much higher for *B. muscorum* than for *B. jonellus*. Pairwise comparisons similarly showed that *B. jonellus* populations retain genetic cohesion over much greater distances. There are three likely explanations for the observed differences, none of which are mutually exclusive. Firstly and perhaps most intuitively is a difference in dispersal range and/or dispersal propensity between species. Clearly, more frequent long-range dispersal will reduce population structuring. Secondly, inter-specific differences in effective population sizes could lead to different rates of genetic drift, which under some circumstances could reduce the extent of interspecific differences. Thirdly, substantial differences in gene flow between (source) mainland populations and the islands could have a homogenising effect, leading to apparent differences between species.

In the absence of gene flow, small populations should diverge more rapidly (through drift) than equivalent larger populations. However, the effects of sufficient migration (>1 successful migrant per generation) are manifest irrespective of population size, so the observed isolation

by distance points to the importance of dispersal. Population size might only be contributing towards the overall effect if *B. jonellus* were consistently and significantly more abundant. In reality *B. muscorum* was the most numerous of all of the bumblebee species on many of the islands, and was generally widespread, whereas *B. jonellus,* although at times locally abundant, was only found on heathland. The nest sizes of the two species are thought to be similar (*B. jonellus* 50-120 workers and *B. muscorum* < 100 workers; Benton 2006). The effective population size depends on the number of nests that successfully reproduce (rear males and new queens), and we know very little about the proportion of successful nests, even for the common species. Of the two species *B. muscorum* would seem more likely to have a larger effective population size, which does not explain the observed inter-specific differences in population structuring.

The observed isolation by distance suggests that gene flow between islands is relatively more important that gene flow from the mainland. Nevertheless, occasional gene flow from the mainland in just one species could have a homogenising effect. Both *B. muscorum* and *B. jonellus* are still present on Skye, Mull and the adjacent mainland but, following recent declines in the former, *B. jonellus* (although uncommon) is now marginally more abundant in these locations. The presence of stepping-stone populations down the coast may in part explain low genetic distances between some populations, notably Colonsay and Canna. These islands are 105km apart, and in *B. jonellus* are separated by a genetic distance (θ) of just 0.009, whereas *B. muscorum* shows a distance of 0.148. Colonsay also provides an opportunity to make a comparison between species in the absence of the confounding effects of other islands. Both species are present on Colonsay and the adjacent island of Mull, where they were formerly more abundant. Although now scarce, samples were collected from locations which are likely to reflect the historical gene frequencies of the island. *B. muscorum* were still numerous on the adjacent island of Iona which (given its proximity; <1km) is likely to be representative of the allele frequencies on Mull. *B. jonellus* was found in abundance in a single valley in the south-east of the island. The genetic distances (θ) between these populations and Colonsay were 0.0831 for *B. muscorum* and 0.0015 for *B. jonellus,* suggesting substantially more dispersal from the latter species.

The most compelling evidence for greater dispersal by *B. jonellus* comes from an analysis of system as a whole. *B. jonellus* appears to adhere closely to the classic model of isolation by distance, with a regional equilibrium between gene flow and drift, and linearly increasing F_{ST}

variance (Hutchison & Templeton 1999). Heterozygosity does not decrease between the Inner and Outer Hebrides, and instead is related to population size, in accordance with neutral theory (Kimura 1983). Allelic richness, as one would also expect, decreases with distance from source populations due to founder effects. Comparisons between the Inner and Outer Hebrides reveal relatively little population structuring, and indicate low but significant levels of gene flow between the island groups (Table 4.4). Pairwise differences suggest that dispersal is most frequent between Inner Hebridean islands (notably Eigg, θ = 0.0312) and Barra, with increasing genetic differentiation north and south from here. The genetic distance between Skye and the Uists (0.0488) suggests that despite the shorter distance this is a less important route for gene flow. *B. jonellus* is now scarce on Skye, and it may be that it is no longer an important source of migrants.

Table 4.4 The genetic differentiation (θ) between populations of *B. muscorum* and *B. jonellus* on Inner Hebridean islands with those in the Outer Hebrides. *B. muscorum* shows greater structuring on average, and considerably more variance, suggesting lower levels of gene flow.

Inner Hebridean Populations		Outer Hebridean Populations: Monachs	Uists	Barra	Muldoanich	Sandray	Pabbay	Mingulay
B. jonellus	Skye	-	0.0488	0.0377	-	0.0502	0.0631	0.0626
	Canna	-	0.0541	0.0406	-	0.0512	0.0678	0.0743
	Rum	-	0.0501	0.0413	-	0.0530	0.0655	0.0707
	Eigg	-	0.0373	0.0312	-	0.0445	0.0548	0.0557
	Muck	-	0.0598	0.0411	-	0.0519	0.0673	0.0613
	Mainland	-	0.0500	0.0365	-	0.0537	0.0559	0.0578
	Mull	-	0.0472	0.0417	-	0.0489	0.0672	0.0712
	Colonsay	-	0.0503	0.0398	-	0.0527	0.0639	0.0668
	Lunga	-	0.0488	0.0357	-	0.0502	0.0528	0.0508
	Staffa	-	0.0832	0.0688	-	0.0873	0.1012	0.0959
	Coll	-	0.0511	0.0385	-	0.0493	0.0653	0.0721
	Tiree	-	0.0662	0.0594	-	0.0752	0.0820	0.0980
	Inner Hebrides Average	**-**	**0.0539**	**0.0427**	**-**	**0.0557**	**0.0672**	**0.0698**
	Std Error	-	0.0033	0.0031	-	0.0036	0.0038	0.0042
B. muscorum	Canna	0.2351	0.1114	0.1370	0.1293	0.1424	0.1040	0.1215
	Rum	0.2948	0.1861	0.2304	0.2022	0.2439	0.1991	0.2109
	Eigg	0.2247	0.1330	0.1575	0.1334	0.1825	0.1457	0.1460
	Muck	0.2086	0.1431	0.1778	0.1808	0.1978	0.1601	0.1748
	Iona	0.1468	0.0801	0.0838	0.0550	0.1025	0.0825	0.0842
	Colonsay	0.2188	0.1732	0.1754	0.1376	0.1785	0.1615	0.1766
	Lunga	0.1886	0.1128	0.1325	0.1384	0.1491	0.1141	0.1272
	Staffa	0.2085	0.1494	0.1411	0.1428	0.1539	0.1268	0.1535
	Coll	0.2771	0.2306	0.2278	0.1946	0.2444	0.2189	0.2387
	Tiree	0.2458	0.2108	0.2052	0.1722	0.2238	0.2041	0.2221
	Inner Hebrides Average	**0.2249**	**0.1531**	**0.1669**	**0.1486**	**0.1819**	**0.1517**	**0.1655**
	Std Error	0.0134	0.0149	0.0146	0.0134	0.0147	0.0145	0.0154

By contrast, *B. muscorum* appears to more closely follow Hutchison and Templeton's (1999) 'type IV' model. Regional equilibrium is approximated between relatively close populations, but non-equilibrium conditions and high variance in F_{ST} at greater distances indicate a transition from gene flow to drift effects. Close adherence to isolation by distance within both the Inner and Outer Hebrides suggest a discontinuity between the two island groups, which is borne out by the estimates of genetic distance (Table 4.4). Greater variance at all levels suggests the increasing importance of drift in determining genetic distances at this scale.

Changes in genetic diversity between the two island groups also indicate very limited gene flow. The significant reduction in heterozygosity (despite the large populations) in the Outer Hebrides suggests a substantial bottleneck during initial founding events, which has not been redressed by subsequent immigration (Table 4.3). The absence of a concordant drop in allelic richness is at first puzzling, until viewed in the context of recent genetic bottlenecks in the Inner Hebrides. The non-equilibrium excess of heterozygotes detected in several of the Inner Hebridean islands indicates a recent drop in allelic richness (Cornuet & Luikart 1996). Previous Inner Hebridean levels are probably best approximated by Iona (adjacent to the source population of Mull), where allelic richness is 4.22.

4.5.4 Dispersal range

On balance it seems most likely that *B. jonellus* has a greater dispersal range (or an increased propensity for dispersal), at least over water. Over-land dispersal is likely to occur over larger distances because dispersing queens can forage and rest *en route*, and although we assume the two will correlate, this may not necessarily be the case. It is relatively clear from the lack of genetic structuring between Mull and Colonsay that *B. jonellus* frequently disperses over distances in excess of 16km. Dispersal between the Inner and Outer Hebrides is less common but still regular, and implies unbroken flights in excess of 27km (Skye - Uists). Genetic distances imply that this route is not the most frequently used, and flights of between 50 and 70km are more common (Canna - Barra and Eigg - Barra).

The genetic distances Mull (Iona) - Colonsay, and Uists – Monachs suggest that dispersal in *B. muscorum* is infrequent over unbroken distances in excess of 8km. The presence of *B. muscorum* on the Outer Hebrides in itself implies occasional dispersal of 27km or more, which confirms anecdotal accounts from elsewhere (see Benton 2006). However, the considerable structuring between Inner and Outer Hebridean populations indicates that such events are rare.

It is interesting to speculate as to why *B. jonellus* might disperse further than *B. muscorum.* As a heathland species, it has probably always had a somewhat fragmented distribution, with colonisation of new habitats requiring dispersal over lowland areas between ranges of hills or mountains. In the afternoons and evenings the mountain ranges of Barra and the Uists (383m and 620m altitude, respectively) are very conspicuous on the westerly horizon (pers. obs.), and

it is conceivable that this visual cue attracts migrants. It is noteworthy that *B. jonellus* belongs to the subgenus *Pyrobombus* (Dalla Torre), of which three other species occur in the UK: *B. pratorum*; *B. monticola* and *B. hypnorum*. Bumblebees have been studied in detail in the UK since the beginning of the 20th century, and colonisation events are rare. However, in recent years *B. hypnorum* arrived from continental Europe (Goulson & Williams 2001) and both *B. monticola* and *B. pratorum* have colonised Ireland. It is possible that long-range dispersal is a trait common to this subgenus. *B. muscorum* by contrast is generally a lowland species, and in the UK frequently occurs along the coastline (Benton 2006). Long range dispersal may historically have conferred little advantage, and would clearly have increased the probability of movement into unsuitable habitat.

4.5.5 Implications for conservation

B. muscorum is in the subgenus *Thoracobombus* (Dalla Torre), along with *B. ruderarius*, *B. pascuorum*, *B. humilis* and *B. sylvarum*. Of these, all but *B. pascuorum* have declined considerably in recent decades and persist only in small population fragments. Just as long-range dispersal may feature throughout the *Pyrobombus*, it is equally possible that short-range dispersal is a trait common to the *Thoracobombus*, and that this is accelerating declines by limiting the scale at which metapopulations can persist. Bumblebees are frequently observed several kilometres offshore from ferries, yachts or at lightships, but *Thoracobombus* are seldom or never observed (Mikkola 1984; Goulson 2003; pers. obs.). In the UK, *B. humilis* and *B. sylvarum* now persist only where substantial areas of flower-rich grassland remain. It has been estimated that an area of at least 10 square kilometres of suitable habitat is necessary for populations to persist (Edwards, pers. comms, in Benton 2006). The local extinction of *B. humilis* and *B. sylvarum* from smaller habitat fragments of apparently high quality suggests that inbreeding may be occurring, and that stochastic extinctions are not followed by re-colonisation, both of which suggest that dispersal is a limiting factor.

A better understanding of inter-specific differences in dispersal range will be crucial in devising successful conservation strategies for threatened species. Clearly, the scale at which efforts should be targeted may depend directly on the species of concern. For example, the fragmented populations of *B. humilis* and *B. sylvarum* in the Thames estuary (UK) are of particular concern, as development plans threaten several strongholds. Given limited dispersal,

the loss of even a few important sub-populations to the planned developments could lead to metapopulation break-down and the local extinction of these species. On a more positive note, agri-environment schemes have the potential to create abundant habitat for rare species (Carvell *et al.* 2004; 2006; 2007), but will only be successful if targeted. It should be considered a priority to consolidate remnant populations by increasing the area of suitable habitat in the adjoining land, and therefore the effective population size of each fragment. Habitat corridors or stepping-stone populations should be provided, at a suitable scale, to re-instate genetic linkage between isolated populations and increase the probability of re-colonisation following stochastic local extinction. Vertebrate conservationists routinely employ these techniques, and make use of translocations and re-introductions where natural dispersal is proving inadequate, but invertebrate conservation is lagging behind. Urgent action is needed to prevent the extinction of several more bumblebee species, which are following the characteristic pattern seen in *B. subterraneus*, last recorded at Dungeness in 1988.

Chapter 5 - Diploid males and their triploid offspring in fragmented bumblebee populations

5.1 ABSTRACT

Hymenopteran species with single-locus complimentary sex-determination (sl-CSD) theoretically face additional costs of inbreeding, due to a loss of diversity at the sex determining locus. Individuals that are homozygous at this locus develop into diploid males, effectively leading to the mortality of 50% of a colony's work force. However, studies of several species which regularly inbreed or have low levels of genetic diversity have found only low frequencies of diploid males. It is possible that frequency dependent selection maintains diversity at the sex-determining locus, or that inbreeding-avoidance mechanisms such as sex-allele signalling minimise the frequency of 'matched-matings'. Alternatively, by screening males caught during worker sampling, previous studies may have underestimated the frequency of diploid males, perhaps because their colonies are short-lived, or because the diploid males are destroyed during the non-reproductive phase of the colony cycle. Laboratory studies have found that a small percentage of diploid males produce viable diploid sperm, and that if these males mate then the resultant colonies produce triploid offspring. Assessing the frequency of triploid colonies could provide an alternative minimum estimate of the relative frequency of diploid males. Here we use microsatellite markers to determine the frequency of triploid individuals of *B. muscorum* and *B. jonellus* in a model island system. We find triploids in populations of both species, the first time that triploids have been detected in any wild bee populations. Observed triploid frequencies of up to 8% are detected, and estimated total frequencies peak at 20% with respect to normal diploid workers. For both species, triploid frequency is negatively correlated with population size, with populations limited to less than 13km^2 of suitable habitat particularly at risk. Estimated total triploid frequencies are higher in *B. muscorum* than in *B. jonellus*, perhaps due to the greater dispersal range of the latter species. Despite relatively high triploid frequencies, few diploid males were detected in these populations. Possible reasons for the under-detection of diploid males are discussed, along with implications for the conservation of rare social hymenopterans.

INTRODUCTION

Hymenopterans use a reproductive system called arrhenotokous parthenogenesis in which females develop from fertilised diploid eggs whereas unfertilised eggs produce males (but see Beukeboom *et al.* 2007). The sex of an individual depends on one of several sex-determination mechanisms (Cook 1993; van Wilgenburg *et al.* 2006a), best understood of which is the single-locus complementary sex determination system (sl-CSD) thought to be ancestral to the Hymenoptera (Crozier 1977; Periquet *et al.* 1993; Crozier & Pamilo 1996). Under this system, individuals which are heterozygous at the poly-allelic sex locus develop into females, whilst hemizygous (or homozygous) individuals develop into males (Cook & Crozier 1995). These males are generally costly as they have low viability, are unable to mate or are sterile (reviewed in Cowan & Stahlhut 2004). There are additional knock-on costs for females that choose to mate with diploid males, because no fertile offspring result from such matings. Clearly, this cost is greatest for monoandrous taxa such as bumblebees. Both inbreeding and genetic drift in small populations increase the frequency of 'matched-matings' (Zayed & Packer 2001). The resultant diploid male production (DMP) is thought to substantially increase the extinction proneness of inbred populations (Zayed & Packer 2005), and has been proposed as an indicator of pollinator declines (Zayed *et al.* 2004).

In species with sl-CSD, the high fitness costs of DMP should favour mechanisms which minimise inbreeding, particularly in social species where diploid males are produced at the expense of female workers (Cowan & Stahlhut 2004). This is perhaps the reason why, in the Hymenoptera, both sexes commonly disperse from the natal area prior to mating (Michener 1974; Antolin & Strand 1992), and why males and reproductive females are frequently produced asynchronously or leave the nest at different times (Holldobler & Bartz 1985; Holldobler & Wilson 1990). In bumblebees, young males are forced from the colony by the workers after 4 or 5 days, presumably to avoid sib-mating (Plowright & Pallett 1979). Additionally, in wasps, bumblebees and other eusocial bees (*Lasioglossum* sp.) kin-recognition systems result in preferential mating between unrelated individuals (Smith 1983; Smith & Ayasse 1987; Foster 1992; Ode *et al.* 1995).

Despite these mechanisms, diploid males have been detected in more than 40 species of social hymenopterans (van Wilgenburg *et al.* 2006a). Two species of bumblebees have been studied in the laboratory to date; *B. terrestris* (Duchateau *et al.* 1994; Duchateau & Marien 1995;

Ayabe *et al.* 2004) and *B. atratus* (Garófalo & Kerr 1975; Plowright & Pallett 1979), and in accordance with sl-CSD expectations, 50% of brother-sister matings led to colonies which produced diploid males at the expense of half of the workers. However, recent studies in small populations of rare bumblebees found very few diploid males despite low genetic diversity at neutral markers, so the extent of DMP and the consequences for wild populations remains uncertain (Darvill *et al.* 2006; Ellis *et al.* 2006). Many rare bumblebee species now exist only in small isolated populations, so elucidating the importance of genetic factors in their continuing declines is a conservation priority.

The diploid males of some hymenopteran species have been found to produce viable unreduced diploid sperm (Whiting 1961; Naito & Suzuki 1991; Crozier & Pamilo 1996; Yamauchi *et al.* 2001). If these males mate, viable colonies of triploid individuals are sometimes produced. These colonies are unable to reproduce, so although the cost of the initial matched mating is delayed, it remains a reproductive dead-end. Triploids have now been found in several Hymenoptera species including sawflies, wasps, ants and bees (Table 5.1). The presence of triploids indirectly indicates that diploid males were produced in the previous generation (Crozier & Pamilo 1996), which is significant because in some social species diploid males are not detectable at all stages of the colony cycle (Pamilo *et al.* 1994; Henshaw *et al.* 2002).

Table 5.1 Hymenopteran species in which triploids have been detected.

Taxon	Origin	References
Sawflies		
Athalia rosae ruficornis	Lab	Naito & Suzuki 1991
Wasps		
Bracon hebetor	Lab	Bostian 1934; Torvik 1931; Whiting 1961
Habrobracon "pectinophorae"	Lab	Inaba 1939, cited in Whiting 1961
Cotesia vestalis	Lab	de Boer *et al.* 2007
Ropalidia revolutionalis	Wild	Henshaw, unpublished (in Liebert *et al.* 2004)
Polistes fuscatus	Wild	Liebert *et al.* 2004
Polistes dominulus	Wild	Liebert *et al.* 2004; Liebert *et al.* 2005
Polistes aurifer	Wild	Liebert *et al.* 2004
Ants		
Solenopsis invicta	Wild	Krieger *et al.* 1999
Camponotus sp. 5 (tetraploid)	Wild	Imai *et al.* 1977
Crematogaster sp. 2	Wild	Imai *et al.* 1977
Bees		
Apis mellifera	Lab	Chaud-Netto 1975
Bombus terrestris	Lab	Duchateau & Marien 1995; Ayabe *et al.* 2004

Adapted and expanded from Liebert *et al.* 2004

The low frequencies of bumblebee diploid males found to date may be the result of high diversity at the sex-determining locus, or inbreeding-avoidance mechanisms such as sex-allele signalling (Paxton *et al.* 2000). Alternatively, by screening males caught incidentally during worker sampling, previous population genetic studies may have underestimated true diploid male frequencies, perhaps because DMP colonies are short-lived, or because diploid males are not released at all stages of the colony cycle. Here we determine the frequency of triploid individuals within previously studied populations of *B. muscorum* and *B. jonellus* in a model island system. Additionally, we establish whether triploid (and therefore diploid male) frequencies correlate with population size, as predicted by mutation-drift equilibrium expectations (Yokoyama & Nei 1979).

5.2 MATERIALS AND METHODS

5.2.1 Sample collection and genotyping

As part of a wider study of the population genetics of *B. muscorum* and *B. jonellus* (Chapters 3 and 4), workers and males of these species were collected from islands in the Inner and Outer Hebrides (Scotland, UK). The islands were visited between late June and early September in 2003-2005, and spanned a range of sizes and degrees of isolation. DNA samples were taken from workers following the non-lethal method of Holehouse *et al.* (2003) to minimise harm to the populations. Males of both species were lethally sampled so that their sex could be established beyond doubt. Samples were preserved in pure ethanol and stored at ambient temperature. In total 1072 *B. muscorum* (978 ♀ & 96 ♂) and 768 *B. jonellus* (764 ♀ & 4 ♂) were collected (Table 5.2). DNA was extracted from macerated tarsi using the HotShot protocol (Truett *et al.* 2000), and individuals were then genotyped at up to 9 microsatellite loci (see Chapter 3).

5.2.2 Detection of triploids

Following visualisation on a slab-gel sequencer (ABI PRISM™ 377), fragment sizes were manually scored using Genotyper (Applied Biosystems). Triploids were, in principle, identified by the presence of three distinct alleles at one or more loci. The presence of abnormally asymmetric peak intensities in heterozygote genotypes was also deemed indicative (although not conclusive), following Liebert *et al.* (2004, 2005).

In order to control for type I errors (false triploids) produced as a consequence of genotyping errors or contamination, a conservative approach to scoring was employed. The ABI PRISM™ 377 system produces two types of artefact which have to be accounted for. These effects, generally referred to as 'pull-up' (sometimes as bleed-through), lead either to the appearance of false peaks, or to increased intensity of genuine peaks. Firstly, overlapping dye fluorescence wavelengths affect other dyes within the same lane. Secondly, internal reflections affect lanes neighbouring strongly amplified products. In the absence of pull-up, the larger allele in a heterozygote genotype will ordinarily appear slightly less intense due to differential amplification during PCR. However, pull-up can cause either the larger or smaller allele of a

heterozygote genotype to appear disproportionately bright. These pull-up effects were identified and accounted for by careful inspection of the allele sizes both between and within lanes. Any uncertainly scored individuals were re-amplified and re-genotyped, several times if necessary, until a consensus was reached.

Where three peaks consistently appeared at one or more loci and abnormally asymmetric heterozygote genotypes were present at others, the individual was recorded as a putative triploid. Repeat DNA extractions were then carried out on sub-samples of the original tarsi, in order to control for the possibility of contamination, and the process of PCR amplification and genotyping was repeated at all loci. Only when genotypes remained unchanged were individuals deemed to be triploid, following Liebert *et al.* (2004). We believe this method to be conservative with respect to triploid frequency because some genuine triploids may have been discounted due to the confounding effects of pull-up.

5.2.3 Estimating total triploid frequencies

Triploids were primarily detected by the presence of three distinct alleles at one or more loci. Uneven band intensities, although of corroborative use, are not such reliable indicators of ploidy (Ridout 2000). The probability of a triploid having three different alleles at one or more loci, and therefore of it being correctly identified, depends on the number of alleles present at each locus within the population. Paradoxically therefore, in small (genetically depauperate) populations where triploids are likely to be more frequent, they are also more difficult to detect. Similarly, where two species differ in overall genetic diversity, as was the case here, the power with which triploids can be resolved differs accordingly. For each species, the total frequency of triploids in each population was estimated from the detected frequency following the iterative method of Krieger & Keller (1998).

5.2.4 Population size

Bumblebee population sizes are difficult to determine because their nests, the reproductive unit, are hard to find. Established methods estimate nesting density indirectly, based on the frequency at which sisters are detected within a sample of workers (Darvill *et al.* 2004).

However, this method relies on genetic markers of sufficient variability to distinguish sisters from unrelated individuals, and also requires an estimate of foraging range. Levels of genetic variation in these populations, particularly for *B. muscorum,* are too low to reliably resolve sisters with a practical number of microsatellites, and the foraging ranges of both species are unknown. Population sizes were instead ranked on an ordinal scale from 1-5 (as in Chapter 4). The area of suitable habitat per island was estimated using 1:50,000 Ordnance Survey maps and field observations of where each species was foraging. Population sizes were then ranked based primarily on these habitat areas (1-3, 3-10, 10-30, 30-60 and > 60 km^2), but where foragers were found to be particularly abundant or scarce, populations were moved up or down a single category accordingly. A Spearman's rho correlation was performed using SPSS version 15.0 to determine the relationship between triploid frequency and population size.

5.3 RESULTS

5.3.1 Observed triploid frequencies

In total, 12 (1.2%) *B. muscorum* and 6 (0.8%) *B. jonellus* workers were found to be triploid, out of a total of 978 and 764 respectively (Table 5.2). The frequencies of observed triploids did not differ significantly between species (Fisher's exact test, two-tailed, $P = 0.48$). No triploid males were detected from either species. Triploids were detected in 6 of 17 sampled populations for *B. muscorum,* and in 4 of 17 *B. jonellus* populations. Triploids were most frequent on Staffa and the Monachs, both of which are small and relatively isolated islands (see Figure 2.1).

Table 5.2 The number of triploids detected in each of the populations. Also shown are the total sample sizes, average heterozygosity (H_E), allelic richness, population size (category) and habitat area. For *B. muscorum,* allelic richness and H_E were calculated using all loci except B131.

	Bombus muscorum						*Bombus jonellus*					
Population	Sample size	Triploids Detected	Allelic Richness	H_E	Population Size	Habitat Area (km^2)	Sample size	Triploids Detected	Allelic Richness	H_E	Population Size	Habitat Area (km^2)
Barra	50 ♀ 3 ♂	-	3.10	0.39	5	67	82 ♀ 0 ♂	-	8.63	0.77	5	65
Mingulay	49 ♀ 1 ♂	-	2.99	0.37	3	5	34 ♀ 1 ♂	**2** ♀	7.50	0.70	2	5
Muldoanich	25 ♀ 6 ♂	-	3.63	0.42	1	1	-	-	-	-	-	-
Pabbay	38 ♀ 16 ♂	**1** ♀	3.33	0.40	3	3	36 ♀ 0 ♂	-	7.55	0.73	2	3
Sandray	59 ♀ 1 ♂	**1** ♀	3.05	0.37	3	4	25 ♀ 0 ♂	-	9.00	0.76	3	4
Monachs	50 ♀ 49 ♂	**4** ♀	2.22	0.31	1	2	-	-	-	-	-	-
Uists	87 ♀ 3 ♂	-	3.32	0.40	5	400	92 ♀ 0 ♂	-	9.30	0.75	5	221
Colonsay	67 ♀ 0 ♂	-	3.21	0.42	4	32	28 ♀ 0 ♂	-	10.24	0.76	3	30
Lunga	36 ♀ 6 ♂	-	3.43	0.51	1	1	38 ♀ 0 ♂	-	9.90	0.74	1	1
Staffa	56 ♀ 0 ♂	**4** ♀	3.33	0.48	1	0.5	48 ♀ 0 ♂	**2** ♀	8.19	0.70	1	0.5
Iona	50 ♀ 5 ♂	-	4.22	0.55	2	6.5	-	-	-	-	-	-
Canna	62 ♀ 3 ♂	-	3.11	0.43	3	5.75	45 ♀ 0 ♂	**1** ♀	9.58	0.76	3	5.75
Coll	70 ♀ 0 ♂	-	3.46	0.50	5	73	45 ♀ 1 ♂	**1** ♀	9.05	0.74	4	51
Eigg	64 ♀ 2 ♂	-	3.30	0.53	2	10.75	36 ♀ 1 ♂	-	9.87	0.76	2	10.8
Muck	53 ♀ 0 ♂	**1** ♀	2.91	0.43	2	4.25	47 ♀ 0 ♂	-	8.81	0.75	2	4.25
Rum	43 ♀ 1 ♂	**1** ♀	2.91	0.45	2	13	32 ♀ 0 ♂	-	10.00	0.75	2	27
Tiree	119 ♀ 2 ♂	-	3.27	0.50	5	75.25	54 ♀ 1 ♂	-	8.18	0.72	3	24
Skye	-	-	-	-	-	-	43 ♀ 0 ♂	-	10.03	0.75	4	200
Mull	-	-	-	-	-	-	37 ♀ 0 ♂	-	10.68	0.77	4	200
Mainland †	-	-	-	-	-	-	42 ♀ 0 ♂	-	10.02	0.76	5	>200

5.3.2 Estimated total triploid frequencies

Triploids are less likely to be detected in populations with relatively low levels of genetic diversity, which results in an underestimate of true frequencies. Predicted total triploid frequencies are given in Table 5.3.

Table 5.3 Observed and estimated total triploid frequencies in the subset of populations in which triploids were found. Total frequencies were estimated using the multilocus procedure of Krieger & Keller (1998). The triploid percentages which we estimate remained undetected are also shown.

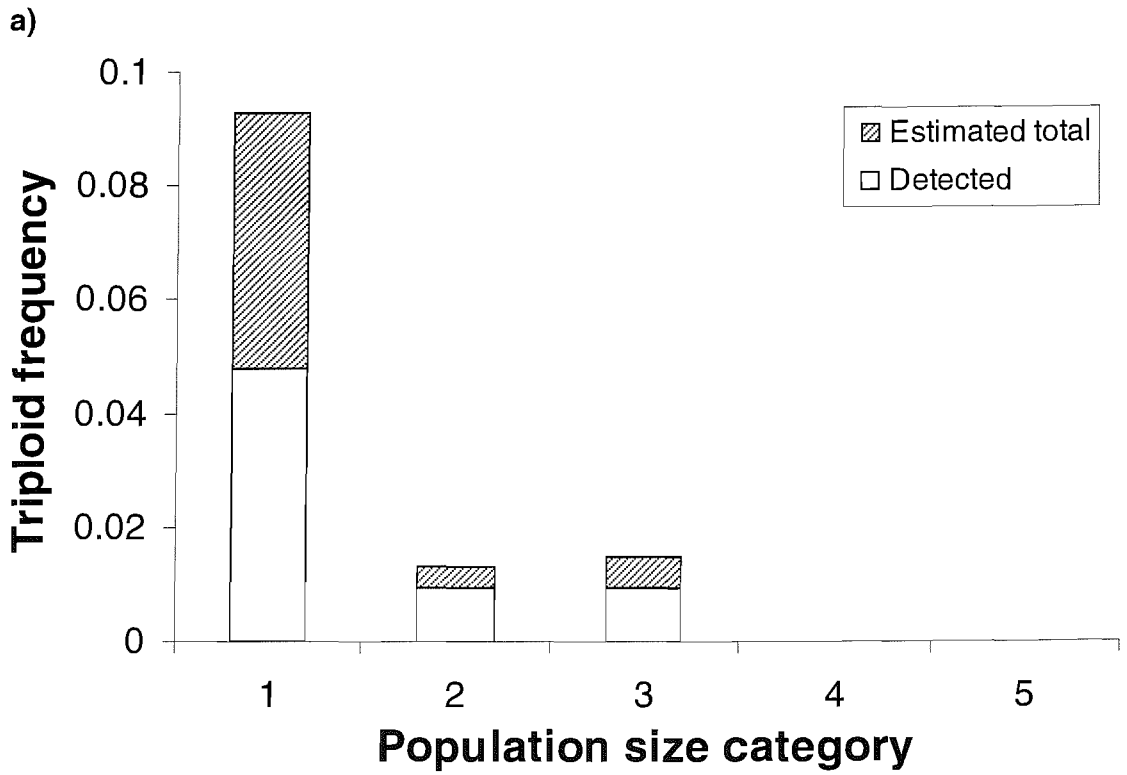
	Population	Obs. triploid frequency	Estimated total frequency	% undetected
B. muscorum	Staffa	0.0714	0.0981	27.2
	Monachs	0.0800	0.2000	60.0
	Muck	0.0189	0.0256	26.3
	Rum	0.0233	0.0334	30.4
	Pabbay	0.0263	0.0372	29.3
	Sandray	0.0169	0.0279	39.3
B. jonellus	Staffa	0.0417	0.0421	1.0
	Mingulay	0.0588	0.0604	2.6
	Canna	0.0222	0.0223	0.4
	Coll	0.0222	0.0223	0.4

The four *B. muscorum* triploids detected on the Monachs represent 8% of the total worker sample. However, there were (on average) just 2.22 alleles per locus in this population, so a high proportion of triploids (estimated at 60%) will have remained undetected. Indeed, a further two *B. muscorum* workers from the Monachs were identified as putative triploids on the basis of abnormally asymmetric peaks at several loci, but neither possessed 3 alleles at any loci. The estimated true frequency of triploids in this population is 20%, relative to ordinary diploid workers. In other populations of *B. muscorum* between 26% and 39% of triploids remained undetected. It is not possible to calculate the true frequency of triploids in populations where none were detected, but it seems likely that in many cases zero frequencies are underestimates. The greater genetic diversity of *B. jonellus* populations led to a larger

proportion (> 97%) of triploids being detected. Based on these figures it follows that approximately 11 triploid *B. muscorum* individuals remained undetected, whereas all *B. jonellus* triploids are likely to have been resolved. If these estimates accurately reflect true triploid frequencies, then *B. muscorum* triploids occurred significantly more frequently than those of *B. jonellus* (Fisher's exact test, two-tailed, $P = 0.0289$).

5.3.3 Population size and triploid frequency

For both species, there was a significant negative correlation between triploid frequency and population size (One-tailed Spearman's rho, N = 5, $P = 0.027$ and $P = 0.019$ for *B. muscorum* (Figure 5.1a) and *B. jonellus* (Figure 5.1b) respectively). Triploids were most frequent in the smallest populations (category 1), and were absent from the largest (category 5).



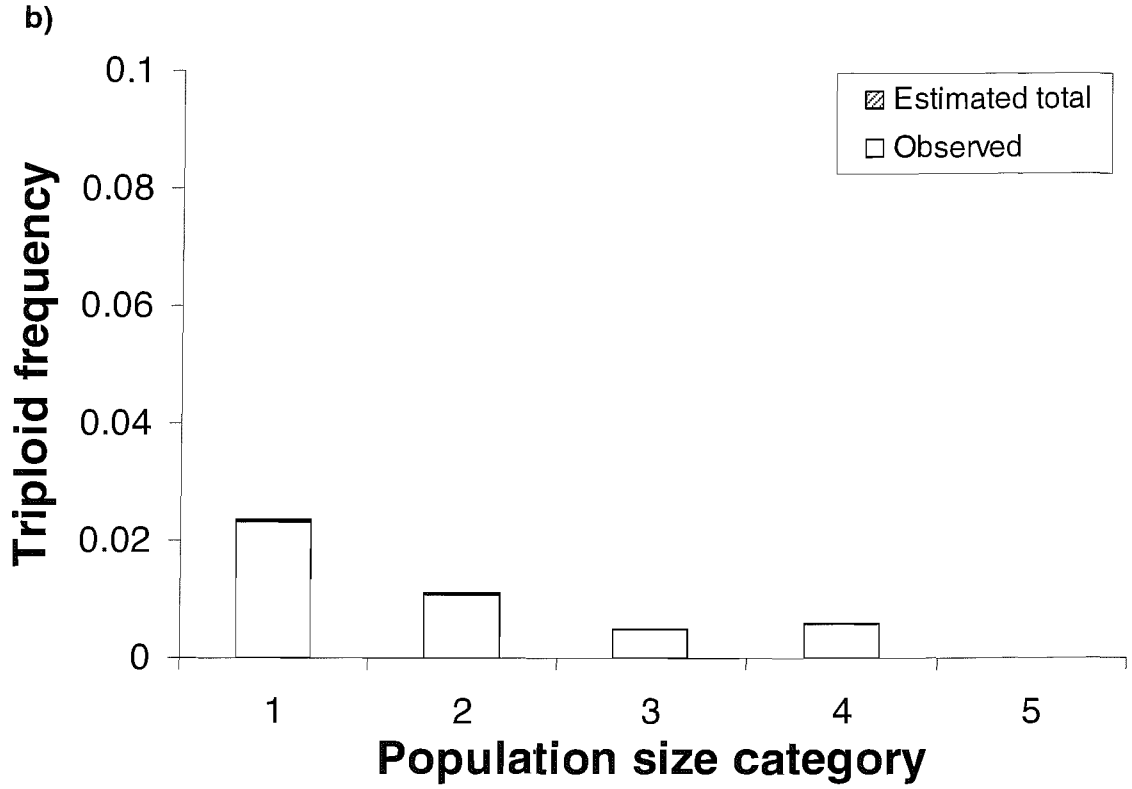


Figure 5.1 The relationship between population size (category) and triploid frequency for a) *B. muscorum* and b) *B. jonellus*. The values shown are overall frequencies within each population size category, rather than average values. The entire bar represents the estimated total triploid frequency, with the detected proportion shown in white.

5.3.4 Diploid male frequencies

A total of 98 *B. muscorum* males were caught and genotyped from the Hebrides. Two of these were found to be diploid. In *B. jonellus* only 4 males were caught, and none were diploid.

5.4 DISCUSSION

It has been argued that the costs associated with DMP favour high mutation rates at the sex-determining locus (M. Brown, pers. comms.), with new alleles benefiting from frequency dependent selection (Yokoyama & Nei 1979). Therefore, unlike at neutral markers, levels of diversity might remain high even in small and isolated populations. DMP might also be avoided or minimised by mechanisms such as kin-recognition or sex-allele signalling. The low frequencies of diploid males found to date in rare bumblebee populations (Darvill *et al.* 2006; Ellis *et al.* 2006) might be seen as confirmation that, relative to other factors, DMP is an insignificant component of population fitness. However, here (for the first time in any species of bee) we find triploid workers in wild populations, indirectly indicating the presence of diploid males. Triploid frequencies peak at an estimated 20% of sampled workers, demonstrating a considerable genetic load carried by small inbred populations. Triploid colonies are significantly smaller than normal colonies (Ayabe *et al.* 2004), so these relative worker frequencies probably underestimate the proportion of triploid nests. Furthermore, only a small percentage of diploid males are thought to be fertile (Duchateau & Marien 1995; Ayabe *et al.* 2004), indicating frequencies far in excess of previous estimates, with associated costs and consequences for population fitness (Zayed & Packer 2005).

Our data also demonstrate a direct link between population size and triploid frequency. Under equilibrium conditions (i.e. in the absence of significant immigration), the number of sex-determining alleles in a population should depend on the effective population size, reflecting a balance between mutation and drift (Yokoyama & Nei 1979). It follows that diploid male frequencies, and therefore triploid frequencies should be negatively correlated with population size; a prediction confirmed here. The production of diploid males in inbred hymenopteran populations substantially increases local extinction rates (Zayed & Packer 2005). This study therefore provides clear evidence of inbreeding depression, and provides empirical support for inbreeding theory. Estimated total triploid frequencies were significantly higher in *B. muscorum* than in *B. jonellus*, in accordance with observed differences in genetic diversity at neutral markers (see Chapter 4).

The discovery of triploid workers indirectly confirms that diploid males were present in the previous generation. Yet, of a total of 98 genotyped *B. muscorum* males, only two were

diploid, representing an overall frequency of just 0.21% (with respect to 966 sampled workers). Within populations (Pabbay and Tiree) diploid male frequencies peaked at 2.6% and 0.8% respectively (of diploid workers). In *B. jonellus* only 4 males were caught, and none were diploid, therefore all diploid male frequencies were zero. These values contrast with triploid frequencies which, across all populations, were 1.2% and 0.8% for *B. muscorum* and *B. jonellus* respectively. In individual populations, triploid frequencies of up to 8% were detected, and total frequencies were estimated at up to 20%, far in excess of observed diploid male frequencies. It is thought that only a small percentage of diploid males are capable of mating successfully (estimated at 5% in *B. terrestris* (Ayabe *et al.* 2004), and 2.4% in *Solenopsis invicta* (Krieger *et al.* 1999)) so a higher frequency of diploid males is expected. Indeed, in the case of introduced (and therefore inbred) populations of the fire ant *S. invicta,* 73-100% of males were diploid, resulting in a triploid frequency of 12% with respect to non-reproductive females. It is interesting to speculate why we found the reverse situation, with triploid frequencies exceeding those of diploid males.

a) Changes in DM frequency between years

It is likely that DMP is not constant over time, and occurs more frequently in some years than in others. Frequencies might fall following migration events or increase in response to periodic bottlenecks. In certain years diploid males could therefore be at low frequency or absent altogether, but the triploid progeny of the previous generation would still be detectable. Sampling for this study occurred over three summers (2003-2005), and triploids were detected in every year. It seems unlikely that in each case these populations were visited, by chance, in the year following DMP.

b) Changes in DM frequency during the season

DMP colonies grow more slowly than normal colonies (Plowright & Pallett 1979; Ayabe *et al.* 2004) and in some species exhibit higher mortality in the wild (Ross & Fletcher 1986). It is therefore likely that the relative frequency of diploid males declines throughout the season. However, observed frequencies suggest that diploid males were scarce when samples were collected, but were abundant when new gynes were emerging later in the season. Honeybees are known to destroy diploid male eggs or larvae (Woyke 1963; Drescher & Rothenbuhler

1964; Woyke 1979; Ratnieks 1990), with their recognition and removal mediated by differing cuticular hydrocarbons (Santomauro *et al.* 2004). Colonies of both *Polybiodes* wasps (Henshaw *et al.* 2002) and *Formica* ants (Pamilo *et al.* 1994), recognise and remove males from non-sexual broods but then release diploid males when colonies are producing reproductive offspring (new males and queens). In laboratory studies, diploid males of *B. terrestris* and *B. atratus* were reared to adulthood, but colonies were fed *ad libitum* and wild resource-stressed colonies may behave differently (Garófalo & Kerr 1975; Duchateau *et al.* 1994; Duchateau & Marien 1995; Ayabe *et al.* 2004).

c) High fecundity among diploid males

Laboratory studies of *B. terrestris* found that only 5% of queens that mated with diploid males produced viable colonies (Ayabe *et al.* 2004). However, 78% of these queens produced a few (presumably triploid) offspring, but the colonies did not survive. Only 22% of queens did not lay any eggs. We cannot yet rule out higher rates of diploid male viability in *B. muscorum* and *B. jonellus,* which would contribute to triploid frequencies. It is also possible that diploid males benefit from emerging earlier than their haploid counterparts. Queens produced early in the season may find diploid males at high frequency relative to haploids.

Bumblebee sex-ratios are heavily male biased (Bourke 1997) and most species are monoandrous (Estoup *et al.* 1995b; Schmid-Hempel & Schmid-Hempel 2000; Sauter *et al.* 2001; Payne *et al.* 2003), so the vast majority of males do not reproduce. It may be beneficial for males to attempt to mate with their sisters (the new gynes) prior to leaving the nest (see chapter 6). Workers usually police this behaviour by ejecting males from the nest (Plowright & Pallett 1979), but it is plausible that in DMP colonies this system breaks down. However, 50% of such couplings (where successful) should result in colonies that produce both triploid males and females, but only triploid females were found.

d) Underestimation of diploid male frequencies

Males of both species were caught when encountered, but the primary focus of field work was to collect sufficient samples of workers for population genetic analyses. Relatively few males of either species were caught, presumably because sampling was carried out prior to the reproductive phase of most colonies. However, perhaps too few males were genotyped to

accurately assess diploid frequencies, particularly in *B. jonellus*. It is also possible that males in general (or diploid males in particular) exhibited atypical foraging behaviour and were therefore not caught. However, populations were surveyed extensively in the course of sampling and it seems unlikely that a spatial foraging niche was overlooked. Triploid frequencies reached a maximum amongst *B. muscorum* populations on the Monachs, and yet of 49 males sampled from here, none were diploid.

e) Overestimation of triploid frequencies

Assuming that all colonies were of equal size, and that foraging workers were thoroughly mixed (or sampling efforts were sufficiently dispersed) then the relative abundance of workers should accurately reflect the ratio of triploid to normal colonies. If triploid workers were for some reason more easily caught or were disproportionately abundant, then triploid frequencies could have been overestimated. However, triploid colonies of species studied to date are significantly smaller than normal (Ayabe *et al.* 2004), meaning that worker ratios probably underestimate relative frequencies of nests. Additionally, many triploid colonies may not have survived for long enough to be detected. Triploids were caught during the date range 6th August – 2nd September, so evidently some wild triploid colonies were able to establish and persist throughout the season. However, a high proportion of queens that mated with diploid males may have failed to establish nests, so it seems more likely that triploid frequencies are underestimated.

f) Triploids without diploid males

Triploids could be produced as the result of a meiotic abnormality at some stage during development. However, it is hard to envisage why this would occur more frequently in small populations. Many of the triploids showed 3 peaks at several of the (unlinked) loci, so any such abnormality would have to be genome-wide.

These theories are not mutually exclusive, but the most likely explanation seems to be that DMP colonies were present within these populations, but the males themselves remained undetected. It is possible that diploid males are removed during the non-reproductive phase of colony growth, but are released alongside new reproductives. Workers may be unable to distinguish diploid males from haploids, or mechanisms for their removal may be less

effective once the colony has reached the competition point (Van der Blom 1986; Van Doorn & Heringa 1986; Duchateau & Velthuis 1988). However, while it is interesting to speculate, further empirical work is required to resolve the perceived disparity. It would be highly informative to return to these populations and determine whether diploid male frequencies peak late in the season.

5.4.1 Implications for conservation

Bumblebees provide keystone pollination services in temperate regions across the world, and their conservation is therefore extremely important (Allen-Wardell *et al.* 1998). However, owing to habitat loss, populations of many species have declined and fragmented to the extent that they now only exist in a series of small habitat fragments. This study confirms what theory has, for some time now, predicted; that inbreeding and genetic drift in small populations leads to an increased frequency of diploid males. The frequency of diploid males has previously been proposed as an indicator of the health of pollinator populations (Zayed *et al.* 2004). However, here we find that diploid males are difficult to detect directly during the course of routine worker sampling, so assumptions about the behaviour of diploid male producing nests may need to be revised. Instead we find that triploid frequency is a useful correlate of effective population size, and suggest that it could be used in future conservation work.

The principal reason underlying continued bumblebee declines is thought to be the loss of suitable habitat (Goulson *et al.* 2005; Williams 2005). As a result of their sociality, effective population sizes are small (Chapman & Bourke 2001) so relatively large areas of habitat are needed to support viable populations. However, it has not previously been possible to estimate the minimum area needed by any method other than scrutiny of (imperfect) distribution maps. If low triploid frequencies are indicative of low diploid male frequencies, and if these in turn are a prerequisite for healthy populations, then it is now possible to make cautious inferences about acceptable habitat areas. All twelve *B. muscorum* triploids were found in populations containing between 0.5 and 13km^2 of suitable habitat. Similarly, *B. jonellus* triploids were (with one exceptional outlier at 51km^2) all found in populations with between 0.5 and 5.75km^2 of suitable habitat. *B. jonellus* seems capable of dispersing over greater distances than *B.*

muscorum, and perhaps as a consequence retains higher levels of genetic diversity (at neutral microsatellite markers) than *B. muscorum* (see Chapter 4). It is possible that allelic diversity at the sex-determining locus is higher in *B. jonellus,* resulting in lower overall levels of triploid production and a lower threshold habitat area.

Of course, effective population size depends on more than just the area of suitable habitat, but it is likely to be a limiting factor. It seems that populations limited to less than 13km^2 of suitable habitat are particularly likely to suffer from diploid male production in addition to other problems associated with small population size. Very few nature reserves in the UK are sufficiently large. Conservationists and policy makers should therefore recognise the importance of managing habitat on an appropriate scale if further extinctions are to be prevented.

Chapter 6 - Aggregations of male *Bombus muscorum* (Hymenoptera: Apidae) at mature nests. Incestuous brothers or amorous suitors?

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6.1 ABSTRACT

Aggregations of male bumblebees are occasionally observed at the nest entrances of conspecifics. It has always been assumed that they originate from nearby nests and are hoping to mate with emerging gynes. However, it is possible that they are males from within the nest that have not yet dispersed, or are hoping to mate with their sisters. Inbreeding in Hymenoptera with single locus complimentary sex determination (sl-CSD) is costly and perhaps should be avoided. Nevertheless, other taxa with sl-CSD have been found to inbreed in this way. We use microsatellites to compare aggregating males with workers from within the *B. muscorum* nest. All males have genotypes inconsistent with origination from within the nest. Estimates of F_{IS} are near zero, indicating low rates of non-random mating. Clearly male bumblebees can detect mature nests, and congregate in the hope of mating with virgin queens. These data suggest that mechanisms may exist to avoid brother-sister matings at the nest, and perhaps beyond.

6.2 INTRODUCTION

Much is still to be learned about the mating behaviour of many bumblebee species, with successful copulations only seldom observed (Goulson 2003). A great diversity of male mating strategies exist, and are broadly grouped by Williams (1991) into 4 categories. *Patrolling* males leave scent marks on a number of prominent objects and visit them sequentially, hoping that a queen will be attracted by the pheromone. In *racing* behaviour, males choose a perch and pursue potential mates from this look-out, but do not compete with other males for preferred perches, unlike *territorial* males. Finally, *cruising* males hover in mid-air, rather than perching, and pursue potential mates as they pass.

In addition to these strategies, a number of authors have documented the chance observation of aggregations of male bumblebees in close proximity to nest entrances (Smith 1858; Tuck 1897; Frison 1930; Krüger 1951; Alford 1975; Lloyd 1981; Free 1987; Villalobos & Shelly 1987; Foster 1992). It has always been assumed that these males are individuals from other nests, hoping to mate with newly emerging gynes. It is, however, possible that these males have recently emerged from within the nest and are lingering prior to dispersal, or are waiting at the exit in the hope of mating with one of their sisters. One might predict that selection would act against this behaviour, as the single-locus complementary sex determination system (sl-CSD) in bumblebees results in 50% of brother-sister mating leading to nests producing (sterile) diploid-males in place of half of the work force (Cook & Crozier 1995). These nests suffer significant costs and are likely to produce few reproductives, resulting in significant individual and population-wide fitness effects (Zayed & Packer 2005). However, many more males are produced in any one season than are queens, so many males may fail to mate (reviewed in Bourke 1997). Given that males can mate multiple times, mating with a sister prior to dispersal may well be a good insurance strategy (Cook & Crozier 1995). Indeed, Cowan (1979) found that in the Vespid wasp, *Euodynerus foraminatus*, males routinely wait at their natal nest and mate with their sisters as they emerge. It was not clear whether the females were complicit in this behaviour, although those females that emerged in the male's absence dispersed immediately. Subsequent work has concluded that this species does have sl-CSD (Stahlhut & Cowan 2004b), and that despite diploid male production, inbreeding is frequent (Stahlhut & Cowan 2004a).

However, recent work suggests that, in addition to diploid male production, brother-sister mating has significant fitness costs for bumblebees. Hibernation survival and colony foundation success were both significantly reduced in inbred lines, and nest sizes were on average smaller (Gerloff & Schmid-Hempel 2005). One might therefore predict that inbreeding would be avoided, and indeed some bumblebee species have been found to exhibit such behaviour. Foster (1992) found that queens of *B. frigidus* and possibly *B. bifarius* avoided mating with nestmates based on individually-borne cues. However, the same study found that *B. californicus* and *B. rufocinctus* mated indiscriminately with nestmates. These latter species are thought to congregate at the entrances of conspecific nests and compete for access to emerging virgin queens. Foster (1992) argues that species with this mating system eject young males from the colony, and in so doing avoid inbreeding. However, this system does not preclude the possibility that males may linger in the region of the nest. Dispersal carries its own inherent costs and risks (Bengtsson 1978), not least that no mates may ever be found.

Here we use molecular techniques to assess whether males of *B. muscorum* aggregated around a nest are brothers of the new gynes emerging from within, or are unrelated males from different nests.

6.3 MATERIALS AND METHODS

6.3.1 Study species

Once widespread on the mainland, *Bombus muscorum* (L.) now survives only in a series of small fragmented populations. It is, however, still relatively abundant on some Scottish islands (Edwards & Broad 2005) where it thrives on heath and machair (Goulson *et al.* 2005). Within the UK, a number of different subspecies are recognised, differentiated on the basis of coat colour, with the race found on the island of Pabbay being *B. muscorum agricolae* Baker.

6.3.2 Sample collection

On the 17th August 2003, whilst visiting the island of Pabbay (Hebrides, NW Scotland; Figure 2.1) an aggregation of up to 20 male *B. muscorum* was discovered concentrated within a few square metres. Upon closer inspection it became evident that they were localised around the entrance to a mature *B. muscorum* nest. Their behaviour was quite distinctive, with each bee attempting to perch close to the nest entrance. When another bee flew close to a perched male, the in-situ male would set off in pursuit and a chase lasting several seconds would ensue. It was not possible to tell whether the objective was to drive away a competitor, or whether it was an attempt to catch and mount a potential mate. Shortly after the chase had ended one of the males would land back at a spot close to the nest.

The chance discovery of this rare phenomenon offered a unique opportunity to investigate the relatedness of the male bees to the nest. As many of the swarming males as possible were caught using a butterfly net before the disturbance caused them to disperse. Nine were caught in total. Non-lethal tissue samples were taken for DNA analysis following the method of Holehouse *et al.* (2003). A sample of 12 workers was then collected as they left the nest to forage. In addition, a population genetic sample was taken by collecting DNA samples from 36 foraging workers from random locations across the island. This sample was used to assess the suitability of the chosen molecular markers for use in this study, and to quantify certain population genetic parameters. Samples were preserved in pure ethanol and stored at ambient temperature.

6.3.3 Molecular methods

DNA was extracted using the HotShot protocol (Truett *et al.* 2000). Workers were genotyped at 7 microsatellite loci: B132, B118, B96, B10, B11, B124, B126 (Estoup *et al.* 1995b; Estoup *et al.* 1996). B11 was found to be monomorphic. Microsatellites were amplified by polymerase chain reaction (PCR) in 10μL volumes using QIAGEN Multiplex PCR kits. Each reaction contained approximately 10ng template DNA, 1μL Q-solution, 5μL PCR Master Mix and 0.2μM of each primer. Samples were initially denatured at 95°C for 15 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 51°C for 90 s and extension at 72°C for

90 s. A final extension step at 72°C for 10 min then followed. PCR products were visualised on an ABI PRISM™ 377 semi-automated sequencer using an internal size standard (GeneScan ROX 350, Applied Biosystems). Fragment sizes were scored using Genotyper (Applied Biosystems). Repeat PCRs were carried out on any samples that had failed to amplify or were uncertainly scored.

6.3.4 Statistical methods

The dataset was first checked for unexpected mutation steps, large gaps in the data or unusually sized alleles using MSA (Dieringer & Schlotterer 2003). Tests for genotypic linkage disequilibrium and departure from Hardy-Weinberg equilibrium (HWE) were performed using GENEPOP version 3.4 (Raymond & Rousset 1995). Sequential Bonferroni corrections (Rice 1989) were applied to minimise Type I errors. Population genetic parameters were calculated using FSTAT version 2.9.3 (Goudet 2001). The coefficient F_{IS} (Wright 1951) was calculated using Weir & Cockerham's (1984) estimator (f).

6.3.5 Assessing relatedness

Bumblebees exhibit haplodiploidy, with diploid queens producing diploid workers and new queens from fertilised eggs, and haploid males from unfertilised eggs. The vast majority of bumblebees so far studied are monoandrous, with the queen mating only once (Estoup *et al.* 1995b; Schmid-Hempel & Schmid-Hempel 2000; Sauter *et al.* 2001; Payne *et al.* 2003). Maternal and paternal genotypes can therefore be estimated based on the frequencies of alleles in a sample of worker offspring. Parental genotypes can be deduced in most cases, with the exception being when the queen is homozygous, but for a different allele to that found in the male genotype. In this case both alleles will be present at a frequency of 0.5, and it will not be possible to determine which allele came from which parent.

As males are produced from an unfertilised egg, if any alleles are present in a male genotype that are not present in the nest-queen genotype then it is certain that the male is not the offspring of that queen. Workers are also able to lay unfertilised eggs, which develop into males, so it was also necessary to determine whether congregating males could be their

offspring. Males were excluded as being potential workers-sons if they exhibited any alleles not present in the inferred queen *or* paternal genotypes.

6.4 RESULTS

Neither a global test nor tests by locus detected any significant deviation from HWE. Similarly, no two locus-pairs demonstrated significant linkage disequilibrium.

Based on the alleles found at each locus in the sample of workers, the parental genotypes were estimated (Table 6.1). At B132, it was not possible to determine whether the queen was homozygous for allele 155 or 157, so both possibilities were considered.

Table 6.1 The alleles present in the sample of workers from the nest, and the parental genotypes estimated on the basis of their frequencies.

Locus	**B132**		**B118**		**B96**		**B10**		**B11**		**B124**		**B126**	
Alleles	155	157	201	203	226	228	176		128		250	254	141	
Frequency	0.5	0.5	0.75	0.25	0.625	0.375	1		1		0.25	0.75	1	
QUEEN	155/155 ***or*** 157/157		201	203	226	228	176	176	128	128	250	254	141	141
MALE	155 ***or*** 157		201		226		176		128		254		141	

The genotypes of all of the sampled males contained alleles *not* present in the maternal genotype (Table 6.2) confirming that the males were not the offspring of the nest-queen. Similarly, no male had a genotype consistent with being the offspring of a worker from the nest. Within the male genotypes, the most polyallelic locus (B96) contained 4 alleles, suggesting that the males originated from at least 2 different nests.

Table 6.2 The genotypes of the sampled males found aggregated around the nest.

Locus	B132	B118	B96	B10	B11	B124	B126
Male 1	157	207*	226	176	128	258*	141
Male 2	157	207*	226	176	128	250	141
Male 3	165*	203	228	176	128	250	141
Male 4	157	207*	228	176	128	250	141
Male 5	157	207*	232*	176	128	258*	141
Male 6	155	201	232*	176	128	258*	141
Male 7	157	201	226	176	128	258*	139*
Male 8	157	201	224*	176	128	256*	141
Male 9	157	201	232*	176	128	250	141

* alleles that are not present in the inferred maternal or paternal genotypes. These indicate that these males did not originate from the sampled nest.

Of the 7 microsatellite loci amplified, 6 were found to be polymorphic with a range of 2-6 alleles per locus (Table 6.3). Average expected heterozygosity (H_E) for the polymorphic loci was 0.465 ± 0.12 and the estimate of F_{IS} (f) was -0.004 ± 0.039.

Table 6.3 Observed and expected heterozygosity for the 6 loci used, along with the number of alleles per locus and f, Weir & Cockerham's (1984) estimator of F_{IS}.

	b132	b118	b96	b10	b124	b126	Mean	Std. Error
H_E	0.413	0.597	0.721	0.237	0.769	0.053	0.465	± 0.115
H_O	0.405	0.541	0.757	0.216	0.892	0.054	0.478	± 0.130
No. of alleles	4	4	6	2	5	2	3.83	± 0.654
f	0.018	0.096	-0.051	0.089	-0.162	-0.014	-0.004	± 0.039

6.5 DISCUSSION

To date it was not known whether male bumblebees congregating outside mature nests were the offspring of that nest, or males from other nests in the area. In this case, none of the 9 sampled males had genotypes consistent with being the offspring of either the queen or the workers from the nest. They must, therefore, have emerged from other nests on the island, and congregated at the entrance. Analysis of their genotypes suggests that the males originated from at least 2 different nests. Males must therefore be receptive to as yet unidentified cues emitted from mature colonies.

Based on this small study it is not possible to say whether male bumblebees avoid congregating at their own nests in all cases. However, the population as a whole was found to be in Hardy-Weinberg equilibrium, and estimates of the inbreeding co-efficient (f) were very low (-0.004), both of which are consistent with the avoidance of frequent brother-sister matings. Cameron *et al.* (2004) recently studied male congregations of eusocial Asian stingless bees (*Trigona collina*), which are well known for gathering outside nests. They found that none of the males in drone aggregations of up to 42 males were flying in front of their natal nest. Given the high costs of inbreeding depression (Gerloff & Schmid-Hempel 2005) and the production of diploid males (Zayed & Packer 2005) it is perhaps unsurprising that brother-sister matings are avoided. Whether this behaviour is mediated by the willing dispersal of males or is policed by workers remains unknown. If the costs of inbreeding are sufficiently high then male bumblebees may choose to focus their efforts elsewhere. Bumblebees are known to mark their nest entrances with colony-specific odours (Foster & Gamboa 1989; Pouvreau 1996) and one function of this may be to help males avoid congregating at their own nests.

As part of a wider study of the population genetics of *B. muscorum* (Darvill *et al.* 2006), samples from several small isolated islands were collected. Some islands were as small as 0.5km^2, but despite this no diploid males were found on most islands. It is possible that diploid male producing nests are very short-lived and therefore diploid males are at very low frequency. However, it is also possible that the absence of diploid males may be indirect evidence for an inbreeding avoidance mechanism. In the Field Cricket (*Gryllus bimaculatus*), females reared in isolation have been found to show a clear preference for mating with

unrelated individuals, with mating preference increasing as relatedness decreased (Simmons 1991). Simmons (1989) suggests that females use their own cuticular compounds as a template. Pheromone composition is known to correlate with kinship in primitively eusocial sweat bees (*Lasioglossum* spp.) (Smith & Ayasse 1987). It would be of great interest to determine whether bumblebees are similarly able to determine relatedness on the basis of cuticular compounds and avoid inbreeding on this basis.

Chapter 7 - Forager size variation

7.1 ABSTRACT

Bumblebee workers vary in size considerably, and different sized workers are known to specialise in different tasks (alloethism). Small bees perform with-nest tasks, while larger individuals specialise in foraging for pollen and nectar. Even within foragers, thorax widths of some species range from 3.3 to 6.8mm, and it is thought that this variation is adaptive. It has previously been demonstrated that *B. terrestris* foragers of different sizes specialise in visiting flowers which match their tongue-length. However, forager size-variation will only be of benefit in a heterogeneous foraging landscape; under some circumstances variation could lead to deviations from a local optimum. Previous studies have yet to reach a consensus over the relative size of pollen and nectar collectors, and it is not yet clear whether forager size changes throughout the season. Less still is known about whether forager size differs between populations. Limited evidence suggests that bees at more northerly latitudes are larger, but it is not known whether inter-population differences exist at a smaller scale. A local reduction in mean forager size could reflect a differing local optimum, competition effects, or a costly response to resource scarcity or inbreeding. Here we explore patterns of size variation in *B. muscorum* and *B. jonellus* foragers on 18 oceanic islands (the Hebrides, UK), and on the adjacent mainland. Within islands, foragers of both species specialised in visiting flowers of an appropriate size. Pollen collectors did not differ in size significantly from nectar collectors, and no seasonal change in forager size was detected. Highly significant differences were found between islands, and an exploratory analysis reveals that *B. muscorum* were larger on islands where the foragers predominantly used heath and moorland. No effect of inbreeding was detected for either species. Differences between *B. jonellus* populations simultaneously correlated with population size, foraging niche breadth and niche overlap with *B. muscorum*: foragers were smaller in small populations, where niche breadth and niche overlap were greatest. Intriguingly, the extent of forager size variation within populations was closely correlated between the two bumblebee species, and in both cases correlated with the niche breadth of *B. muscorum*. Mechanisms underlying the observed patterns of inter-population forager size variation are discussed, including the possible role of competitive displacement.

INTRODUCTION

Bumblebee workers vary in size considerably, especially when compared to closely related species such as honeybees. Individual workers, even from the same nest, have thorax widths ranging from 2.3 to 6.7mm, representing a 10-fold increase in mass (Alford 1975; Goulson *et al.* 2002). It is thought that this size variation is adaptive, allowing different sized workers to specialise in different tasks (alloethism). In particular, the foragers of several bumblebee species are known to be larger on average than those that stay in the nest (Colville 1890; Sladen 1912; Meidell 1934; Richards 1946; Cumber 1949; Brian 1952; Free 1955; Goulson *et al.* 2002). A number of theories exist to explain the adaptive benefits of this (reviewed in Goulson 2003). It is possible, for example, that larger foragers are more efficient foragers (Goulson *et al.* 2002), perhaps due to greater visual acuity (Spaethe & Chittka 2003; Kapustjanskij *et al.* 2007) or longer foraging range (Cresswell *et al.* 2000). Others have suggested that thermoregulation may be a factor, as larger bees are better able to maintain adequate body temperature for foraging on cold days (Free & Butler 1959; Heinrich 1979).

However, even within foragers there remains considerable size variation, with foragers of *B. terrestris*, for example, having thoraxes ranging from 3.3 to 6.8mm in width (Goulson *et al.* 2002). Peat *et al.* (2005b) argue that this variation is adaptive, as differently sized foragers specialise in visiting different plant species (which they handle more efficiently), and in so doing they maximise colony foraging efficiency whilst minimising intracolony competition. However, such variation will only be adaptive in a heterogeneous foraging landscape, and it remains to be seen whether the extent of variation is, itself, variable. There is also some evidence that pollen-gathering foragers are larger than nectar-gathers (Brian 1952; Free 1955; Miyamoto 1957; Pouvreau 1989), although Goulson *et al.* (2002) found the reverse. Finally, some authors have reported changes in forager size throughout the season, although the results have been highly variable (Knee & Medler 1965; Plowright & Jay 1968; Röseler 1970). Clearly these relationships are not well understood.

Less still is known about whether forager sizes differ between populations. Factors such as thermoregulation, foraging range or floral diversity are likely to be of variable importance in different populations, but are colonies able to respond to variation in the local optimum? There is some evidence that, within-species, bees at more northerly latitudes are larger (Peat *et al.*

2005a), but whether differences exist at a more local scale remains unknown. Furthermore, it is not known whether size variation is at all times adaptive. It is possible, for example, that in populations where resources are scarce or patchily distributed, colonies grow slowly and workers are smaller. Inbreeding may also play a role, and a loss of genetic diversity could conceivably lead to a costly loss of forager size variation (if size has a genetic component).

Within-species, larger foragers have longer tongues, although the relationship is non-linear (large bees have disproportionately short tongues) (Goulson *et al.* 2002). Resource partitioning with respect to tongue length is thought to be an important factor in determining community composition and species coexistence (Heinrich 1976; Teräs 1976; Inouye 1978, 1980; Pyke 1982; Barrow & Pickard 1984; Harder 1985; Johnson 1986; Graham & Jones 1996). The role of competition in shaping bumblebee communities has proved somewhat controversial (reviewed in Goulson 2003). However, recent work suggests that where bumblebees forage alongside honeybees, foragers are smaller than in otherwise identical control sites (Goulson *et al. unpublished data*). Whether this is evidence of competition remains to be seen, but it is unknown whether similar changes in forager size are observed in the presence of congeners.

Here we explore patterns of size variation in bees collected, as part of a wider study, from 18 oceanic islands (the Hebrides, UK), and from the adjacent mainland. We determine whether different sized foragers specialise on certain flowers, or collect pollen versus nectar, and whether mean forager size changes throughout the season. We also look for changes in mean forager sizes between populations, and attempt to identify whether observed patterns represent an adaptive response to local conditions or a costly response to external factors.

7.2 MATERIALS AND METHODS

7.2.1 Sample collection

Studies were carried out between 15th June and 2nd Sept 2003-2005. Islands throughout the Hebrides were visited with the primary aim of sampling workers of *B. muscorum* and *B. jonellus* for population genetic analyses. In addition to taking DNA samples, data concerning the foraging ecology of these species were collected. Habitats were searched systematically to

avoid recording the same bees more than once, but this occurred occasionally (tarsally sampled bees were recaptured). These individuals were released immediately, and foraging data were not recorded. The majority of bees were actively foraging, in which case the plant species was recorded and the individual was briefly observed to determine whether it was collecting nectar or pollen. Nectar collectors were those which were not actively grooming pollen into their corbiculae, but it should be noted that the majority of pollen collectors were also collecting nectar. Bees were caught and transferred into a queen-marking plunger (Watkins & Doncaster Ltd.), for tarsal sampling. Additionally, vernier callipers were used to record the mean thorax width at the widest point following Goulson *et al.* (2002). The presence/absence of all *Bombus* species on the island was recorded, along with the date and the habitat type in which the bee was caught. Habitat types were classified as: marsh, lowland meadow, grazed field, heath, machair, garden and track/hedge. Since the main aim of the sampling was to obtain material for genetic studies, habitats were not sampled equally. Effort was instead concentrated in habitats or areas where experience suggested bees were most likely to be found. However, it seems likely that the patterns of forage use observed during sampling will broadly reflect actual habitat and forage use.

7.2.2 Differentiating workers from queens

Prior to carrying out an analysis of worker size variation it was necessary to separate queens and workers. It is likely that a few queens were sampled along with workers, and in some cases (e.g. *B. jonellus* on Colonsay and Skye) it was necessary to take tarsal samples (and thorax width measurements) from queens early in the season, for practical reasons. Frequency histograms (Figure 7.1) were plotted and used to pick cut-off points, above which individuals were categorised as queens.

7.2.3 Exploring forager size variation

To investigate patterns of forager size variation (thorax width), a multifactorial General Linear Model (GLM) was developed for each species (using SPSS version 15.0), using the starting model:

Size = Flower + Pollen + Island + Year + Day + Flower*Pollen + Flower*Day + Pollen*Day

Plant species with less than 10 recorded visits in total were excluded. The variable 'Day' was coded from 1 through to 80, representing the date range 15th June – 2nd Sept. 'Year' was coded from 1 to 3 (2003-2005). Individuals were either recorded as nectar collectors or as pollen collectors, although as noted earlier, many pollen collectors were probably also collecting nectar. The design was both unbalanced and non-orthogonal, and in particular it should be noted that most islands were visited in only one year (see Table 7.4). GLMs involving interactions were performed using sequential sums of squares (type 1) to avoid contravening hierarchical assumptions, following Grafen and Hails (2002). Interaction terms were entered in all possible combinations, and were removed from the model if significance was not consistent (unless *adjusted* R^2 values were improved by their retention). Non-significant main effects were retained in the final model if their inclusion improved the overall fit of the model (i.e. if they produced higher *adjusted* R^2 values). Post-hoc tests were performed in SPSS to aid interpretation, using the 'simple main effects' comparison. This is analogous to Tukey's post-hoc test, but (unlike other tests) compares differences between estimated marginal means, rather than observed means (Winer *et al.* 1991).

7.2.4 Inter-population size variation

Where mean forager sizes (or variation thereof) differed between islands, a number of possible explanatory variables were investigated in a second series of analyses, where 'island' was the unit of replication. It is important to note, of course, that exploratory analyses such as these have an inherently higher risk of Type I error. For each species, the estimated marginal mean values for thorax width (retained from the previous GLM) and standard deviations of thorax width on each island were defined as dependent variables. Estimated marginal means were

used in preference to raw means, as the former have been standardised for differences attributed to other variables. However, as a result of the non-orthogonality of the data, there may have been a degree of uncertainty in the fitting of the estimated marginal means, and therefore all analyses were repeated using the raw size data.

A number of explanatory variables were considered (Table 7.1), with the aim of investigating whether the observed variation was the result of: beneficial adaptation to local conditions; competition with the other species; or a costly consequence of sub-optimal conditions. Additionally, the mean values for thorax width and variation were compared between species, to determine whether there were correlations between the two.

It is possible that the mean size or variation of foragers is influenced by the range of plant species that they forage from, or the range of habitats that they use. Niche breadth values for both forage and habitat use were calculated following Goulson and Darvill (2004), using the standard Simpson's index formula:

$$D_s = \sum_{i=1}^{s} \frac{(n_i(n_i - 1))}{(N(N-1))}$$

where (for forage use) n_i is the number of flowers of the ith species that were visited, N is the total number of flowers visited, and s is the total number of flower species visited. As is usual, results are presented as 1/D, so that larger values indicate higher diversity. This index is insensitive to sample size (Magurran 1988). Only workers which were foraging (as opposed to caught mid-flight) were included in the calculation, and (for forage use) separate values were calculated for nectar and pollen collection.

Under some circumstances, the two species use the same habitats (Chapter 2). It is possible that the mean size or variation of one species may be influenced by competition for resources with the other species. Niche overlap was calculated for both forage use and habitat use, following Colwell and Futuyma (1971). For forage use niche overlap separate measures were calculated for pollen and nectar visits.

$$\text{Niche overlap between bee species } i \text{ and } h \;=\; 1 - 0.5\sum\nolimits_k (P_{ik} - P_{hk})$$

$$\text{Where } P_{ik} = \frac{\text{No. bee species } i \text{ visiting plant species } k}{\text{Total no. bee species } i}$$

Table 7.1 The variables used in an exploratory analysis of inter-island size variation.

Heterozygosity †	Presence of machair
Allelic richness †	Forage use niche breadth
Population size category †	Habitat use niche breadth
Distance to nearest neighbouring island †	Forage use niche overlap
Probability of a recent genetic bottleneck †	Habitat use niche overlap
Triploid frequency †	Forage use niche breadth of the other species
Year*	Habitat use niche breadth of the other species
Inner or Outer Hebrides	

* Few islands were visited in more than one year (Table 7.4). In order to include year as a potential explanatory variable in these analyses (where island was the unit of replication), some data points were deleted. A total of 38 data points were deleted for *B. muscorum*, and 46 for *B. jonellus*. See Table 7.4 for final sample sizes.

† These variables were calculated in Chapters 4 & 5 (see these chapters for more detail).

Exploratory analyses were performed using a GLM (in SPSS version 15.0), or where assumptions were not met, using equivalent but more conservative non-parametric tests (Mann-Whitney U and Spearman's rank correlations).

7.3 RESULTS

7.3.1 Differentiating workers from queens

The distribution of thorax widths for both *B. muscorum* and *B. jonellus* were bimodal (Figure 7.1). Individuals of *B. muscorum* with thorax widths ≥ 6.4mm were classified as queens, and excluded from analyses of worker size variation. Similarly, *B. jonellus* with thorax widths ≥ 5.6mm were classified as queens.

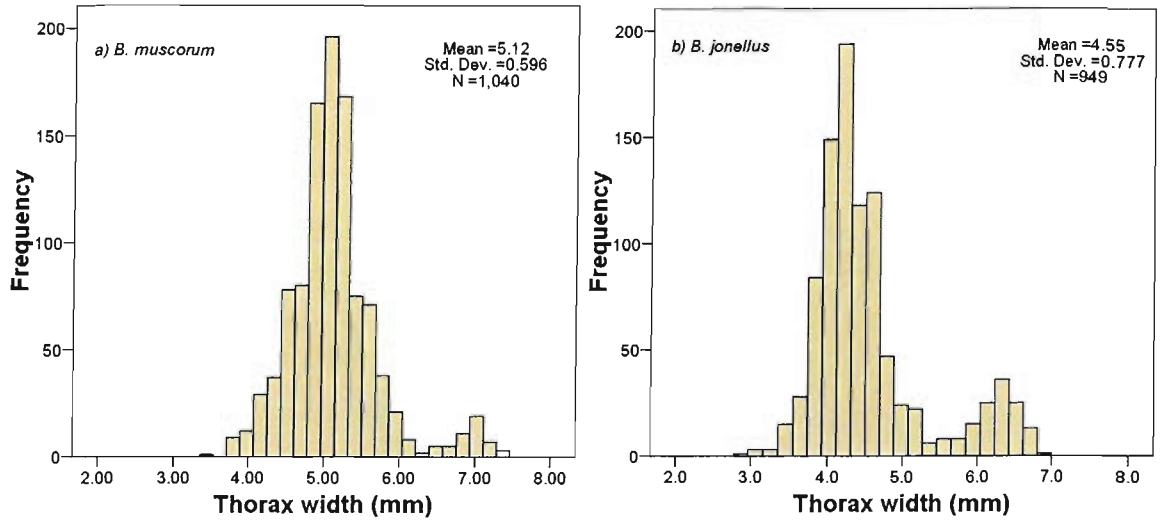

Figure 7.1 The frequency distributions of thorax widths for a) *B. muscorum* and b) *B. jonellus*. For *B. muscorum*, queens were defined as individuals with a thorax width in excess of 6.4mm, whereas 5.6mm was selected as the cut-off point for *B. jonellus*.

The average thorax widths of *B. muscorum* and *B. jonellus* queens were significantly different ($F_{1, 172} = 199.57$, $P < 0.00001$). Mean thorax widths were: *B. muscorum*: 6.950 ± 0.0330 S.E. (95% C.I. = 6.884 - 7.016; Range = 6.5 – 7.3); *B. jonellus*: 6.303 ± 0.0259 S.E. (95% C.I. = 6.251 - 6.354; Range = 5.6 - 6.9). Worker thorax widths also differed significantly between species ($F_{1,1813} = 1433.2$, $P < 0.00001$). Mean thorax widths were: *B. muscorum*: 5.024 ± 0.013 S.E. (95% C.I. = 4.997 - 5.051; Range = 3.5 - 6.33); *B. jonellus*: 4.284 ± 0.014 S.E. (95% C.I. = 4.257 - 4.310; Range = 2.9 - 5.59).

7.3.2 Exploring worker size variation

The best fitting GLM models for both *B. muscorum* and *B. jonellus* are given in Table 7.2. For both species, significant differences in worker size (thorax width) were found between islands. Additionally, a significant component of the overall thorax width variation was explained by differences between forage plant species (flower). For *B. jonellus*, differences were also found between years. For both species, pollen foragers did not differ in size from nectar foragers ($P > 0.05$), and no correlation was found between thorax width and capture date.

Table 7.2 Best-fitting GLM models explaining variation in forager thorax width for a) *B. muscorum* and b) *B. jonellus*.

a) *B. muscorum*

Source	Type III Sum of Squares	Df	Mean Square	F	*P*
Island	8.530	16	.533	3.237	0.00002
Flower	4.533	11	.412	2.502	0.00425
Year	.470	1	.470	2.855	0.09148
Error	135.882	825	.165		
Total	160.926	854			

$R^2 = 0.156$ (Adjusted $R^2 = 0.126$)

b) *B. jonellus*

Source	Type III Sum of Squares	Df	Mean Square	F	*P*
Island	13.980	14	.999	8.268	< 0.00001
Flower	1.319	3	.440	3.639	.01264
Year	7.701	2	3.850	31.881	< 0.00001
Error	80.678	668	.121		
Total	102.557	687			

$R^2 = 0.213$ (Adjusted $R^2 = 0.191$).

B. muscorum foragers vary in size considerably between forage plants (Figure 7.2). *Trifolium repens*, *Calluna vulgaris* and *Odontites vernus* attracted the smallest foragers, whereas the largest visited *Centaurea nigra* and *Trifolium pratense*.

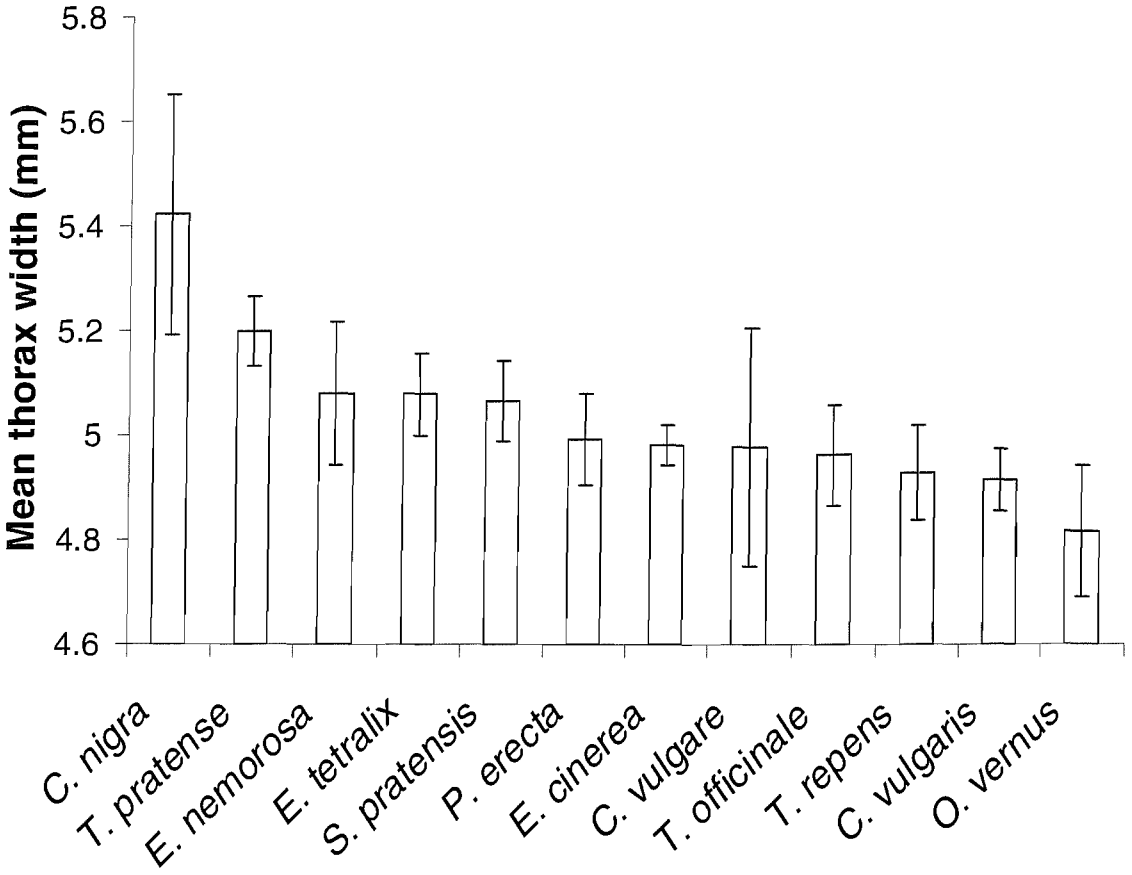

Figure 7.2 The mean thorax widths of *B. muscorum* foragers using a range of forage plants. The plotted values are estimated marginal means (± SE). Species abbreviations represent *Centaurea nigra, Trifolium pratense, Euphrasia nemorosa, Erica tetralix, Succisa pratensis, Potentilla erecta, Erica cinerea, Cirsium vulgare, Taraxacum officinale* agg, *Trifolium repens, Calluna vulgaris,* and *Odontites vernus.*

Post-hoc ('simple main effects') pairwise comparisons indicate several significant differences, although none were significant after strict Bonferroni corrections for multiple tests (Table 7.3). There is a suggestion that visitors to *Trifolium pratense* and *Centaurea nigra* may be larger than those visiting other plant species.

Table 7.3 Pair-wise comparisons of the average size of *B. muscorum* foragers using a range of forage plant species. These post-hoc comparisons are 'simple main effects', based on estimated marginal mean values of thorax width. Values shown are un-adjusted *P*-values, with significance nominally indicated where $P < 0.05$. However, following strict Bonferroni correction (Rice 1989), only *P*-values < 0.00076 remain significant.

	Odontites vernus	*Calluna vulgaris*	*Euphrasia nemorosa*	*Taraxacum officinale* agg	*Erica cinerea*	*Erica tetralix*	*Centaurea nigra*	*Trifolium pratense*	*Succisa pratensis*	*Cirsium vulgare*	*Potentilla erecta*	*Trifolium repens*
Odontites vernus	-											
Calluna vulgaris	0.481	-										
Euphrasia nemorosa	0.134	0.263	-									
Taraxacum officinale agg	0.298	0.686	0.441	-								
Erica cinerea	0.214	0.269	0.488	0.852	-							
Erica tetralix	0.077	0.091	0.992	0.352	0.281	-						
Centaurea nigra	0.030	0.042	0.223	0.084	0.073	0.182	-					
Trifolium pratense	0.002	0.002	0.388	0.010	0.007	0.230	0.387	-				
Succisa pratensis	0.111	0.115	0.927	0.441	0.330	0.908	0.136	0.234	-			
Cirsium vulgare	0.537	0.801	0.708	0.947	0.989	0.696	0.200	0.357	0.739	-		
Potentilla erecta	0.263	0.426	0.588	0.827	0.911	0.464	0.093	0.071	0.515	0.960	-	
Trifolium repens	0.391	0.906	0.324	0.758	0.598	0.215	0.062	0.001	0.293	0.841	0.630	-

These values have not been adjusted for multiple tests. Following strict Bonferroni correction, none of these pairwise differences remain significant (all $P > 0.05$).

B. jonellus regularly used just two plant species (*Centaurea nigra* and *Succisa pratensis* were only used on Staffa and Lunga). Those using *Erica cinerea* were significantly larger than those using *Calluna vulgaris* (Figure 7.3; 'main effects' post-hoc test, $P < 0.05$ after strict Bonferroni correction). Other pairwise comparisons were not significant.

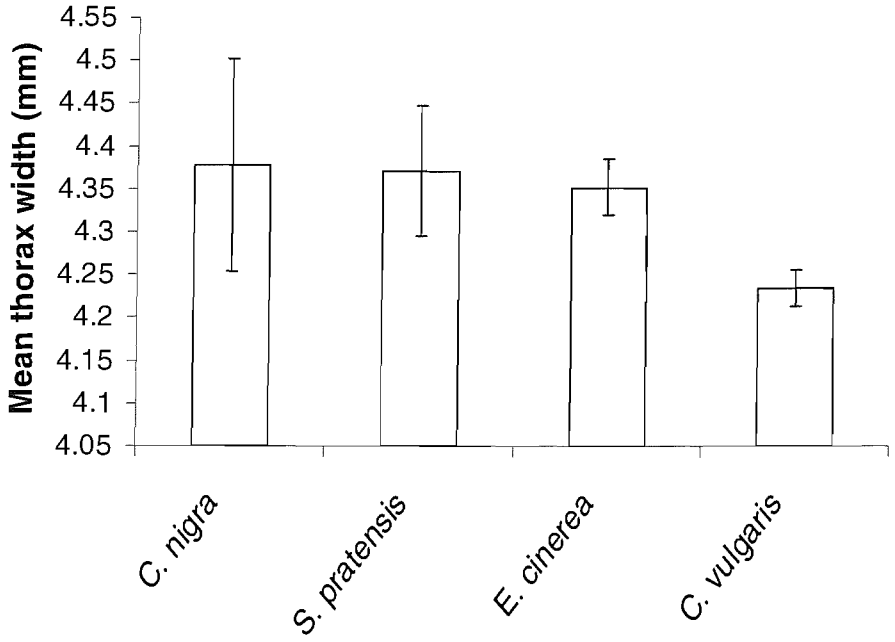

Figure 7.3 The mean thorax widths of *B. jonellus* foragers using a range of forage plants. The plotted values are estimated marginal means (± SE).

7.3.3 Inter-population size variation

Although a significant component of size variation was explained by forage plant preferences, for both species the largest differences were found between populations (Table 7.2). In exploring inter-island size variation, ideally estimated marginal means would have been retained following implementation of the full model (Size = Island + Flower + Year), allowing comparisons between islands once the effects of 'flower' and 'year' had been taken into account. Regrettably, this was not possible due to insufficient overlap in sampling within islands between years. Instead, estimated marginal means for island were calculated following implementation of the sub-model 'Size = Island + Flower'. The variable 'year' was then included in subsequent analyses of inter-island variation to determine the importance of inter-year variation relative to other factors. In order to assign a single year to each island, a small number of data points were deleted (see Table 7.4).

For *B. muscorum*, estimated means ranged from 5.24mm (± 0.065 SE) to 4.47mm (± 0.041 SE), representing a 14.7% reduction in thorax width. Similarly, for *B. jonellus*, values spanned the range 4.65 (± 0.057 SE) to 4.07 (± 0.053 SE), or a 12.5% reduction.

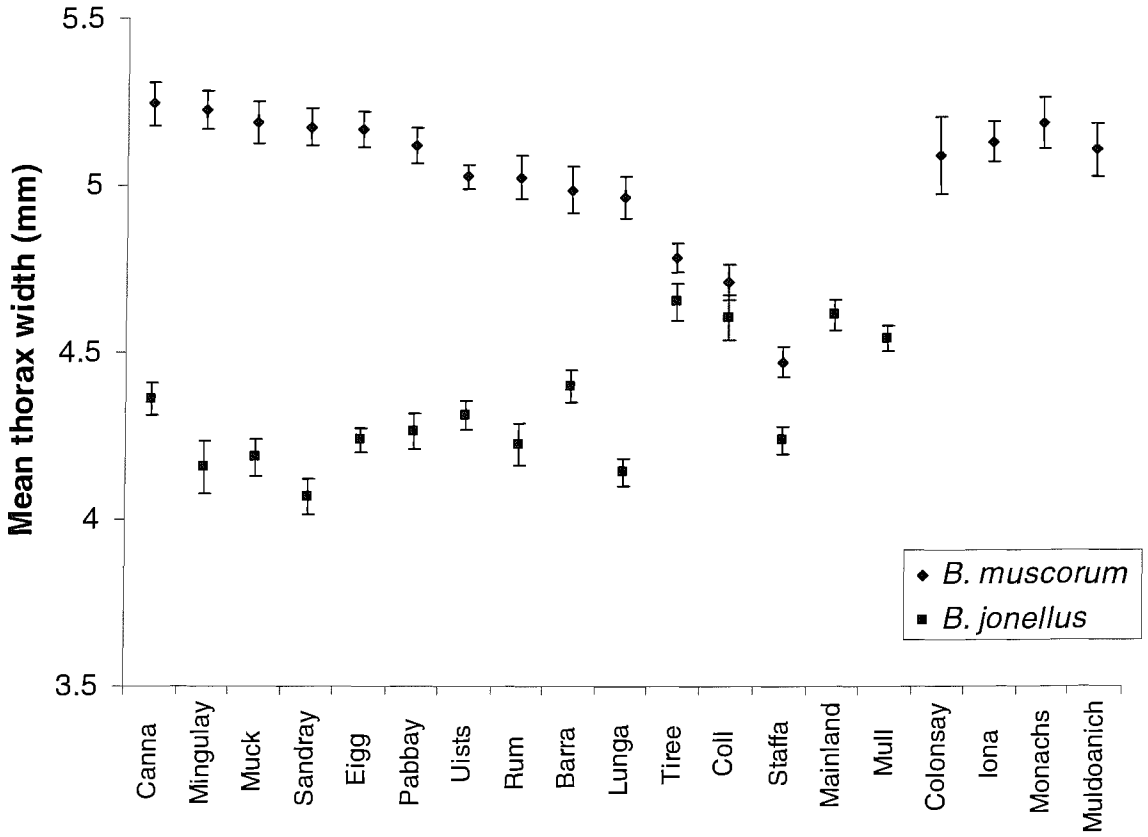

Figure 7.4 Estimated marginal mean thorax widths for both *B. muscorum* and *B. jonellus* in each of the sampled populations (± SE). *B. muscorum* were very scarce on Mull and the Mainland, and could not be sampled. Similarly, *B. jonellus* were not found on Iona, Muldoanich and the Monachs. *B. jonellus* were present on Colonsay and Skye, but workers were not sampled (see main text).

For each species, exploratory analyses were performed to investigate which of several hypotheses best explained the observed inter-population variation (Table 7.1). Fitted values for niche breadth and niche overlap (all foraging visits combined) are given in Table 7.4, along with total sample sizes.

Table 7.4 Summary statistics for foraging workers caught during the three sampling years. Simpson's index and niche overlap values are calculated from all records of foraging workers, and the values shown here are calculated for all foraging visits combined. Separate measures for pollen-only visits and nectar-only visits were also calculated, but all correlations with these variables were weaker than the corresponding correlations using the combined values shown here.

	Sample sizes of foraging workers						Simpson's Index 1/D				Niche overlap	
	B. muscorum			*B. jonellus*			*B. muscorum*		*B. jonellus*			
Island	**2003**	**2004**	**2005**	**2003**	**2004**	**2005**	**Forage use**	**Habitat use**	**Forage use**	**Habitat use**	**Forage use**	**Habitat use**
Barra	45	-	-	92	-	-	4.50	1.49	1.39	1.00	0.43	0.80
Canna	13*	42	-	5*	40	-	1.53	1.00	1.69	1.00	0.48	1.00
Coll	67	-	-	14*	-	37	3.56	1.26	1.00	1.00	0.00	0.08
Colonsay	-	22	-	-	-	-	4.53	1.74	-	-	-	-
Eigg	5*	61	-		60		1.00	1.00	2.03	1.00	0.53	1.00
Ioua	-	-	36	-	-	-	1.82	1.24	-	-	- / 0 †	- / 0 †
Lunga	-	36	-	-	43	-	1.60	1.49	2.73	2.04	0.66	0.67
Mainland	-	-	-	-	11*	53	-	-	1.17	1.00	- / 0 †	- / 0 †
Mingulay	45	-	-	22	-	-	2.05	1.00	1.94	1.00	0.86	1.00
Monachs	-	-	56	-	-	-	2.01	1.00	-	-	- / 0 †	- / 0 †
Muck	20*	32	-	6*	44	-	2.28	1.00	1.84	1.00	0.53	1.00
Mull	-	-	-	-	7*	60	-	-	1.00	1.00	- / 0 †	- / 0 †
Muldoanich	25	-	-	-	-	-	2.10	1.00	-	-	- / 0 †	- / 0 †
Pabbay	33	-	-	20	-	-	3.22	1.07	1.37	1.00	0.33	0.97
Rum	-	39	-	-	31	-	2.32	1.94	2.87	1.00	0.56	0.62
Sandray	56	-	-	29	-	-	3.04	1.00	2.05	1.00	0.50	1.00
Staffa	-	56	-	-	50	-	1.08	1.00	1.48	1.27	0.86	0.88
Tiree	113	-	-	59	3*	-	5.15	1.80	1.33	1.00	0.15	0.17
Uists	-	-	92	-	-	30	1.32	1.00	1.33	1.00	0.03	0.00

* These individuals were removed from the data set prior to analyses where 'island' was the unit of replication in order that each island contained data from only a single year. For consistency, Simpson's Index and Niche overlap values were also calculated using data from only a single year.

† Where one species was absent, and therefore niches were not overlapping, values of zero were substituted.

For *B. muscorum*, of the tested variables only habitat niche breadth correlated significantly with mean thorax width (Figure 7.5a; Correlation coefficient = -0.587, P = 0.013, N = 17). Inspecting outlying points suggested that, for each island, it was the proportion of foraging visits to heath and bog habitats that best explained differences in mean thorax width. This new variable (proportion of visits to heath and bog) correlated strongly with mean thorax width (Figure 7.5b; Correlation coefficient = 0.688, P = 0.002, N = 17). If raw means of thorax width are used, instead of estimated marginal means, then this correlation is not significant, although it remains significant in a parametric regression (Figure 7.5d; $F_{1,15}$ = 5.481, P = 0.033). None of the other explanatory variables investigated here significantly explained the observed inter-island differences in mean thorax widths (all $P > 0.05$).

Of note also, foraging diet breadth (pollen and nectar visits combined) was positively correlated with population size, (Figure 7.5c; Correlation coefficient = 0.549, P (two-tailed) = 0.022, N = 17).

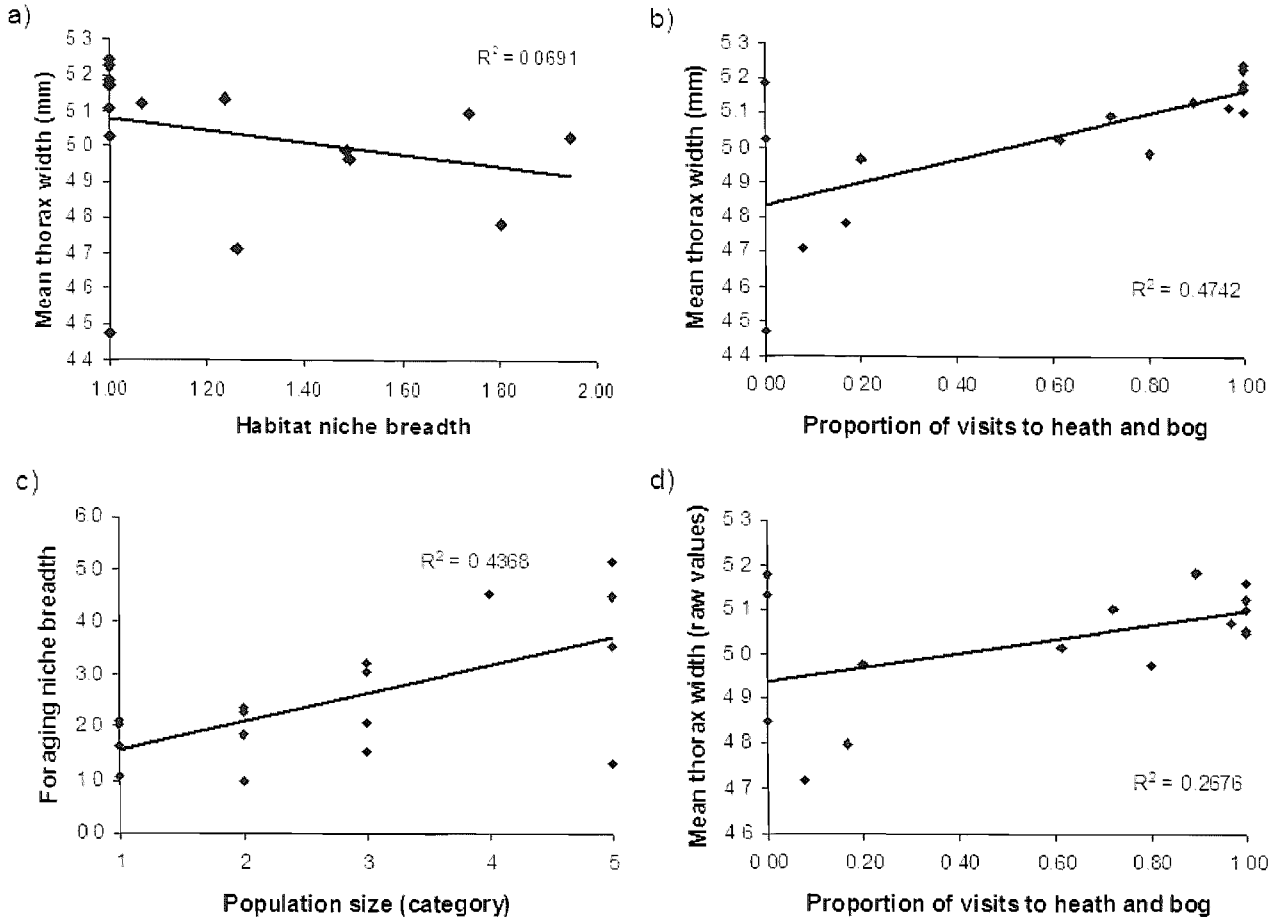

Figure 7.5 The significant correlations revealed by an exploratory analysis of inter-population size variation in *B. muscorum*. a) mean thorax width was weakly correlated with habitat niche breadth, b) mean thorax widths were significantly higher in populations where foragers used heath and bog habitats, c) diet breadth increases significantly with population size, d) raw (unstandardised) mean thorax widths are also significantly higher in populations where foragers used heath and bog habitats, but the relationship is not so strong.

For *B. jonellus,* an interesting pattern of significant correlations was revealed by the exploratory analyses (Table 7.5). Foragers were larger on average in large populations, but smaller when there was more foraging niche overlap with *B. muscorum.* Foraging niche overlap was highest in small populations. When foraging niche overlap was at its highest, *B. jonellus* had a wide diet breadth. When *B. jonellus* had a wide diet breadth, it was significantly smaller than when it used a narrow range of forage plants. When populations of *B. jonellus* were large, its foraging niche breadth was at its narrowest (which is when the foragers themselves were at their largest). These relationships are illustrated graphically in Figure 7.6.

Table 7.5 Significant (2-tailed Spearman's rank) correlations, as revealed by an exploratory analysis of inter-population size variation in *B. jonellus.* All variables are correlated at the $P < 0.01$ level. All results remain qualitatively unchanged when raw mean thorax widths are used instead of estimated marginal means.

	Spearman's rho	Foraging niche breadth*	Population size	Foraging niche overlap	Thorax width
Thorax width	Coefficient	-.835	.761	-.806	-
	Significance	$P < 0.001$	$P < 0.001$	$P < 0.001$	-
	N	15	15	15	-
Foraging niche overlap	Coefficient	.854	-.823	-	
	Significance	$P < 0.001$	$P < 0.001$	-	
	N	15	15	-	
Population size	Coefficient	-.674	-		
	Significance	$P = 0.006$	-		
	N	15	-		
Foraging niche breadth	Coefficient	-			
	Significance	-			
	N	-			

* Foraging niche breadth values are for pollen and nectar visits combined. Separate measures for pollen-only and nectar-only correlated less strongly with the other variables shown here.

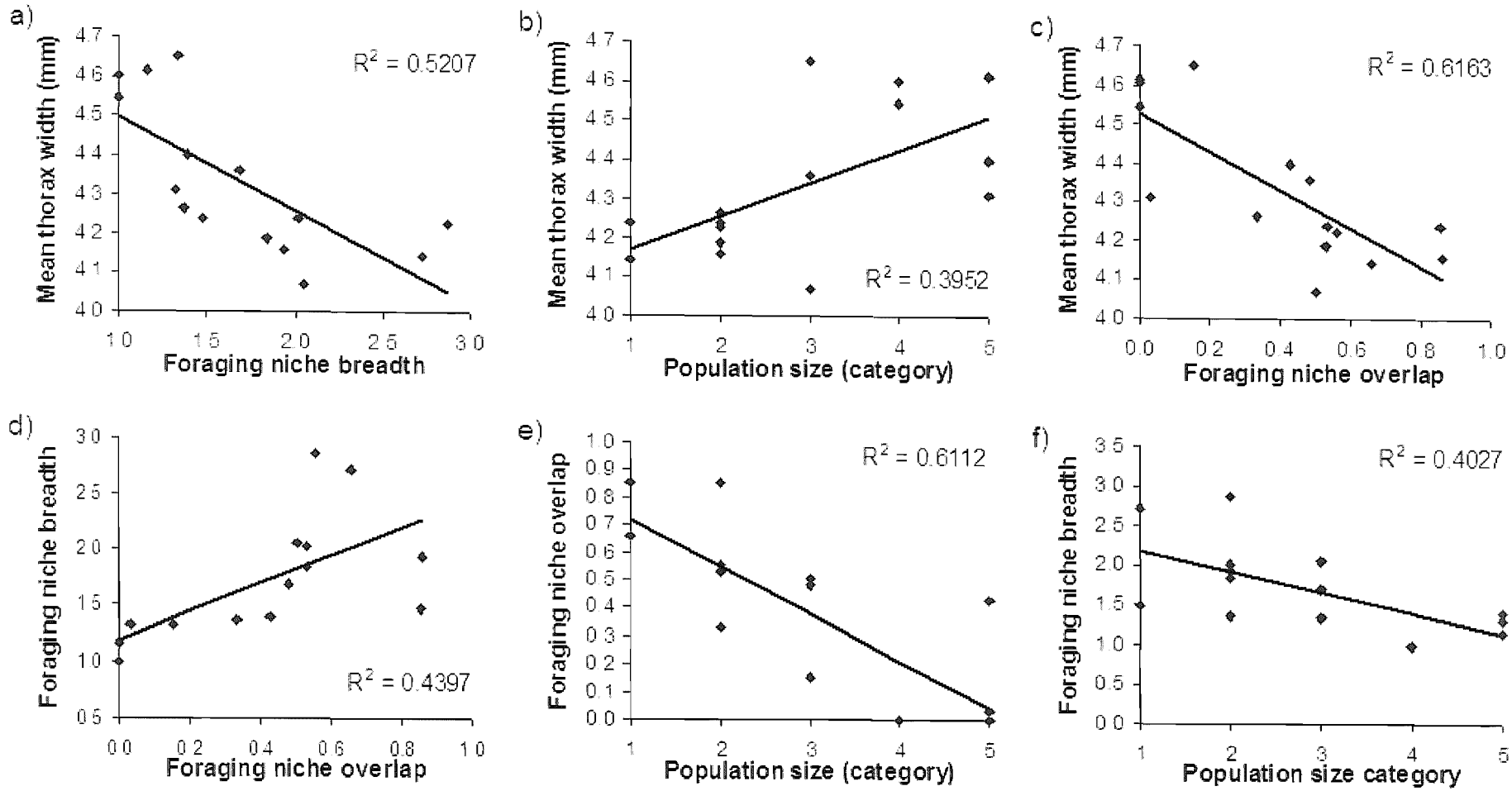

Figure 7.6 Significant correlations relating to inter-population variation in *B. jonellus* mean thorax width. All correlations are significant at the $P < 0.01$ level.

Finally, in order to investigate factors affecting variation within populations, similar exploratory analyses were performed for each species with the standard deviation of thorax width as the dependent variable.

Of the potential explanatory variables, for *B. muscorum* only foraging diet breadth correlated significantly with the standard deviation of forager size (Figure 7.7a; Spearman's correlation coefficient= 0.498, P = 0.042 (2-tailed), N = 17). Interestingly, it was also *B. muscorum's* forage diet breadth which was found to correlate with *B. jonellus* forager size variation (Figure 7.7b; Spearman's correlation coefficient = 0.571, P = 0.041 (2-tailed), N = 13). *B. jonellus*' own forage diet breadth values did not correlate significantly with standard deviation of forager sizes, and nor did any other putative explanatory variables (all $P > 0.05$).

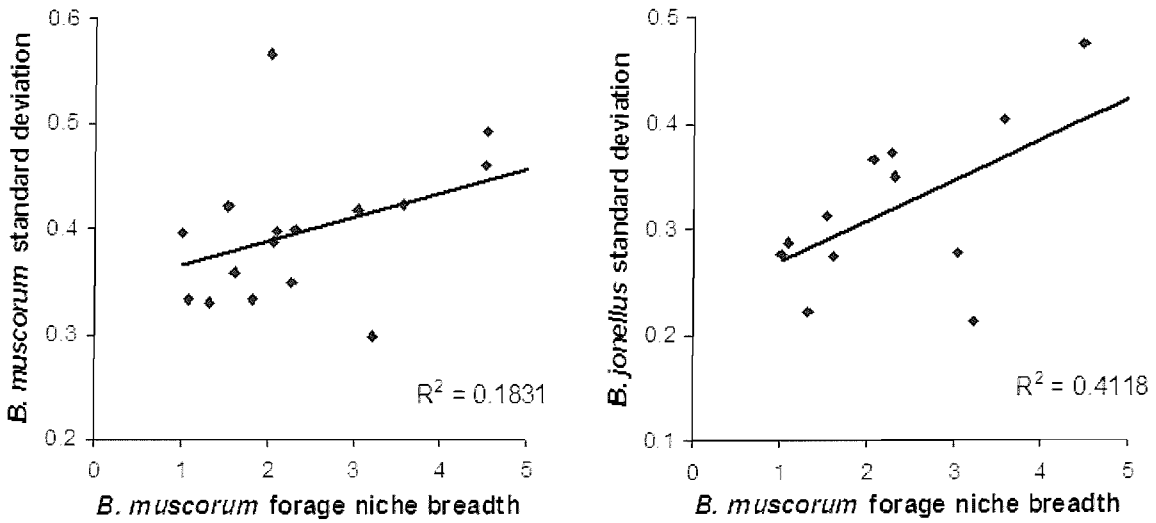

Figure 7.7 The relationship between the foraging niche breadth of *B. muscorum* and standard deviations of thorax width in populations of a) *B. muscorum* and b) *B. jonellus.*

Interestingly, it was found that the standard deviations of the two species were highly correlated (Figure 7.8; 2-tailed Spearman's correlation coefficient = 0.747, P = 0.003, N = 13).

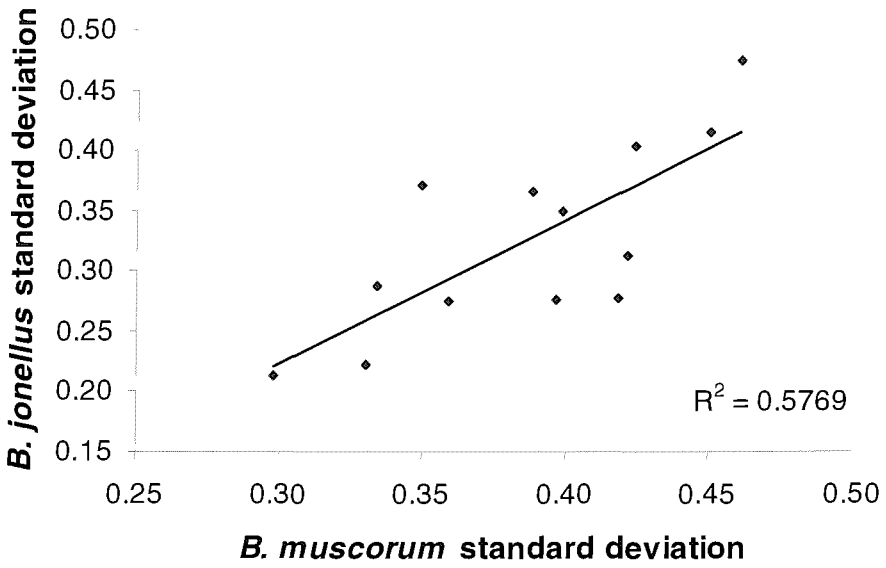

Figure 7.8 The relationship between the standard deviations of *B. muscorum* thorax widths and the corresponding standard deviations for *B. jonellus*. Each data point represents one of the thirteen populations in which these species co-occur.

7.4 DISCUSSION

Here we confirm the findings of Peat *et al.* (2005b) by demonstrating that different sized foragers specialise in visiting different plants. Plowright and Plowright (1997) had previously shown that short-tongued bees are able to forage more quickly on shallow flowers than long-tongued congeners. However, nectar foraging rate slows significantly when nectar levels approach the limit of reach of a bee's tongue (Harder 1983). It seems that a long tongue confers significant benefits when feeding from a deep flower, but is unwieldy when drinking from a shallow flower. Tongue length is known to increase with thorax width (Goulson *et al.* 2002; Peat *et al.* 2005b), so by producing foragers of a range of sizes a bumblebee colony presumably increases the range of forage plants it is able to efficiently exploit. In the case of *B. jonellus,* small foragers visited *Calluna vulgaris* whereas larger workers chose to visit *Erica cinerea.* The former have a corolla (calyx) depth of 4mm, compared to the 5-6mm typical of *Erica cinerea* (Rose 1981). Similarly, large *B. muscorum* foragers tended to visit plant species with deep corollae such as *Trifolium pratense* and *Centaurea nigra*, whilst small foragers visited shallower flowers (including *Trifolium repens* and *Calluna vulgaris*).

In common with the second experiment of Goulson *et al.* (2002), but in contrast to the first, we find no evidence of a size difference between pollen collectors versus those collecting only nectar. It is possible that different sized bees specialise in each of these tasks only when visiting flowers of a certain structure. Stout (2000) found that small foragers of *B. terrestris* and *B. lapidarius* were better able to trigger flowers of *Cytisus scoparius*, but this relationship may not generally be true.

Previous studies have, in general, reported an increase in forager size throughout the season. Knee and Medler (1965) found that worker size late in the season increased for three American species, and similarly Röseler (1970) found that, after an initial decline, forager size generally increased in *B. terrestris*. The same pattern was observed by Plowright and Jay (1968) for some species but not in others. We find no effect of sampling date, with size variation better explained by other factors, although the sampling method was probably insensitive to subtle changes.

Peat *et al.* (2005a) found that bumblebees from southern England were smaller than populations of the same species in Scotland, which they argued was an adaptive response to the colder climate. Here we find inter-population variation in forager size on a much smaller geographic scale. Islands were generally only visited in a single year, but for both species differences between years were unimportant relative to other factors. *B. muscorum* workers were biggest on islands where they were predominantly found foraging on heath and bog (having taken into account the flower species on which they were caught). There was no evidence of an affect of niche overlap with *B. jonellus* (although competition with other species cannot be ruled out), and no suggestion of an involvement of genetic factors. Within islands, foragers using several machair plants (notably red clover, the most visited plant) were actually larger than those using heathland plants. The increased mean size therefore seems most likely to correlate with an unidentified variable which itself correlates with an abundance of heath and bog habitats (or to a paucity of machair type habitats). Environmental and climatic factors are obvious candidates. For example, heathland is likely to be on higher ground than machair (which is invariably close to sea level) and hence may be more exposed to high winds or lower temperatures, favouring larger workers.

For *B. jonellus,* inter-population differences of a similar magnitude were observed, but the underlying causes seemed to be quite different. In large populations of *B. jonellus* there was

little or no niche overlap between the two species, with *B. jonellus* specialising on *Calluna vulgaris,* and *B. muscorum* either using *Erica cinerea* or foraging elsewhere on machair. However, observed correlations perhaps suggests that in small populations *B. jonellus* are forced to forage from the same flowers as *B. muscorum*, and that they feed from a broader range of (perhaps sub-optimal) food plants. Perhaps as a result they are smaller, either because of the cost of competition, or in order to avoid it. In large populations they are able to specialise in a narrow range of (presumably preferred) forage plants, and perhaps as a consequence are larger (Figure 7.6f). This relationship contrasts with that of *B. muscorum*, which instead increases its foraging niche breadth in larger populations, perhaps taking advantage of a greater diversity of suitable resources (Figure 7.5c).

Of course it is difficult to infer causative relationships from observed correlations, but there is some suggestion of niche contraction or competition with *B. muscorum*. The role of competition in shaping bumblebee communities has proved somewhat controversial (reviewed in Goulson 2003). All bumblebee species occupy a broadly similar niche, so one might intuitively expect competition between species. They exhibit remarkably little morphological variation other than differences in size, generally have an annual colony cycle and are all active throughout the summer. Crucially, all species feed almost exclusively on nectar and pollen at all stages of the life-cycle. These similarities have caused many authors to question how so many species manage to coexist. The one character which obviously varies between species is the length of their tongues, and this variation leads to differences in floral preferences (e.g. Harder 1985; Graham & Jones 1996). Resource partitioning with respect to tongue length has been proposed as a mechanism allowing several species to coexist (Heinrich 1976; Teräs 1976; Inouye 1978, 1980; Pyke 1982; Barrow & Pickard 1984; Harder 1985; Johnson 1986; Graham & Jones 1996).

Inouye (1978), studying bumblebee populations in Colorado (USA), found that *B. appositus* (a long-tongued species) preferentially foraged on *Delphinium barbeyi*, while *B. flavifrons* (medium-tongued) used *Aconitum columbianum*. However, when foragers of one species were experimentally removed, the remaining species visited the other plant more frequently. Under normal circumstances *B. appositus* chose *D. barbeyi* because they obtained greater rewards per flower, but in the absence of their competitor equal rewards were obtained from both flower species (Graham & Jones 1996). This is perhaps evidence of competitive release, whereby each bee species is ordinarily restricted to one preferred flower, but profitably uses

two or more species in the absence of competition. Again in Colorado, Pyke (1982) found that the seven species in the area could be categorised into one of four groups according to tongue length: long-tongued, medium-tongue and short tongued, plus short tongued bees that robbed nectar from bird-pollinated flowers. He found that, at any one site, no more than four species could be found, and there was only one species from each group. He interpreted this pattern as evidence of inter-specific competition leading to the competitive exclusion of certain species.

However, subsequent studies in Europe have failed to find similar patterns, and bumblebee communities here frequently consist of up to eleven species, with overlapping tongue-lengths (Ranta & Vepsäläinen 1981; Ranta 1982). Several studies of these populations failed to find any evidence for the role of tongue-length in shaping community composition (Ranta 1982; Ranta & Tiainen 1982; Ranta 1983; Williams 1988). Nevertheless, one of the most convincing examples of competition in bumblebees appeared independent of tongue-length, suggesting that other factors are important. Sympatric and allopatric populations of *B. flavifrons* and *B. rufocinctus* were experimentally generated in subalpine meadows in Utah (USA). These two species are very similar in both tongue length and size (Bowers 1985). In the absence of competition both species used a very similar suite of flowers. However, where both species co-occurred, *B. rufocinctus* no longer used their (previously) preferred flower species and were smaller. This was perhaps because they had received less food during development, but could also indicate a shift in optimum forager size in response to competition. Interestingly, no effects of competition were detected in *B. flavifrons,* so the two species differed in some way, leading to an asymmetry in the effects of competition. The only obvious difference is in emergence time, with *B. flavifrons* becoming established several weeks before *B. rufocinctus.* Emergence time has also been implicated in explaining differential rarity and species-coexistence in the UK (Goulson *et al.* 2005) and Ireland (Fitzpatrick *et al.* 2007). By the time the first workers of *B. rufocinctus* appear, *B. flavifrons* workers are already numerous, but exactly how early emergence provides a competitive advantage is not yet clear.

The differential effects of increased foraging niche overlap for *B. jonellus* and *B. muscorum* show similarities to those found in *B. flavifrons* and *B. rufocinctus* (Bowers 1985). *B. muscorum* (like *B. flavifrons*) showed no change in size or in foraging niche breadth in populations where niche overlap with *B. jonellus* increased. *B. jonellus*, by contrast, were smaller in populations where foraging niche overlap with *B. muscorum* was greatest. Unlike *B. rufocinctus, B. jonellus* were not displaced from their preferred forage plant (*Calluna*

vulgaris), but rather increased their foraging niche breadth to encompass a greater range of forage plants. Whether this is evidence of competitive displacement by *B. muscorum,* or relates instead to a scarcity of preferred resources in small populations is not clear from this study. It is noteworthy though that *B. muscorum* emerges earlier than *B. jonellus*, and it is possible that the abundance of *B. muscorum* workers in some way affects the size or foraging preferences of subsequent broods of *B. jonellus.*

The observed inter-population differences in the extent of forager size variation raise interesting questions about the adaptive benefits of, and mechanisms underlying this variation. The fact that variation in both species is highly correlated (Figure 7.8) is even more intriguing, and not adequately explained. The correlation between variation and foraging niche breadth for *B. muscorum* (Figure 7.7a), makes a degree of sense, although separating cause and effect is not possible. It is much harder to explain the relationship between variation in *B. jonellus* populations and the niche breadth of *B. muscorum* (Figure 7.7b). The extent of variation in *B. jonellus* populations is not related to niche overlap with *B. muscorum* for either forage use or habitat use (and neither is it correlated with *B. jonellus* diet breadth). Perhaps the most likely explanation is that some unidentified factor is affecting the size variation of both species, to which *B. muscorum* responds by increasing its niche breadth.

The fact that different sized bumblebee foragers visit different flowers implies that, for each plant species, there is an optimum forager size to efficiently handle it. Peat *et al.* (2005b) suggest that this variation maximises colony foraging efficiency, whilst minimising intracolony competition. However, forager size variation will only be of benefit if, within the foraging range of the colony, a range of forage plants exist to which each class of forager is well suited. In the absence of such floral diversity, variation may be costly, resulting in deviation from an optimal mean forager size. It is not known whether colonies are able to respond to temporal or seasonal changes in the available floral resources by adjusting the sizes of workers that they rear, but it would be a relatively straightforward area to study. In genetically isolated populations, selection would presumably produce one or more optimums for size, or an optimum degree of variation. However, changes in variation here appeared independent of physical or genetic isolation (see Chapter 4) raising the exciting possibility that adaptive variation may be controlled at the colony level.

Chapter 8 - General discussion

Prior to the start of this thesis (June 2003), very little was known about the effect of habitat fragmentation on the population genetics of bumblebees, particularly the rarer species. Less still was known about the relative importance of genetic factors for species conservation. Theory predicted that, as social hymenopterans, bumblebees should be particularly susceptible to losses of genetic diversity due to small population sizes (Chapman & Bourke 2001). Furthermore, inbreeding in small and isolated populations was thought likely to lead to the production of diploid males, further reducing population fitness (Zayed & Packer 2005). However, it was unclear at what scale these effects would be manifest, since almost nothing was known about the dispersal capabilities of bumblebees, even for the common species.

Until recently, studying the population genetics of rare species was extremely difficult, as lethal sampling was necessary in order to obtain template DNA. Work in this area was greatly facilitated by the development of a non-lethal DNA sampling technique (Holehouse *et al.* 2003). Subsequent studies, including chapters presented in this thesis, have advanced our understanding of bumblebee ecology, and informed conservation strategies. The first such study (Darvill *et al.* 2006; Chapter 3) found that some *B. muscorum* populations as little as 3km apart were significantly differentiated, as were all populations >10 km apart. In the Hebrides, a model system of oceanic islands, the overall extent of genetic structuring was moderately high ($\theta = 0.12$) (for a discussion of levels of genetic differentiation see Wright 1978b; Hartl & Clark 1997; Balloux & Lugon-Moulin 2002). On the mainland, in the south of the UK the two studied populations were also significantly differentiated from one another, although to a lesser extent ($\theta = 0.04$).

B. muscorum has recently been added to the UK's Biodiversity Action Plan (UKBAP) in recognition of its rarity. The population genetics of other UKBAP species have been simultaneously studied over the last few years. Ellis (2005) found comparable levels of genetic structuring in *B. humilis* ($\theta = 0.06$) and *B. sylvarum* ($\theta = 0.08$) in remaining fragmented populations in the south of the UK. No evidence of genetic isolation by distance was found, suggesting that little or no gene flow occurs between populations. It seems likely that these populations are now genetically isolated, but remain relatively similar (when compared to the structuring observed in the Hebrides) due to their relatively recent isolation. Habitat loss due to agricultural intensification has been greatest in the south, and until recently these sites may have been linked by stepping-stone populations. Charman (2007) studied *B. distinguendus* on the Hebrides and Orkney, and also found significant genetic structuring ($\theta = 0.08$). In general

it seems that (fragmented) populations of UKBAP species, unlike common and widespread species, exhibit genetic structuring.

The scale over which genetic structuring will be observed, and therefore over which metapopulations can persist, will depend on dispersal range. As noted previously, all populations of *B. muscorum* >10 km apart were significantly differentiated. Pairwise comparisons between Coll/Tiree and the Uists showed high levels of differentiation (average pairwise $\theta = 0.22$). The same pairwise comparisons for *B. distinguendus* demonstrate less genetic differentiation, despite the absence of stepping-stone populations (average pairwise $\theta = 0.10$; Charman 2006). Does this suggest that dispersal range differs between species? *B. jonellus* occurs on many of the same islands as *B. muscorum*, facilitating a comparison between these two species (Chapter 4). This comparison suggests that *B. jonellus* disperses over greater distances than *B. muscorum*. In support of this, members of the subgenus *Pyrobombus* (which includes *B. jonellus*) have been involved in a number of recent colonisations, with *B. hypnorum* arriving in the UK, and both *B. monticola* and *B. pratorum* reaching Ireland. It is possible that *Pyrobombus* are generally long-range dispersers, and that *Thoracobombus* (which includes *B. muscorum*) disperse only relatively short distances. It is notable that, with the exception of the ubiquitous *B. pascuorum*, the *Thoracobombus* species in the UK (*B. muscorum, B. humilis, B. sylvarum* and *B. ruderarius*) have faired badly in the wake of agricultural intensification. Perhaps reduced dispersal renders them sensitive to inbreeding, or maybe they are simply unable to recolonise areas vacated by stochastic extinctions.

In consort with increased genetic differentiation one would expect a loss of genetic diversity as a consequence of population subdivision, particularly when the resulting populations are small. It is important to exercise a degree of caution when making direct comparisons between species based on a small number of microsatellites. Certain species, or subgenera, may by chance exhibit less variation at the chosen markers. Indeed, markers are generally not selected at random, but are a subset of those initially trialled, selected for a useful amount of variability. In addition, allelic richness is sensitive to sample size, frequently confounding comparisons (Leberg 2002). Furthermore, a rigorous comparison should correct for differences in microsatellite length (Petit *et al.* 2005). However, as data amass, covering a wide range of species and degrees of rarity, a casual comparison becomes increasingly valid (Table 8.1). Data from the literature are combined with unpublished data from previous

studies, and where possible are re-analysed applying a rarefaction correction to allelic richness (Leberg 2002; Kalinowski 2005).

Table 8.1 Genetic diversity estimates for *Bombus* species which had been genotyped at the time of submission. N_S is the total (or average) sample size, and N_a is the minimum samples size used in allelic richness calculations. For single samples, average values across multiple loci are presented (± S.E.). Where several populations were genotyped, the value presented is the average of averages (± S.E.).

	Species	Population	N_S	N_a	Allelic Richness	He	Source
Unprotected populations	*B. ignitus*	Zhejiang, China	21	-	9.44 ± 1.34*	0.84 ± 0.03	Shao *et al.* 2004
	B. ignitus	Beijing, China	33	-	12.22 ± 1.53*	0.85 ± 0.02	Shao *et al.* 2004
	B. ignitus	Nagano, Japan	26	-	8.22 ± 0.72*	0.83 ± 0.03	Shao *et al.* 2004
	B. ignitus	Niigata, Japan	19	-	8.33 ± 0.97*	0.82 ± 0.03	Shao *et al.* 2004
	B. terrestris	Continental Europe	37.5 average	-	5.96 ± 0.12*	0.61 ± 0.01	Estoup *et al.* 1996
	B. terrestris	Landford, UK	79	23	6.39 ± 1.15	0.74 ± 0.06	Darvill *et al.* 2004
	B. terrestris	Rothamsted, UK	158	23	7.57 ± 1.32	0.72 ± 0.06	Knight *et al.* 2005
	B. lucorum	Landford, UK	52	23	10.16 ± 1.58	0.80 ± 0.04	Darvill, unpublished data
	B. lucorum	Bern, Switzerland	40	-	7.00 ± 2.00*	0.60 ± 0.12	Estoup *et al.* 1996
	B. lapidarius	Rothamsted, UK	269	23	6.40 ± 0.61	0.72 ± 0.03	Knight *et al.* 2005
	B. hypnorum	Various, Sweden	10	-	6.75 ± 1.03*	0.72 ± 0.14†	Paxton *et al.* 2001
	B. jonellus	Outer Hebrides, UK	53.4 average	23	8.20 ± 0.36	0.74 ± 0.01	Chapter 4
	B. jonellus	Inner Hebrides, UK	40.8 average	23	9.26 ± 0.24	0.74 ± 0.01	Chapter 4
	B. jonellus	Mainland Scotland	42	23	9.76 ± 1.89	0.76 ± 0.07	Chapter 4
	B. pratorum	Rothamsted, UK	125	23	5.84 ± 0.99	0.69 ± 0.05	Knight *et al.* 2005
	B. pascuorum	Continental Europe	22.7 average	-	5.49 ± 0.16*	0.56 ± 0.01	Widmer & Schmid-Hempel 1999
	B. pascuorum	Landford, UK	195	23	6.13 ± 1.15	0.55 ± 0.11	Darvill *et al.* 2004
	B. pascuorum	Rothamsted, UK	125	23	5.65 ± 0.99	0.55 ± 0.11	Knight *et al.* 2005
	B. pascuorum	Foix, France	29	23	6.57 ± 1.14	0.54 ± 0.12	Ellis *et al.* 2006
	B. sylvarum	Epenede, France	10	10	4.00 ± 0.85	0.53 ± 0.09	Ellis *et al.* 2006
UKBAP populations	*B. muscorum*	Outer Hebrides, UK	50.0 average	23	3.13 ± 0.17	0.38 ± 0.01	Chapter 4
	B. muscorum	Inner Hebrides, UK	61.4 average	23	3.35 ± 0.12	0.46 ± 0.02	Chapter 4
	B. muscorum	Southern UK	35.5 average	23	4.14 ± 0.03	0.51 ± 0.01	Chapter 4
	B. sylvarum	Southern UK	25.6 average	21	3.12 ± 0.10	0.39 ± 0.02	Ellis *et al.* 2006
	B. humilis	Southern UK	31.0 average	11	3.19 ± 0.11	0.44 ± 0.02	Ellis 2005
	B. distinguendus	Outer Hebrides, UK	231	15	2.63 ± 0.03	0.37 ± 0.01	Charman 2007
	B. distinguendus	Inner Hebrides, UK	119	15	2.40 ± 0.10	0.35 ± 0.00	Charman 2007
	B. distinguendus	Orkney	25	15	3.18 ± 0.37	0.42 ± 0.07	Charman 2007

* Allelic richness was not available, and the average number of alleles per locus is presented. Measures of allelic richness are sensitive to sample size. Where original data were available for re-analysis, allelic richness values were standardised for a sample size of 23.

† Expected heterozygosity (H_E) was not available, and observed heterozygosity is given. These measures are expected to be very similar for populations that are in Hardy-Weinberg equilibrium.

All four of the rare (UKBAP) species studied to date show lower levels of genetic diversity (allelic richness and heterozygosity) than common species. Within-species, island populations also show reductions, both for comparisons between Eurasia and the UK/Japan, and between the UK mainland and off-shore islands. This presumably indicates that large bodies of water present a substantial barrier to dispersal, as might be expected. Wherever species are widespread and abundant, they appear to maintain a relatively high level of genetic diversity,

consistent with an accumulation of mutations and a low rate of loss through drift. The rare UKBAP species, for which population sizes are smaller, demonstrate lower levels of both allelic richness and heterozygosity, consistent with the effects of genetic drift and inbreeding. It is striking that these species exhibit the lowest levels of both allelic richness and heterozygosity of any species so far studied.

If reductions in the genetic diversity (and increased homozygosity) of neutral markers are indicative of changes at functional genes, then it seems likely that population fitness and evolutionary potential will be reduced. However, there is some debate over the importance of inbreeding depression in haplodiploid Hymenoptera due to the potential for purging of deleterious alleles by haploid males (Bruckner 1978; Crozier 1985; Werren 1993; Antolin 1999; Packer & Owen 2001). As I have previously argued, purging will be ineffective against female sex-limited traits. Gerloff & Schmid-Hempel (2005) demonstrated reductions in colony foundation success and hibernation survival (reductions of 23% and 6% respectively) in response to brother-sister mating. Similarly, Beekman *et al.* (1999) found that inbred queens laid fewer eggs, suggesting that colony growth might be slower. It is possible that haplodiploid insects suffer less from inbreeding than diploid insects, but substantial inbreeding depression does occur (Henter 2003). Rather than questioning whether bumblebees are susceptible to inbreeding depression, it is perhaps more relevant to consider at what scale it is likely to occur. Unlike at neutral loci, in sizable populations selection will favour the fittest genotypes, and may maintain diversity at coding loci for certain traits. When populations become small, stochastic events and genetic drift become more important than selection in shaping the genetic composition of the next generation, and beneficial alleles may be lost. However, the threshold population size at which inbreeding depression in the conventional sense becomes important is unknown, and it remains to be seen whether its effects are important, relative to other factors.

One of the primary aims of this thesis was to determine the minimum population size (or habitat area) needed to avoid a high rate of stochastic local extinctions, and to maintain sufficient genetic diversity to avoid inbreeding depression. Answering this question proved difficult, not least because it is unknown whether reduced genetic diversity at neutral markers necessarily correlates with reduced population fitness. In *B. muscorum*, no clear pattern of reduction in genetic diversity in relation to population size was evident, perhaps as a result of the confounding effects of recent bottlenecks. Changes in heterozygosity tended to indicate

that, for this species, isolation from other populations was a more important factor than population size or habitat area. For *B. jonellus* (which showed less frequent signs of genetic bottlenecks), heterozygosity was negatively correlated with population size (Figure 4.5). How though can an acceptable drop in heterozygosity be quantified? Populations of all sizes show reduced heterozygosity relative to the largest (category 5) populations, but without knowing whether this causes a reduction in population fitness it is not possible to say whether this should be a cause for concern.

In addition to conventional inbreeding depression, many hymenopteran species are susceptible to the additional costs of diploid male production. Unlike at neutral loci, selection ought to strongly favour diversity at the sex determining locus, given that individuals possessing rare alleles will have a very low probability of producing diploid male offspring. As with conventional inbreeding depression, in small populations stochastic events and drift become more important factors than selection, and sex-determining alleles may be lost. In the absence of any inbreeding avoidance mechanisms, a reduced pool of sex determining alleles will result in an increased frequency of diploid males, which theoretically initiates an extinction vortex (Zayed & Packer 2005). For *B. muscorum*, single diploid males were found in three of the 16 populations studied (Chapter 3). Similarly, single diploid males of each species were found during population genetic analyses of *B. humilis* and *B. sylvarum* (Ellis 2005). No diploid males were found in populations of *B. distinguendus* (Charman 2007). However, despite the relatively small numbers of diploid males detected, triploid workers were found in both *B. muscorum* and *B. jonellus* (Chapter 5). The presence of triploid colonies indicates that, although not detected, diploid male producing colonies were present during the previous generation. The reason why diploid males were not detected is both intriguing and puzzling, and a number of possibilities are discussed. More work is urgently needed in this fascinating area, as the ramifications are far-reaching. Although triploidy had been previously demonstrated in the lab (Ayabe *et al.* 2004), this was the first time that triploids had been detected in wild populations. Furthermore, the frequency of triploids correlated negatively with population size, demonstrating a direct cost of habitat fragmentation for both species. Estimated total triploid frequencies were higher in *B. muscorum*, perhaps due to the lower levels of genetic diversity in this species. *B. muscorum* triploids were all found in populations containing between 0.5 and 13km^2 of suitable habitat. Similarly (with one exceptional outlier at 51km^2), all *B. jonellus* triploids were found in habitat areas in the range 0.5 and 5.75km^2.

Population sizes are likely to depend on more than just the area of suitable habitat, but these figures are likely to be of use to conservationists and policy makers alike.

Peripheral studies in this thesis also demonstrated that the male bumblebees aggregating around a mature *B. muscorum* nest were unrelated to workers within. Given that 50% of brother-sister matings result in diploid male producing nests, it is perhaps unsurprising that these matings are avoided, but it is interesting nonetheless. Much is still to be learned about the role of olfactory cues in mating and mate choice. Clearly, male bumblebees are able to detect mature nests and aggregate in the hope of mating with emerging gynes, but we do not know what cues they are responding to, and over what range they can be detected.

Finally, this thesis explored patterns of forager size variation within and between populations. Foragers were found to specialise in visiting flowers of an appropriate size, presumably to maximise colony foraging efficiency and minimise intracolony competition. Average forager size differed between populations, for both *B. muscorum* and *B. jonellus*. The variation in *B. muscorum* seemed to relate to the proportion of foraging visits to heath and bog type habitats, suggesting that environmental or climatic factors influence the local optimum for forager size. Patterns of variation in *B. jonellus* suggested the possibility of competitive displacement where foraging niche overlapped with that of *B. muscorum*. Foragers of *B. jonellus* were smallest in small populations, where niche breadth was widest and niche overlap with *B. muscorum* was greatest. Fascinatingly, the extent of forager size variation within populations was strongly correlated between the two species, but the underlying cause of this variation could not be identified.

8.1 Conservation strategies and future management

If fragmented populations of rare bumblebee species suffer from reduced fitness as a consequence of a loss of genetic diversity then it seems likely that further local extinctions will occur. Local extinctions are a normal part of metapopulation dynamics, be they purely stochastic or accelerated by genetic factors. The population of *B. muscorum* in the Hebrides seems to be functioning as a metapopulation, so local extinctions are likely to be balanced by recolonisation. The relatively recent loss of what was presumably a source population on the adjacent mainland gives some cause for concern, as do suggestions of genetic bottlenecks in

the Inner Hebrides. However, in the short-term, the future of *B. muscorum* on the Western Isles seems relatively secure. The apparent long-range dispersal abilities of *B. jonellus* would seem to guarantee its persistence, so long as suitable habitat remains available.

It is the isolated populations of *B. muscorum* in the south of the UK which give greatest cause for concern. Unlike in the Hebrides where fragmentation is on a relatively small scale, these southern UK populations are isolated by much greater distances. It seems likely that they are no longer part of a wider metapopulation. The same is almost certainly true of many of the remaining populations of *B. sylvarum* and *B. humilis* (Ellis 2005). In recent decades, many similarly isolated populations have disappeared. Whether genetic factors were involved in these local extinctions is unknown, and in some respects it is irrelevant. The crucial parameters for the long-term persistence of these species are population size and dispersal. Large populations are more likely to withstand short-term perturbations, and less likely to lose genetic diversity. Adequate dispersal will maintain genetic diversity and will ensure that vacant but suitable patches are re-colonised. Whether either population sizes or dispersal ranges are adequate to ensure the continued survival of these species in the south is debatable, but the available evidence perhaps suggests not.

If conservationists are keen to preserve the remaining populations of *B. muscorum* in the south of the UK then the conservative assumptions are that population sizes are unsustainably small and dispersal abilities insufficiently large. We can speculate that the same is true for *B. humilis* and *B. sylvarum*, although we lack such clear evidence for dispersal range. What then should be done by way of intervention? Conservation strategies for vertebrates routinely consider genetic factors, and it seems we may need to adopt similar measures in the management of rare bumblebee populations.

Translocations perhaps provide the simplest solution to the perceived problem. Queens could be collected from one population, either in spring or autumn, and released elsewhere. The offspring of subsequent crosses between inbred populations are expected to show significantly increased fitness (Hedrick 1995; Hedrick *et al.* 1997), a phenomenon known as heterosis (Keller & Waller 2002), or genetic rescue (Tallmon *et al.* 2004). It is likely that, by chance, different deleterious alleles will have been fixed in each population, and their effect will be masked when mixing occurs (Whitlock *et al.* 2000). Crosses between isolated populations have been found to produce dramatic increases in fitness in wild populations (Heschel & Paige

1995; Westemeier *et al.* 1998; Evans *et al.* 1999; Madsen *et al.* 1999). There are those that would argue, from a philosophical point of view, that we should be aiming for self-sustaining populations that need no intervention. That aside, there are clear potential benefits of translocations, perhaps particularly as a short-term interim measure.

However, before considering translocations, conservationists should assess the likelihood of outbreeding depression. This is a decrease in the fitness of progeny resulting from matings between individuals from genetically differentiated populations (Tallmon *et al.* 2004). Genetic costs may arise from the break up of coadapted gene complexes, the re-introduction of deleterious mutations (Rhymer & Simberloff 1996), or from the mixing of alleles which confer advantages under different environmental conditions (Beebee & Rowe 2004). An extreme example of the latter was observed during attempts to reinvigorate European populations of the mountain ibex (*Capra ibex*) in the Tatra Mountains of (then) Czechoslovakia. Individuals from Turkish and Egyptian populations were released in the Tatra Mountains, but the hybrid offspring mated in autumn, and gave birth in February, a period of extreme cold. In this case outbreeding depression resulted in the complete extinction of the entire European sub-species, as the introduction of maladapted alleles led to 100% juvenile mortality. Clearly outbreeding depression has important implications for conservation when genetic management involves the translocation of individuals (Frankham 1996; Storfer 1999; Allendorf *et al.* 2001). In the case of bumblebees, we have seen that the foragers in different populations differ in mean size, and in the extent of size variation (Chapter 7). These changes seem to relate to the habitats and plants which the colonies use. If these size differences, or other variable traits (e.g. emergence time) are under genetic control, then by introducing maladapted genotypes, these translocations could have negative consequences for recipient populations.

A more conservative option is to work towards increasing the habitat available to remaining populations in order to increase effective population sizes. Habitat restoration strategies might also seek to introduce stepping-stone populations, restoring gene flow between previously isolated sites. Edwards (1999) suggested that a healthy bumblebee population requires at least 10 km^2 of suitable habitat (this was an educated guess since at the time little was known about the nesting densities or foraging ranges of rare bumblebees). Observed triploid frequencies also suggest that, if diploid males are to be avoided, habitat areas of this order of magnitude are required. Indeed, no surviving populations of *B. sylvarum* or *B. distinguendus* in the UK

are known from areas smaller than this. It is easy to see why bumblebee species with specialized habitat requirements have become rare. Nature reserves have preserved some fine examples of natural and semi-natural habitats, but in densely populated countries such as the UK most of these reserves are tiny fragments of the original area, often of just a few hectares. Very few are large enough to support viable populations of rare bumblebee species. However, the introduction of agricultural stewardship schemes offers hope for threatened species. Certain options within these schemes allow farmers to receive payments in return for managing field margins for wildlife. By increasing the local abundance of suitable perennial flower species, notably legumes, these schemes have been shown to increase bumblebee abundance and species richness (Croxton *et al.* 2002; Carvell *et al.* 2004; Pywell *et al.* 2005; Carvell *et al.* 2006; Carvell *et al.* 2007). If conservationists are successful in encouraging farmers in sensitive areas to incorporate these options into their farm plans, then rare bumblebee populations may expand from remaining habitat fragments and on to adjacent farmland.

Chapter 9 - References

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