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Correlates of rarity in UK bumblebee (Bombus spp.) populations

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Abstract

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Sarah Elizabeth Rustage

The decline of bumblebee (*Bombus* spp.) populations in the UK and worldwide has been well reported. It has been generally assumed that such declines result in the genetic impoverishment of some species, potentially leading to reduced fitness and increased extinction risk. This study tested the fundamental assumption linking population fragmentation with fitness, in a model system of two *Bombus* species native to the UK. *Bombus monticola* has declined significantly in range across the UK in recent years and occupies fragmented upland areas, while *Bombus pratorum* has remained abundant and widespread over many habitat types. The effects of genetic diversity on fitness have been addressed in wild *Bombus* species, but this is the first study to explicitly compare data from species of differing levels of population connectivity and hence test the assumptions of traditional population genetic theory.

As genetic diversity has often been linked with immunocompetence, aspects of the innate immune response were quantified, together with parasite load. These empirical measures of fitness showed lower than expected variability between the two study species, and no evidence was found to support the theory of lower fitness in fragmented populations. However, the considerable variability between sample sites in both species for all parameters measured raised interesting questions as to the underlying evolutionary processes; it is postulated that *B. monticola* populations may maintain a higher than expected N_e, despite their fragmented distribution. This study also provided methodological developments. An alternative method for the quantification of wing wear as a proxy for age was proposed, which could be easily applied to other *Bombus* species and possibly adapted for use in other flying insects. In addition possible sources of error in AFLP analysis were highlighted which have not been adequately discussed in the current literature, namely the effects of sample storage. Given the utility of AFLPs for non-model species, this is an important avenue for future research, and would be applicable to studies in other systems.

Overall, the data presented here emphasise the challenges of studying fitness in wild populations, and underline the requirement for research into the fundamental principles underlying many assumptions made by conservation genetic theory.

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Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

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Abbreviations, Symbols and Acronyms

Abbreviation	Definition
AFLP	Amplified fragment length polymorphism
AMP	Anti-microbial peptide
BAP	Biodiversity action plan
BLAST	Basic Local Alignment Search Tool
BWARS	Bees, Wasps and Ants Recording Society
CSD	Complementary sex determination
ΔK/Delta K	Change in log probability of K clusters in STRUCTURE
f	Individual inbreeding coefficient
F_{ST}	Fixation index value
HFC	Heterozygosity-fitness correlation
H_j	Expected heterozygosity
Imd	Immune deficiency pathway
IPCC	Intergovernmental Panel on Climate Change
JNCC	Joint Nature Conservation Committee
K	Number of clusters assigned by STRUCTURE analysis
L-DOPA	3,4-Dihydroxy-L-phenylalanine
LnP(D)	Log probability of K clusters in STRUCTURE
LOCPRIOR	Determines whether to use prior sampling location information when assigning K in STRUCTURE analysis
LPS	Lipopolysaccharide
MHC	Multi-histocompatability complex
NBN	National Biodiversity Network
N_e	Effective population size
PAMP	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGRP	Peptidoglycan Recognition Protein
PLP	Percentage polymorphic loci
PO	Phenoloxidase

pro-PO	Pro-phenoloxidase
qPCR	Quantitative polymerase chain reaction
RAPD	Randomly amplified polymprphic DNA
RFLP	Restriction fragment length polymorphism
sl-CSD	Single locus complementary sex determination

Chapter 1

Introduction

In the last few decades genetic methods have become an important tool for conservation biologists. The genetic structure of populations can indicate levels of inbreeding (Keller & Fournier 2002, Zayed et al. 2004, Darvill et al. 2006), highlight populations which may be isolated (Ellis et al. 2006), suggest levels of past and present migration (Bergl & Vigilant 2007), and define populations for the purposes of management (Coates 2000, Lara-Ruiz et al. 2008). This information may be particularly important for the conservation of rare and endangered species. As well as being a useful tool, genetic diversity is itself of conservation importance. The loss of genetic diversity may act to exacerbate existing problems such as habitat loss, increasing the rate of population decline. If realised too late genetic threats may cause extinction even if a species appears to be surviving in areas of suitable habitat such as nature reserves, as is believed to have been the case for *Bombus subterraneus*, the latest bumblebee species to go extinct in the UK (Goulson et al. 2008b).

By definition, declining and rare species occur in smaller populations, which are likely to be fragmented and isolated from each other. Small populations may be able to continue functioning as a larger meta-population if migration is possible, maintaining a higher total effective population size (N_e) than the population size in any one patch of habitat (Hanski 1998). However if populations are trapped in "islands" of suitable habitat, surrounded by hostile landscapes which are difficult or impossible to cross, migration becomes reduced and causes a subsequent reduction in gene flow (the exchange of genes between individuals and populations; Newman & Pilson 1997, Frankham 2005). Where N_e is low, genetic diversity can become reduced by genetic drift. This is the random sub-sampling of alleles within a population each generation, and can allow alleles to become fixed in the population (for example one allele at one locus present at both chromosomes in all individuals in a diploid organism) or to disappear entirely; thus genetic diversity is lost through successive generations, and as a direct result heterozygosity is reduced (Newman & Pilson 1997, Frankham 2005). Genetic drift may be counteracted by the process of natural selection, but in small populations it can alter allele frequencies even for non-neutral alleles, as the process of selection is weaker (Campbell et al. 1999). The relaxation of selection may allow mildly deleterious mutations to persist and accumulate, leading to a reduction in the fitness of the population as a whole (Hedrick & Kalinowski 2000; also see Chapter 6).

In addition to the effects of drift, genetic diversity can become reduced in isolated populations due to increased levels of inbreeding. Under inbreeding, individuals

are more likely to be homozygous and are likely to show reduced fitness, either by losing any heterozygote advantage, or due to an increase in the presence of recessive deleterious alleles in the homozygous state (Charlesworth & Willis 2009). While major effect deleterious alleles may be purged (the individuals carrying them will die, and therefore not pass on the genes to the next generation) an accumulation of small effect deleterious alleles may accumulate in the homozygous form, causing a reduction in fitness known as inbreeding depression. Inbreeding depression may be lower in some haplo-diploid organisms when compared with diploid organisms (Henter 2003, Charlesworth & Willis 2009), as the haploid state of males may act to increase the action of selection and allow purging of mutational load (Schmid-Hempel 2005). However, this may not apply for female sex-limited traits (Henter 2003). While purging of genetic load may be more frequent in haplo-diploid systems, eusocial species of bees, wasps, and ants are at greater general risk of reduced genetic diversity. As the reproductive unit is the colony, not the individual, N_e is naturally lower than in diploid organisms (Chapman & Bourke 2001, Zayed 2004). This may make populations more vulnerable to genetic drift, and the effects of demographic and environmental stochasticity.

The short-term effects of inbreeding can vary between family lines; this might occur if, for example, one family happens to be well adapted to the current environmental conditions and comes to dominate in a particular area for a time (Gerloff & Schmid-Hempel 2005). Under prolonged periods of inbreeding, negative effects can be felt at the population level, increasing the risk of population extinction (Roulston 2000). Inbreeding depression can have stronger negative effects on fitness under situations of environmental stress (Roulston 2000, Fox & Reed 2011). Natural populations may therefore be more severely affected, as they are likely to experience greater environmental stochasticity than populations in captivity or laboratory conditions (Hedrick & Kalinowski 2000). The fitness costs of inbreeding depression are potentially strong selectors for kin-avoidance mating behaviour, as the costs of inbreeding outweigh any apparent kin selective advantages such as increased worker relatedness in social insects (Schmid-Hempel & Schmid-Hempel 1998). Kin-recognition and inbreeding avoidance has been observed in a number of insect species, including the termite *Zootermopsis nevadensis* (Shellman-Reeve 2001), the cockroach *Blattella germanica* (Lihoreau et al. 2007), and the ant *Iridomyrmex humilis* (Keller & Passera 1993). Experiments using crickets have shown that negative effects of inbreeding may be avoided by polyandry (Tregenza & Wedell 2002). Largely monoadrous groups such as bum-

blebees (Schmid-Hempel & Schmid-Hempel 2000, Strassmann 2001) may be more susceptible to the effects of inbreeding, as there are no opportunities for post-copulatory mechanisms such as sperm competition. Kin-recognition mechanisms and avoidance behaviour are therefore likely to be under strong selection, and this has been demonstrated in *Bombus terrestris* females which show significant reluctance to mate with siblings (Whitehorn et al. 2009a).

1.1 Measuring genetic diversity

Given the importance of genetic health for the continued survival and adaptability of species, it is vital to be able to accurately assess levels of genetic diversity and variation. As previously discussed, a high level of allelic diversity and heterozygosity should, in theory, allow a population to adapt more easily to change, so it seems intuitive that levels of marker heterozygosity should correlate with fitness. Reed and Frankham (2003) found heterozygosity at neutral markers to be significantly positively correlated with fitness, and such heterozygosity-fitness correlations (HFCs) have subsequently been observed in a number of species groups, including molluscs (Pogson & Zouros 1994), fish (Borrell et al. 2004, Pujolar et al. 2005, Lieutenant-Gosselin & Bernatchez 2006), insects (Luong et al. 2007, Reber et al. 2008), birds (Hansson et al. 2004, Markert et al. 2004, Brouwer et al. 2007), and mammals (Da Silva et al. 2006, Rijks et al. 2008, Da Silva et al. 2009, Fitzpatrick & Evans 2009).

There are three theories which may explain the HFCs which have been observed. The "direct effect", or "functional overdominance", hypothesis suggests that HFCs occur because the markers themselves are under selection, and are responsible for any heterozygote advantage that is observed. This may be important in studies using allozyme markers (Borrell et al. 2004), but does not explain HFCs observed when using selectively neutral (non-coding) markers such as microsatellites. The "local effect" hypothesis assumes that the marker loci are tightly linked with fitness loci which are in close proximity. Linkage disequilibrium between the markers and fitness loci would therefore produce HFCs. Local effects have been supported as an explanation for HFCs (Hansson et al. 2004), but in practice can be difficult to distinguish from general effects (Brouwer et al. 2007). The "general effect" hypothesis suggests that any heterozygote advantage is due to genome-wide effects, assuming that marker loci are representative of heterozygosity at all

loci. In this case, high heterozygosity at the markers would be interpreted as a low value for the individual inbreeding coefficient (f). Although there is support for this theory (Rijks et al. 2008), a large number of marker loci need to be used and it may be difficult to confirm that any observed HFCs are not a result of local effects (Slate & Pemberton 2002). The theory behind the general effect hypothesis has been criticised, and there has been suggestion that the widespread reporting of general effect HFCs may be largely due to publication bias (Coltman & Slate 2003). In natural populations, individuals with a detectable level of inbreeding are generally uncommon (Balloux et al. 2004, Slate et al. 2004) unless N_e is very small and the populations are isolated, as may be the case for some endangered species (Fitzpatrick & Evans 2009). Given that the general effect hypothesis is based around variation in f, which has been linked only weakly, if at all, with multilocus heterozygosity (Pemberton 2004, Slate et al. 2004, DeWoody & DeWoody 2005, Fitzpatrick & Evans 2009), it seems unlikely that it is the major mechanism underlying HFCs.

1.2 Genetic diversity, immunity, and parasite load

Immunocompetence (the ability of an individual to mount a general immune response to a pathogen; Schmid-Hempel 2003, Wilson-Rich et al. 2009) is assumed to vary between genotypes. Although adaptive immunity is restricted to vertebrates, invertebrate species are still capable of generating specific immune responses (Schmid-Hempel 2005, Schulenburg et al. 2007; see Chapter 4). High genetic diversity has been described as a prerequisite for specific immune responses (Schulenburg et al. 2007), so genetic diversity is expected to correlate with an increased ability to resist and recover from infection. A reduction in genetic diversity may result in the loss of specific resistance genes for example, and cause a subsequent increase in susceptibility (Spielman et al. 2004). However, results from studies in invertebrates have been mixed. Inbred individuals of *Drosophila melanogaster* show a reduced level of disease resistance (Spielman et al. 2004), while studies in the bush cricket *Metrioptera roeseli* (Berggren 2009), and in the bumblebees *B. terrestris* (Gerloff et al. 2003) and *B. muscorum* (Whitehorn et al. 2010), have found no decrease in overall immunocompetence with decreased heterozygosity. Immune responses in gynes of the ant *Formica exsecta* have even been observed to increase under inbreeding (Vitikainen & Sundström 2010). Haploid

males may be particularly susceptible to infection, as they are not able to benefit from any advantages of heterozygosity at immune-related loci (Baer & Schmid-Hempel 2006). However, experiments have also found haploid males to show no significant difference in levels of parasite infection when compared to diploid females (Ruiz-González & Brown 2006a), shedding doubt on the theory of haploid susceptibility.

A decrease in immunocompetence as a result of inbreeding and loss of genetic diversity may cause increased susceptibility to parasitism (see Chapter 5). Social organisms provide an ideal environment for the transmission of parasites and other pathogens, as many individuals living in close proximity and sharing food sources provide ample opportunities for infections to spread (Schmid-Hempel & Schmid-Hempel 1998, Hughes & Boomsma 2004, Evans et al. 2006, Tarpy & Seeley 2006, Wilson-Rich et al. 2009). The presence of parasites has been observed to regulate populations of some species. For example, *B. terrestris* populations in Tasmania have spread and rapidly become invasive (despite having low genetic diversity owing to founder effects), due to the absence of the parasites which would restrict colony growth in their native Europe (Allen et al. 2007). By contrast, parasitic infection is believed to have contributed to large scale population declines of *Bombus* species in North America (Cameron et al. 2011). Levels of antimicrobial secretions increase with increasing degrees of sociality in bee species (Stow et al. 2007), suggesting that parasites place a strong selection pressure on social species for evolving higher resistance (Schmid-Hempel 1994).

It has been suggested that parasites encourage genetic diversity within colonies by exerting negative frequency-dependent selection on their hosts, putting common genotypes at a disadvantage (Shykoff & Schmid-Hempel 1991a). It is therefore possible that polyandry in social insects such as ants and honeybees may have evolved as a mechanism for increasing genetic diversity, and increasing the resistance of a colony to pathogens (Schmid-Hempel 1998, Baer & Schmid-Hempel 2003, Palmer & Oldroyd 2003, Hughes & Boomsma 2006, Tarpy & Seeley 2006). Correlative tests have shown that colonies of ants which are genetically heterogeneous have lower levels of parasite infection than colonies with greater intra-colony genetic similarity (Schmid-Hempel & Crozier 1999). Although this evidence was not supported by some studies in honeybees (Woyciechowski et al. 1994, Neumann & Moritz 2000), subsequent experimental studies have found similar results - colonies of honeybees founded by multiply mated queens show lower intensity of infection by highly virulent pathogens such as *Paenibacillus larvae* (Seeley &

Tarpy 2007), and *Ascosphaera apis* (Tarpy 2003). Females of the bumblebee *B. terrestris* show the same trend when artificially inseminated with sperm from multiple drones; under polyandry reproductive output was increased and parasite load in workers was decreased, in both laboratory (Baer & Schmid-Hempel 2003) and field conditions (Baer & Schmid-Hempel 1999). This trend was observed even though males were chosen at random to increase genetic diversity, and not chosen specifically for quality. *A. mellifera* colonies sired by many males have also been shown to maintain more constant temperatures (Jones et al. 2004), higher productivity and colony longevity (Mattila & Seeley 2007), compared with less genetically diverse colonies (Hughes et al. 2008).

Despite the clear advantages of increased genetic diversity, polyandry is rare among social Hymenoptera (Estoup, Tailliez, Cornuet & Solignac 1995, Schmid-Hempel & Schmid-Hempel 2000, Strassmann 2001). This may be due to costs associated with multiple matings such as the energy and time required, and the increased exposure to predation (Crozier & Fjerdingstad 2001). Competion between males is also a likely cause, as males can act to prevent multiple matings. Monoandry is imposed on *B. terrestris* females for example, by the deposition of a "mating plug" by males; the main function of this plug appears to be to prevent the female from engaging in multiple matings (Baer & Schmid-Hempel 1999, Sauter et al. 2001). There is also likely to be strong selection on the workers of a colony to prevent a queen mating more than once, as this would reduce the relatedness of the workers to future progeny, thus reducing their inclusive fitness (Strassmann 2001). An alternative explanation is provided by Baer and Schmid-Hempel (2001), who observed a decrease in fitness of *B. terrestris* females when a shift was made from single mating to low levels of polyandry. Although the cause of the fitness reduction could not be determined (artificial insemination eliminated the possibility of environmental factors such as increased predation or increased energetic costs, as well as any possible mechanism for imposed monandry), the need to bridge this gap in fitness to achieve the benefits of polyandry may explain why many social Hymenoptera species remain monandrous, despite the apparent advantages that polyandry offers as a result of increased intra-colony genetic diversity.

Genetically programmed social behaviours may also confer advantages to susceptible individuals, limiting and controlling the spread of infections. Such behaviours may include cuticular grooming to remove parasites and microbes, detection and removal of diseased brood, and the removal of dead adult bees from the colony

(Calleri et al. 2006, Wilson-Rich et al. 2009). It has been shown that these behaviours in many species have a genetic component. In populations of the termite *Zootermopsis angusticollis* decreased heterozygosity correlates with significantly higher loads of cuticular microbes and higher mortality, due to a negative effect of inbreeding on hygiene behaviour, namely cuticular grooming (Calleri et al. 2006). These behavioural traits may play a vital role in colony immunity, complementing individual immune defences. The effectiveness of such behavioural immune responses in *A. mellifera* may have reduced pathogen pressure to the extent that individual immunity is of secondary importance, allowing the loss of some genes related with immunity in other organisms (Evans et al. 2006).

1.3 Diploid male production

The maintenance of genetic diversity is particularly relevant in haplodiploid organisms which are, as discussed, potentially more at risk of reduced genetic diversity (Chapman & Bourke 2001, Zayed 2004). In addition to the general fitness benefits of genetic diversity, in haplodiploid species low diversity may impose the additional cost of diploid male production. Complementary sex determination (CSD) means that the sex of offspring is decided by heterozygosity or homozygosity at the sex determining locus or loci. In haplodiploid systems such as bees, wasps, and ants, sex is determined by heterozygosity at a single locus (sl-CSD); males are haploid and produced from unfertilised eggs, while females are diploid and produced from fertilised eggs (Cook & Crozier 1995). However, if the CSD locus is diploid and homozygous, an inviable or effectively sterile diploid male will result (Cook & Crozier 1995). A small number of diploid males are expected to be produced by chance after matings between individuals sharing the same allele at the sex locus, but diploid male production is more likely if allelic diversity is low at the CSD locus. Populations with a low N_e often display reduced genetic diversity (Ellis et al. 2006), and may therefore show higher levels of diploid male production (Darvill et al. 2012). This has been observed in populations of *B. florilegus*, which has experienced declines after the introduction of *B. terrestris* in Japan (Takahashi et al. 2008a), in neotropical Euglossid (orchid) bees (López-Uribe et al. 2007, although see Souza et al. 2010), and in island populations of *B. muscourum* and *B. jonellus* (Darvill et al. 2012). Reduced genetic diversity and elevated levels of diploid male production are often observed in populations

experiencing high levels of inbreeding (Zayed & Packer 2005). Levels of diploid male production can therefore be used to indicate declines in genetic diversity, and increased inbreeding in populations of haplo-diploid organisms (Packer & Owen 2001, Zayed & Packer 2001, Zayed et al. 2004, Whitehorn et al. 2009b).

Increased diploid male production imposes a heavy cost on a colony. Diploid males do not function as workers and replace on average 50% of female worker offspring when produced, effectively increasing the rate of female mortality in that generation (reviewed by Harpur et al. 2013). Diploid males in *Bombus* are reared to adulthood, in contrast to honeybees for example, which remove diploid male larvae from the nest (Gerloff & Schmid-Hempel 2005). They therefore represent a significant investment with no returns in the form of labour or reproduction. If a diploid male succeeds in mating with a female, the resulting offspring will be triploid and inviable (Ayabe et al. 2004, de Boer et al. 2007, Cournault & Aron 2009, Darvill et al. 2012); diploid males therefore have the potential to increase levels of mortality over two successive generations, negatively affecting colony growth. A reduction in worker numbers may lead to reduced foraging efficiency and competitiveness for resources, and subsequently reduce colony survival (Ross & Fletcher 1986, Duchateau et al. 1994, Gerloff & Schmid-Hempel 2005, Zayed & Packer 2005, de Boer et al. 2007). The production of such individuals could contribute to the development of an extinction vortex (Zayed & Packer 2005), hastening the decline of a population and exacerbating the effects of genetic drift and demographic and environmental stochasticity to reduce N_e. The production of sterile males in solitary bee species can increase the base extinction rate by as much as 63%, which is more than an order of magnitude higher than the increased extinction rate observed in diploids under inbreeding depression (Zayed & Packer 2005). Significant fitness effects have also been observed in social haplo-diploid species under both laboratory conditions (Plowright & Pallett 1979), and in the field where *B. terrestris* colonies containing diploid males produce fewer offspring and show reduced survival (Whitehorn et al. 2009b). The production of diploid males is therefore a significant component of the risks associated with reduced genetic diversity in organisms with CSD.

1.4 Bumblebees as a model system

Bumblebees (*Bombus* spp.) are eusocial, haplodiploid insects (Goulson 2003, Benton 2006). They provide an important ecosystem service in temperate regions such as the UK and mainland Europe, serving a central role in pollination networks (Memmott et al. 2004). Some bumblebees can forage kilometres away from their nest (Kreyer et al. 2004, Knight et al. 2005, Osborne et al. 2008), so one nest may provide a source of pollinators for a wide area. The offspring are fed on pollen, and unlike honeybees only a few days' worth of resources are stored in the nest at any one time (Goulson 2003, Benton 2006); bumblebees therefore need to maintain a high and constant foraging rate. The hairy bodies of bumblebees and their large size relative to honeybees means they are likely to be more efficient as pollinators, coming into contact with the reproductive parts of flowers more frequently during foraging (Kells et al. 2001, Goulson 2003).

Although some species remain abundant and widespread, many have declined severely in abundance in the last 50 years, and survive only in a few isolated locations (reviewed in Goulson 2003, Kosior et al. 2007). Three of the 25 species in the UK are now known to be extinct, and seven have declined so severely they have been placed on the UK Biodiversity Action Plan (BAP) list as a high conservation priority (Goulson 2003, Goulson et al. 2005, Williams 2005, Benton 2006, Carvell et al. 2006, JNCC 2007).

1.5 Reasons for bumblebee declines

Habitat loss and land use change

Although the decline of bumblebees is likely due to a combination of factors, habitat loss has been widely implicated as the major driver (Luig et al. 2005, Williams & Osborne 2009). A loss of unimproved grasslands, especially chalk grasslands, to agricultural intensification and urbanisation has resulted in a reduction of floral diversity across Europe and North America (Robinson & Sutherland 2002, Goulson 2003, Carvell et al. 2004, Goulson et al. 2005, Williams 2005, Benton 2006), meaning that both the quality and quantity of forage available for bumblebees has been reduced (Carvell et al. 2006, 2007). Intensive management may also reduce the structural variation of habitats, affecting not only the foraging possibilities

for bumblebees, but also the quantity of suitable nesting and over-wintering sites (Carvell 2002).The increased specialisation of farms has reduced landscape heterogeneity, impacting the diversity of wild bee populations and the presence of more specialised species (chiefly long-tongued species; Goulson et al. 2005, Pywell et al. 2006). Remaining high quality habitats exist in isolated fragments, subdividing previously contiguous populations (Goulson et al. 2006, Fitzpatrick et al. 2007).

The restoration of habitats using environmental stewardship schemes may be beneficial for bumblebee populations, mitigating the effects of intensive agriculture to some extent and providing "stepping-stones" of habitat which could re-connect neighbouring populations (Kremen et al. 2002). Sowing field margins with nectar and pollen-rich wild flower seed mixes can provide both foraging and nesting opportunities for pollinators (Kells et al. 2001), and can significantly increase the abundance of invertebrates including *Bombus* (Meek et al. 2002, Pywell et al. 2005, 2006, Heard et al. 2007). The abundance and diversity of bumblebees at a site is linked with the abundance of preferred foraging plants (Carvell 2002). Fabaceae species are particularly favoured by bumblebees for pollen collection (Carvell et al. 2001, Goulson et al. 2005, 2008a), as bumblebees are entirely dependent on pollen as their source of protein (Smeets & Duchateau 2003). The nitrogen-fixing abilities of Fabaceae mean that they produce pollen with a higher protein content than other plant groups (as suggested by Hanley et al. 2008); they are therefore a popular choice for wild flower mixes in environmental stewardship schemes (Pywell et al. 2006). However, these approaches would need to be widely adopted, as the foraging range of a bumblebee colony is likely to exceed the boundaries of any one farm (Goulson et al. 2002, Knight et al. 2005).

Exposure to pesticides

While some environmental stewardship measures successfully increase bumblebee densities, the stress of habitat loss from land use may be compounded by the associated increase in exposure to pesticides and other agrochemicals. Such chemicals are broad-scale, killing beneficial insects such as pollinators as well as pest species (Mommaerts et al. 2006), particularly after repeated applications (Brittain et al. 2010). Exposure can occur by direct topical contact, and via ingestion of contaminated nectar and pollen (Goulson et al. 2008b, Krupke et al. 2012), and may be prolonged as some chemicals can persist in the environment for long periods (reviewed by Goulson 2013, Jones et al. 2014). Chemical drift may mean that exposure occurs even in areas set aside for conservation (de Snoo & van der Poll 1999). While incidents of large-scale pesticide poisoning in bees have reportedly

decreased (Barnett et al. 2007), records of incidents involving wild bees are likely to be less accurately recorded than those involving commercial species such as *A. mellifera* (Thompson 2001). The impact of pesticides (notably neonicotinoids) on wild bees has received increasing attention in recent years, resulting in a recent moratorium on the use of neonicotinoids within the EU (European Commission 2013). There is increasing focus on the possible sub-lethal effects of exposure to pesticides, which may include impaired navigational and learning abilities (Krupke et al. 2012, Palmer et al. 2013), reduced foraging efficiency and feeding behaviour (Morandin et al. 2005, Mommaerts et al. 2010, Gill et al. 2012, Henry et al. 2012, Elston et al. 2013, Laycock et al. 2012, 2014), reduced immune function (Di Prisco et al. 2013), reduced production of sexuals (Whitehorn et al. 2012), reduced worker survival (Henry et al. 2012), and slower rates of colony growth (Whitehorn et al. 2012).

Introduced pests, pathogens and invasive species

Invasive species have been described as second only to habitat loss in terms of driving extinction (Ruesink et al. 1995, Thomson 2004), and competition with introduced species may be a major factor explaining the decline of bumblebees across the world (Ings et al. 2005, Cameron et al. 2011, Meeus et al. 2011). Examples include dramatic declines in populations of *B. florilegus* in its native Japan since the introduction and naturalisation of *B. terrestris* (Takahashi et al. 2008a), and reduced reproductive success in *B. occidentalis* due to foraging competition with the introduced *A. mellifera* in California (Thomson 2004). The stress of competition with *A. mellifera* may also make nests more vulnerable to brood parasitism (Forup & Memmott 2005).

Infection by parasites and other pathogens can seriously impact populations, and has been implicated in large scale population declines in *Bombus* species (Cameron et al. 2011; also see Chapter 5). While low levels of infection occur naturally, wild bee populations could be at risk of increased rates of infection from outside sources (Goka et al. 2001, Colla & Packer 2008, Otterstatter & Thomson 2008, Meeus et al. 2011, Szabo et al. 2012, Arbetman et al. 2013). High levels of bumblebee-specific parasites such as the protozoa *Crithidia bombi* and *Nosema bombi*, and the tracheal mite *Locustacarus buchneri*, have been observed in commercially reared colonies compared with wild populations (Whittington & Winston 2003, Otterstatter & Thomson 2008, Yoneda et al. 2008). Such colonies may facilitate pathogen spill-over, increasing parasite load in wild bees (Morandin et al. 2005, Colla et al. 2006, Szabo et al. 2012, Arbetman et al. 2013) and potentially

introducing novel parasites, as observed in Argentina with the introduction of *Apicystis bombi* (Plischuk et al. 2011). Parasites may also act as vectors for other pathogens, facilitating inter-specific infection with other diseases including viruses (Fürst et al. 2014).

1.6 Variation in population declines

Several theories have been proposed to explain why some bumblebee species appear to be at greater risk of decline than others; generally speaking it appears that some species are able to increase their range due to higher levels of tolerance to anthropogenic pressures (Kosior et al. 2007). However, the specific causes of increased extinction risk are a subject of debate.

Declines have been correlated with tongue length, with long-tongued species being more at risk (Goulson & Darvill 2004, Goulson et al. 2005). This may be due to a higher degree of dietary specialisation in long-tongued species. Dietary specialists have been highlighted at high risk of decline in a study across the UK and the Netherlands (Biesmeijer et al. 2006), and by comparisons between specialist and generalist bee species (Zayed & Packer 2005). However, this theory has been strongly contested by studies finding no correlation between dietary specialisation, tongue length, and risk of decline (Williams 2005, Williams & Osborne 2009). A subsequent 3-year study of feeding behaviour in two nationally rare species (*B. sylvarum* and *B. humilis*), and two widespread and abundant species (*B. lapidarius* and *B. pascuorum*), found that feeding habits were inconsistently linked with the rate of decline (Connop et al. 2010). The conflicting results of dietary specialisation studies have led others to suggest alternative hypotheses for the differential rates of declines in bumblebee species. Habitat and climate specialisation, using range size as an indicator of niche breadth, has been suggested as an indicator of risk (Williams 2005), as well as emergence time from hibernation (Fitzpatrick et al. 2007, Williams et al. 2009). Later emerging species rely on floral resources being present later in the year, but this behaviour is in conflict with changing farming practices such as silage production replacing traditional fodder crops such as hay. As well as containing a lower floral diversity, silage crops are harvested earlier in the year than hay (Vickery et al. 2001), meaning that there is a sudden drop in the quantity of forage available for bees and other pollinators; this puts later-emerging species at a disadvantage, having fewer floral resources

available to them.

A decline in pollinators could have serious consequences for crop plants, and therefore human food supplies (Carvell et al. 2006). Although the relevance for crop plants has been questioned by some (Ghazoul 2005), the general consensus is that a decline of pollinators such as bumblebees could have serious consequences for agriculture (Carvell et al. 2006). Although the highest volume food crops such as rice and wheat are wind-pollinated, 75% of food crop species are reliant on insect pollinators (Potts et al. 2010). It has been found that distance from natural or semi natural habitats has a negative impact on pollinator diversity and abundance (Kremen et al. 2002, Steffan-Dewenter & Schiele 2008, Ricketts et al. 2008), and fruit set within monocultures has been shown to vary depending on the availability of wild pollinators (Steffan-Dewenter et al. 2002). Many wild plants are also likely to be affected; around 80% of wild plant species depend directly on insect pollination for fruit or seed set, while up to 73% show pollinator limitation at least some of the time (Potts et al. 2010). Obligate outcrossing plant species which are reliant on insect pollinators, especially bees, have shown declines in abundance and diversity in both the UK and the Netherlands in tandem with pollinator declines (Biesmeijer et al. 2006), while increased fragmentation of plant populations has been shown to cause changes in pollinator behaviour which may lead to reduced pollen transfer and reduced fitness in the plants (Goverde et al. 2002). A loss of species diversity is likely to reduce the levels of redundancy in a pollination system (Kremen et al. 2002), meaning that ecosystem services are less well buffered against fluctuations in pollinator populations. A plant community may remain relatively unchanged if declining pollinator species are replaced by functionally similar species (Memmott et al. 2004), but lower species richness may result in a loss of functional diversity, particularly specialist interactions (Fontaine et al. 2005, Biesmeijer et al. 2006).

1.7 Study aims

A suite of potential stressors are acting on wild bumblebee populations in the UK and worldwide, and have already reduced populations (Fitzpatrick et al. 2007, Kosior et al. 2007, Cameron et al. 2011). While the direct effects of factors such as pesticide exposure and invasive species are well-studied (Ings et al. 2005, Cameron et al. 2011, Gill et al. 2012, Whitehorn et al. 2013), it is important to under-

stand the potential knock-on effects of population reduction and fragmentation on future population viability. Given that bumblebees are haplodiploid, and may therefore be more susceptible to the loss of genetic diversity (Chapman & Bourke 2001, Zayed 2004), they may be particularly vulnerable to the effects of low genetic diversity. As some bumblebee species are more severely affected than others (Goulson et al. 2005, Williams 2005, Fitzpatrick et al. 2007), levels of population fragmentation vary. In addition to the conservation relevance of their ecological and economic importance as pollinators, bumblebees therefore provide an ideal system for testing fundamental assumptions linking population isolation, genetic diversity, and fitness. This study aimed to assess the fitness in terms of immunocompetence (and by extension the population viability) of two *Bombus* species native to the UK, which differ in their distributions, levels of population isolation, and degree of dietary and habitat specialisation (see Chapter 2).

Chapter 2

Sample collection and comparison of foraging between *B. monticola* and *B. pratorum* workers

2.1 Introduction

Bumblebees perform a vital ecosystem service as pollinators, especially in temperate climates like that of the UK (Memmott et al. 2004). Many bumblebee species have experienced widespread declines in recent decades, in the UK and worldwide (Williams 1982, Kosior et al. 2007, Williams & Osborne 2009, Cameron et al. 2011). This is widely believed to be the result of a combination of factors including habitat loss (Biesmeijer et al. 2006, Carvell et al. 2006), introduced species, diseases, and parasites (Ings et al. 2005, Takahashi et al. 2008a, Cameron et al. 2011), land use change and intensification of agriculture (Robinson & Sutherland 2002, Goulson et al. 2005, Williams 2005, Benton 2006, Carvell et al. 2007), with increasing attention focussed on the use of certain pesticides (Gill et al. 2012, Henry et al. 2012, Whitehorn et al. 2012, Di Prisco et al. 2013). There is still debate as to why some bumblebee species have been more severely affected in terms of population declines than others. It has been suggested that susceptibility may be linked to climatic range size (Williams et al. 2007), and dietary breadth (Goulson & Darvill 2004, Goulson et al. 2008a, although see Connop et al. 2010).

Rarer species, which tend to exist in more isolated populations, may be at risk of a reduction in genetic diversity as migration between populations is reduced (Darvill et al. 2006, Ellis et al. 2006). This may lead to knock-on effects as populations become less able to adapt to change, and may result in lower fitness (Frankham 2005). This may be due to a loss of immunocompetence, and a subsequent increase in parasite susceptibility. However, while genetic diversity may be lost as populations become more isolated, functionally important genes (like those involved in immunity) may be under purifying selection (Aguilar et al. 2004). Thus traits such as immunocompetence, which relate strongly to fitness, may not correlate with overall neutral genetic diversity. While the link between genetic diversity and immunocompetence has been investigated in *Bombus* (Gerloff et al. 2003, Whitehorn et al. 2010), the assumed link between genetic diversity, level of population isolation, and immunocompetence has not been empirically tested.

This study aims to assess the link between relative levels of population isolation and fitness (assessed using immunocompetence and parasite load), in two bumblebee species native to the UK. As a group bumblebees are highly variable in their foraging preferences and behaviours, which may be related to factors such as tongue length (Goulson et al. 2005, Goulson & Darvill 2004). This variabil-

ity is important for ecosystem functioning as bumblebees will forage on a wide range of flowering plants, both wild and commercial (Corbet & Osborne 1992). However, foraging behaviour (and therefore diet) can also influence other aspects of bumblebee biology. For example, damage to the wings is most likely to occur due to collisions with vegetation during foraging (Foster & Cartar 2011), and may go on to influence mortality (Cartar 1992). Variation in foraging preferences may determine the frequency and severity of collisions, and therefore influence lifespan, as well as factors estimated using wing wear such as individual age (see Chapter 3). Variation in diet, especially dietary protein availability, can also affect immunocompetence, for example by affecting the expression of antimicrobial peptide genes (Alaux et al. 2010, Brunner et al. 2014). Variability in foraging may therefore influence the results of immunocompetence studies.

These possibilities highlight the need to consider foraging preferences when making inter-species comparisons of factors such as immunocompetence. This study therefore addressed the following aims:

1. To characterise the foraging patterns of two congeners, *B. monticola* and *B. pratorum*, at various sites across the UK and Ireland.

2. To compare the forage breadth and preferences of *B. monticola* and *B. pratorum* within each sample site.

2.1.1 Study species

Figure 2.1. Foraging *Bombus* workers photographed during fieldwork in 2011. A = *B. pratorum* (photo courtesy of R. Billington); B = *B. monticola* (photo taken by S. Rustage).

Two *Bombus* species were selected for this study; *Bombus pratorum*, commonly known as the early bumblebee (Fig. 2.1), and *B. monticola*, commonly known as the bilberry bumblebee (Fig. 2.1), as the focal species. The two species are relatively closely related, both belonging to the subgroup *Pyrobombus* (Fig. 2.2). However, *B. monticola* has a much narrower range than *B. pratorum* across mainland Europe, being restricted to upland regions and higher latitudes (Fig. 2.3; Rasmont & Iserbyt 2013, Williams 2014). This is also the case in the UK; *B. monticola* is restricted in its distribution, particularly in the south of England, but is more widespread in Scotland (Fig. 2.4). By contrast *B. pratorum* is widespread across much of the UK and Ireland, but shows a slightly more limited distribution in Scotland (Fig. 2.4). Both species have colonised Ireland relatively recently; *B. pratorum* was first recorded there in 1947, and *B. monticola* slightly later in 1974 (National Biodiversity Data Centre 2014). It is uncertain whether their arrival resulted from natural colonisation events, and the source populations are currently unknown. As in the UK and mainland Europe, Irish populations of *B. monticola* are more patchy in their distribution than *B. pratorum* populations (Fig. 2.5). The two species also show different levels of habitat specialisation (Edwards & Jenner 2005). While *B. pratorum* is a generalist in both habitat and diet (Goulson et al. 2005), *B. monticola* is an upland specialist, and reportedly has more particular dietary preferences (Edwards & Jenner 2005). While dietary specialisation could be a product of a more restricted habitat range, to the author's knowledge no specific dietary preferences have been formally reported for *B. monticola*.

2.2 Methods

2.2.1 Selection of sampling sites

Samples were collected at sites where *B. monticola* and *B. pratorum* were known to co-occur. This minimised the variation in habitat, climate, and other environmental conditions such as land use, which may cause differences in behaviour and population parameters between the two species. Using records from the Bees, Wasps and Ants Recording Society (BWARS) database (BWARS 2010), sample sites were located where records showed both species to be present after the year 2000. Sites were located to cover as much of the UK and Irish range of *B. monti-*

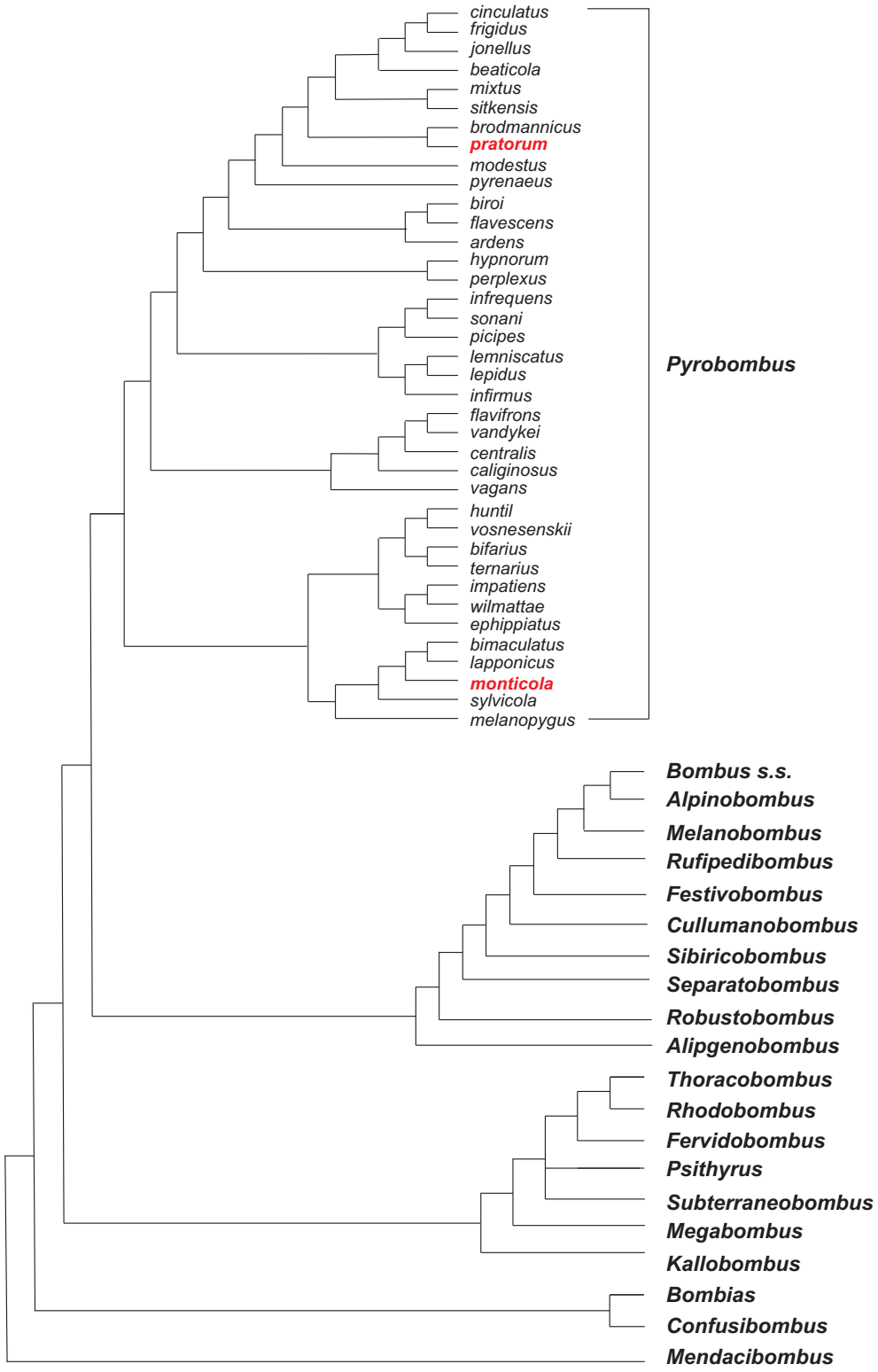

Figure 2.2. Phylogeny of *Bombus* subgroups, modified from Hines et al. (2006). Subgroups are indicated in bold text. Species are listed for Pyrobombus only; the focal species for this study are indicated in red.

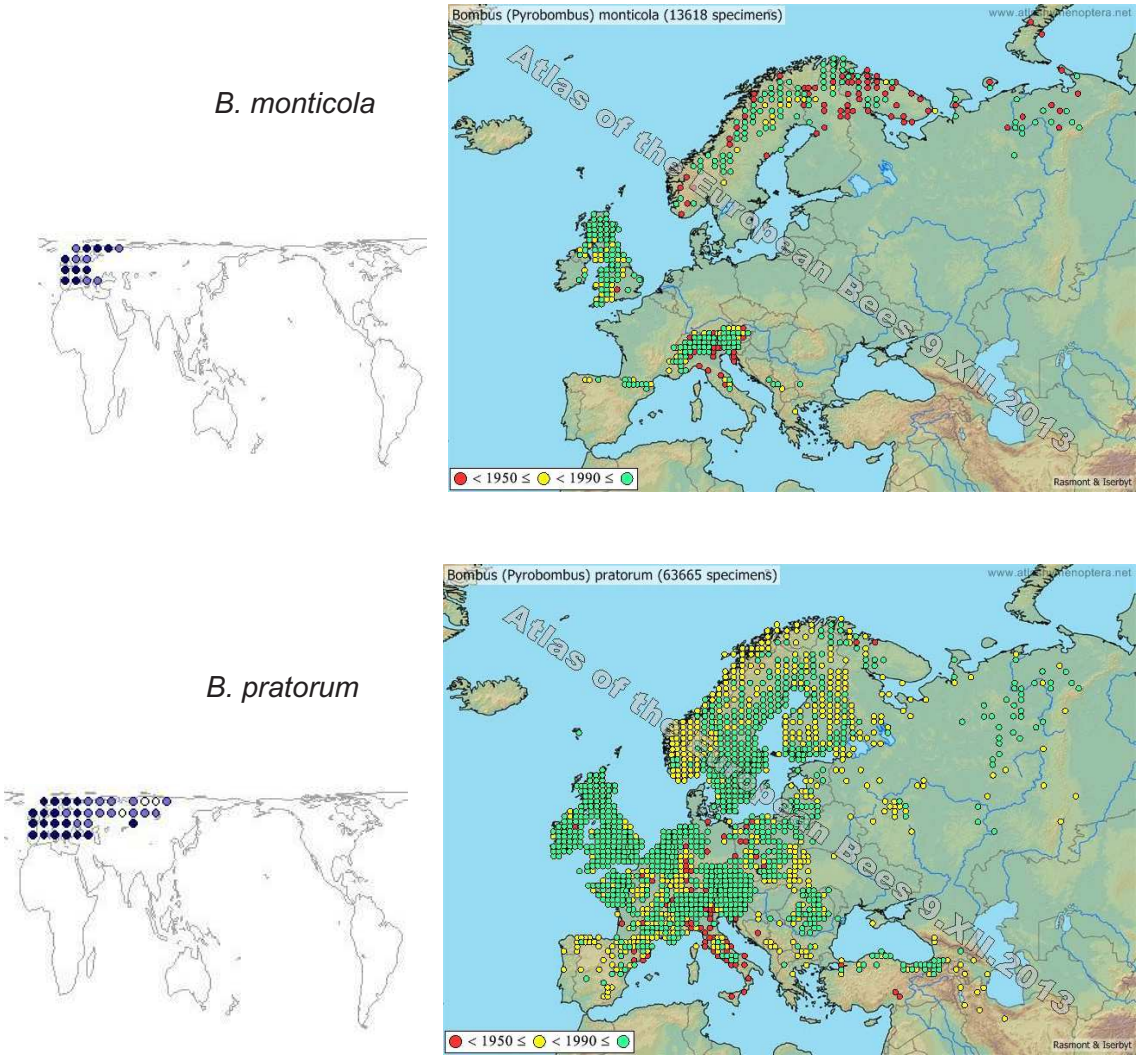

Figure 2.3. The global and European distributions of *B. monticola* (top) and *B. pratorum* (bottom). Global distribution maps are from the Natural History Museum (Williams 2014); permission to reproduce these images has been granted by P. Williams. European distribution maps are from the Atlas Hymenoptera (Rasmont & Iserbyt 2013); permission to reproduce these images has been granted by P. Rasmont.

Figure 2.4. UK distributions of *B. monticola* (left) and *B. pratorum* (right). Distributions are based on records from the UK Bees Wasps and Ants Recording Society (BWARS) database from 1960-2014, downloaded on 17/08/2014. Records are shown at 10km resolution. Maps were compiled using the National Biodiversity Network Gateway (data.nbn.org.uk; Dataset key: GA000055).

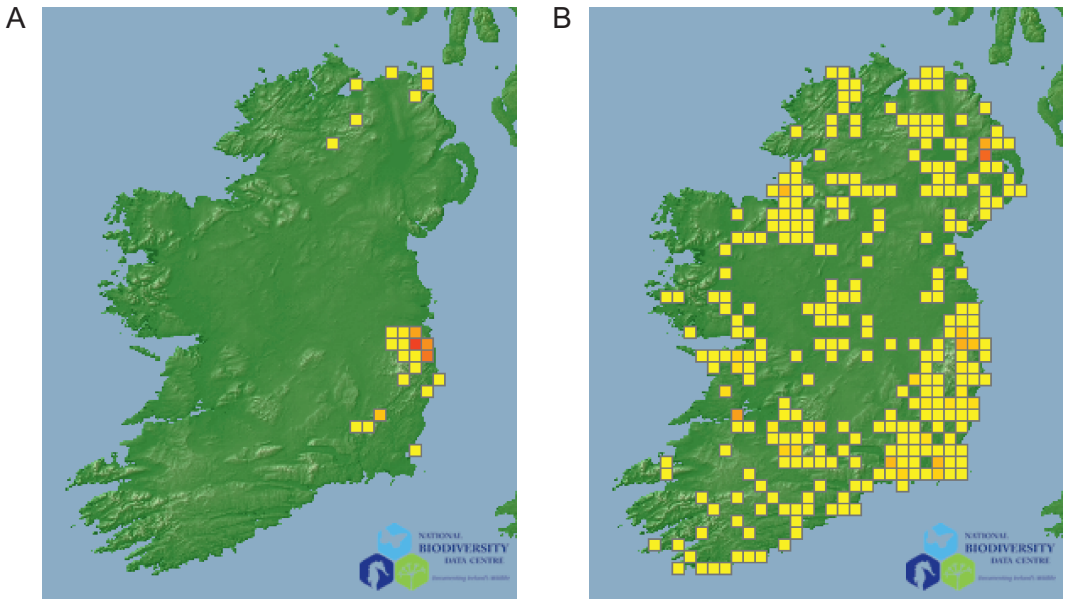

Figure 2.5. Distributions of A) *B. monticola*, and B) *B. pratorum* in Ireland. The colour gradient from yellow to red shows the number of records for the species in the grid square, yellow being lowest and red being highest. The range for *B. monticola* is 1-27 records per square. The range for *B. pratorum* is 1-52 records per square. Maps were downloaded from the National Biodiversity Data Center on 17/08/2014, and show data from the Bees Of Ireland dataset (Fitzpatrick & Murray 2014).

cola as possible (Fig. 2.4; Fig. 2.6). Where possible, 40 individuals of each species were collected at each site (Table 2.1); however, at some sample sites only one species was present (Fig. 2.6). The maximum sample collected of one species at any site was 50 individuals, with the minimum being 19 individuals (Table 2.1). Only worker bees were collected; reproductive individuals were not included. As bumblebees are eusocial this ensured minimal impacts on colony success, and thus populations, in the subsequent season.

Table 2.1. List of sampling sites and number of individuals collected at each site. At the Peak District sample site only *B. pratorum* was present. At the Ben Lawers and Glen Shee sample sites only *B. monticola* was present. Co-ordinates indicate the central point around which sampling was conducted. Sampling month indicates the timing of sample collection in 2011. Site numbers correspond to those in Fig. 2.6.

Site Name	Site No.	Co-ordinates	*B. pratorum* collected	*B. monticola* collected	Sampling month
Dartmoor	1	50°36'49.15" N 3°51'51.11" W	50	40	May-June
Long Mynd	2	52°32'52.70" N 2°49'33.37" W	40	40	May-June
Stiperstones	3	52°34'49.55" N 2°56'05.76" W	40	40	May-July
Snowdonia (Aber Falls)	4	53°13'41.54" N 4°00'15.93" W	40	40	June
Peak District (Swallow Moss)	5	53°10'21.22" N 1°57'40.73" W	40	-	June
Lake District (Ennerdale)	6	54°31'33.24" N 3°22'39.64" W	19	40	June
Ben Lawers	7	56°30'45.71" N 4°15'46.94" W	-	30	July-August
Glen Shee	8	56°51'16.64" N 3°25'49.97" W	-	36	August-September
Dublin	9	53°13.746 N 6°16.197 W	40	40	June
Antrim	10	55°05.405' N 6°07.204' W	40	31	June

2.2.2 Sample collection

The central point for each sampling site was taken as the grid reference listed in the BWARS database (Table 2.1). Sample collection was conducted within a 10x10km area around this point at each site. The area was initially searched for foraging patches. Between 3-5 individuals were then collected from a patch of forage using a butterfly net, and stored in individual tubes (Thermo-Fisher Scientific) which were kept on ice. Wherever possible, the patches sampled were at least 200m apart, to avoid the possibility of over-sampling any one colony. The reasons for this were

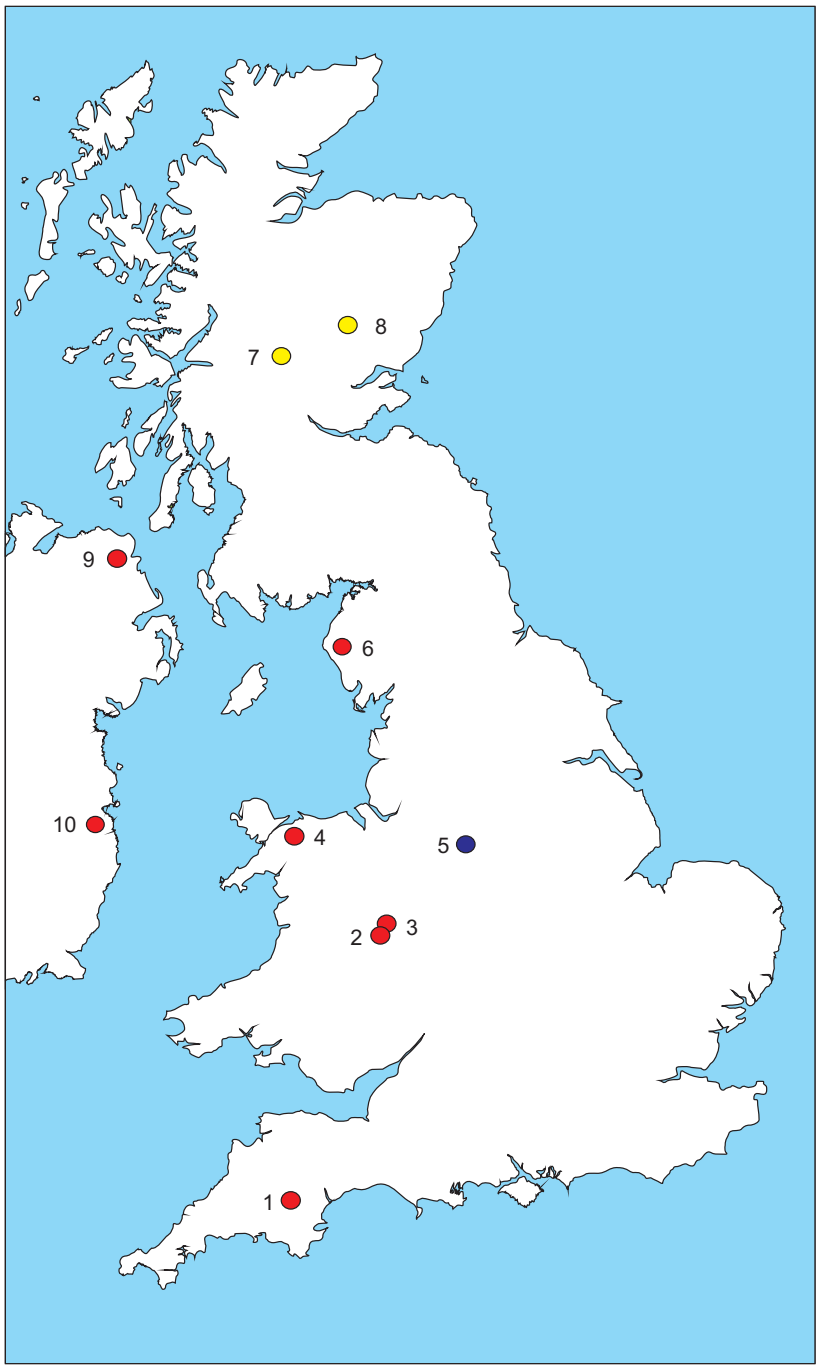

Figure 2.6. Sampling sites: Red icons indicate sites where samples of both species were collected; blue icons indicate *B. pratorum* collection only; yellow icons indicate *B. monticola* collection only. Site numbers correspond to those in Table 2.1

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twofold; firstly, to avoid any negative effects on colony success by removing too many workers, and secondly to avoid influencing population genetic parameters by sampling a lot of related individuals. A GPS reading was taken at the point of collection of each sample, along with notes of weather conditions (temperature, wind speed, cloud cover and precipitation) and forage plant if applicable. After collection, samples were kept on ice before being freeze-killed and stored at -24°C using a portable freezer (Heavy Duty 22 Litre Compressor Fridge/Freezer T0022, Euroengel). Samples were stored at a maximum of -10°C throughout fieldwork, before being dissected and stored long-term at -20°C.

2.2.3 Comparison of foraging preferences

To ascertain whether *B. monticola* and *B. pratorum* were choosing different forage plants, the plant on which each individual was collected (where applicable) was recorded. Dietary breadth and evenness were defined for the two species on a site-by-site basis, by calculating the Shannon-Weiner diversity of observed forage plant usage. Data were tested for normality using the Shapiro-Wilk test, then dietary breadth and evenness were compared between *B. monticola* and *B. pratorum* using paired t-tests. As sampling of both species at each site was completed at the same time, the range of flowers available for foraging was the same for each species at each site. However, due to the differences in sampling times floral resources would have varied significantly between sample sites, hence why inter-specific comparisons were only made within sample sites.

2.3 Results

Both *B. monticola* and *B. pratorum* workers were found foraging on 22 plant species. A total of 28 forage plant species were noted overall, though the forage plants used varied considerably between sample sites (Fig. 2.7).

No significant differences were obvserved between *B. monticola* and *B. pratorum* in terms of diet breadth (t=0.216, df=6, p=0.836) or dietary evenness (t=-0.558, df=6, p=0.597).

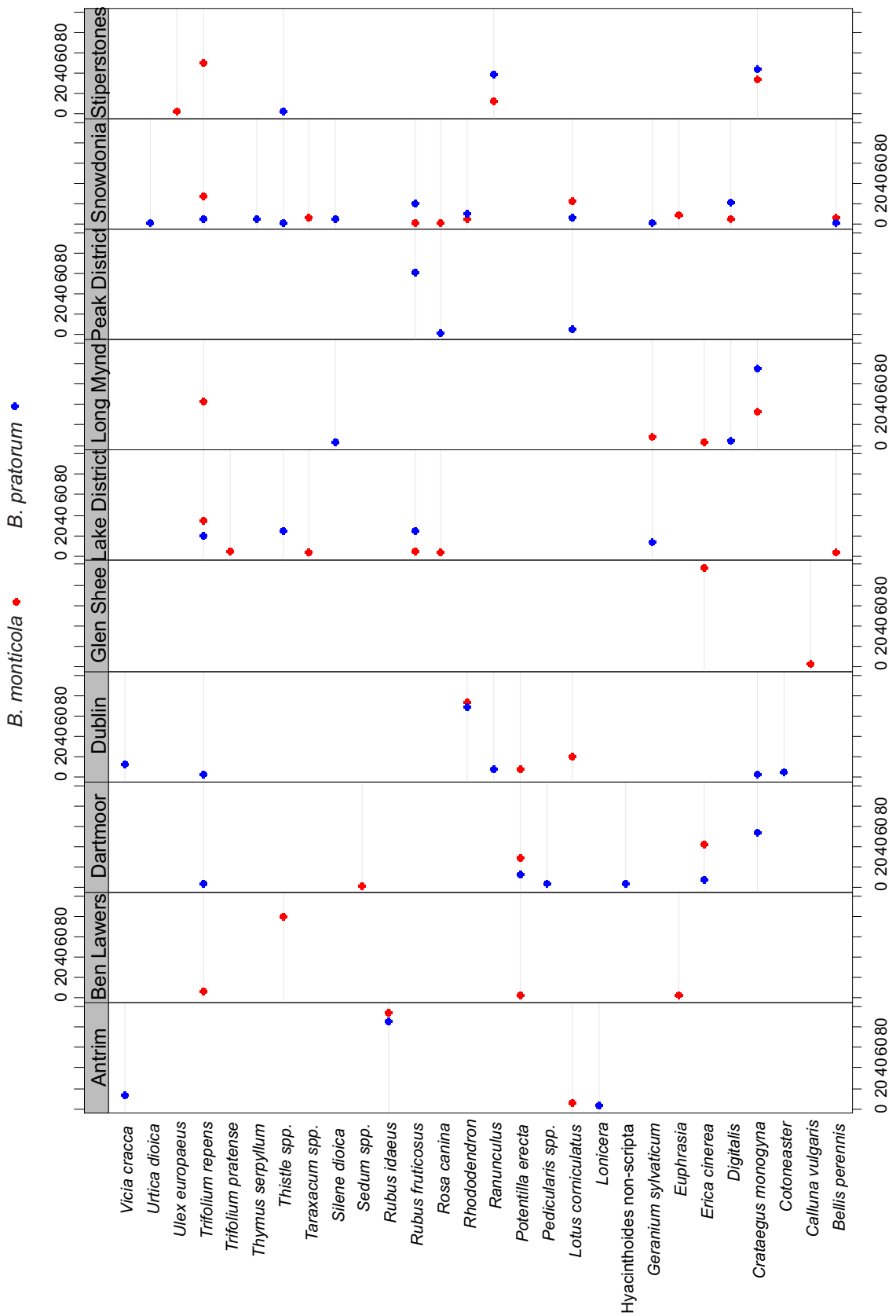

Figure 2.7. Comparison of the plant species used for foraging by *B. pratorum* and *B. monticola* workers sampled during fieldwork in 2011. Although diet breadth and evenness did not vary significantly between the two species, there are indications of considerable site-by-site variability in forage plant use.

2.4 Discussion

Foraging preferences are highly variable between bumblebee species (Goulson et al. 2005). Those preferences will change over the course of the year as different plants begin and finish flowering, and may also be related to factors such as pollen quality (Hanley et al. 2008) and tongue length (Goulson & Darvill 2004). Differences in foraging preferences and diet are likely to be linked with other processes such as the accumulation of wing wear (Foster & Cartar 2011), the chances of mortality (Cartar 1992), and levels of immunocompetence (Alaux et al. 2010). When making comparisons between species it is therefore important to assess whether there is a marked difference in foraging behaviour.

Workers of each species were found foraging on a variety of plants at all the sample sites visited, and dietary breadth was highly similar between the the two species at each sample site. While dietary breadth (the number of forage plant species recorded) was similar between *B. monticola* and *B. pratorum* within sample sites, there was considerable variation in the variety of forage plants observed between sample sites for each species. For example, *B. monticola* was observed foraging on ten plant species at the Snowdonia sample site, but only two plant species at the Antrim sample site (Fig. 2.7). Variation between sample sites may simply be due to the timing of sampling, or due to differences in the plant communities between sites. The availability of flowering plants will change over the course of a season, and subsequently influence the foraging behaviour of pollinators. While all sample sites were reasonably similar upland and moorland habitat, plant communities could vary significantly at a local scale, influencing foraging behaviour within species at different sites. Dietary breadth has been linked with immunocompetence in honeybees (Alaux et al. 2010), while dietary protein content has been linked with antimicrobial peptide expression in *Bombus* (Brunner et al. 2014). Inter-site variability in foraging may therefore need to be considered when assessing variation in immunocompetence in both *B. monticola* and *B. pratorum*.

Rarity and risk of decline in *Bombus* has been linked with dietary specialisation (Goulson & Darvill 2004, Goulson et al. 2008a); however, it does not appear from the results reported here that this is the case for *B. monticola*. The restricted range of *B. monticola* in mainland Europe (Fig. 2.3) as well as in the UK and Ireland (Fig. 2.4) may suggest support for the theory that rarity and risk of

decline correlates with climatic range size (Williams et al. 2007). While the UK is not particularly close to the southern limit of the overall range for *B. monticola*, it does not contain areas of equivalent altitude to the Alps for example; the UK may therefore be an area at the limit of the climatic tolerance of *B. monticola*. This species may experience further reductions in its range in the UK under predicted climate change conditions, as there are few areas which would allow altitudinal migration to compensate for an increase in temperature. However, this is purely speculative. More research covering a larger portion of the species range would be required to test this hypothesis, and is therefore beyond the scope of this study.

Although there was considerable inter-site variability in dietary breadth and forage plant choice, the results of this study suggest that *B. monticola* and *B. pratorum* show similar foraging preferences at the majority of sites visited. There was considerable overlap in the plant species visited by both species at most sites, and the plants that accounted for the largest proportion of workers sampled at any one site were generally the same between the two study species (Fig. 2.7). Plants associated with only one of the study species at a given site were generally at a low frequency (<20%; Fig. 2.7). A notable exception was *Trifolium repens*, on which larger proportions of *B. monticola* were generally found. This apparent preference for *T. repens* may be explained by the relatively later time of emergence of *B. monticola* compared to *B. pratorum*. As a legume, the pollen of *T. repens* is high in protein (Hanley et al. 2008), which is beneficial for immune function (Brunner et al. 2014). Later emerging *Bombus* species may show a preference for higher-protein pollen than early emerging congeners, as they have a shorter time in which to forage and provision the larvae in the nest, and for the larvae to develop (Goulson & Darvill 2004).

2.5 Conclusions

The results of this study suggest considerable inter-site variability in foraging behaviour in both the study species. This may be caused by variation in plant communities between sites, or simply be a result of the time of sampling. In either case, differences in foraging behaviour may need to be considered when comparing factors such as wing wear and immunocompetence between sample sites for each species.

No significant differences in dietary breadth or evenness were detected between *B. monticola* and *B. pratorum* within some sample sites. The plant species accounting for the greatest proportion of sampled workers were the same for *B. monticola* and *B. pratorum*. Thus, there may not be a biologically significant difference in foraging behaviour between *B. monticola* and *B. pratorum*. It therefore seems reasonable to assume that any differences subsequently found between *B. pratorum* and *B. monticola* in terms of immunocompetence are unlikely to be attributable simply to differences in foraging behaviour.

It must be recognised at this point that this is a limited assessment of differences in foraging behaviour, as workers have only been assessed at one point in time and were not observed for long periods before capture. Differences in foraging preference may become apparent at other points in the season.

Chapter 3

A comparison of two methods for assessing wing wear in *Bombus* species

3.1 Introduction

Under laboratory conditions it is possible to know the precise age of individuals, as they can be observed from the point of emergence. However, under field conditions this is rarely possible, particularly when studying bumblebees as the nests are difficult to locate in the first instance, and are often concealed underground (Edwards & Jenner 2005). The accumulation of irreparable damage over the course of a lifetime is inevitable, and in the case of flying insects this damage may be in the form of wing wear (with wings becoming increasingly damaged over time; Mueller & Wolf-Mueller 1993). The extent of wear to the wings has been used as a measure of relative age in a number of insect groups including Hymenoptera (Mueller & Wolf-Mueller 1993, Allen et al. 2007, Whitehorn et al. 2010), Lepidoptera (Stjernholm et al. 2005, Kemp 2006, Peixoto & Benson 2008), Zygoptera (Plaistow & Tsubaki 2000), and Empididae (LeBas et al. 2004). There are many circumstances when it is important to know, at least approximately, the age of an individual. For example, some immune parameters in *Bombus* such as phenoloxidase (PO) activity (see Chapter 4) are known to be significantly correlated with age (Doums et al. 2002, Whitehorn et al. 2010). Finding an effective way to get this information is therefore highly relevant to any study of bumblebee immunity.

The extent of wing wear in Hymenoptera, especially *Bombus*, has frequently been quantified using a categorical scale developed by Muller & Wolf-Muller (1993). The method was originally developed for carder bees (*Anthidium manicatum*), and assigns samples to categories depending on the number of nicks, tears and excisions in the trailing edges of the forewings (Fig. 3.1; Table 3.1). This allows age to be estimated quickly, easily, and without requiring a great deal of specialist training or equipment. However, the categorisation of wings is somewhat subjective, meaning that the estimated age of an individual is likely to be dependent on the person conducting the research; results may therefore not be truly comparable across studies. The method also assumes that wing wear accumulates at a constant rate over the lifetime of an individual, and that the rate of accumulation is comparable across individuals both within and between colonies. There are several reasons why such assumptions may be invalid, a notable example being the influence of body size on the rate of wing wear (Mueller & Wolf-Mueller 1993). *Bombus* workers vary greatly in size depending on their species (Alford 1975, Peat et al. 2005a), and within the same colony (Goulson

et al. 2002). It is not possible to account for this when using a categorical scoring method, but individual body size is positively related to the accumulation of wing wear (Mueller & Wolf-Mueller 1993, Foster & Cartar 2011). This may be directly, through increased wing loading in larger individuals (Danforth 1989, Fischer & Kutsch 2000), or indirectly through different behaviours in individuals of varying size; larger individuals spend more time foraging for example (Goulson et al. 2002), and are therefore subject to increased predation risk and more frequent collisions with vegetation that can damage wings (Foster & Cartar 2011). The influences of body size (both direct and indirect) may also cause variation in wing wear accumulation between species, and this may be further compounded by the highly variable foraging and nesting preferences in different *Bombus* species, as well as potential differences in wing morphology relative to body size.

Many studies have used variations on the wing wear scale (Whitehorn et al. 2010, Goulson et al. 2012), but few have discussed the recommendations of Mueller & Wolf-Mueller (1993) that the scale be calibrated for different species and populations, or that body size should be taken into account in some way. The likely influences of body size and foraging habits on wing wear (Mueller & Wolf-Mueller 1993, Foster & Cartar 2011) shed doubt on whether a universal scale can be applied across populations, and even within colonies. This study aimed to define an alternative method for quantifying wing wear in *Bombus*, accounting for body size variation. This was tested in two *Bombus* species, *B. monticola* and *B. pratorum*, sampled from multiple sites across the UK (see Chapter 2). *B. pratorum* workers are generally smaller than *B. monticola* workers (Alford 1975), so may be expected to accumulate wing wear more slowly (all other factors being equal). While the two species reportedly have different habitat and nesting preferences (Edwards & Jenner 2005; see Chapter 2), sampling from the same sites aimed to minimise the effects of interactions with different vegetation types. This allowed a comparison of the relationship of wing morphology to body size in the two species, giving an indication of how suitable a universal scale of wing wear would be across *Bombus* species.

This study addressed the following questions and aims:

1. Do species differ in body size, and does body size affect wing wear?

2. To define a method for assessing wing wear on a continuous scale, accounting for body size variation.

3. Is there congruence between categorical and continuous estimations of wing wear?

4. Are there inter-site differences in wing wear profiles within species?

3.2 Methods

3.2.1 Categorical age scale

After sample collection (as described in Chapter 2), each individual bee was defrosted and dissected. The forewings were removed and stored in individual tubes, and the thorax was separated from the abdomen and weighed using an analytical balance. Each wing was inspected using a 10X magnifying lens, and scored according to the criteria described by Mueller & Wolf-Mueller (1993; Fig. 3.1; Table 3.1). Left and right wings for each bee were scored separately, and the mean of the two values was taken. The means were rounded to the nearest whole number; this value was then taken as the estimate of individual age. Wings showing a large area of damage likely to be the result of a single trauma were discounted. Age categories 4-6 were pooled into one category of 4+ for analysis, due to the low number of individuals in those groups.

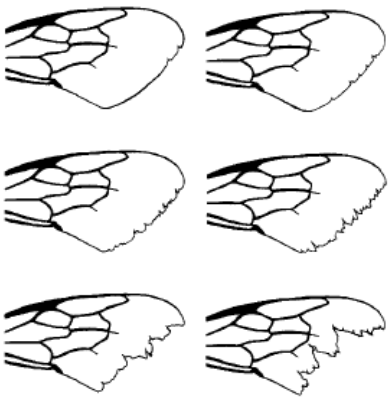

Figure 3.1. Image from Mueller & Wolf-Mueller (1993), illustrating the guidelines for each age category. Top left = category 1; top right = category 2; center left = category 3; center right = category 4; bottom left = category 5; bottom right = category 6 (categories fully described in Table 3.1). Permission to reproduce this image has been granted by U. Mueller.

Table 3.1. Definitions of wing wear categories as described by Mueller & Wolf-Mueller (1993).

Age Category	Description
0	Wing margin completely intact.
1	Wing margin showing one or two nicks.
2	Wing margin showing 3 to 10 nicks.
3	Wing margin almost completely serrated with more than 10 nicks, but at least some original margin intact.
4	Wing margin completely serrated with excisions less than half the width of the distal submarginal cell. No original margin intact.
5	Wing margin completely serrated with excisions more than half, but less than the entire width of the distal submarginal cell.
6	Wing margin showing major excisions greater than the width of the distal submarginal cell. Excisions may reach distal cross veins.

3.2.2 Morphometric analysis

Each wing was photographed at 10X magnification using a USB microscope camera and Infinity Capture software (Lumenera Corporation). The images were then loaded into SigmaScan Pro (Systat Software Inc.). The perimeter of the distal end of each wing was outlined using the track edges tool to obtain measurements of both wing perimeter and wing area (Fig. 3.2). As with the categorical method, the left and right wings were measured individually, and the mean values were used for subsequent analysis. Samples were scored by one observer; however, to assess how consistent scoring would be if conducted by multiple observers, samples from one site were assessed by three people. The measurements of wing area and wing perimeter from each person were then compared.

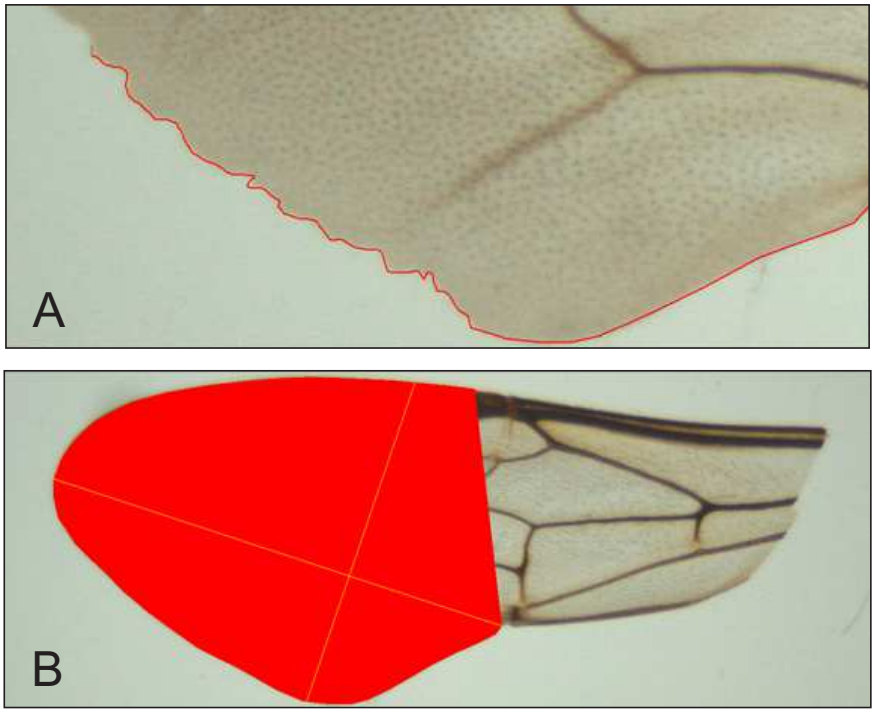

Figure 3.2. Screen shots of wing measurement using SigmaScan Pro (Systat Software Inc.). Panel A indicates how the wing perimeter was measured; panel B shows section of the wing measured. The method was only applied to the end portion of the wing, which has been shown to accumulate the most wear during the lifetime of an insect (Mueller & Wolf-Mueller 1993). The measurements were normalised for body size (approximated by thorax weight) before any further analysis.

3.2.3 Statistical analysis

The mean thorax weight for each species was compared using ANOVA. Thorax weight was compared between sample sites for each species using ANOVA and post-hoc Tukey's tests. Thorax weight between age categories was analysed separately for *B. pratorum* and *B. monticola* on a site-by-site basis, again using ANOVA and post-hoc Tukey's tests. For all ANOVA analyses, the residual values were visually checked for normality and homogeneity of variance. The relationship of thorax weight to wing area and wing perimeter was determined using linear regression for each species.

To determine the extent to which human error may affect the reliability of the morphometric data, the wing area and wing perimeter of all samples from the Snowdonia sample site were measured by three observers. The raw data from each person were compared using ANOVA. For further analysis of the morphometric data, it was necessary to account for the variation in body size between

samples. Data for each species were treated separately, as the relationship of wing size to body size may differ between species. However, it was assumed that the relationship of body size to wing size (and therefore to overall wing area and perimeter) would be reasonably consistent within a species, even if the average body size varied slightly between sample sites. Data from all sample sites for a species were therefore pooled. The raw measurements of wing perimeter and wing area were regressed against thorax weight, to determine the general relationship of body size to wing area and wing perimeter for each species. The residual values around the best fit line were then calculated. For ease of analysis, the residuals from each site were increased by the inverse of the smallest value; this ensured that all data points were positive. The adjusted residual values (referred to from this point simply as residuals) for each data point were used for subsequent analysis, meaning that any influence of variation in body size was effectively removed.

To compare the results of categorical scoring with the morphometric data, the residual values of wing area and wing perimeter were compared across age categories for each species on a site-by-site basis. This was achieved using Kruskal-Wallis tests, due to non-normality and unequal variance between categories in some cases. When significant differences were detected post-hoc pairwise Mann-Whitney tests were used, adjusted for multiple testing using Holm's correction.

Age structure as assessed using the categorical scale (Mueller & Wolf-Mueller 1993) was analysed between sample sites for each species using Chi-squared tests, and with Fisher's Exact test or Freeman-Halton tests if the frequencies were insufficient for Chi-squared tests. The residuals for wing area and wing perimeter were compared across sites using ANOVA, or Kruskal-Wallis tests where data were non-normal and could not be successfully transformed.

All statistics were performed using R (v. 2.15.2).

3.3 Results

3.3.1 Variation in body size within and between species, and the influence of body size on wing wear

The samples collected for this study illustrate both inter- and intra-specific variation in size (Fig. 3.3). Mean thorax weight was significantly different between the two species ($F_{1,610}$=122.1, $p<0.001$), and also varied significantly between sample sites in both species (*B. pratorum*: $F_{7,283}$=2.21, $p<0.05$; *B. monticola*: $F_{8,312}$=8.27, $p<0.001$).

To determine whether wing wear is linked to body size, the mean thorax weight of each age category was analysed using ANOVA in both study species. Thorax weight varied significantly between age categories in both species (*B. pratorum*: $F_{4,255}$=3.00, $p<0.05$; *B. monticola*: $F_{4,275}$=2.53, $p<0.05$). Post-hoc tests showed the significant differences in both species to lie between the lowest and two highest age categories (Fig. 3.4), indicating that the heaviest bees had the most wing wear. A significant effect of sample site was also observed for *B. monticola* ($F_{8,275}$=11.50, $p<0.001$); this is likely to be due to the significant variation in thorax weight between sample sites (Fig. 3.3). While this is not proof of causation, these results support the theory that larger bodied individuals accumulate more wing wear, and highlight the need for an effective age estimation method to account for variation in body size.

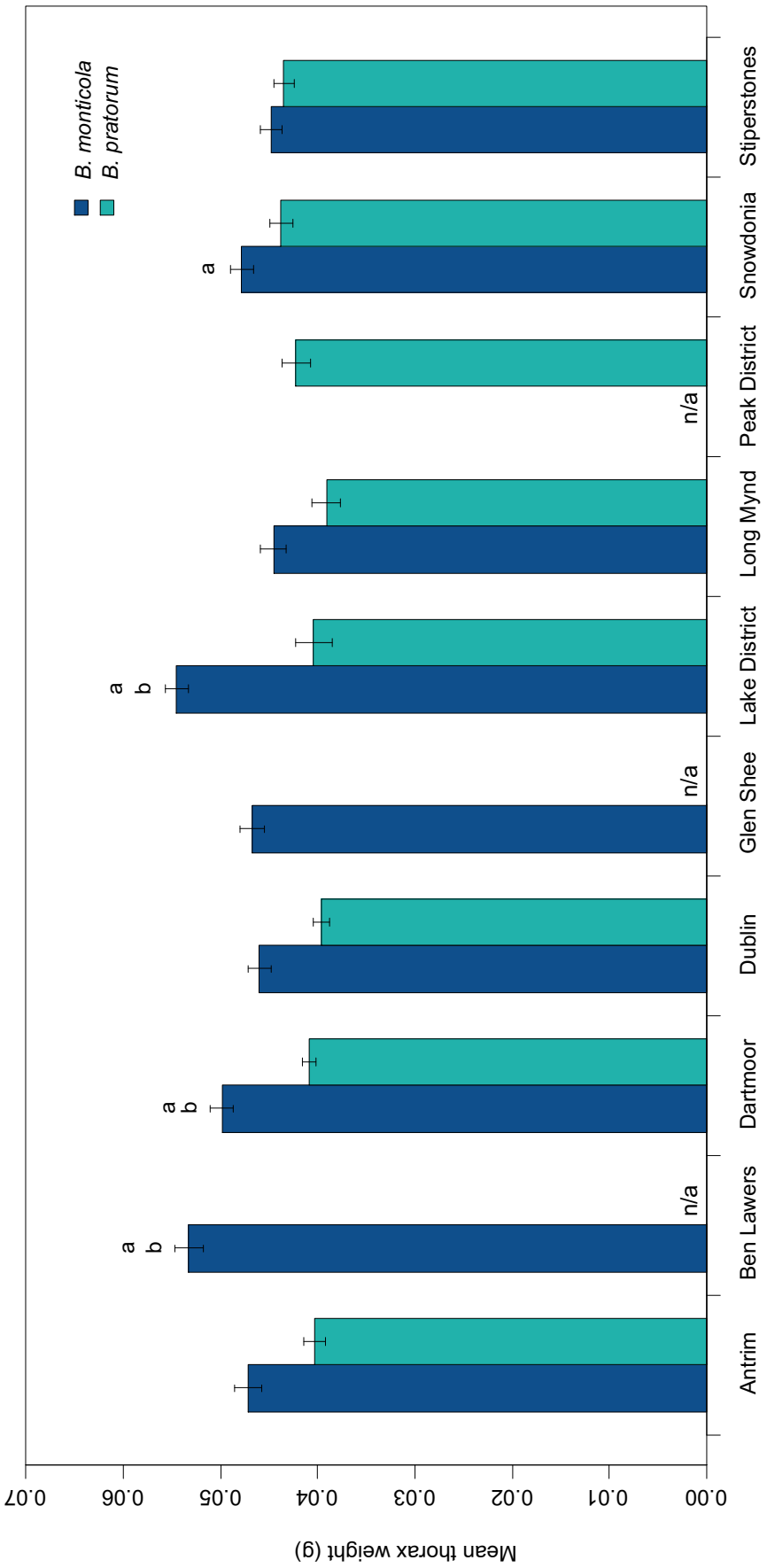

Figure 3.3. Variation in thorax weight between sample sites in *B. monticola* and *B. pratorum*. Bars indicate the mean thorax weight ± standard error. No significant differences were observed between sites in *B. pratorum*. Significant pairwise comparisons for *B. monticola* are indicated above the relevant bars.

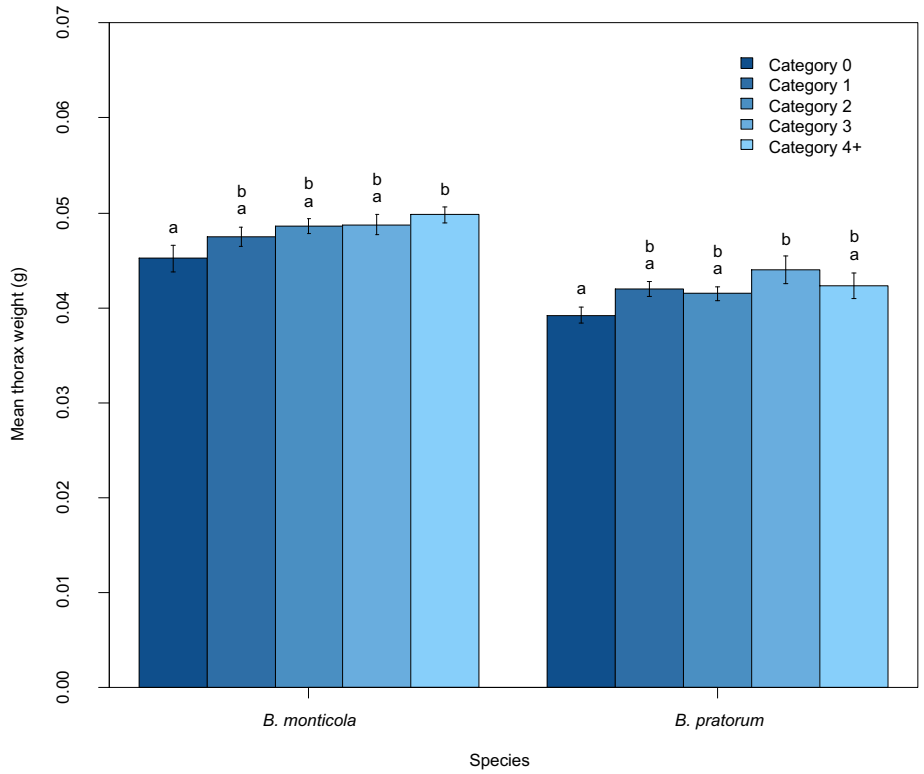

Figure 3.4. Variation in thorax weight between age categories in *B. monticola* and *B. pratorum*. Bars show mean thorax weight ± standard error. Letters above bars indicate results from post-hoc Tukey's tests; note that post-hoc tests were performed separately for each species.

3.3.2 Reliability of morphometric data collection

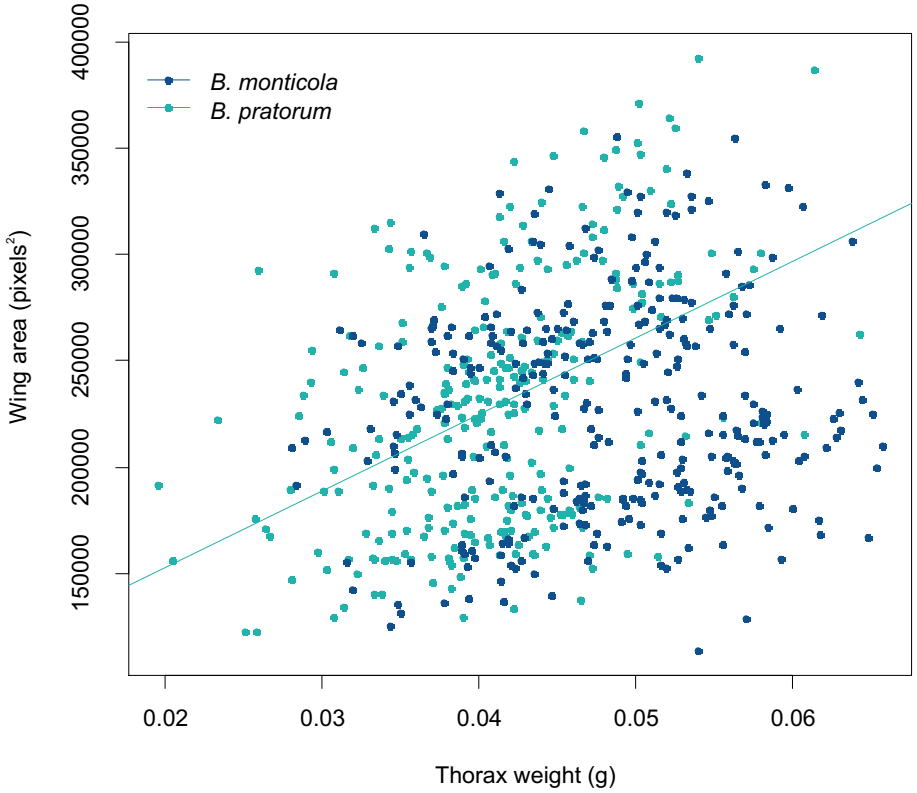

Figure 3.5. Correlation of wing area with thorax weight in *B. monticola* and *B. pratorum*. Best fit lines show linear regressions of wing area against thorax weight.

To determine how reliably wing wear could be measured using the method described in this study, individuals collected at the Snowdonia sampling site (39 *B. pratorum* workers and 40 *B. monticola* workers) were processed by three observers. No significant differences were found in the measurements of wing area ($F_{2,219}$=2.88, p=0.059) or wing perimeter ($F_{2,219}$=1.36, p=0.258) between observers. This suggests that morphometric data collected as described in this study could be reliably compared and/or pooled between observers. The data were adjusted to account for body size before further analysis.

A linear relationship was observed in both species between thorax weight and wing area (Fig. 3.5), and thorax weight and wing perimeter (Fig. 3.6). The relationship was significant in *B. pratorum* (wing area: t=8.27, p<0.001, R^2=0.19; wing perimeter: t=5.71 , p<0.001, R^2=0.10), but was not significant in *B. monticola* (wing area: t=1.51, p= 0.133, R^2=0.004; wing perimeter: t=1.40, p=0.162, R^2=0.003). The difference in the relationship between wing size and body size in

the two species meant that formal comparisons could not be made between them.

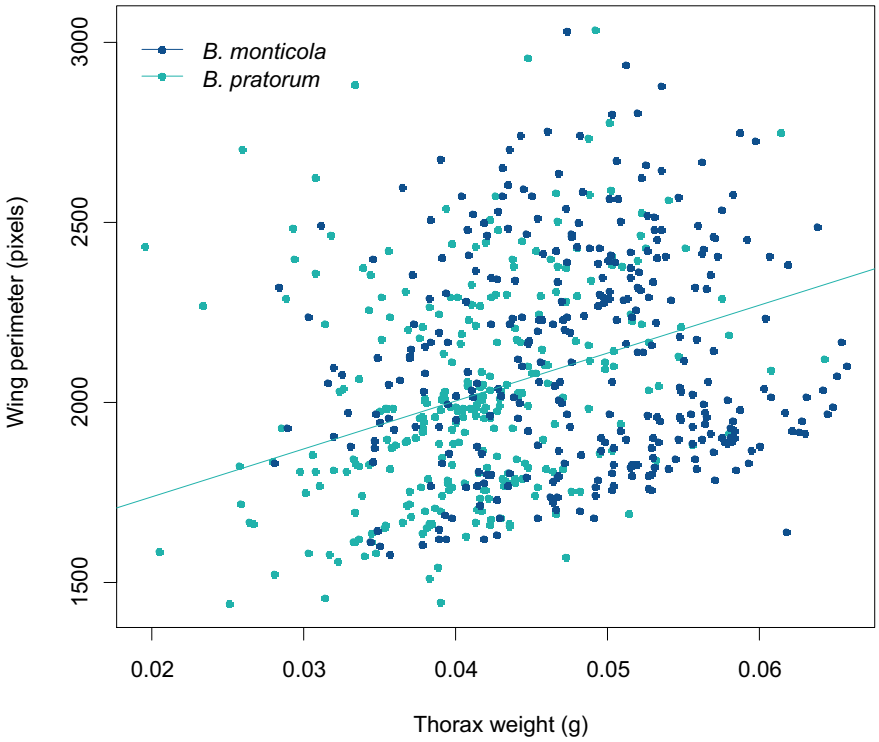

Figure 3.6. Correlation of wing perimeter with thorax weight in *B. monticola* and *B. pratorum*. Lines of best fit show linear regressions of wing perimeter against thorax weight.

3.3.3 Determining the level of congruence between categorical and continuous measurements of wing wear

Wing area

As expected, wing area residuals in both study species decreased as the wing wear category (assigned according to Mueller & Wolf-Mueller 1993) increased, indicating that in higher categories wing area is lower relative to body size. This indicates a certain amount of agreement between the categorical estimation of wing wear and the morphometric data. However, in both species there was a lot of variation in wing area residuals within each wing wear category, and a lot of overlap between categories (Fig. 3.7; Fig. 3.9).

For *B. monticola* there was no significant variation in wing area residuals between wing wear categories at the Antrim, Dublin, Lake District, Long Mynd, or Snowdonia sample sites (Table 3.2). Significant variation between categories was observed at the Ben Lawers, Dartmoor, Glen Shee, and Stiperstones sample sites

(Table 3.2). For *B. pratorum* the wing area residuals did not vary significantly between wing wear categories at the Lake District, Peak District, Snowdonia, or Stiperstones sample sites (Table 3.3). Significant variation between categories was observed at the Antrim, Dartmoor, Dublin, and Long Mynd sample sites (Table 3.3).

In both study species, significant differences in wing area were rare between sequential categories (Table 3.2; Table 3.3). Significant differences were generally between the lowest and highest categories (between individuals with the least damaged and most damaged wings respectively). This is most likely to be the result of a large amount of variation in the wing area residuals contained within each category, and overlap between categories (Fig. 3.7; Fig. 3.9). This makes it difficult to distinguish the categories from each other, except at the extremes of the overall range.

Wing perimeter

The general trend across all sites was for higher residual perimeter values in the higher wing wear categories; this is in agreement with the expectations of the study, as tears and nicks accumulating in the wings will cause the perimeter to increase.

For *B. monticola*, no significant variation between categories was observed at the Antrim, Ben Lawers, or Lake District sample sites (Table 3.2). Significant differences between categories were observed at the Dartmoor, Dublin, Glen Shee, Long Mynd, Snowdonia, and Stiperstones sample sites (Table 3.2). For *B. pratorum*, no significant differences in the residuals for wing perimeter were observed between categories at the Antrim, Lake District, and Peak District sample sites (Table 3.3). Significant variation between categories were observed at the Dartmoor, Dublin, Long Mynd, Snowdonia, and Stiperstones samples sites (Table 3.3).

As with the analysis of wing area, post-hoc tests showed the significant differences in both species to be largely between the extremes of the categorical scale (Table 3.2; Table 3.3). This is most likely to be the result of a large amount of variation and overlap in the wing area residuals contained within each category (Fig. 3.8; Fig. 3.10). As with the wing area data, this makes it difficult to distinguish categories from each other, apart from at the extreme ends of the overall range of the data.

Table 3.2. Comparisons of wing area and wing perimeter residual values across categories of wing wear (assigned according to Mueller & Wolf-Mueller 1993) for *B. monticola*. Kruskal-Wallis χ^2 values indicate overall significance of variation between categories. Significant pairwise interactions indicate which wing wear categories were found to be significantly different from each other. The level of significance is indicated with asterisks (* = p<0.05; ** = p<0.01). In some cases no significant pairwise comparisons were found, even though the initial Kruskal-Wallis test indicated significant differences between categories; this is likely to be due to the conservative nature of the correction for multiple testing (Holm's correction). Sites where no samples were collected are indicated using n/a.

Wing area	Site	Kruskal-Wallis χ^2	df	p-value	Post-hoc tests
	Antrim	8.38	4	0.079	-
	Ben Lawers	9.51	4	<0.05	-
	Dartmoor	15.78	4	<0.01	0-4 **
	Dublin	4.55	4	0.337	-
	Glen Shee	12.04	4	<0.05	2-3 *
	Lake District	1.25	2	0.536	-
	Long Mynd	4.47	4	0.346	-
	Peak District	n/a	n/a	n/a	n/a
	Snowdonia	3.55	4	0.470	-
	Stiperstones	13.43	4	<0.01	1-4 *
Wing perimeter	Site	Kruskal-Wallis χ^2	df	p-value	Post-hoc tests
	Antrim	6.96	4	0.138	-
	Ben Lawers	7.43	4	0.115	-
	Dartmoor	14.51	4	<0.01	0-3 *
	Dublin	26.90	4	<0.001	-
	Glen Shee	14.88	4	<0.01	1-3 *
	Lake District	4.15	4	0.386	-
	Long Mynd	10.45	4	<0.05	-
	Peak District	n/a	n/a	n/a	n/a
	Snowdonia	21.56	4	<0.001	0-2 *
					0-3 *
					1-2 **
					1-3 *
	Stiperstones	22.10	4	<0.001	0-2 *
					0-3 **
					1-2 *
					1-3 **

Table 3.3. Comparisons of wing area and wing perimeter residual values across categories of wing wear (assigned according to Mueller & Wolf-Mueller 1993) for *B. pratorum*. Kruskal-Wallis χ^2 values indicate overall significance of variation between categories. Significant pairwise interactions indicate which wing wear categories were found to be significantly different from each other. The level of significance is indicated with asterisks (* = p<0.05; ** = p<0.01). In some cases no significant pairwise comparisons were found, even though the initial Kruskal-Wallis test indicated significant differences between categories; this is likely to be due to the conservative nature of the correction for multiple testing (Holm's correction). Sites where no samples were collected are indicated using n/a.

Wing area	Site	Kruskal-Wallis χ^2	df	p-value	Post-hoc tests
	Antrim	10.50	3	<0.05	0-2 *
	Ben Lawers	n/a	n/a	n/a	n/a
	Dartmoor	16.48	4	<0.01	0-3 *
	Dublin	18.27	4	<0.01	1-3 *
					1-4 *
	Glen Shee	n/a	n/a	n/a	n/a
	Lake District	1.25	2	0.536	-
	Long Mynd	10.51	4	<0.05	-
	Peak District	6.70	4	0.153	-
	Snowdonia	7.57	4	0.109	-
	Stiperstones	4.24	3	0.237	-

Wing perimeter	Site	Kruskal-Wallis χ^2	df	p-value	Post-hoc tests
	Antrim	6.17	3	0.104	-
	Ben Lawers	n/a	n/a	n/a	n/a
	Dartmoor	17.21	4	<0.01	-
	Dublin	20.31	4	<0.001	0-3 *
					0-4 *
					1-3 *
					1-4 *
	Glen Shee	n/a	n/a	n/a	n/a
	Lake District	3.07	2	0.216	-
	Long Mynd	14.06	4	<0.01	0-2 *
	Peak District	7.28	4	0.122	-
	Snowdonia	19.81	4	<0.001	0-3 *
					1-3 **
	Stiperstones	11.05	3	<0.05	0-3 *
					1-3

Figure 3.7. Comparison of wing area residual values across age categories in *B. pratorum* samples. No significant differences were found between sequential age categories. There was considerable overlap in the range of wing area measurements assigned to each category, as illustrated by the large whiskers in the figure. The amount of variation within sites is also markedly different between sample sites, hence the requirement for non-parametric testing of the data. Boxes show upper and lower quartile, with the heavy line showing the median. Whiskers are determined by the median ± 1.5 quartiles. Outliers are suppressed for clarity.

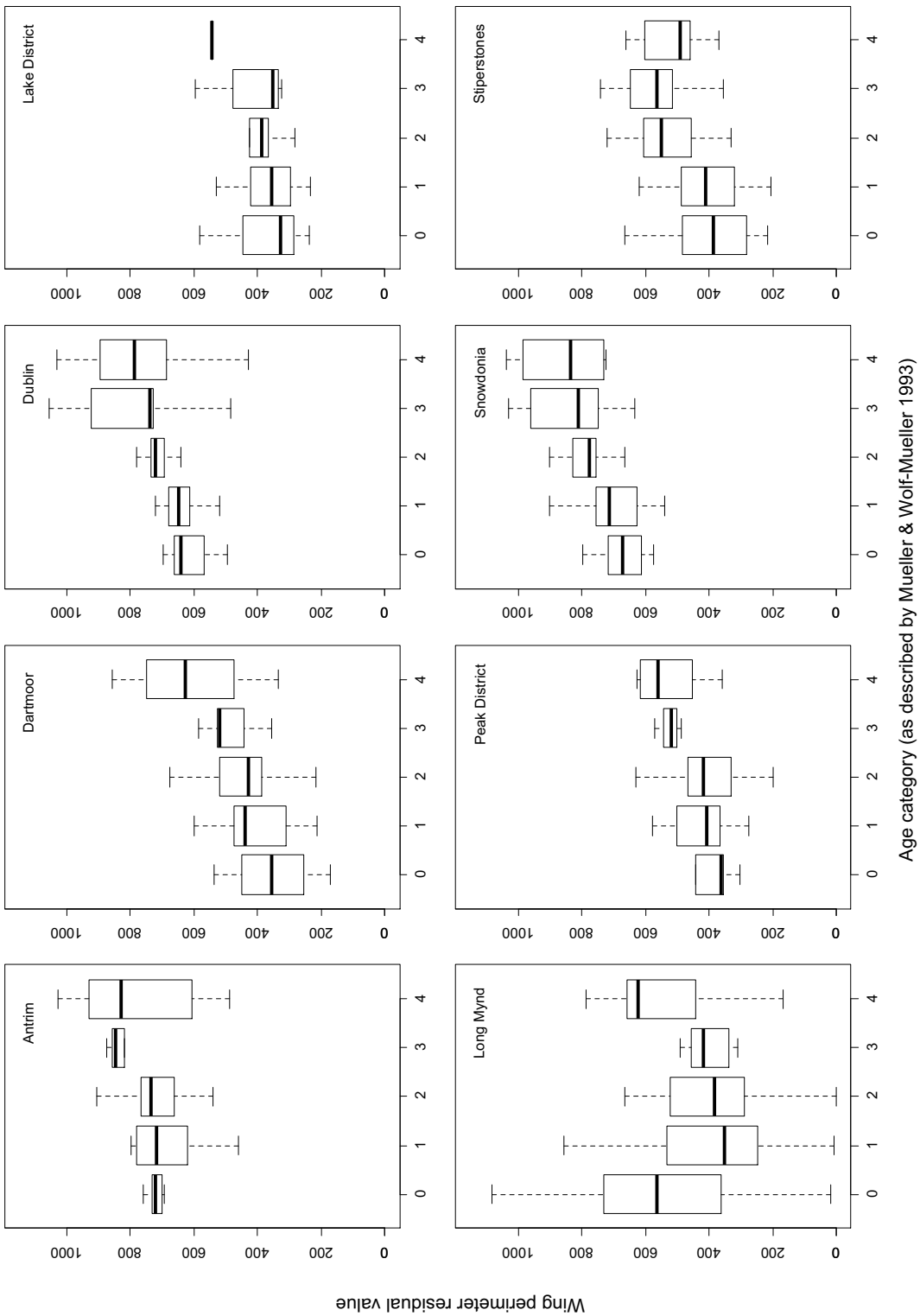

Figure 3.8. Comparison of wing perimeter residual values across age categories in *B. pratorum* samples. No significant differences were found between sequential age categories. There was considerable overlap in the range of wing area measurements assigned to each category, as illustrated by the large whiskers in the figure. The amount of variation within sites is also markedly different between sample sites, hence the requirement for non-parametric testing of the data. Boxes show upper and lower quartile, with the heavy line showing the median. Whiskers are determined by the median $\pm$ 1.5 quartiles. Outliers are suppressed for clarity.

Figure 3.9. Comparison of wing area residual values across age categories in *B. monticola* samples. No significant differences were found between sequential age categories. There was considerable overlap in the range of wing area measurements assigned to each category, as illustrated by the large whiskers in the figure. The amount of variation within sites is also markedly different between sample sites, hence the requirement for non-parametric testing of the data. Boxes show upper and lower quartile, with the heavy line showing the median. Whiskers are determined by the median ± 1.5 quartiles. Outliers are suppressed for clarity.

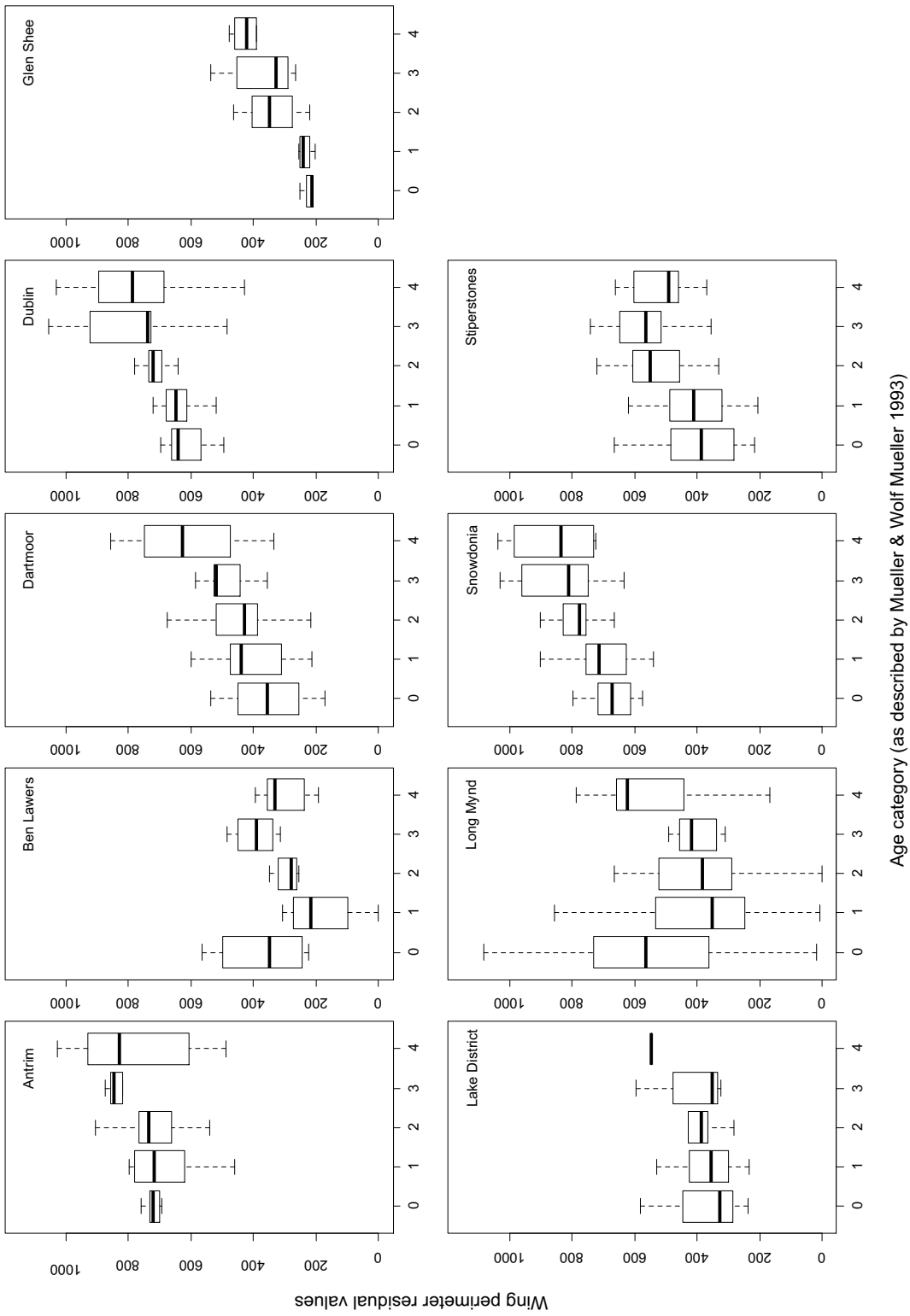

Figure 3.10. Comparison of wing area residual values across age categories in *B. monticola* samples. No significant differences were found between sequential age categories. There was considerable overlap in the range of wing area measurements assigned to each category, as illustrated by the large whiskers in the figure. The amount of variation within sites is also markedly different between sample sites, hence the requirement for non-parametric testing of the data. Boxes show upper and lower quartile, with the heavy line showing the median. Whiskers are determined by the median $\pm$ 1.5 quartiles. Outliers are suppressed for clarity.

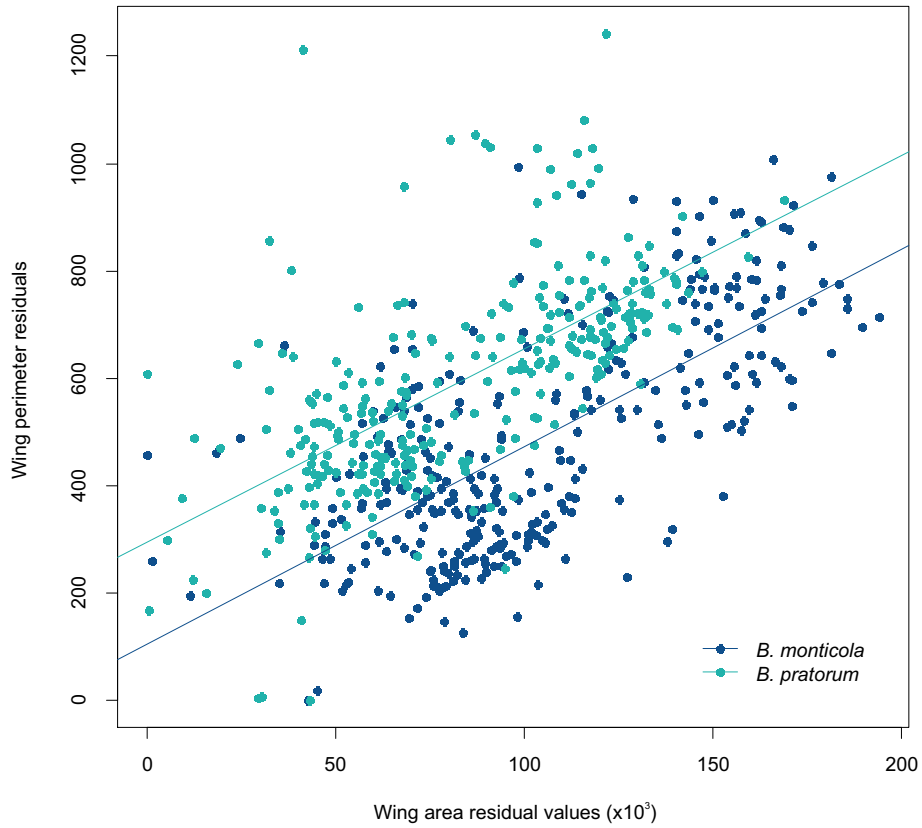

Figure 3.11. Correlation of wing area and wing perimeter residual values in *B. pratorum* and *B. monticola*. Trend lines show regression lines calculated separately for each species. In both cases there was a significant relationship between the wing area and wing perimeter residual values (*B. monticola*: t=17.11, adjusted R^2=0.48, p<0.001; *B. pratorum*: t=14.25, adjusted R^2=0.41, p<0.001). This indicates that it would be possible to use just one of these metrics as an indicator of wing wear in future work.

Both wing area and wing perimeter showed consistent trends across age categories, in accordance with the expectations of the study (wing area decreased in the higher categories, while wing perimeter increased). When wing area residuals were regressed against wing perimeter residuals for each species, a significant relationship was found in each case (*B. monticola*: t=17.11, adjusted R^2=0.48, p<0.001; *B. pratorum*: t=14.25, adjusted R^2=0.41, p<0.001; Fig. 3.11). Given this correlation, it would be possible to use just one of the measures to represent wing wear in future. The results from this study suggest that either metric would be appropriate; however, using wing area may be a more consistent option than wing perimeter. With increased wing damage, wing area will always decrease. Wing perimeter will initially increase as the edge of the wing becomes more ragged; however, after a certain degree of wing damage, sufficient area could be lost to cause the perimeter to decrease. This would lead to anomalous results

for individuals with a very high level of wing wear.

3.3.4 Variability in wing wear between sites in *B. monticola* and *B. pratorum*

The level of wing wear estimated using the categorical scale (Mueller & Wolf-Mueller 1993) differed significantly between sites in both species (*B. pratorum*: $\chi^2(28)= 42.04$, N = 293, $p<0.05$; *B. monticola*: $\chi^2(32)= 70.99$, N = 321, $p<0.01$). The two study species had significantly different wing wear profiles overall ($\chi^2(4)= 56.09$, N = 614, $p<0.001$). *B. pratorum* generally fell into the lower categories, while *B. monticola* showed a more even distribution between categories (Fig. 3.12).

As with the results collected using the categorical scale, there was considerable variation in the morphometric data between sites; this was true for both *B. monticola* and *B. pratorum*. Both species showed significant inter-site variation in wing area residuals (*B. monticola*: $F_{8,311}=87.79$, $p<0.001$; *B. pratorum*: $F_{7,279}=90.08$, $p<0.001$) and wing perimeter residuals (*B. monticola*: $F_{8,311}=62.86$, $p<0.001$; *B. pratorum*: Kruskal-Wallis $\chi^2(7)=150.18$, $p<0.001$). The variability in the data strongly suggests that mechanisms other than body size are influencing the accumulation of wing wear in both study species.

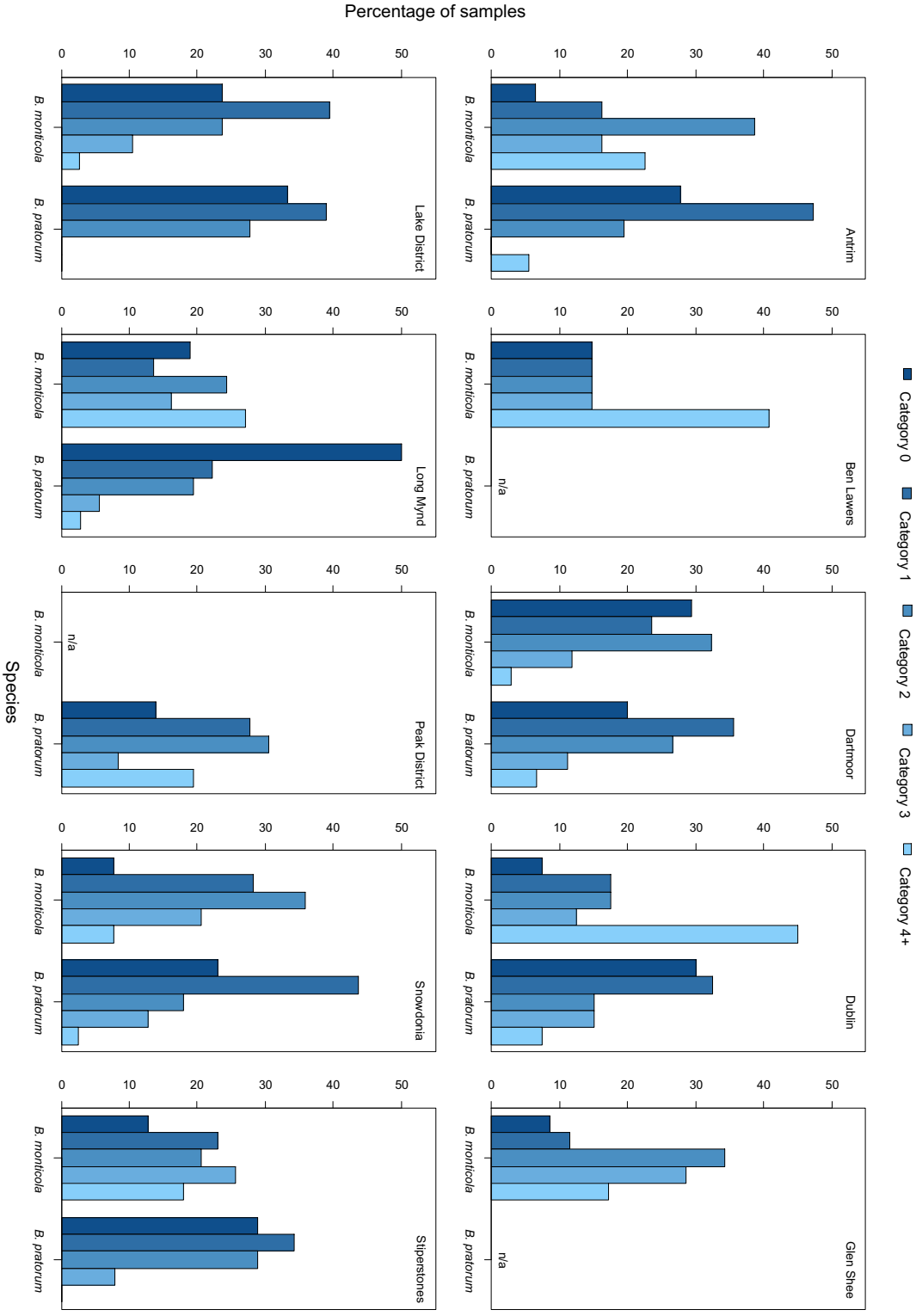

Figure 3.12. Wing wear profiles of *B. monticola* and *B. pratorum* estimated using the categorical scale developed by Mueller & Wolf-Mueller (1993). Results are expressed as percentages for each species.

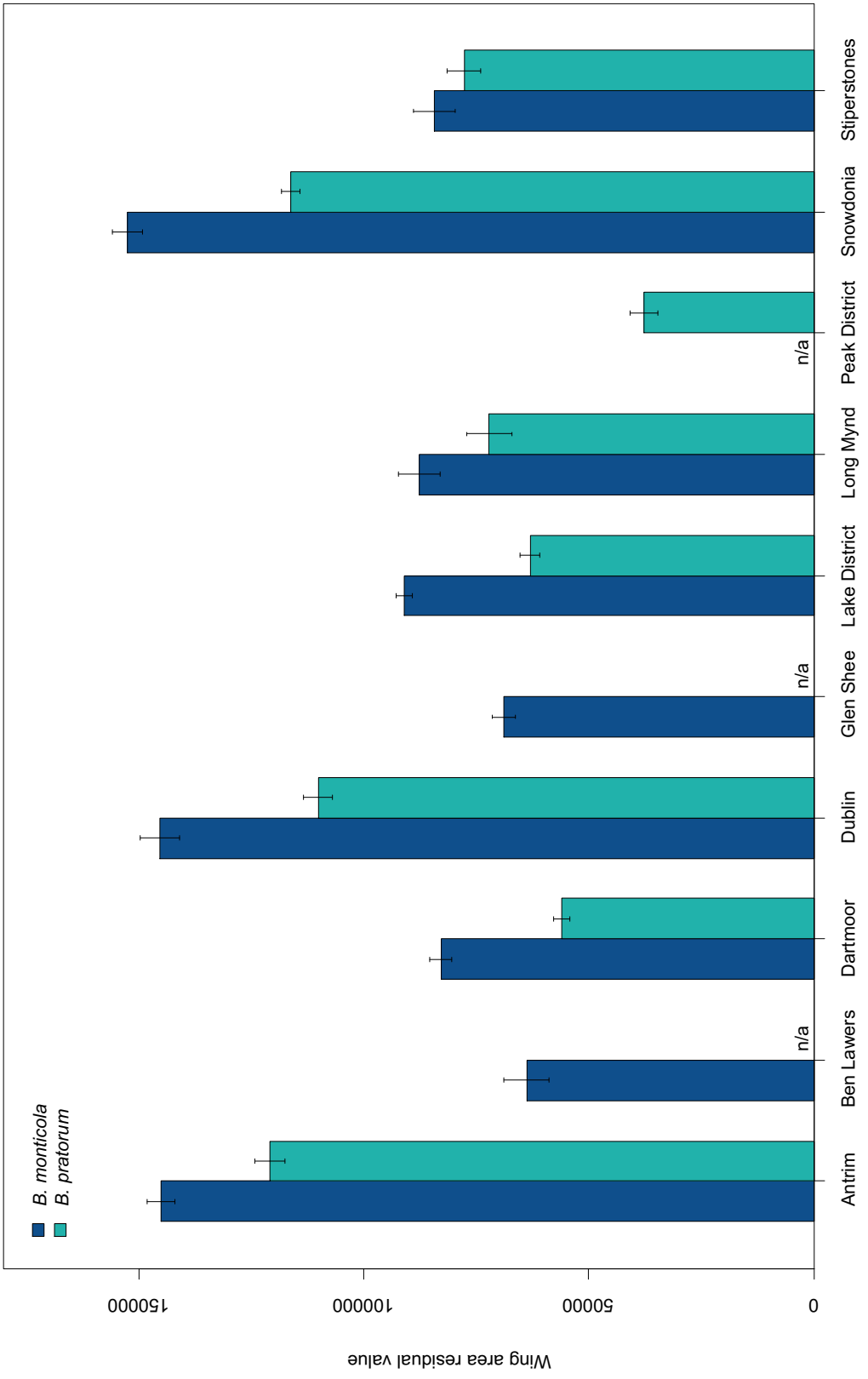

Figure 3.13. Comparison of residual values for wing area across sample sites in *B. monticola* and *B. pratorum*. Bars show mean values ± standard error.

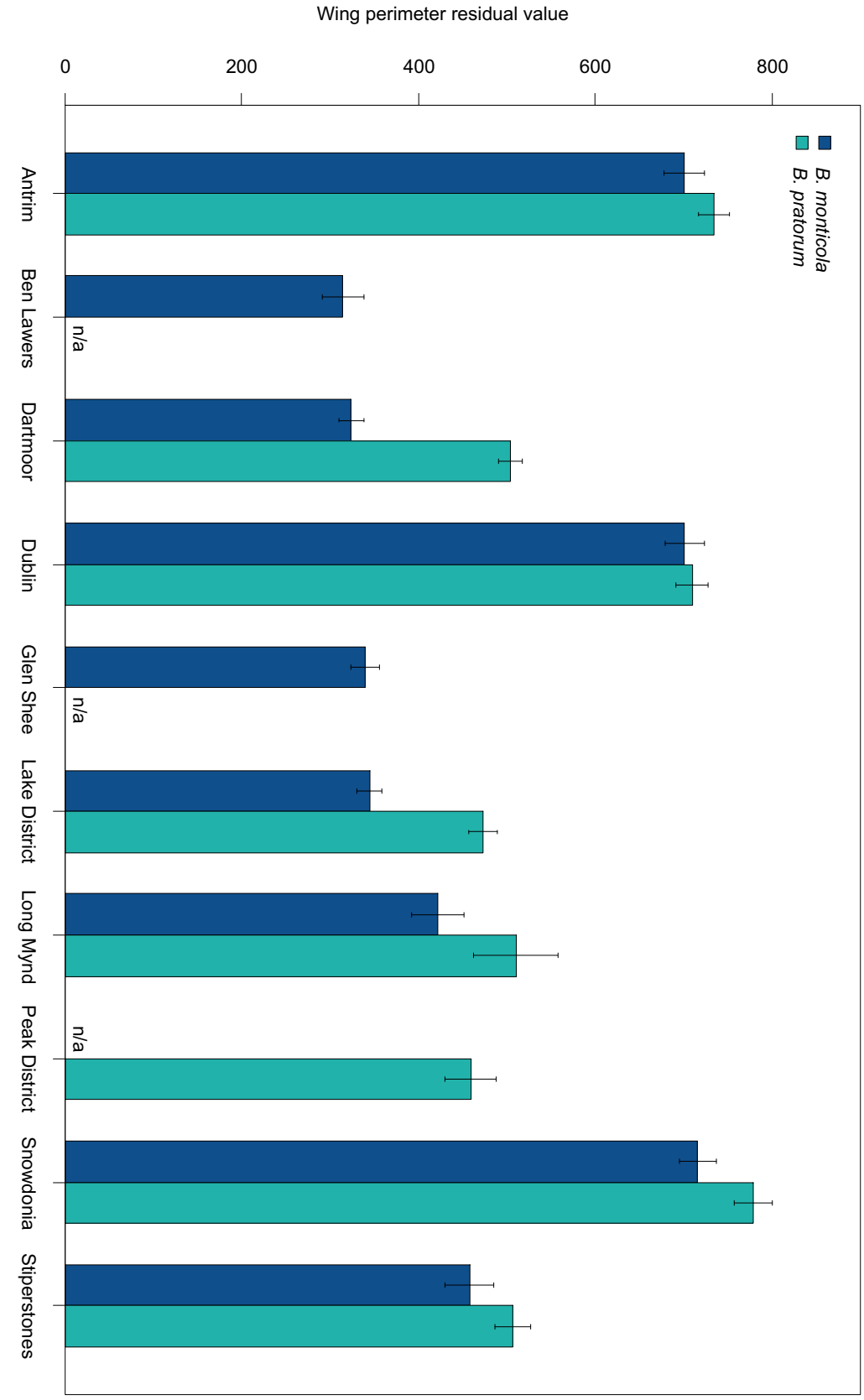

Figure 3.14. Comparison of residual values for wing perimeter across sample sites in *B. monticola* and *B. pratorum*. Bars show mean values ± standard error.

3.4 Discussion

Being able to reliably estimate the relative ages of sampled individuals is vital for a number of applications, notably studies of immunity which could be confounded by age variation (Doums et al. 2002, Whitehorn et al. 2010). However, knowing the exact age of individual insects is often not possible, particularly in the case of *Bombus* species which generally nest underground (Edwards & Jenner 2005). Flying insects accumulate wing damage throughout their lives, making wing wear an attractive proxy for individual age. Categorical scales of wing wear similar to that proposed by Mueller & Wolf-Mueller (1993) have been used for this purpose in various Hymenopteran species (López-Uribe et al. 2008, Whitehorn et al. 2010, Goulson et al. 2012). However, this method does not account for variation in body size, which is known to influence the rate of wing wear accumulation (Mueller & Wolf-Mueller 1993, Foster & Cartar 2011).

This study defined an alternative method for quantifying the level of wing wear in two *Bombus* species, and to the author's knowledge is the first to explicitly account for body size while doing so.

3.4.1 The influence of body size on wing wear

There is a great deal of size variation in *Bombus* workers, within and between colonies as well as between species (Goulson et al. 2002, Peat et al. 2005a, Couvillon et al. 2010). This variability was reflected in the results of this study. Significant inter-site variation in body size was observed in *B. monticola*, in addition to significant size differences between *B. pratorum* and *B. monticola* (*B. pratorum* workers being significantly smaller). This inherent variability in size at the colony, population, and species level, is potentially problematic when using wing wear as a proxy for age. Wing wear accumulates faster in larger individuals (Mueller & Wolf-Mueller 1993, Foster & Cartar 2011), possibly due to increased wing loading (Danforth 1989, Fischer & Kutsch 2000) or due to differences in behaviour. Larger workers are likely to spend more time foraging (Pouvreau 1989, Goulson et al. 2002), possibly due to being more tolerant to lower temperature and adverse weather (discussed by Goulson et al. 2002), or being more efficient at foraging due to increased olfactory and optical sensitivity (Spaethe et al. 2007, Kapustjanskij et al. 2007). Foraging exposes individuals to a higher risk of wing

damage via collisions with vegetation (Foster & Cartar 2011) and increased exposure to predation (Cartar 1992); thus body size may influence wing wear both directly and indirectly. This violates the assumption of a constant rate of wing wear accumulation between individuals, which is fundamental to the use of wing wear as a proxy for age. The need to account for body size is therefore clear, as acknowledged by Mueller & Wolf-Mueller (1993); however, to date there has been no simple, standard method to achieve this in the context of measuring wing wear in *Bombus*.

3.4.2 Benefits of measuring wing wear using morphometric data

Wing wear provides an easily measured proxy for age in flying insects, and using a categorical scale to grade the extent of wing damage is common for this purpose (Mueller & Wolf-Mueller 1993, Whitehorn et al. 2010, Goulson et al. 2012). An advantage to the use of a categorical scale is the speed at which samples can be processed, with no need for specialist equipment. However, the scoring process can be subjective and therefore variable between observers. To be sure of consistent results, all samples would ideally need to be scored by the same person; this may prove very time consuming, particularly for studies using large numbers of individuals. Categorical scoring is also vulnerable to the confounding effects of variable body size. This may be particularly relevant for studies of *Bombus* which, as discussed, vary widely in size both within and between colonies and species (Goulson et al. 2002, Peat et al. 2005a, Couvillon et al. 2010).

The method proposed by this study provides an alternative technique for quantifying wing wear in *Bombus*. Slightly (but not considerably) more time is required to train observers to collect the morphometric data described here. However, the low levels of variability between observers, as the subjectivity of category assignment is removed, means that scoring could be performed by several people. This would reduce the overall time required to process samples, making the overall investment of time comparable to that required for categorical scoring by one person. The other possible drawback to the use of morphometric data is obtaining high-resolution images for scoring. This study used lethally sampled individuals, so the wings could be separated from the body for ease of imaging; however, this may not be possible when studying rare and/or endangered species. Photography

has been used to measure wing morphometrics in live bees during mark-recapture studies (such as Foster & Cartar 2011), so it may be possible to refine the method in future to remove the need for lethal sampling. The use of thorax weight could also be substituted with another measure of body size, such as thorax width (as in Whitehorn et al. 2010), if lethal sampling is not possible.

There was some agreement between the results of the two methods. Higher categories showed higher levels of wing wear; wing area was negatively correlated with category, while wing perimeter was positively correlated. However, the high degree of overlap in the morphometric data between categories also highlights the difficulties of partitioning a continuous process into discrete groups. The use of continuous data, which can be easily obtained using digital images, may therefore provide a better representation of the degree of wing damage experienced by different individuals. In addition to the more precise and repeatable nature of the measurements, the main advantage of the method detailed by this study is the ability to account for variation in body size. As discussed, *Bombus* workers vary significantly in size within and between colonies (Goulson et al. 2002, Peat et al. 2005a, Couvillon et al. 2010), and this can have both direct and indirect effects on the rate of wing wear accumulation (Mueller & Wolf-Mueller 1993, Cartar 1992, Foster & Cartar 2011). Accounting for body size variation therefore allows more effective comparisons of relative levels of wing wear between individuals within and between populations. What has not been discussed by this study is inter-specific variation in body size and wing wear accumulation. The relationship of body size to both wing area and wing perimeter was markedly different in *B. monticola* and *B. pratorum*, although the correlation was positive in both cases (Fig. 3.5; Fig. 3.6). As the ratio of wing size (in terms of area and perimeter) to body size may not be consistent between species, it cannot be assumed that wing wear would accumulate at the same rate or in the same way. For this reason no inter-specific comparisons were made in this study; more research would be required into the mechanisms underlying wing wear in different species before such comparisons could be made.

The method detailed in this study still assumes a constant rate of wing wear accumulation between individuals once body size is accounted for; however, this assumption is fundamental to any method using wing wear as a proxy for age. It would not be recommended to make direct comparisons between *Bombus* species, as wing wear is likely to accumulate at different rates between species (for example due to variation in foraging and habitat preferences); this is a limitation that was

acknowledged by Mueller & Wolf-Mueller (1993), and requires more research to be resolved. However, with these caveats, the author believes that the data obtained using the method described here is of a higher quality, more accurate, and more reliable than data obtained using a categorical method. The ability to account for body size variation, shown to be an important confounding factor in this and other studies (Mueller & Wolf-Mueller 1993, Foster & Cartar 2011), means that more valid comparisons can be drawn between populations. However, even after body size has been accounted for there is still a great deal of variation between sample sites, indicating that body size is not the only contributing factor to wing wear.

3.4.3 Using wing wear as a proxy for age

While wing damage is often used as a proxy for age, in some cases the correlation between these factors may be weak (López-Uribe et al. 2008). This is likely to be due to the influence of external factors such as foraging preferences. Observational studies have suggested that wing wear accumulation is mainly caused by collision with vegetation during foraging rather than by ageing per se (Foster & Cartar 2011). This does seem intuitive, given that flying insects, especially pollinators such as bumblebees, forage in a complex and three-dimensional landscape. The large variety of habitats that bumblebees inhabit means that these effects are likely to vary widely on a site-by-site basis, and also depending on the time at which samples are collected. The change in available foraging plants over the course of the season is likely to cause the rate of wing wear to vary with time; harder vegetation including twigs and thorns is likely to cause a higher degree of damage than low-lying soft vegetation. This theory appears to be supported by the results reported here, which showed significant inter-site variability. *B. pratorum* and *B. monticola* workers in this study were found to be foraging on similar species at each sample site, though foraging preferences appeared to vary widely between sample sites (see Chapter 2). While no formal comparisons were made between the two species (for reasons already discussed), sites of particularly high or low wing wear show similar patterns in *B. monticola* and *B. pratorum*. While foraging data could only be collected at the point of sampling, and foraging behaviour may change over the course of a season, the similarities suggest a correlation between foraging behaviour and wing wear in both species. It is therefore possible that the rate of wing wear accumulation is not constant throughout the year, a factor

which may need to be considered if samples are being collected at multiple time points. A difference in nesting behaviour may also account for wing wear to some degree, as a preference for different nest sites may influence the types of vegetation that an individual routinely encounters. Without very detailed observation of the foraging and nesting behaviour of a given species, it would be difficult to account for such variation when using wing wear as a measure of age.

While there are certainly caveats to the use of wing wear, it is still a useful measure for fieldwork-based studies. While the amount of wing wear or number of injuries may not correlate with an individual worker's precise age, it would almost certainly correlate with how much time that individual has spent outside the nest and foraging. This may in fact be more relevant to consider than age itself. Flying, foraging, and other behaviours are costly to perform, and foraging rate in particular can be linked to life span (König & Schmid-Hempel 1995, Doums & Schmid-Hempel 2000). If this is the case, rather than interpreting wing wear as age specifically, it may instead be interpreted as representative of the overall effort an individual has expended over its lifetime. If wing wear is to be interpreted in this way, further study would be required into the patterns of wing wear among the castes of bees which have different life histories from workers, such as queens and males; wing wear is likely to accumulate differently, and depend on different factors, in these groups of individuals. As illustrated by the data collected in this study, the accumulation of wing wear is likely to vary considerably between *Bombus* species, due to the variability in *Bombus* as a group in terms of body size (Peat et al. 2005a), wing to body size ratio, habitat and foraging preferences (Edwards & Jenner 2005).

3.5 Conclusions

The estimation of age is crucial to effectively study many aspects of invertebrate biology, and the quantification of wing wear is a convenient proxy to use in flying insects such as *Bombus*. The categorical scale developed by Mueller & Wolf-Mueller (1993) provides a quick and easy way to do this (Whitehorn et al. 2010, Goulson et al. 2012), but is vulnerable to confounding variables such as body size variability within and between populations. The need for calibration of the method is discussed at some length by Mueller & Wolf-Mueller (1993); however, many subsequent studies have failed to do this.

The use of morphometric data as detailed in this study provides a more effective way to measure wing wear in wild-caught *Bombus* workers. By using higher resolution data about the extent of wing wear, accounting for body size, and reducing observer variability, some of the sources of error inherent to a categorical age scale are removed. However, this study also reinforces the call by Mueller & Wolf-Mueller (1993) for wing wear measures to be calibrated for use in individual species.

The data presented here highlight the complex nature of wing wear as a biological process, illustrated by the variability between populations of both *B. monticola* and *B. pratorum* after body size was accounted for. Given the likely link with foraging activity (López-Uribe et al. 2008; Foster & Cartar 2011), interpreting wing wear as indicative of foraging effort or energy expenditure, rather than age, may be a more relevant application of the data.

Chapter 4

A comparison of innate immune responses in *B. monticola* and *B. pratorum*

4.1 Introduction

Theory suggests that a reduction in genetic diversity should correlate with a reduction in fitness (Reed & Frankham 2003), and may play an important role in the extinction risk facing many populations of rare species (Frankham 2005, O'Grady et al. 2006). Heterozygosity-fitness correlations (HFCs) have been observed in a number of species including birds (Markert et al. 2004, Brouwer et al. 2007), fish (Borrell et al. 2004, Lieutenant-Gosselin & Bernatchez 2006), mammals (Da Silva et al. 2006, 2009), and insects (Tarpy 2003, Calleri et al. 2006, Baer & Schmid-Hempel 2001, Haag-Liautard et al. 2009). Small, isolated populations are known to experience reduced genetic diversity (Ellis et al. 2006), so may be expected to show lower levels of fitness. This may occur due to a reduction in immunocompetence (the ability of an individual to mount a general immune response to a pathogen or parasite; Schmid-Hempel 2003, Wilson-Rich et al. 2009). Immunocompetence is likely to have a strong impact on individual fitness by affecting susceptibility and survival, and is considered a key determinant of colony-level fitness in social insects (Baer & Schmid-Hempel 2006).

Social insect colonies provide ideal environments for the transmission of parasites and other pathogens, because many individuals live in close proximity in a stable environment (in terms of temperature, humidity etc.) and share food sources (Schmid-Hempel 1998, Hughes & Boomsma 2004, Evans et al. 2006, Tarpy & Seeley 2006, Wilson-Rich et al. 2009). An effective immune system is therefore vital to protect against infection. Unlike the adaptive immune system found in vertebrates, invertebrates rely on an innate immune response to protect against infection and parasitism (Schmid-Hempel 2005, Schulenburg et al. 2007). There are a number of key components to this innate response, including haemolymph coagulation (Muta & Iwanaga 1996, Iwanaga 2002, Theopold et al. 2004), melanisation (Söderhäll & Cerenius 1998, Cerenius & Söderhäll 2004, Cerenius et al. 2010,b), and the production of antimicrobial substances (Tzou et al. 2002, Schmid-Hempel 2005).

The process of melanisation is important for combating pathogens (particularly parasites) via encapsulation. This involves coating the pathogen in melanised haemocytes, forming a capsule which kills the pathogen inside. Other substances produced by the melanisation cascade (such as quinones and reactive oxygen species) can also act to kill pathogens (Nappi & Ottaviani 2000). The encap-

sulation response can be measured in a laboratory environment using implants of sterile nylon wire (König & Schmid-Hempel 1995). However, this requires live specimens which can be problematic for logistical reasons; this is particularly true for studies sampling over wide areas and time periods, where storing bees alive would be close to impossible. Many studies have therefore used an *in vitro* method to quantify this immune response, using extracted haemolymph to measure the activity of the enzyme phenoloxidase (PO), which plays a vital role in the melanisation cascade (Söderhäll & Cerenius 1998). While some active PO is present in the haemolymph, some is also present as the inactive precursor to PO, pro-phenoloxidase (pro-PO). This becomes activated in response to an immune challenge or cell damage, responding to the presence of pathogen associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) and peptidoglycans (Cerenius et al. 2008, Bidla et al. 2009; Fig. 4.1). By combining a sample of haemolymph with 3,4-Dihydroxy-L-phenylalanine (L-DOPA) and measuring the rate of conversion of L-DOPA to dopachrome using a spectrophotometer (Moret & Schmid-Hempel 2009) it is possible to quantify the activity of PO and, by proxy, the melanisation response. By adding bovine α-chymotrypsin to the reaction to activate residual pro-PO, it is also possible to compare the potential vs active immune response.

The melanisation process is an effective defence against parasites and other pathogens, but is usually limited to a rapid, localised response (Cerenius & Söderhäll 2004, Korner & Schmid-Hempel 2004, Cerenius et al. 2008; discussed later). Other responses, which are slower to activate, provide protection over a longer time period. This includes the production of antimicrobial peptides (AMPs). While the structure of AMPs in insects varies greatly, they can be broadly grouped into three classes; cysteine-rich peptides (the defensins), proline-rich peptides (such as abaecin and apidaecin), and glycine-rich peptides (hymenoptaecins; Bulet et al. 1999), which are targeted to different forms of pathogen (Bulet et al. 1999, Imler & Bulet 2005). The process by which AMP production is activated is still not well understood in invertebrates; the majority of studies of insect antimicrobial peptide production have been conducted using the fruit fly *Drosophila melanogaster* (Choe et al. 2002, Lemaitre et al. 1996), but the mechanisms are likely to be highly conserved across taxa (Schmid-Hempel 2005, Lebestky et al. 2000). After infection, pathogens must be recognised as non-self. This occurs through the action of recognition proteins; these recognition molecules are currently poorly understood, although some have been described in *D. melanogaster*, such as Peptidoglycan

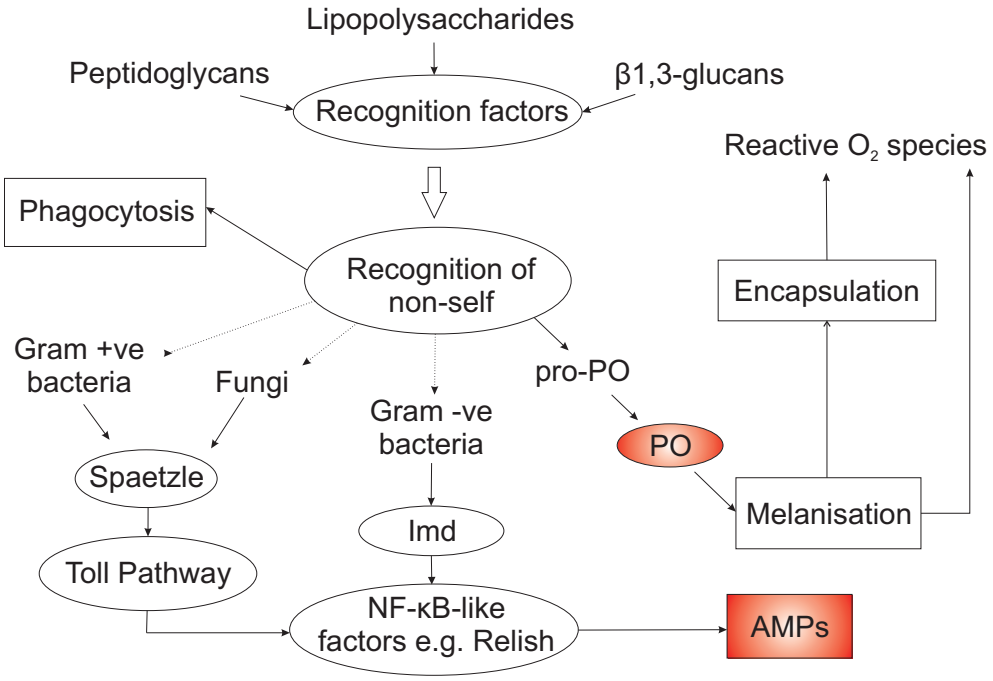

Figure 4.1. Illustration of the key aspects of the insect innate immune response (based on Tzou et al. 2002). The responses targeted by this study are highlighted in red. The final responses are shown in boxes, while other processes, receptors and pathways are shown in ellipses. The assays used in this study focus on the activity of phenoloxidase (PO) and antimicrobial peptides (AMPs); this combination will show how well an individual would be able to respond to a variety of pathogens, as the assays cover the main responses for both microbial and parasitic challenges.

Recognition Protein (PGRP), necessary for activation of the Relish transcription factor and initiation of AMP production (Choe et al. 2002). The recognition factors act on pathogen surface molecules such as peptidoglycans and LPS, and subsequently combine with a cytokine-like protein (Spaetzle) to cause a cascade of reactions through the Toll pathway (triggered by gram-positive bacteria and fungi) and/or immune deficiency (Imd) pathway (triggered by gram-negative bacteria). This results in the activation of AMP production genes via NF-κB-like factors such as Dorsal, Dif and Relish (reviewed in Khush et al. 2002; see Fig. 4.1). AMPs are then produced in the fat body (the functional equivalent of the liver in mammals) and released into the haemolymph (Bulet et al. 1999). The presence of these peptides can be measured *in vitro* using extracted haemolymph for a zone of inhibition assay (Moret & Schmid-Hempel 2000, Whitehorn et al. 2010).

Initiating and maintaining an immune response is costly to the individual, and has been correlated with a reduction in reproductive output in *B. terrestris* (Moret & Schmid-Hempel 2000), reduced body condition in Lepidoptera (Cotter et al. 2004,

Talloen et al. 2004), and reduced life expectancy in the beetle *Tenebrio molitor* (Armitage et al. 2003). In some cases the costs of the immune response may be hidden, as individuals may change behaviours to compensate. For example, *B. terrestris* workers show no negative effects when responding to an immune challenge, unless they are denied the opportunity to increase their foraging activity and therefore their food intake; under starvation conditions, mounting an immune response is correlated with a significant reduction in life span (Moret & Schmid-Hempel 2000). Some immune responses can also have autoreactive (self-harming) effects (Nappi & Ottaviani 2000). This is particularly true of the melanisation response; many of the by-products produced, such as reactive oxygen species and quinones, are effective at killing pathogens but are also toxic to the host (Nappi & Ottaviani 2000; Sadd & Siva-Jothy 2006). For this reason, melanisation has been observed to be a localised, fast-acting but short-lived response (Cerenius & Söderhäll 2004; Cerenius et al. 2008). AMP production, while slower acting, is not cytotoxic (Bulet et al. 1999) but does increase the requirement for dietary protein (Brunner et al. 2014). All these costs mean that a balance must be struck between maintaining resistance, minimising the energetic cost, and preventing harm to the host. There is suggestion that trade-offs may exist between different immune functions to achieve this. Under laboratory conditions *B. terrestris* workers showing increased AMP activity have reduced levels of PO activity (Moret & Schmid-Hempel 2001), while increased PO activity in the cotton leafworm *Spodoptera littoralis* is correlated with reduced lysosyme activity (Cotter et al. 2004). The relative levels of investment in the different defence mechanisms can vary depending on environmental cues. For example, *Spodoptera* larvae in higher density populations show an elevated level of PO activity due to the increased likelihood of parasitism; individuals at lower densities have a lower risk of infection, and lower their investment in the PO mechanism accordingly (Wilson et al. 2001).

B. monticola exists in isolated populations across the UK and Ireland, while *B. pratorum* is widespread and continuous in its distribution across the same area (BWARS 2014; Fitzpatrick & Murray 2014; see Chapter 2). As these species coexist at the same sites with relatively similar foraging habits (see Chapter 2), they provide an ideal system in which to test the assumptions linking population isolation with fitness. Assuming population isolation to be linked with genetic diversity (which has been demonstrated in *Bombus*; Zayed et al. 2004, Packer et al. 2005, Zayed et al. 2005, Ellis et al. 2006), it may be expected that *B. monticola*

would show reduced immunocompetence relative to its more ubiquitous congener *B. pratorum*.

This study aimed to address the following questions:

1. Is there significant variation in immuncocompetence between *B. monticola* and *B. pratorum*?

2. Is there significant intra-specific variation in immunocompetence in *B. monticola* and/or *B. pratorum*?

4.2 Methods

4.2.1 Haemolymph extraction

The majority of studies on immunocompetence in *Bombus* species have used samples of haemolymph collected from live bees (Brown et al. 2003; Moret & Schmid-Hempel 2009). Due to the problem of sample storage in remote locations, it was not feasible to use live bees for this project. Samples were therefore freeze-killed and stored at $-24°C$ using a portable freezer unit (Heavy Duty 22 litre Compressor Fridge/Freezer T0022, Euroengel). Existing protocols were then adapted to allow the use of freeze-killed specimens. Collecting haemolymph from the abdomen using micro-capillary tubes (Brown et al. 2003; Moret & Schmid-Hempel 2009) was found to be extremely time-consuming, and in many cases ineffective when using freeze-killed specimens (less than 1μl collected). There was also the risk that invasive methods of collection could damage internal tissues and organs required for the assessment of parasite load. It was therefore decided to extract haemolymph by homogenising the thorax on ice in phosphate-buffered saline (PBS: 8.74 g NaCl, 1.78g Na_2HPO_4 $2H_2O$, 1000 ml distilled H_2O, pH 6.5) as in Whitehorn et al. (2010). To account for variation in body size within and between the two study species, the thorax was weighed using an analytical balance, and the volume of PBS added was adjusted to give 500mg thorax per ml. Each thorax was homogenised on ice using a sterile micropestle, then centrifuged at 4°C for 10 minutes at 13,200g. The supernatant was then removed and snap frozen in liquid nitrogen before being stored at $-20°C$.

4.2.2 Quantifying phenoloxidase (PO) activity

Previous studies have used 20μl of haemolymph solution for assays of PO activity (Moret & Schmid-Hempel 2009; Whitehorn et al. 2010), but the volume of haemolymph extracted from a frozen specimen was generally not sufficient to allow both active and total PO to be tested in this way. This was especially true for *B. pratorum* given the small body size of workers. Existing protocols were therefore adapted to use a smaller volume of haemolymph. Samples were defrosted on ice, and diluted by adding additional sterile PBS. The final protocol used 5μl of sterile PBS added to 10μl of haemolymph solution before use. For active PO, 5μl of diluted haemolymph was added to 175μl of PBS and 20μl of L-DOPA solution (4mg ml^{-1}, Sigma) in a flat-bottomed 96-well plate (Starlab). For total PO, 5μl of the haemolymph dilution was added to 155μl PBS and 20μl bovine α-chymotrypsin solution (2.1mg ml^{-1}, Sigma). This was allowed to incubate for 5 minutes at room temperature before the addition of 20μl L-DOPA solution as previously described. The reaction was then allowed to proceed for 88 minutes in the spectrophotometer (FLUOstar OMEGA 415-1244, BMG Labtech) at 30°C. Readings were taken at 480nm every 2 minutes throughout. Three replicates were run of each assay – the mean of the three values was used for analysis. The rate of reaction (V) was taken to be the linear phase of the reaction. Any reactions showing no linear phase or a downwards linear phase were taken to have a rate of 0. Controls were run in each plate to check for the oxidation of L-DOPA over time. For the active PO assays the control used was PBS with L-DOPA. For total PO, the control was L-DOPA, PBS and α-chymotrypsin. Any reaction observed in the control wells was subtracted from the rate of reaction in the sample wells before analysis.

4.2.3 Measuring antimicrobial peptide (AMP) activity

The protocol for the zone of inhibition assay was adapted from a standard protocol (Moret & Schmid-Hempel 2000). All haemolymph samples were initially centrifuged for 2 minutes at 8000g to remove any residue from the haemolymph extraction process and reduce the risk of contamination. The supernatant was then carefully removed and used for the assays. *Arthrobacter globiformis* (NCIMB Ltd.) was grown in a sterile brain-heart infusion broth, and adjusted to give 10^8 cells ml^{-1}. 100μl of bacterial suspension was mixed with 10ml of sterile Mueller-

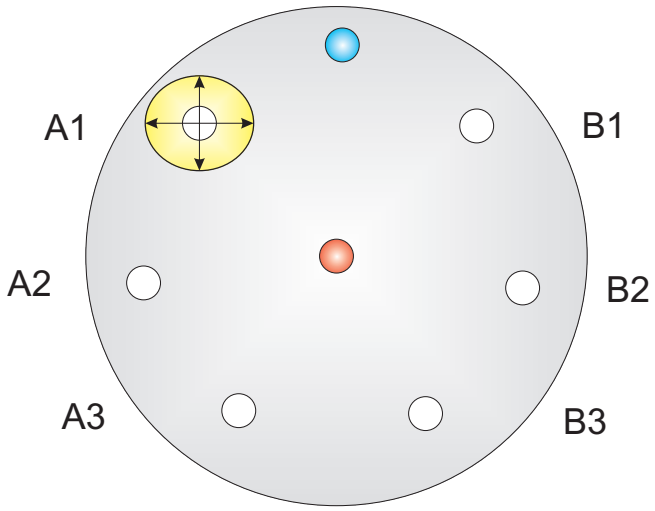

Figure 4.2. Layout of agar plates used for zone of inhibition assays. Agar plates were all 9cm in diameter, containing 10ml of Muller-Hinton agar. White circles indicate wells containing haemolymph samples. Wells A1-A3 represent three replicates of one sample, wells B1-B3 represent three replicates of a second sample. The red circle indicates the well containing the tetracyclin positive control; the blue circle indicates the location of the well containing PBS as a negative control. All wells were 2mm in diameter. The yellow area indicates a zone of inhibition, with arrows showing where measurements would be taken.

Hinton agar (Oxoid) at 48°C, and poured into a sterile 9cm petri dish (Sterelin). The dish was swirled to create a thin layer of agar and ensure even dispersal of the bacteria. Once the agar had set, eight evenly spaced 2mm wells were created. 3μl of haemolymph solution was added to six of the wells (three replicates of each of two samples, one from each study species). Each plate also contained one well of 3μl sterile PBS as a negative control and one well of 3μl of tetracyclin (0.125mg ml^{-1}) as a positive control (Fig. 4.2). The plates were sealed with Parafilm® to prevent drying out, and incubated overnight at 27°C. Following incubation, the diameter of any clear zone around each well was measured using digital callipers at the widest and narrowest points. The mean was taken of all measurements for each sample. To account for any differences between plates, such as slight temperature variation within the incubator, average readings were adjusted before analysis to be a percentage of the clear zone produced by the positive control.

4.2.4 Statistical analysis

All tests were performed using R (v 2.15.2). The rate of reaction with and without the addition of α-chymotrypsin was compared separately for each species using Welch's t-tests; data were square-root transformed before testing to meet the as-

sumption of normality. PO activity data was analysed separately for each species using ANOVA, with thorax weight, age and sample site as fixed factors. Interactions were included between all terms. Residuals from all ANOVA analyses were visually checked for normality and homogeneity; in the event of the assumptions of ANOVA not being met, data were square-root transformed and re-checked. Comparisons between the two species were made on a site-by-site basis, again using ANOVA.

The proportion of samples with a zone of inhibition present was initially compared between *B. monticola* and *B. pratorum* overall using a χ^2 test. Comparisons were then made between the two species on a site-by-site basis, using χ^2 tests or Fisher's exact tests when values were too small to allow χ^2. Factors influencing the presence/absence of zones of inhibition were analysed separately for *B. monticola* and *B. pratorum*, using a GLM with a binomial distribution. Thorax weight, age, and sample site were treated as covariates. Interactions between all terms were also included. Covariates were mean-centred before analysis to reduce any possible correlation of effects. Models were simplified using step-wise likelihood ratio testing based on the AIC value. When zones were present, factors influencing the zone diameter were analysed separately for each species using ANOVA with thorax weight, age, and sample site as fixed effects. The results for zone diameter were then compared between species on a site-by-site basis, again using ANOVA. The residuals of all ANOVA analyses were visually checked for normality and homogenetiy. Age in all cases was approximated using wing area adjusted for body size (as described in Chapter 3).

4.3 Results

4.3.1 Phenoloxidase (PO) activity

The addition of α-chymotrypsin made no significant difference to the rate of reaction in either species (*B. monticola*: t=-1.4247, df=603, p=0.155; *B. pratorum*: t=-0.2094, df=501, p=0.834; Fig. 4.3). This may be because pro-PO present in the haemolymph was activated during the process of haemolymph extraction; PO activation has been observed to occur as a response to the presence of PAMPs, but also as a response to heat stress and cell damage (Cerenius et al. 2010). The

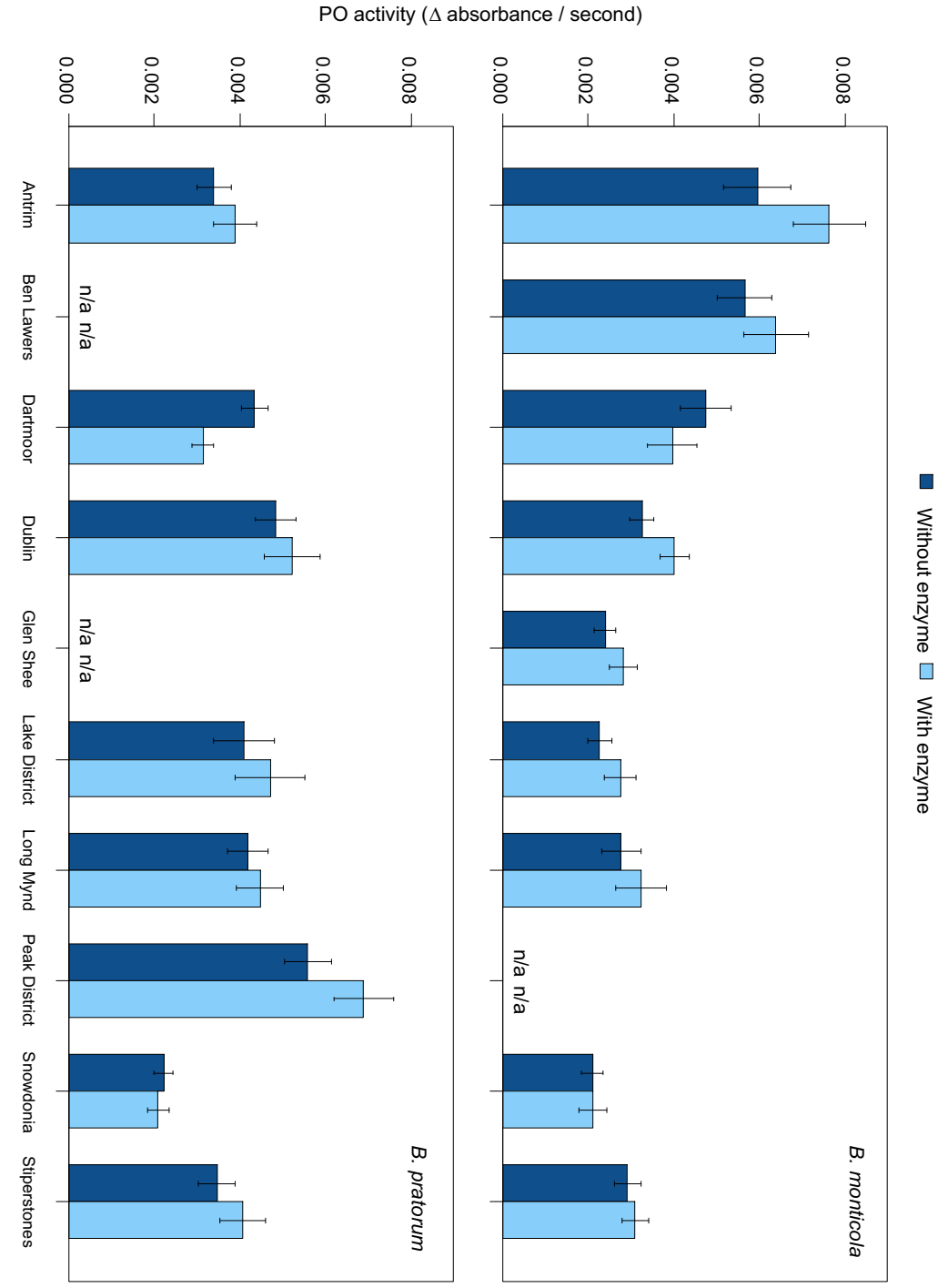

Figure 4.3. Comparison of PO activity with and without the addition of α-chymotrypsin to activate any pro-PO present in the sample. Bars show mean values $\pm$ standard error.

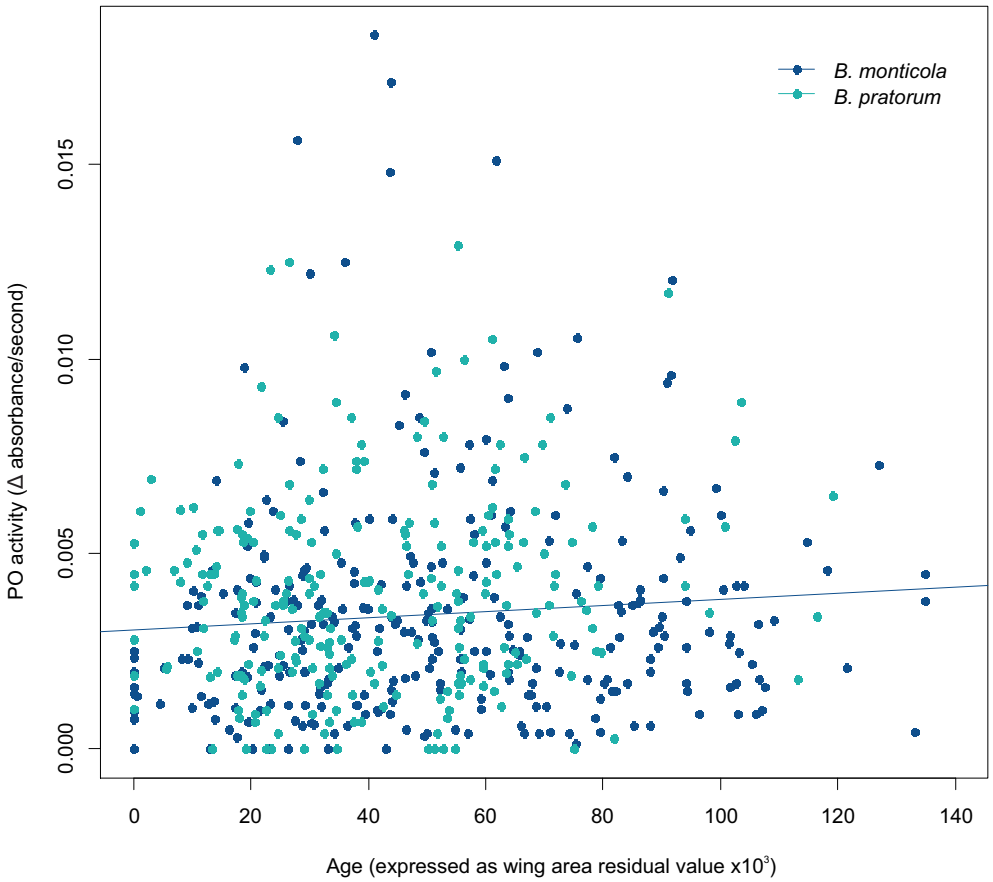

Figure 4.4. A negative relationship was observed between PO and age in both species, with older individuals showing slightly lower levels of PO activity. This trend was significant in *B. monticola*, but only close to significant in *B. pratorum*. Age was estimated using a measurement of wing area adjusted for body size (see Chapter 3). As bees accumulate wing wear throughout their lifetime, older bees would be expected to have a relatively smaller wing area for their body size when compared to younger bees. Trend lines show the least squares regression lines from the original ANOVA.

process of crushing the thorax to release haemolymph is likely to have triggered this process, even though the extraction was performed on ice to reduce the likelihood of heat activation. It was also noted that the reactions containing bovine α-chymotrypsin were less consistent; this is probably because the addition of an extra component increased the variation in background absorbance of the samples. Due to the lack of any significant effect of the enzyme, combined with the reduced reliability of the assay containing bovine α-chymotrypsin, only the active PO assay results were analysed further.

For *B. pratorum* there was no significant effect of thorax weight on PO activity ($F_{1,233}$=1.31, p=0.253). There was also no significant effect of age, although the results were close to significant ($F_{1,233}$= 3.57, p=0.06). Although the trend was

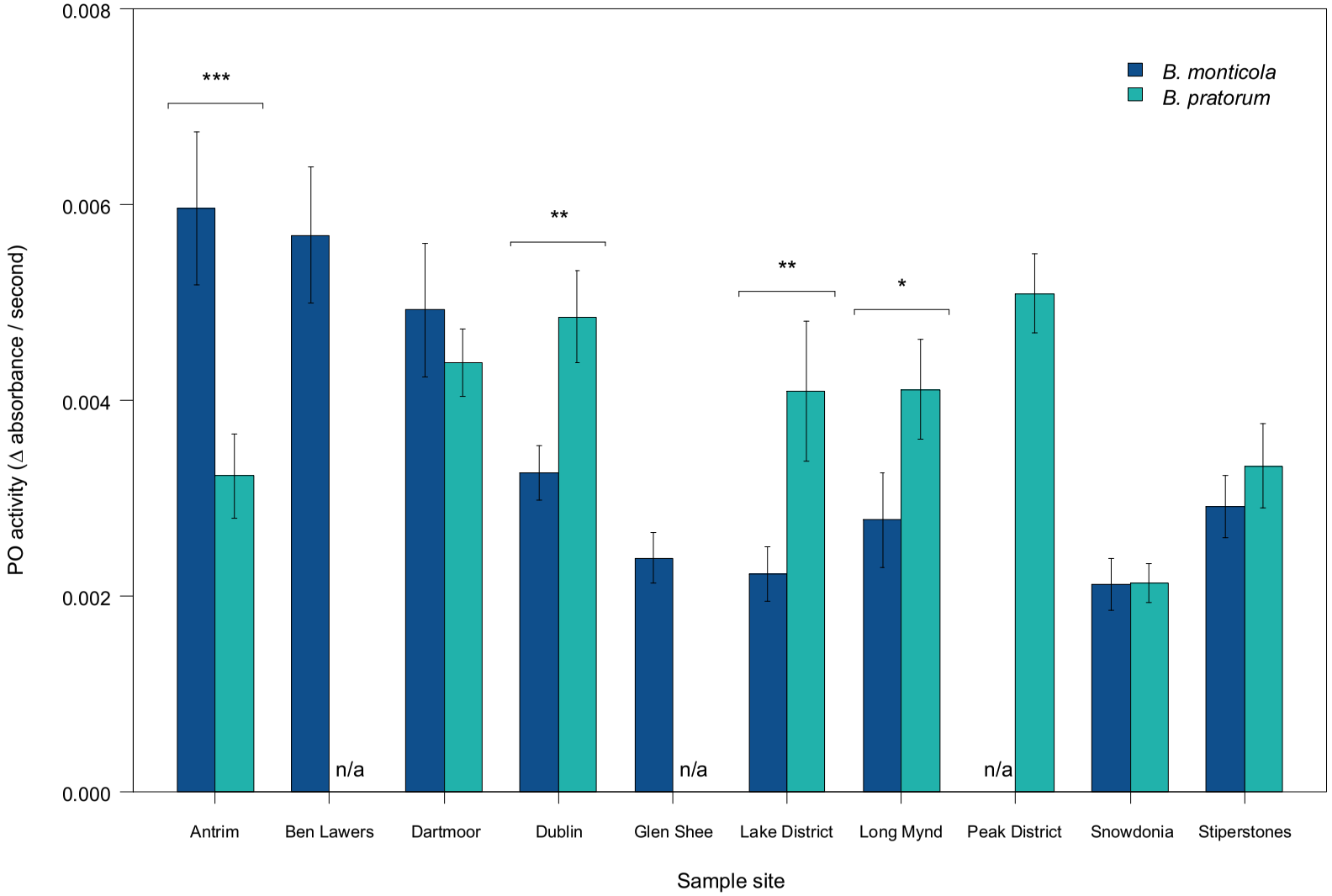

Figure 4.5. Comparison of mean PO activity between *B. monticola* and *B. pratorum*. Bars show mean values ± standard error. Spaces marked as "n/a" indicate where samples were not processed for that species and/or site. Comparisons were made using ANOVA on a site-by-site basis. Significant differences between species are indicated with asterisks above the relevant bars (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

quite weak, PO activity was lower in older bees, which agrees with the results of previous studies (such as Whitehorn et al. 2010). There was a significant variation in PO activity between sample sites ($F_{7,233}$=6.02, p<0.001). There were no significant interactions. Thorax weight had a significant effect on PO activity in *B. monticola* ($F_{1,274}$=7.21, p<0.01); there was also a significant interaction between thorax weight and sample site ($F_{8,274}$=2.66, p<0.01). This is likely to be due to the significant variation in thorax weight in *B. monticola* workers between sample sites (see Chapter 3). Age was found to have a significant effect on PO activity ($F_{1,274}$=35.72, p<0.001), with older individuals showing lower PO activity than younger individuals, as in the *B. pratorum* samples. There was also significant variation in PO activity between sample sites ($F_{8,274}$=4.05, p<0.001). There were no other significant interactions.

Table 4.1. Comparisons of PO activity between *B. monticola* and *B. pratorum* within sample sites

	F value	p value
Antrim	$F_{1,41}$=12.934	<0.001 ***
Dartmoor	$F_{1,62}$=0.096	0.758
Dublin	$F_{1,74}$=9.218	<0.01 **
Lake District	$F_{1,51}$=8.658	<0.01 **
Long Mynd	$F_{1,39}$=4.317	<0.05 *
Snowdonia	$F_{1,66}$=0.041	0.841
Stiperstones	$F_{1,70}$=0.378	0.541

Comparisons of PO activity between *B. monticola* and *B. pratorum* within sample sites. Comparisons were made using ANOVA. Significant results are highlighted with asterisks (* = p<0.05; ** = p<0.01; *** = p<0.001).

Due to the significant variation of PO activity between sample sites in both species, comparisons between species were made on a site-by-site basis using ANOVA. Apart from the samples at the Dublin and Lake District sample sites, all data were square-root transformed to meet assumptions of normality. Significant differences in mean PO activity were observed between species at the Antrim, Dublin, Long Mynd, and Lake District sample sites (Fig. 4.5; Table 4.1). PO activity was higher in *B. pratorum* at Dublin, Lake District and Long Mynd sites, but was substantially higher in *B. monticola* at the Antrim sample site. No significant

differences were observed at the Dartmoor, Snowdonia, or Stiperstones sample sites (Table 4.1).

4.3.2 Antimicrobial peptide (AMP) activity

In both species there was significant variation between sample sites in the proportion of samples with a zone of inhibition present (*B. pratorum*: $\chi^2(7)=37.45$, $p<0.001$; *B. monticola*: $\chi^2(7)=48.05$, $p<0.001$). Significant differences were observed between species in the proportion of samples with a zone present at the Antrim and Dartmoor sample sites (Fig. 4.6; Table 4.2), but no significant differences were observed at the Dublin, Long Mynd, Stiperstones, Snowdonia, or Lake District sample sites (Table 4.2). In *B. pratorum* no significant effect of thorax weight (z=0.81, p=0.420) or age (z=-0.93, p=0.353) was observed on the presence/absence of zones of inhibition. Similarly, in *B. monticola* no significant effect of thorax weight (z=-0.64, p=0.524) or age (z=-1.46, p=0.146) was seen on zone presence. There were no significant interaction terms in the analysis for either species.

Table 4.2. Comparisons of zone of inhibition presence/absence between *B. monticola* and *B. pratorum* within sample sites

	χ^2 value	p value
Antrim	-	<0.05 *
Dartmoor	7.03	<0.01 **
Dublin	-	0.140
Lake District	-	0.538
Long Mynd	-	>0.99
Snowdonia	-	0.359
Stiperstones	-	0.223

Comparisons of the proportion of samples producing a zone of inhibition diameter between *B. monticola* and *B. pratorum* within each sample site. Comparisons were made using χ^2 or Fisher's exact tests. Where χ^2 tests were used, the test statistic is reported. Significant results are highlighted with asterisks (* = $p<0.05$; ** = $p<0.01$).

No significant interaction terms were found in either species for any of the tested

variables with zone diameter, so results are reported for main effects only. In the *B. pratorum* samples there was no significant effect of thorax weight on zone diameter ($F_{1,157}$=0.92, p=0.340), but significant effects were observed for both age ($F_{1,157}$=7.40, p<0.01) and sample site ($F_{7,157}$=2.95, p<0.01). In the *B. monticola* samples a significant effect of thorax weight ($F_{1,171}$=7.99, p<0.01), and age ($F_{1,171}$=8.39, p<0.01), was observed on zone diameter. There was also significant variation between sample sites ($F_{7,171}$=3.99, p<0.001).

Table 4.3. Comparisons of zone of inhibition diameter between *B. monticola* and *B. pratorum* within sample sites

	F value	p value
Antrim	$F_{1,34}$=0.56	0.461
Dartmoor	$F_{1,34}$=0.17	0.681
Dublin	$F_{1,64}$=0.45	0.503
Lake District	$F_{1,32}$=0.03	0.874
Long Mynd	$F_{1,22}$=0.06	0.807
Snowdonia	$F_{1,29}$=2.11	0.158
Stiperstones	$F_{1,44}$=1.36	0.250

Comparisons of zone of inhibition diameter between *B. monticola* and *B. pratorum*. Comparisons were made using ANOVA.

Inter-species comparisons of zone diameter were made on a site-by-site basis (due to strong variability between sites) using ANOVA. No significant differences were observed at any of the sample sites (Fig. 4.8; Table 4.3).

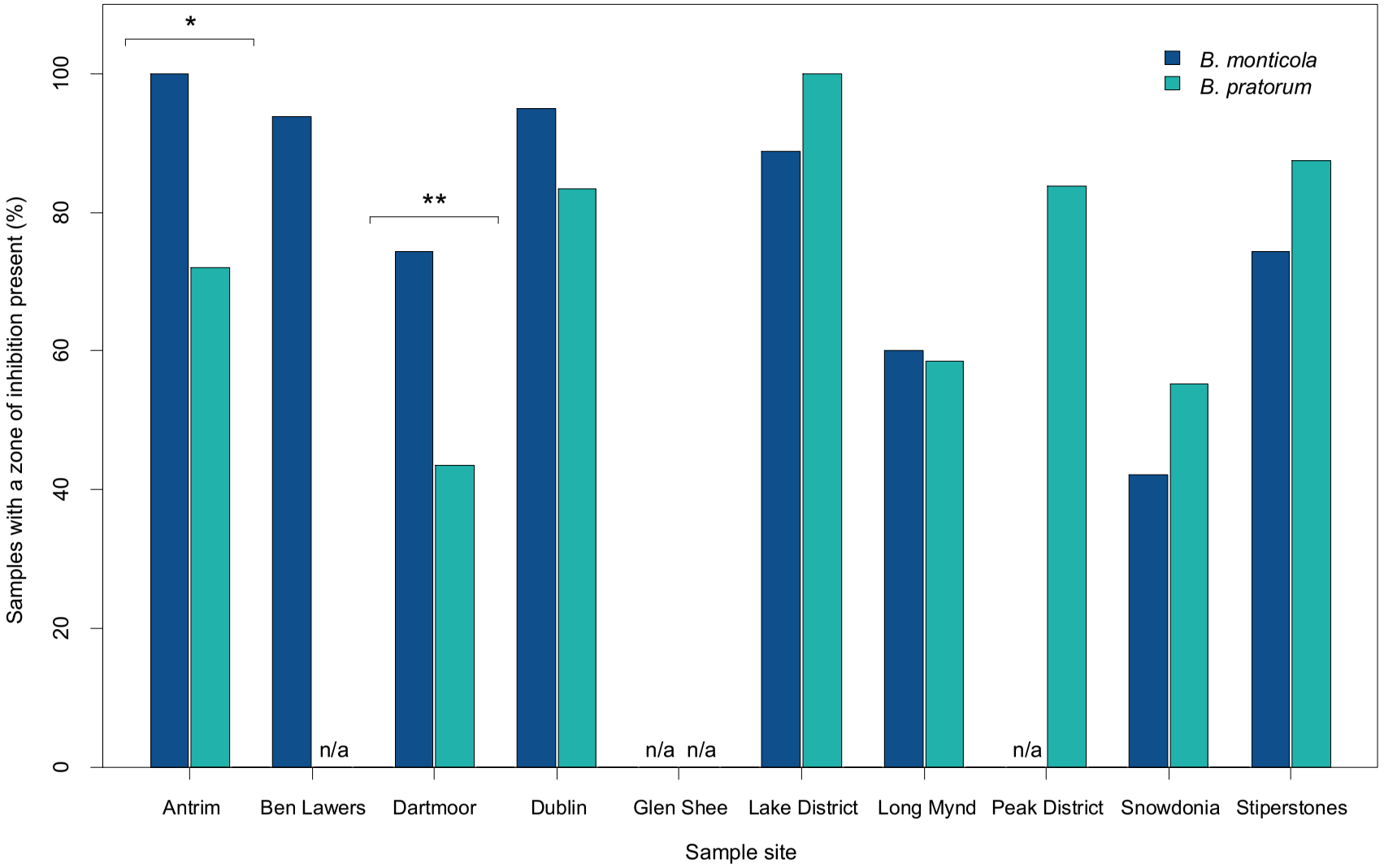

Figure 4.6. Comparison of the proportion of samples which produced a zone of inhibition between *B. pratorum* and *B. monticola*. Results are expressed as a percentage. Significant differences are indicated with an asterisk above the relevant bars (* = $p<0.05$; ** = $p<0.01$). Gaps labelled n/a indicate no samples were processed for that species and/or site.

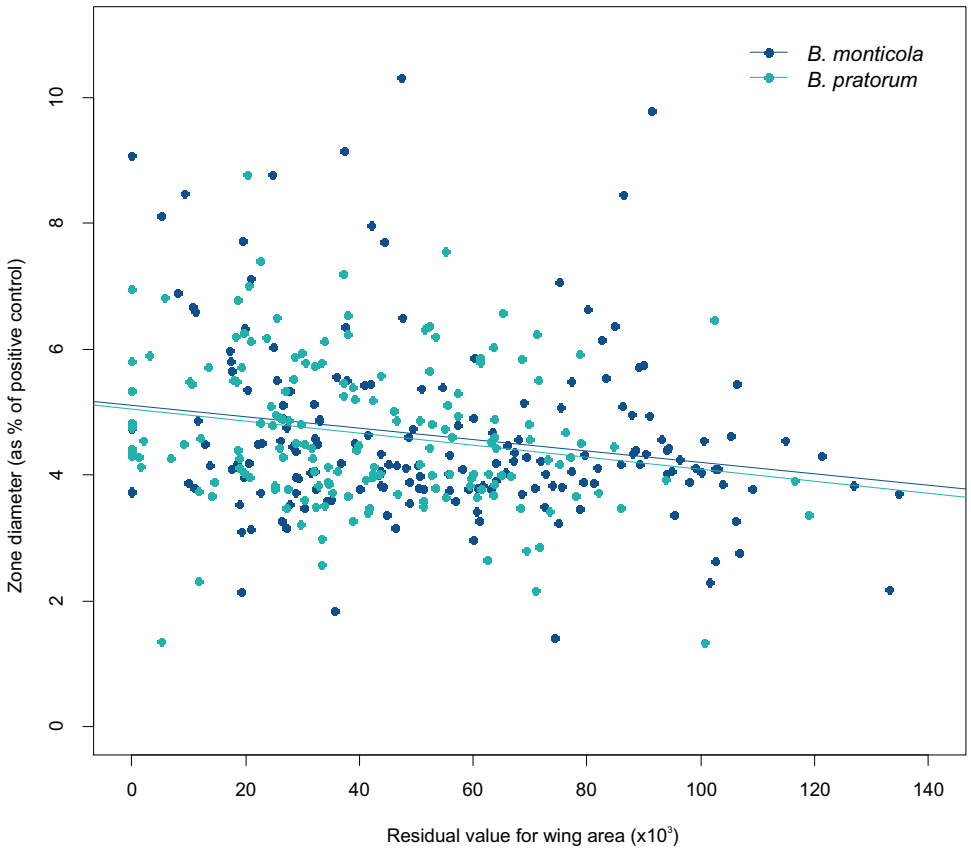

Figure 4.7. Correlation of zone of inhibition diameter with age in *B. pratorum* and *B. monticola*. Zone of inhibition diameter was significantly positively correlated with age in both species. Trend lines show the least squares regression line calculated from the original ANOVA model. Age was approximated by wing area adjusted for body size (see Chapter 3).

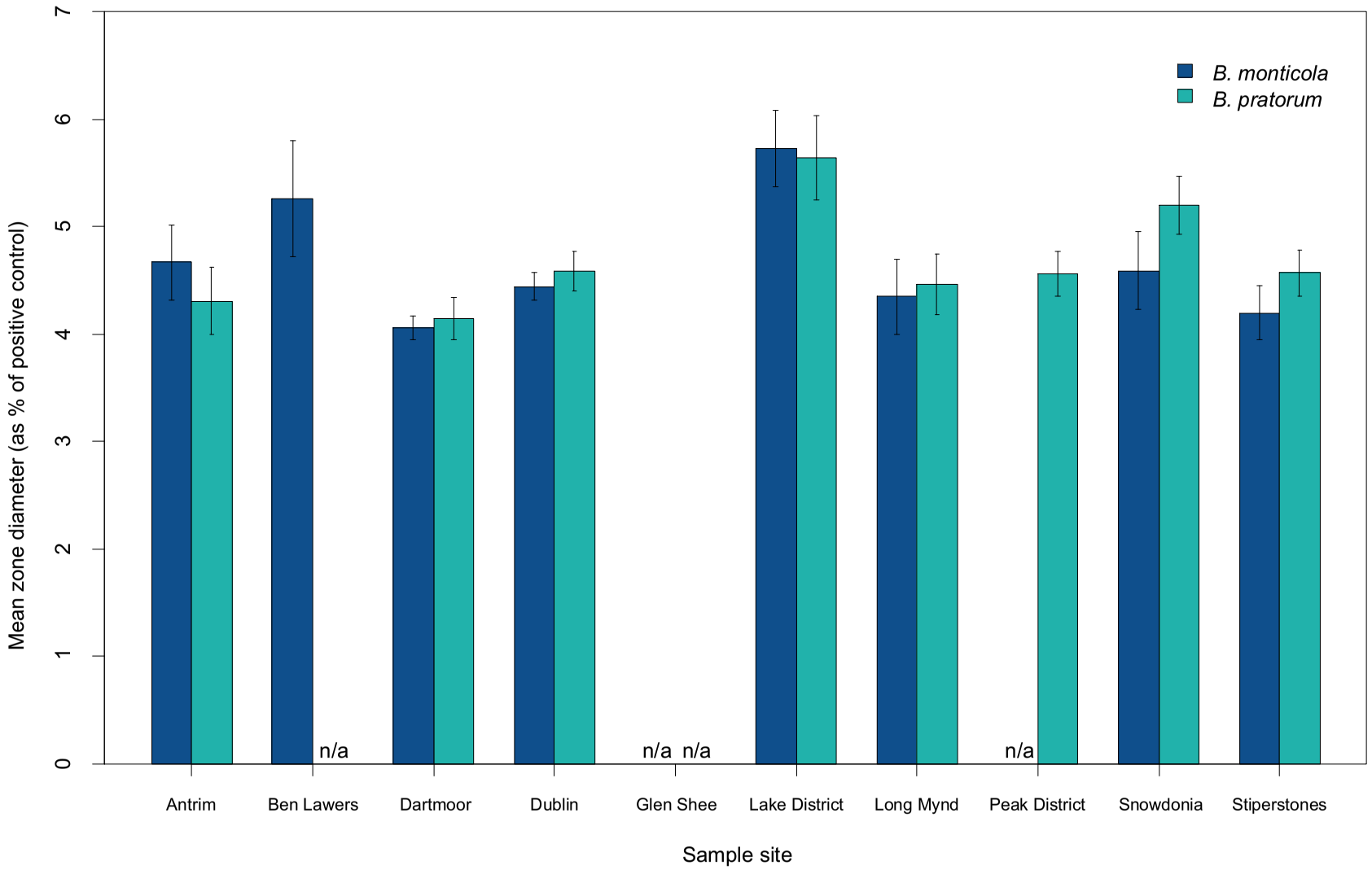

Figure 4.8. Comparison of mean zone of inhibition diameter (expressed as % of the positive control) between *B. monticola* and *B. pratorum*. Data shown are for samples with positive AMP activity only, and displayed as means ± standard error. Spaces labelled n/a indicate where no samples were analysed for that species and/or site (due to lack of samples or insufficient haemolymph available after PO analysis). No significant differences were observed between the two species at any of the sample sites.

4.4 Discussion

The ability to mount an immune response is a crucial component of individual fitness. In the case of eusocial insects such as *Bombus*, individual fitness is inherently linked to colony-level success, and hence to population level viability (Baer & Schmid-Hempel 2006). A reduction in immunocompetence would have significant implications for the long-term survival of populations, as infection by parasites and pathogens can have significant effects on reproduction and colony success (Durrer & Schmid-Hempel 1994, Brown et al. 2003b,c, Otti & Schmid-Hempel 2007, 2008, Rutrecht & Brown 2009). Immunocompetence, including phenoloxidase (PO) and antimicrobial peptide (AMP) activity, has been positively linked with genetic diversity at neutral loci in a number of species including *Bombus* spp. (Hughes & Boomsma 2004, Spielman et al. 2004, Whitehorn et al. 2010). Given that genetic diversity becomes reduced in small, fragmented populations (Packer et al. 2005, Zayed et al. 2005, Ellis et al. 2006), it may be expected that such populations would show a subsequent reduction in immunocompetence. This study compared the immunocompetence of two *Bombus* species native to the UK, which show differing levels of population fragmentation and isolation (see Chapter 2). Given the higher level of isolation of *B. monticola* populations (BWARS 2014; Fitzpatrick & Murray 2014; see Chapter 2), it was initially hypothesised that both the PO and AMP responses would be reduced in comparison to *B. pratorum* workers from the same sample site. Contrary to expectations, no consistent evidence was found for a reduction of immunocompetence in a species with a more fragmented distribution. This suggests that immunocompetence is not linked as simply with population isolation (and by inference with neutral genetic diversity; Ellis et al. 2006) as traditional theory would suggest.

4.4.1 Active vs. total PO activity

The addition of α-chymotrypsin made no significant difference to the rate of reaction in either species. On an individual basis this may be due to a lack of reserves of pro-PO, possibly indicating a compromised immune response. However, this seems unlikely given the universal nature of the observation both within and between species, especially given the high levels of inter-site variability in all other traits measured. It is more likely that part of the haemolymph extraction process caused activation of pro-PO before the assays were run. This is an issue which

has been raised by studies in other organisms (Ashida 1997). Pro-PO can become activated by heat, or by mechanical cell damage (Cerenius et al. 2008, 2010b), and proPO activation has been observed in *Bombus* workers immediately after injection of control substances, indicating a rapid PO response to even minor wounding (Korner & Schmid-Hempel 2004). Although homogenisation was done on ice to minimise the risk of heat activation, the process of crushing the thorax is likely to have caused pro-PO activation via mechanical cell damage. While the results obtained from this assay are still a useful indication of immunocompetence, the possible accidental activation of pro-PO through haemolymph extraction should be considered carefully. Alternative sample preservation and haemolymph extraction methods may be better suited to comparisons of active versus total PO activity.

4.4.2 Phenoloxidase (PO) activity

Phenoloxidase (PO) is a key component of innate immunity in insects, as melanisation has multiple protective effects including wound healing (Theopold et al. 2004), and pathogen and parasite resistance (König & Schmid-Hempel 1995, Schmid-Hempel 2005, Bidla et al. 2009).

Significant variation in PO activity was observed between sample sites in both *B. monticola* and *B. pratorum* in this study. PO activity is known to be variable between genotypes (Lambrechts et al. 2004), and would therefore be expected to vary between populations. Such variability has been observed in the expression of AMP genes in *Bombus* (Barribeau et al. 2014), even in contiguous populations (Brunner et al. 2013), and may therefore offer an explanation for the inter-population variability observed (also see Chapter 5 discussion).

PO activity was also significantly different between species at many sample sites, with levels generally higher in *B. pratorum* (Fig. 4.5). This is consistent with the expectations of traditional population genetic theory; as the more continuously distributed species, *B. pratorum* would be expected to show higher levels of genetic diversity (Newman & Pilson 1997, Reed & Frankham 2003), which has often been correlated with fitness in invertebrates (Hughes & Boomsma 2004, Spielman et al. 2004), including wild populations of *Bombus* (Whitehorn et al. 2010). A notable exception in this study was at the Antrim sample site, where *B. monticola* showed significantly higher PO activity than *B. pratorum*. This

was unexpected, as the fragmented nature of *B. monticola* populations in the UK and Ireland (BWARS 2014, Fitzpatrick & Murray 2014) would be expected to correlate with reduced genetic diversity (Packer et al. 2005, Zayed et al. 2005, Ellis et al. 2006) and therefore reduced fitness (Tarpy 2003, Calleri et al. 2006, Baer & Schmid-Hempel 2001, Haag-Liautard et al. 2009). While *B. monticola* is an expanding species in Ireland (Fitzpatrick & Murray 2014), it may still be expected to show low genetic diversity owing to founder effects and the subsequent maintenance of a fragmented distribution (Darvill et al. 2006, Schmid-Hempel et al. 2007). Although *B. pratorum* also colonised Ireland relatively recently, *B. monticola* is the more recent arrival, and has not expanded to the same extent (Fitzpatrick & Murray 2014; see Chapter 2); it may therefore be expected that *B. monticola* populations would show relatively lower genetic diversity. The data presented here are therefore conflicting as to whether population isolation consistently correlates with PO activity. This may be due to other influences acting at the population level. For example, PO activity may be influenced by exposure to previous infection and/or wounding, as the expression of genes responsible for pro-PO production are upregulated in many invertebrates in response to infection by pathogens and parasites (Bidla et al. 2009, Labbé & Little 2009, Johnston & Rolff 2013). Depending on the pathogen community present at a given sample site, PO activity could be affected in this way at both the individual and population levels. However, the mechanism underlying the difference in PO activity between species remains unclear at this stage.

At the individual level, older bees showed a lower level of PO activity than younger bees (age approximated by wing wear, see Chapter 3). The same trend has been observed in other *Bombus* species (Doums et al. 2002, Moret & Schmid-Hempel 2009, Whitehorn et al. 2010) and other invertebrate species (Siva-Jothy et al. 2005). The PO response is costly for the host, both to initiate and maintain (Moret & Schmid-Hempel 2000, Nappi & Ottaviani 2000, Sadd & Siva-Jothy 2006, Zhao et al. 2011). As individuals age, they may not have the reserves of energy required to produce a strong response. This may be due to a reduced foraging capability for example. Wing damage increases with age (or with foraging effort, see Chapter 3 discussion). While this may not affect the metabolic costs of flight (Hedenström et al. 2001), increased wing wear reduces manoeuvrability and thus increases vulnerability to predation (Cartar 1992, Hedenström et al. 2001). To compensate for the increased risk older individuals may forage less frequently and for less time than younger individuals; this would give less opportunity to

compensate for the costs of immunity. Although thorax weight was not found to be significant in either species, there was in both cases a slight trend towards higher PO activity in larger bodied individuals. It has been noted that larger bees are more likely to play an active foraging role in a colony (Goulson et al. 2002, Spaethe & Weidenmüller 2002). It may be that these individuals are therefore more able to gather the resources needed to buffer against the costs of immunity. Alternatively, those which are unable to maintain effective immunity during foraging may suffer higher mortality, and thus be sampled less frequently.

4.4.3 Antimicrobial peptide (AMP) activity

Antimicrobial peptides (AMPs) form a crucial part of the innate immune response to both microbial and parasitic infection (Schlüns et al. 2010; Deshwal & Mallon 2014). Their production is an inducible response, meaning that AMPs are only produced following an immune challenge (Fig. 4.1). Variation in AMP production is key to the specificity of the invertebrate innate immune response, as the type of AMP produced varies depending on the challenge being faced (Bulet et al. 1999, Choe et al. 2002; reviewed by Khush et al. 2002). However, the production of AMPs can also be affected by factors other than infection, including immune memory (Sadd & Schmid-Hempel 2006, 2007), and nutrition (Brunner et al. 2014).

The data presented in this study show significant variation in the proportion of samples producing AMP activity between samples sites in both *B. monticola* and *B. pratorum*, but very little variation between the two species. However, the presence or absence of a zone of inhibition is not necessarily an indication of whether or not a bee is capable of producing such activity. Given that the production of AMPs is an inducible response (Choe et al. 2002; reviewed by Khush et al. 2002), in order to compare immunocompetence between individuals based on the presence or absence of zones of inhibition it would have to be assumed that all individuals had been exposed to an equal immune challenge. While it seems highly unlikely that bumblebees in the field would not be exposed to microbial pathogens at some point in their lives, there is no way for this to be known for sure. Therefore the lack of a zone of inhibition at the time of testing cannot be taken to mean that no antimicrobial response would ever be produced by that individual; the lack of a zone may be because, however unlikely, that individual had not been exposed to any microbial challenge, and had therefore not initiated AMP production. It may

be more accurate to interpret the results as indicative of the level of threat present at a given site at the time of sampling. The significant variation between sites reported here, and the lack of a consistent difference between species, would be consistent with this hypothesis. To determine whether a true difference between sites and species exists in terms of the capability to initiate AMP production, it would be necessary to conduct a controlled experiment in which individuals from the relevant populations are infected with a known amount of an infectious substance, or a non-infectious immune stimulant such as LPS (as in Moret & Schmid-Hempel 2000, 2001). While the data collected in this study do not indicate the potential for an AMP response, the data can still be usefully interpreted as an indication of the level of immune challenge being faced by both species in various populations.

Different AMPs are produced for specific groups of pathogens, such as gram-positive and gram-negative bacteria, fungi and parasites (Choe et al. 2002; reviewed by Khush et al. 2002, Schmid-Hempel 2005). The assay performed in this study tested the activity of AMPs which had already been produced by the host, quantifying their effectiveness against the growth of *A. globiformis* (a gram-positive bacteria). The response of the assay may therefore be influenced by the pathogens encountered by an individual prior to capture, which are likely to vary depending on the sample site and will affect which AMPs were being produced (for example those which are specific to gram-negative bacteria may not be as effective against *A. globiformis*). The antimicrobial response may be further influenced by immune memory. While invertebrates do not possess an adaptive immune system (Schmid-Hempel 2005, Schulenburg et al. 2007), there is evidence for innate immune memory (sometimes referred to as immune priming; reviewed by Kurtz 2004, Sadd & Schmid-Hempel 2006). Maternal immune memory is known to be passed on to offspring in many systems (reviewed by Grindstaff et al. 2003), and there is evidence for such trans-generational immune priming in invertebrates (Little et al. 2003, Moret 2006, Freitak et al. 2009), including *Bombus* (Sadd et al. 2005, Sadd & Schmid-Hempel 2007). Daughter workers are primed to be more resistant to pathogens already experienced by the queen (Sadd et al. 2005, Sadd & Schmid-Hempel 2007). However, this is at the expense of resistance to other forms of pathogen (Sadd & Schmid-Hempel 2009), and could therefore be maladaptive if daughters were not likely to encounter largely similar suites of pathogens to the queen. This evidence may support the theory of different pathogen communities present at different sample sites, and a subsequent need for differential AMP

production between sample sites. Exposure to the same pathogen communities within sample sites would also explain the highly similar results observed in *B. monticola* and *B. pratorum* workers sampled in the same areas (Fig. 4.8), as workers of both species would be producing the same AMPs. This would result in a consistent interaction with *A. globiformis* between species, while allowing for variation between sample sites.

At the individual level, age (as approximated by wing area, see Chapter 3) was significantly correlated with zone of inhibition diameter in both *B. pratorum* and *B. monticola* (although the relationship was noisy in both species). Contrary to expectation, older bees (i.e. those with a smaller wing area relative to body size) were found to produce larger zones of inhibition than younger bees (those with a larger wing area relative to their body size) in both study species. This was surprising as age has generally been linked with the senescence of the immune response (Doums et al. 2002, Moret & Schmid-Hempel 2009, Whitehorn et al. 2010), as demonstrated in the PO activity data presented here. However it is possible, given that AMP production is induced in response to threat (Choe et al. 2002; reviewed by Khush et al. 2002), that older bees would show higher AMP activity as they have been exposed for longer, or to a greater variety of pathogens. If wing area is indicative of overall lifetime effort rather than specific individual age (see Chapter 3), it may be that individuals which have spent more time outside the nest and foraging (and hence accumulating greater wing damage, reducing their relative wing area) would be exposed to more frequent and more varied pathogens, thus requiring them to produce a stronger AMP response than those bees which may have spent less time outside the nest. It may also be the case, given the costs associated with producing and maintaining the PO response (Moret & Schmid-Hempel 2000, Nappi & Ottaviani 2000, Sadd & Siva-Jothy 2006), that older individuals reduce their PO activity in favour of the AMP response. Trade-offs between the two responses have been observed in lab studies of *Bombus* (Moret & Schmid-Hempel 2001) and other insect species (Wilson et al. 2001, Cotter et al. 2004), and do seem intuitive given the costly nature of the immune response. While AMP production requires an increased amount of protein (Brunner et al. 2014), this can be compensated by behavioural changes such as preferential foraging on high-protein food sources (Moret & Schmid-Hempel 2000). The production of AMPs can also provide a broad-spectrum, long-lasting method of defense (Moret & Siva-Jothy 2003, Korner & Schmid-Hempel 2004), effective against parasitic as well as microbial infection (Schlüns et al. 2010, Deshwal &

Mallon 2014), so may be a more efficient use of restricted resources as an individual ages. The different relationships of the PO and AMP response to individual age may therefore be indicative of an age-related trade-off (see discussion of Chapter 7), and may be due in part to differences in foraging opportunities between sample sites (see Chapter 2). Thorax weight was significant in *B. monticola*, with heavier individuals producing larger zones of inhibition. A slight (though non-significant) trend was similarly observed in *B. pratorum* and in the PO data for both study species, and concurs with similar studies in *Bombus* (Whitehorn et al. 2010). Larger bodied individuals spend a greater time foraging than smaller workers (Goulson et al. 2002). If this is the case, it seems reasonable to assume that larger workers are likely to be exposed to frequent and varied immune challenges during foraging, and would therefore require a stronger AMP response. Given the dietary protein requirements of the AMP response (Brunner et al. 2014), it may also be the case that larger, more frequently foraging individuals are better able to compensate for the costs of immunity, and thus maintain a stronger response.

The PO and AMP responses have thus far been considered independently. However, this is likely to be an over-simplifaction of the overall innate immune system. As mentioned, it is costly to the host to initiate and maintain an immune response (Moret & Schmid-Hempel 2000, Nappi & Ottaviani 2000, Sadd & Siva-Jothy 2006), and this may therefore result in trade-offs between different immune mechanisms (Moret & Schmid-Hempel 2001, Wilson et al. 2001, Cotter et al. 2004), or with other fitness-related traits which have not been measured here. Additionally, the antimicrobial products produced by the melanisation cascade may act synergistically with other innate immune functions such as AMPs to fight infection (Lambrechts et al. 2004). Such dynamics are difficult to interpret when data is only available for a single time point. However, given that in some invertebrate species trade-offs have been observed between immunity and reproduction (Moret 2003, Fedorka et al. 2004, Simmons 2011, Stahlschmidt et al. 2013), these possibilities merit investigation in wild *Bombus* populations as potentially important for colony success and survival, and thus for population viability (see Chapter 7).

4.5 Conclusions

The innate immune system in insects is highly complex. There are many potential influences on both the PO and AMP responses, including prior exposure to infection (Sadd & Schmid-Hempel 2006), trans-generational immune priming (Sadd et al. 2005, Sadd & Schmid-Hempel 2007), nutrition (Brunner et al. 2014) and individual age (Doums et al. 2002; Whitehorn et al. 2010), which are not necessarily linked to population isolation and genetic diversity (for an in-depth discussion of the implications of drift, selection and diversity at neutral and functional loci, and their consequences for proxies of fitness, see Chapter 5 discussion). This study measured the PO and AMP responses separately. However, overall immunocompetence (the ability to mount a response to infection), is in reality the result of interactions between these systems. The possibility of trade-offs between immune mechanisms to reduce the costs of immunity (Moret & Schmid-Hempel 2001), or of synergistic effects of different immune pathways (Lambrechts et al. 2004), contribute further to the complexity of interpreting results.

While there are acknowledged limitations when interpreting data from only one time point, the results of this study show evidence of variability in PO and AMP activity at both the population and individual level. The strong inter-site variability in both the PO and AMP results indicate that the challenges faced by foraging *Bombus* workers are likely to be highly variable between sample sites. However, there is little consistent difference within sites in either of the immune parameters measured between *B. monticola* and *B. pratorum*, particularly in the case of AMP activity. The data presented here do not therefore provide strong support for a link between population isolation and levels of immunocompetence (as measured using PO and AMP activity), but do serve to highlight the complexities of assessing fitness in wild populations.

Chapter 5

Population level comparison of parasite loads in two British *Bombus* species

5.1 Introduction

Genetic diversity has been shown to correlate with immune capacity and resistance to infection in many species, including mammals (Paterson et al. 1998, Siddle et al. 2007), birds (Whiteman et al. 2006), amphibians (Pearman & Garner 2005), fish (Wegner et al. 2003), crustaceans (Ebert et al. 2007), and insects (Calleri et al. 2006). Genetic diversity is also correlated with the degree of isolation of populations (Packer et al. 2005, Zayed et al. 2005, Ellis et al. 2006), as processes such as genetic drift act more strongly in smaller populations to erode genetic variability (Campbell et al. 1999, Frankham 2005). If this is the case, then species with more fragmented populations may show lower immunocompetence, and consequently a higher vulnerability to parasitism (Frankham 2005). This is particularly relevant in a conservation context as many species of conservation concern exist in small, isolated populations, and may therefore have reduced levels of genetic diversity. This may result in greater susceptibility to parasites which can strongly influence population dynamics and extinction risk (Bonsall 2004, De Castro & Bolker 2005, Cameron et al. 2011).

Bumblebees (*Bombus* spp.) perform a vital function as pollinators in temperate climates (Corbet & Osborne 1992, Carvell et al. 2006). However, many species have undergone severe declines across the UK and worldwide (Williams 1982, 1986, Biesmeijer et al. 2006, Fitzpatrick et al. 2007, Williams & Osborne 2009, Brown 2011, Cameron et al. 2011, Schmid-Hempel et al. 2014). Parasites have been implicated as a major driver of population declines in bumblebees (Brown 2011, Cameron et al. 2011, Schmid-Hempel et al. 2014). By contrast, when parasite pressure is released, at least one *Bombus* species has been able to colonise new areas (Allen et al. 2007). Correlations between genetic diversity and parasitism have been observed in *Bombus* in both laboratory and field conditions, both in terms of the number of parasite species present, and also in infection intensity (Baer & Schmid-Hempel 1999, 2001, Whitehorn et al. 2010). The link between genetic diversity and resistance may have been the driving force behind the evolution of multiple mating in many Hymenoptera species, as colonies with higher levels of genetic diversity show lower levels of infection (Liersch & Schmid-Hempel 1998, Baer & Schmid-Hempel 1999, Crozier & Fjerdingstad 2001, Baer & Schmid-Hempel 2001, Brown & Schmid-Hempel 2003a, Tarpy & Seeley 2006, Seeley & Tarpy 2007).

5.1.1 Parasites in *Bombus* species

Social insects such as bumblebees provide ideal hosts for parasites. The inhabitants of a colony live in close proximity and tend to be very genetically similar, particularly in the case of *Bombus* where queens typically mate only once (Estoup, Scholl, Pouvreau & Solignac 1995, Schmid-Hempel & Schmid-Hempel 2000, Brown & Schmid-Hempel 2003a), therefore allowing easy transmission between hosts within the colony. In addition, worker bees from several colonies will forage at the same food sources, allowing horizontal transmission of infection at foraging sites (Durrer & Schmid-Hempel 1994). As well as infection by parasitoids such as *Syntretus* wasps, there are a number of parasite species which exclusively infect bumblebees. These include the tracheal mite *Locustacharus buchneri*, as well as the microparasites *Apicystis bombi*, *Crithidia bombi*, and *Nosema bombi* (discussed later). Infected individuals can have considerably lowered individual fitness (Durrer & Schmid-Hempel 1994, Brown et al. 2000, Otti & Schmid-Hempel 2007, 2008, Rutrecht & Brown 2009) and parasites are thus a highly relevant concern in terms of the short and long term viability of populations.

L. buchneri is an endoparasitic mite, which colonises the trachea and air sacs. The mite is globally distributed, and has four distinct life stages; egg, larviform males and females (the mobile life stages involved in horizontal transmission), and adult females which attach to the walls of the trachea and air sacs, feeding on the haemolymph of the host (Husband & Sinha 1970). While infestations of *L. buchneri* have been associated with a reduced lifespan in workers (Otterstatter & Whidden 2004), it is not clear whether this parasite has significant deleterious effects overall (Yoneda et al. 2008). The same cannot be said of the many microparasite species which infect bumblebees, which can have significant effects on individuals and colonies (and hence populations) at various stages of development (Durrer & Schmid-Hempel 1994, Brown et al. 2003b,c, Otti & Schmid-Hempel 2007, 2008, Rutrecht & Brown 2009).

The neogregarine parasite *A. bombi* (Apicomplexa: Neogregarinorida) infects and degrades adipose tissues (Durrer & Schmid-Hempel 1994, reviewed by Schmid-Hempel 1998). It has been recorded in many species of *Bombus* worldwide (Lipa & Triggiani 1996, Baer & Schmid-Hempel 2001, Rutrecht & Brown 2008a, Plischuk et al. 2011), and has been correlated with high post-hibernation mortality in new queens (Rutrecht & Brown 2008a). Infection initially occurs through ingestion of infective spores, and subsequently spreads either through contact with spores in

faeces, or contact with spores through decomposition of a dead host. Although previously confined to *Bombus* species as hosts, it has recently begun to infect *A. mellifera* in Argentina, where it was introduced via imported commercial colonies of *B. terrestris* (Plischuk et al. 2011). While much of its biology remains poorly understood, the virulence of *A. bombi* makes it a potentially important pathogen.

The globally distributed trypanosome *C. bombi* (Gorbunov 1987, Lipa & Triggiani 1988) is confined exclusively to *Bombus* species, infecting all castes of individuals within a colony (Schmid-Hempel & Tognazzo 2010). Two distinct species of *Crithidia* infect *Bombus* species, *C. bombi* and the recently described *C. expoeki* (Schmid-Hempel & Tognazzo 2010). Due to the recent identification of *C. expoeki*, and subsequent lack of information available, only *C. bombi* will be discussed further in this study. The parasite is spread through contact with infected faeces; horizontal transmission often occurs at shared flowers during foraging, while vertical transmission occurs as gynes come into contact with infectious cells shed in the nest (Durrer & Schmid-Hempel 1994). Horizontal transmission has been shown to occur at a high rate in natural field conditions (Imhoof & Schmid-Hempel 1999), including transmission from one host species to another (Colla et al. 2006, Meeus et al. 2011).

As well as being widespread and easily transmitted, *C. bombi* can have significant fitness implications. Reproductive success of infected queens (i.e. the numbers of reproductive offspring produced) can be reduced by up to 40% (Brown et al. 2003b), while mortality of infected worker bees increases by up to 50% when combined with stressors such as starvation (Brown et al. 2000). High levels of *C. bombi* infection have also been linked to reduced motor skills, leading to a reduced ability to handle novel flower types (Gegear et al. 2005). This is most likely due to impaired learning ability (the time needed to learn and remember flower-handling techniques; Gegear et al. 2006). There are costs associated with learning how to handle novel flower types (Gegear & Laverty 1998). When learning time is increased due to parasitism these costs may be greater than the gain from foraging on that novel flower type, encouraging flower constancy (Gegear & Laverty 1998, Chittka et al. 1999). Bees infected with other parasites such as conopid fly larvae (Diptera: Conopidae) prefer foraging on flowers which require less handling (Schmid-Hempel & Schmid-Hempel 1996, Schmid-Hempel & Stauffer 1998). High prevalence of parasitic infection may therefore affect the reproductive success of some plant species, as infected bees preferentially restrict foraging to certain types of flowers. Changes in host behaviour are not caused by general loss

of condition, as the number and duration of foraging bouts remains unchanged regardless of *C. bombi* infection (Gegear et al. 2006). Secretions from the parasite itself are also unlikely to be the cause (Thomas 2005); however, non-pathogenic activation of the innate immune response is linked to reduced learning ability in *A. mellifera* (Mallon et al. 2003a) and *B. terrestris* (Riddell & Mallon 2006), suggesting that behavioural changes are due to the immune response mounted by the individual. From the point of view of the parasite, such behavioural changes may be beneficial; increased flower handling time will increase the chances of an infection being encountered and/or transmitted. However, learning speed in bees correlates strongly with foraging efficiency and therefore with fitness (Raine & Chittka 2008). Given the increased virulence of *C. bombi* under starvation conditions (Brown et al. 2000) a reduction in foraging efficiency may have colony-wide implications, substantially increase mortality in infected nest bees(Gegear et al. 2006).

N. bombi is an obligate intracellular microsporidian parasite (Fantham and Porter 1914). It infects many *Bombus* species (Tay et al. 2005) but with varying levels of frequency (MacFarlane et al. 1995, Otti & Schmid-Hempel 2007) and intensity (Rutrecht et al. 2007). The spores are usually introduced via ingestion, chiefly infecting larvae and young bees (Rutrecht et al. 2007, Rutrecht & Brown 2008a). After infection the spores are found primarily in the Malpighian tubules, but can extend into the midgut, tracheal tissues, and fat body (MacFarlane et al. 1995), and can also affect the nervous system (Fries 2010). Under laboratory conditions, worker bees infected with *N. bombi* show reduced survival (Schmid-Hempel & Loosli 1998). Infection also affects reproductive individuals; infected males have a lower sperm count, while gynes show reduced willingness to mate (Otti & Schmid-Hempel 2007), as well as swelling and distortion of the abdomen (MacFarlane et al. 1995). Similar results are seen under field conditions, with infected colonies generally being smaller than control colonies, and with reduced reproductive success in both males and gynes (Otti & Schmid-Hempel 2008). Collectively these studies indicate that *N. bombi* has the potential to have significant impacts on the evolutionary dynamics of *Bombus* species.

While the virulence and potential fitness impacts of these parasites is not disputed, an additional component of assessing the impact on hosts is the likelihood of an individual becoming infected. This requires an estimate of the prevalence of various parasites that are present in the environment; a very virulent parasite or pathogen is not as likely to be a significant threat to a population if only present

at very low levels. Unfortunately, the prevalence of any given parasite is difficult to determine in a general sense. *C. bombi* is reportedly present in between 30% and 50% of workers sampled (Tognazzo et al. 2012), *N. bombi* in up to 55% of workers (Durrer & Schmid-Hempel 1994), while *A. bombi* is generally considered to be a low prevalence parasite (Allen et al. 2007, Baer & Schmid-Hempel 2001, Rutrecht & Brown 2008a, Arbetman et al. 2013, Goulson et al. 2012). However, due to logistic and resource limitations the vast majority of studies of parasitism in *Bombus* have been conducted over a single field season, at limited sites, and often in only one or a very few species. There are very few studies which conduct repeated sampling at the same sites over multiple field seasons, despite evidence showing significant inter-annual variation in local parasite prevalence (Durrer & Schmid-Hempel 1995, Ruiz-González et al. 2012), and significant variation between sites and species (Gillespie 2010, Cameron et al. 2011, Cordes et al. 2012). Parasite prevalence also varies within a single season, with infection rates increasing with time as workers are increasingly exposed and pass infections on to others (Goulson et al. 2012, Whitehorn et al. 2013). Extrapolating from such limited data may be misleading, as there is no way to be certain that the prevalence observed is "typical", even for one species or sample site. This study compares the prevalence of various parasite species between the hosts *B. monticola* and *B. pratorum*, to determine whether parasite prevalence is linked with host abundance/rarity. However, data are interpreted with the important caveat that they represent only a snapshot in time at any one site. In order to draw broader conclusions, sampling would need to be repeated across successive months and years at each site.

Determining the potential impacts of various parasite species may be particularly relevant given the reliance of agriculture on commercially reared bumblebee colonies (Velthuis & Doorn 2006). Introduction of commercial colonies has been correlated with declines in native bee populations, increases in parasite prevalence, and the introduction of novel parasites (Goka et al. 2001, Colla et al. 2006, Otterstatter & Thomson 2008, Cameron et al. 2011, Arbetman et al. 2013, Szabo et al. 2012). Commercial *Bombus* colonies have been found to have much higher parasite loads on average than their wild counterparts (Yoneda et al. 2008, Whittington & Winston 2003, Otterstatter & Thomson 2008, Plischuk & Lange 2009), and are often infected with multiple parasite species (Graystock et al. 2013). They also routinely stray from the areas where they are deployed (Morandin et al. 2005, Whittington & Winston 2003); in some cases less than 3% of the pollen collected

by commercially bred workers comes from the target crop, the rest coming from wild plants in the surrounding area (Murray et al. 2013). Commercial bees are therefore able to interact with wild pollinators at shared food sources, giving ample opportunity for parasite transmission (Whittington & Winston 2003, Murray et al. 2013).

It is well established that parasitism can have significant effects at the individual, colony, and population level. The possible link of genetic diversity to immunocompetence, and hence the ability to resist parasitism (Crozier & Fjerdingstad 2001), makes it possible that small, isolated populations may be at higher risk from parasite exposure in comparison to larger, more contiguous populations; this effect may be amplified in *Bombus*, as haplodiploid organisms may be especially prone to the effects of genetic drift (Zayed & Packer 2005, Zayed 2009). It would therefore be expected that rare species, which tend to exist in more isolated populations, would be at higher risk than common species. This study compares the parasite load of a widespread and abundant bumblebee species, *B. pratorum*, with that of a species which exists in isolated but locally abundant populations, *B. monticola*. If population isolation is linked with vulnerability to parasitism through reduced immunocompetence, higher parasite loads would be expected in *B. monticola* relative to *B. pratorum* sampled at the same site.

The study aimed to address the following questions:

1. Is light microscopy or molecular screening more effective for detecting the presence of parasitic infection in *Bombus* species?

2. Does the occurrence of parasitic infection differ between sites and species, and is there evidence for a link between population isolation, relative abundance, and parasite prevalence?

3. Do factors such as age and body size influence infection risk and intensity at the individual level?

4. Is there significant inter-annual variation in the occurrence of parasitic infection?

5.2 Methods

5.2.1 Dissection and light microscopy

After collection (see Chapter 2), individual bees were dissected to separate the legs, thorax and abdomen. After initial separation, abdomens were stored frozen at -20°C. For assessment of parasite load, the abdomen samples were defrosted on ice and opened along the joint between the tergites and stergites using Vanna's iris scissors. Using a dissection microscope at 5X magnification, the tracheal tubes and tissues were inspected for *L. buchneri* and *Syntretus* larvae. The presence or absence of mites and larvae in each sample was recorded, along with the abundance of each. Only the adults of *L. buchneri* were counted.

After inspection for macroparasites, samples of the hind gut, fat body and Malpighian tubules were dissected out and placed in a drop of water on a microscope slide. Three samples of each tissue type were taken from different areas of the abdomen to ensure maximum coverage. The slides were inspected for the presence or absence of *A. bombi*, *Crithidia* spp. and *Nosema* spp. using a phase contrast microscope at 400X magnification. When parasites were present, the intensity of infection was quantified by counting the number of parasites in the field of view for 5 randomly selected points across each sample for each tissue type (5 fields of view across 3 samples = 15 fields of view per tissue type). These counts were then averaged to give a measure of the intensity of infection for each tissue type. After initial scoring, slides were sealed and frozen at -20°C for long-term storage.

Table 5.1. Sequences of diagnostic primers used for parasite screening.

Target species	Primer Name	Primer Sequence (5' - 3')	Region	TM(^{o}C)	Fragment Size (bp)
General Apidae	ApidaeF[a]	AGA TGG GGG CAT TCG TAT TG	18s rDNA	60.5	120
	ApidaeR[a]	ATC TGA TCG CCT TCG AAC CT		59.6	
Apicystis bombi	NeoF[a]	CCA GCA TGG AAT AAC ATG TAA GG	18s rDNA	58.9	260
	NeoR[a]	GAC AGC TTC CAA TCT CTA GTC C		60.3	
Crithidia bombi	SEF [a]	CTT TTG GTC GGT GGA GTG AT	18s rDNA	57.3	420
	SER [a]	GGA CGT AAT CGG CAC AGT TT		57.3	
Crithidia bombi	CB-ITS1-F[b]	GGA AAC CAC GGA ATC ACA TAG ACC	ITS-1	∼60	∼600
	CB-ITS1-B[b]	AGG AAG CCA AGT CAT CCA TCG C		∼60	
Locustacarus buchneri	CI-J-1751[c]	GGA TCA CCT GTA ATA GCA TTC CC	CO1	∼50	555
	LCO1R[d]	CCA ATA GAT GCT ATG GCG TGA		∼50	
Nosema bombi	Nbombi-SSU-Jf1[e]	CCATGC ATG TTT TTG AAG ATT ATT AT	SSU rRNA	55.3	323
	Nbombi-SSU-Jr1[e]	CAT ATA TTT TTA AAA TAT GAA ACA ATA A		50.5	
Nosema bombi	ITSf2[e]	GAT ATA AGT CGA ACA TGG TTG CT	ITS-1	50.0	118/122
	ITSr2[e]	CAT CGT TAT GGT ATC CTA TTG ATC		48.0	
Nosema ceranae	NosemaceranaeF[f]	AAG AGT GAG ACC TAT CAG CTA GTT G	SSU rRNA	61.3	104
	NosemaceranaeR[f]	CCG TCT CTC AGG CTC CTT CTC		63.7	

Primer sequences are from [a]Meeus et al. (2010); [b]Schmid-Hempel & Tognazzo (2010); [c]Simon et al. (1994); [d]Goka et al. (2001); [e]Klee et al. (2006); [f]Bourgeois et al. (2010).

5.2.2 Molecular screening

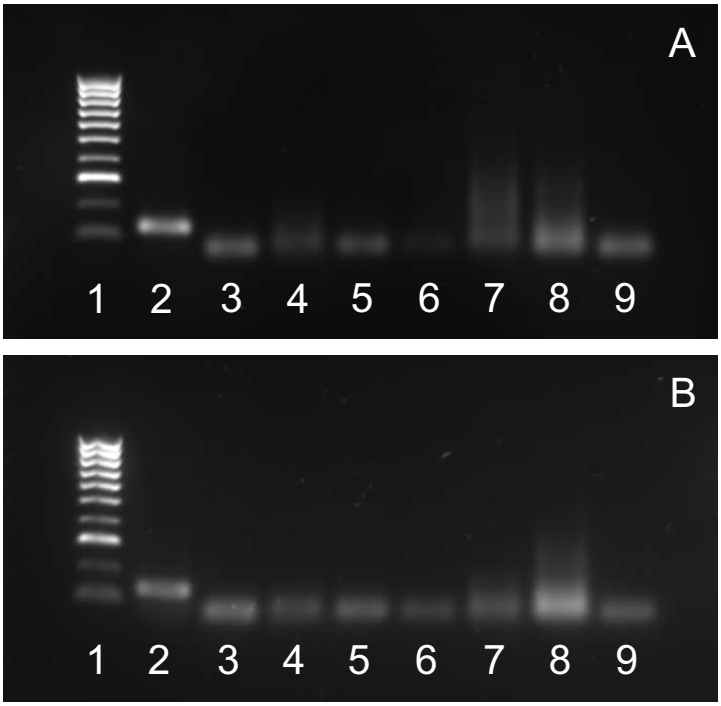

Figure 5.1. Results from PCR using DNA extracted from wing muscle tissue in *B. pratorum* (panel A) and *B. monticola* (panel B). 5μl of each PCR product was run for 1h 10mins on a 1% agarose gel at 100V. Lane 1 = size ladder (Hyperladder IV, Bioline); lane 2 = general Apidae primer (Meeus et al. 2010); lane 3 = *A. bombi* primer (Meeus et al. 2010); lane 4 = *C. bombi* SSU primer (Meeus et al. 2010); lane 5 = *N. bombi* SSU primer (Klee et al. 2006); lane 6 = *N. ceranae* primer (Bourgeois et al. 2010); lane 7 = *C. bombi* ITS primer (Schmid-Hempel & Tognazzo 2010); lane 8 = *N. bombi* ITS primer (Klee et al. 2006); lane 9 = *L. buchneri* primer (Goka et al. 2001; Simon et al. 1994). The band present in lane 2 indicates that the DNA extraction in both cases was successful. No product is present in any other lanes, indicating that no non-specific amplicfication of host DNA occurred during the PCR; this indicates that the primers are suitable for screening in both the study species.

All samples were also screened for the presence or absence of *A. bombi*, *C. bombi*, and *N. bombi* using species-specific molecular markers (Table 5.1). To ensure that the primers used were suitably species-specific, they were tested using extracts of host DNA from both *B. pratorum* and *B. monticola*. Given that most infections occur during foraging (Durrer & Schmid-Hempel 1994), it was decided that leg tissue would be unreliable as a host tissue control due to the possibility of contact with infected pollen. DNA was therefore extracted from wing muscle tissue, as this would not have been exposed to any external contamination. The tissue sample was added to 500μl of 10% Chelex® (10% volume Chelex resin, 50mM TRIS) and homogenised using a sterile micropestle. The samples were heated at 56°C for 30 minutes, then boiled for 8 minutes at 98°C, before being centrifuged

at 13,000g for 5 minutes. The supernatant was then carefully removed and 3μl was used for subsequent PCR reactions. The PCR conditions were as follows: 3μl DNA extract, 1.25μM each primer (0.625μM for *C. bombi* primers), 2X Biomix (Bioline), made up to 10μl using molecular grade H_2O. The PCR cycle was as follows: 98°C for 2 mins; 50 cycles of 98°C for 30 secs, 45 secs annealing (for temperatures see Table 5.1), 72°C for 1 min; 72°C for 5 mins. After PCR, 5μl of the reaction was run for 1 hour at 110V on a 1% agarose gel stained with 5μl of SYBRSafe (Life Technologies) to check for any amplification of host DNA (Fig. 5.3).

The chosen primers were then tested using known positive controls for each parasite species (either a concentrated sample of parasite spores extracted from infected bee faeces, or the abdomen of a bee which had been deliberately infected). When using concentrated spore samples, 50μl of the sample was added to 200μl of 10% Chelex, heated at 56°C for 30 minutes, then boiled at 98°C for 8 minutes before being centrifuged for 5 minutes at 13,000g. When using whole infected bees, the contents of the abdomen were removed and prepared as described before. In all cases 3μl of the supernatant was added to the PCR reactions. The PCR conditions and program were the same as described previously. After PCR, 5μl of the reaction product was run for 1 hour at 110V on a 1% agarose gel. The bands present (Fig. 5.2) were compared with the expected fragment sizes reported in the literature (Table 5.1) to ensure that the reactions had worked correctly.

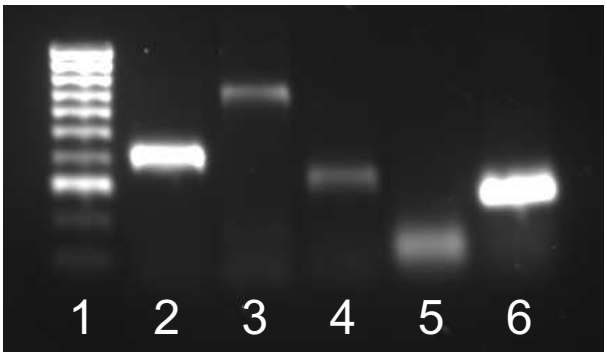

Figure 5.2. Results from PCR reactions using DNA extracted from both concentrated spore samples and whole infected bees. Lane 1 = size ladder (Hyperladder IV, Bioline); lane 2 = *C. bombi* SSU primer (spore sample); lane 3 = *C. bombi* ITS primer (spore sample); lane 4 = *N. bombi* SSU primer (spore sample); lane 5 = *N. bombi* ITS primer (spore sample); lane 6 = *A. bombi* primer (infected abdomen sample). The bands present are at the expected location for all primers, indicating successful amplification of the relevant parasite DNA.

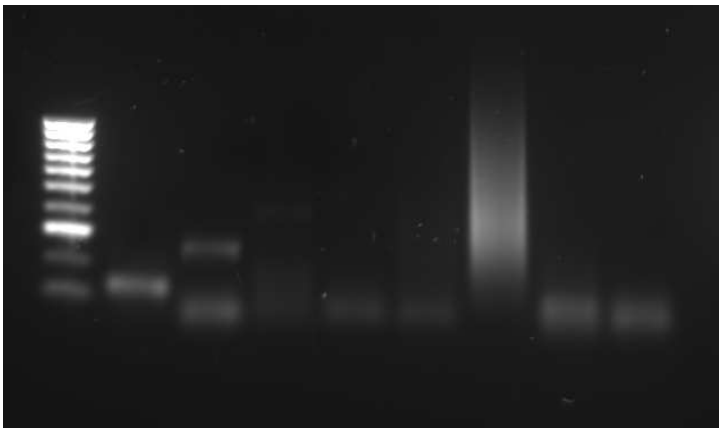

Figure 5.3. Results of negative control PCR reactions using only parasite primers and distilled water. Lane contents after the size ladder are as follows: A = general Apidae primer (Meeus et al. 2010); B = *A. bombi* primer (Meeus et al. 2010); C = *C. bombi* SSU primer (Meeus et al. 2010); D = *N. bombi* SSU primer (Klee et al. 2006); E = *N. ceranae* primer (Bourgeois et al. 2010); F = *C. bombi* ITS primer (Schmid-Hempel & Tognazzo 2010); G = *N. bombi* ITS primer (Klee et al. 2006); H = *L. buchneri* primer (Goka et al. 2001 / Simon et al. 1994). The band present in lane 3 possibly indicates some contamination of the primer stock.

5.2.3 Statistical analysis

Parasitic infection was quantified using the results of dissection for *L. buchneri* and *Syntretus spp.*, and based on molecular screening for *A. bombi*, *C. bombi*, and *N. bombi* (see Results section). The prevalence of each parasite (the proportion of individuals infected) was compared between *B. pratorum* and *B. monticola* at each sample site where the species co-occurred. Samples of the two species were taken to be broadly comparable within each site in terms of foraging behaviour (see Chapter 2) and the background levels of parasites in the environment. Over-sampling any one patch of forage was avoided to minimise the collection of sister bees, which could introduce bias to the results through their high genetic similarity (see Chapter 2). The proportion of infected individuals was compared between species using Chi-squared tests, or Fisher's exact test when the frequencies were insufficient to allow the use of Chi-squared.

Parasite species richness (defined as the number of parasite species infecting an individual) was compared between *B. pratorum* and *B. monticola* at sites where both species co-occurred. Data were checked for normality using Shapiro-Wilks normality test; due to non-normality the data were then analysed using Mann-Whitney U tests.

The relative abundance of *B. monticola* and *B. pratorum* was estimated at each

site where the two species co-occured, using the total sampling time required (in hours) to collect a full sample of each species. As both species were collected simultaneously, and because of the overlap in foraging behaviour (see Chapter 2), it was assumed that whichever was most abundant at a given site would be encountered more frequently, and hence less time would be required to obtain a full sample. Times were taken between the first and last bee caught on a given day; times were then totalled for all sampling days. For further analysis each species was then categorised as either "most abundant", "least abundant", or "equally abundant". In cases where a full sample of 40 individuals could not be collected, that species was classed as least abundant. It must be acknowledged at this point that all estimates of abundance are applicable only to the specific site being studied, and only to the time of year at which samples were collected. The relative abundance of the study species may change over the season; however, this possibility cannot be quantified as it was not possible to repeatedly sample each site during the field season. Fisher's exact test was used to determine whether higher parasite prevalence occurred more frequently in relatively more or less abundant species at each sample site.

To compare parasite prevalence between years, samples of both *B. monticola* and *B. pratorum* were collected at the Dartmoor site in 2011 and 2012. The prevalence of each parasite in *B. monticola* and *B. pratorum* was compared between years using Chi-squared tests, or Fisher's exact test when the frequencies were insufficient to allow Chi-squared. Parasite species richness was compared between years for each species using Welch's t-tests, or Mann-Whitney U tests if Shapiro-Wilks tests showed the data to be non-normal. The prevalence and parasite species richness were also compared between *B. monticola* and *B. pratorum* for both years, to see whether differences between the two species appeared consistent over consecutive field seasons.

Factors potentially influencing parasite prevalence, including thorax weight and individual age, were assessed across sites using generalised linear mixed modelling (GLMM) with a binomial distribution. Parasite species richness was analysed using a GLMM with a quasipoisson distribution for count data. Data for *B. monticola* and *B. pratorum* were analysed separately. For parasite prevalence, separate models were run for *A. bombi*, *C. bombi*, *L. buchneri* (in *B. pratorum* only) and *N. bombi*. In all models thorax weight, age (as estimated using wing wear adjusted for body size, see Chapter 3), and sample site were included as fixed factors, with 2-way interactions between all terms. Sampling month was treated

as a random factor. The majority of samples for both species were collected in June, with the exception of the sites at Ben Lawers and Glen Shee, which were sampled in August/September. Approximate emergence times for each species were compared between sample sites using records from the BWARS database (from 1990-present). The earliest records each year were the same across sites for each species, with the exception of those in Scotland. It was therefore assumed that, as emergence occurred in the same month at all sites, and samples were collected in the same month, that exposure time within species would be similar. Step-wise model simplification was performed using likelihood ratio testing of the AIC values.

The intensity of *L. buchneri* infection was analysed for *B. pratorum* only using ANOVA; there were insufficient infections detected in *B. monticola* to allow further analysis. Thorax weight, age and sample site were treated as explanatory factors. There were insufficient data regarding the intensity of *A. bombi* infection to allow further analysis. All statistical analysis was performed using R (v2.15.2).

5.3 Results

5.3.1 Primer selection and primer suitability

No amplification was observed from host DNA (Fig. 5.1), indicating that all the primers tested were suitable for screening both of the study species. The *N. bombi* ITS region primer (Klee et al. 2006; Table 5.1) was abandoned after the initial testing stage, due to the presence of a bright smear in negative control tests (Fig. 5.1). The expected fragment from this primer was small (118bp), so the presence of a smear would have made accurate detection of a product band difficult. The 18s rDNA primer for *C. bombi* (Meeus et al. 2010; Table 5.1) was also discarded in favour of the ITS region primer (Schmid-Hempel & Tognazzo 2010; Table 5.1), as the expected fragment from the ITS primer (600bp) was more easily distinguishable from those of the other target species. It was also decided not to screen for *N. ceranae*, as the likelihood of detection was very low. Although common in European populations of *Apis mellifera* (Paxton et al. 2007), at the time of sampling *N. ceranae* had only been observed in *Bombus* in South America (Plischuk & Lange 2009, although see Fürst et al. 2014).

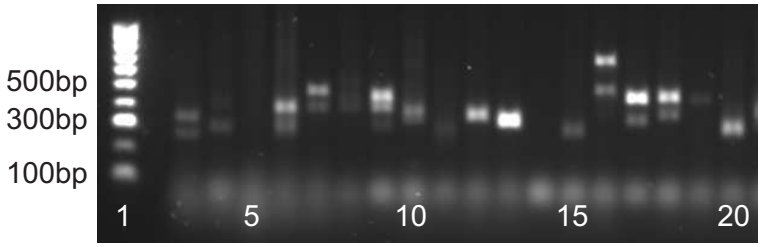

Figure 5.4. Results of a PCR using Chelex-extracted DNA and the SSU rRNA primer for *N. bombi* (Klee et al. 2006; Table 5.1. Lane 1 = size ladder (Hyperladder IV, Bioline); lane 2 = blank; lane 3-20 = 5μl of PCR product. The expected fragment size from the SSU primer is 323bp (Table 5.1); however, many lanes show bands of differing sizes, and multiple bands in some cases. Samples shown in this image are *B. pratorum* from the Long Mynd sample site; similar results were seen in both *B. pratorum* and *B. monticola* at other sample sites. Negative controls run during method development established that the primer does not amplify host DNA, suggesting amplification of a contaminant; however, re-runs of the same samples produced different banding patterns, suggesting that this is unlikely to be the case.

The primers used were generally found to be very effective; many more infections were detected than with light microscopy, in a much shorter time. However, when using the *N. bombi* primer described in Klee et al. (2006), many reactions produced non-specific PCR products, visible as bands of varying sizes when run on agarose gels (Fig. 5.4). Initial tests using *Bombus* muscle tissue established that the primer did not amplify host DNA, suggesting that the bands may be the result of contaminants in the samples. However, the sizes of the non-specific bands were found to vary when multiple reactions were run from the same sample, so are not likely to be the result of consistent amplification of a contaminant. Results for *N. bombi* were scored when a band was present at the expected location on a gel; however, the results are interpreted with a great deal of caution, due to the likelihood of random amplification occurring when these primers are used.

Light microscopy was found to be effective for both *L. buchneri* and *Syntretus* spp. However, when screening for microparasites, PCR-based detection was found to be preferable. While good results were obtained for *A. bombi* and *C. bombi* using PCR, the results for *N. bombi* appeared slightly less reliable; this may indicate a need for further optimisation of the *N. bombi* primers used.

5.3.2 Effectiveness of visual inspection versus molecular screening for detection of parasitic infection

Molecular screening was found to be much more effective for detecting infection by microparasites when compared with light microscopy. This was especially true

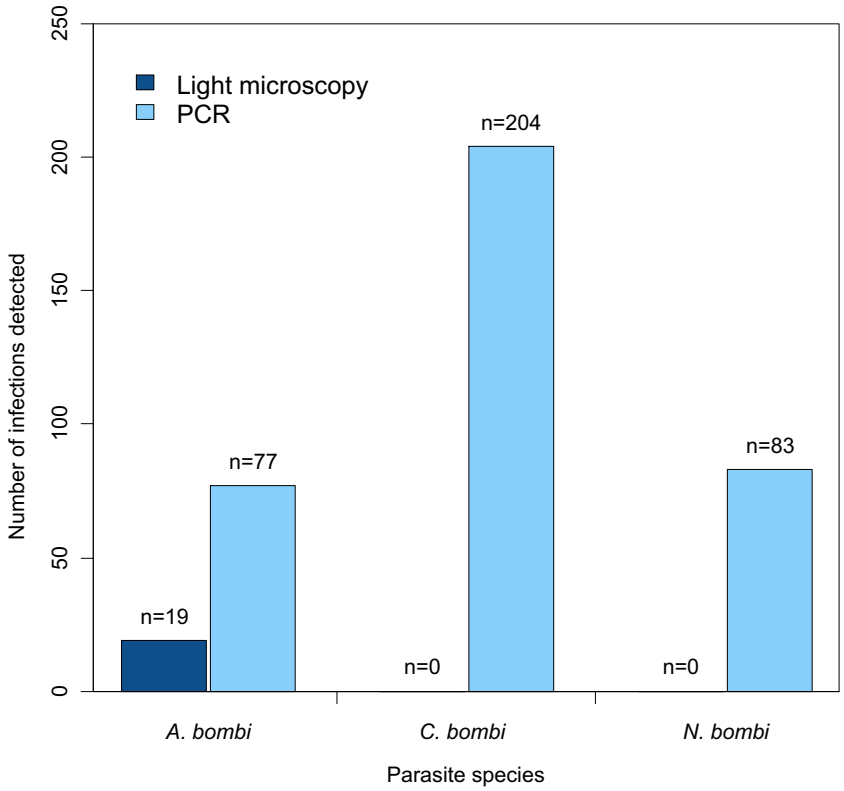

Figure 5.5. Comparison of the frequency of infections detected using light microscopy and PCR-based screening methods. All samples were screened using both methods; the total number of infections detected by each method is indicated above the relevant bar.

for *C. bombi*; no infections were detected using light microscopy, but 204 were detected overall using molecular screening (Fig. 5.5). Infections of *A. bombi* were easily observed under the microscope when the abundance of parasite cells was high, with 19 infections observed. However, when PCR was used, 77 infections were detected, presumably indicating low-level infections which were missed under the microscope (Fig. 5.5). Although results suggested that PCR-based screening was also more effective for *N. bombi* (83 infections detected overall compared with 0 found by microscopy; Fig. 5.5), the primers used for this parasite may not have been performing optimally (as previously discussed). Future studies may therefore require new *N. bombi* primers to be developed, which have a higher degree of specificity.

5.3.3 Variation in parasite prevalence between species and sample sites

In total 341 *B. monticola* (Table 5.3) and 281 *B. pratorum* (Table 5.4) samples were screened for the presence of 5 parasite species.

C. bombi was the most prevalent parasite, with 204 infections detected in total across both species. The infection rate of *C. bombi* ranged from 2.7% (Glen Shee) to 67.5% (Stiperstones) in *B. monticola*, and from 2.5% (Dartmoor) to 57.5% (Dublin) in *B. pratorum*. For both study species, *C. bombi* infection was present in at least one individual at every sample site (Table 5.3; Table 5.4; Fig. 5.6). Significant differences between species in the prevalence of *C. bombi* infection were observed at the Long Mynd (Fisher's Exact Test, $p<0.01$), Stiperstones (Fisher's Exact Test, $p<0.001$), Lake District (Fisher's Exact Test, $p<0.001$), and Dublin ($\chi^2=5.08$, df=1, $p<0.05$) sample sites (Fig. 5.6). No significant differences in *C. bombi* prevalence were observed at the Dartmoor (Fisher's Exact Test, $p=0.20$), Snowdonia ($\chi^2=0.002$, df=1, $p=0.97$), and Antrim ($\chi^2=3.67$, df=1, $p=0.06$) sample sites. Levels of *C. bombi* infection were generally higher in *B. monticola*, although not at the Dublin or Lake District sample sites (Fig. 5.6).

Of the microparasites, *A. bombi* was the least prevalent, with 72 infections detected in total across both species. The rate of *A. bombi* infection ranged from 2.6% (Antrim / Long Mynd) to 32.5% (Stiperstones) in *B. monticola* (Table 5.3), and from 0% (Dartmoor) to 30.5% (Snowdonia) in *B. pratorum* (Table 5.4). For *B. monticola*, *A. bombi* infection was present at all sites apart from Dublin and Glen Shee, but generally at a low frequency (Table 5.3; Fig. 5.6). For *B. pratorum*, *A. bombi* infection was present at all sites apart from Dartmoor; as with *B. monticola* infection rates within each site were generally low (Table 5.4; Fig. 5.6). Significant differences in *A. bombi* prevalence were observed at the Lake District ($\chi^2=3.84$, df=1, $p<0.05$) and Dublin (Fisher's Exact Test; $p<0.01$) sample sites only (Fig. 5.6). No significant differences in the presence of *A. bombi* were found between the study species at the Dartmoor (Fisher's Exact Test; $p>0.99$), Long Mynd (Fisher's Exact Test; $p>0.99$), Stiperstones ($\chi^2=2.30$, df=1, $p=0.13$), Snowdonia ($\chi^2=0.04$, df=1, $p=0.83$) or Antrim (Fisher's Exact Test; $p>0.99$) sample sites. There was a less consistent difference in levels of infection between the two study species than for *C. bombi*; while infection rates were significantly higher in *B. pratorum* at the Dublin and Lake District sample sites, infection rates

appeared higher in *B. monticola* at the Stiperstones site. However, it is difficult to draw conclusions due to the low number of infections encountered; at many sites the frequency of infection is too low to determine which of the study species may be more vulnerable to infection.

A total of 80 *N. bombi* infections were found across both species. The infection rate of *N. bombi* ranged from 0% (Glen Shee, Long Mynd, Stiperstones, Snowdonia) to 62.2% (Dublin) in *B. monticola* (Table 5.3), and from 0% (Dublin) to 45.2% (Antrim) in *B. pratorum* (Table 5.4). Significant differences in *N. bombi* prevalence between the two study species were observed at the Dartmoor (Fisher's Exact Test; $p<0.05$) and Dublin (Fisher's Exact Test; $p<0.001$) sample sites (Fig. 5.6). No significant differences were observed in samples collected at Long Mynd ($\chi^2=0.10$, df=1, $p=0.75$), Stiperstones (Fisher's Exact Test; $p=0.49$), Snowdonia (Fisher's Exact Test; $p=0.11$), the Lake District (Fisher's Exact Test; $p>0.99$), or Antrim ($\chi^2=0.08$, df=1, $p=0.78$). *N. bombi* was recorded as present when a band of the expected size (323bp) was present; however, as previously discussed, bands of varying sizes were produced in a number of cases, indicating that the primers may not be binding exclusively to *N. bombi* DNA. Results for the prevalence of this parasite must not be considered conclusive.

Across both study species a total of 89 *L. buchneri* infections were found. Infection rates ranged from 0% (Dublin, Lake District, Stiperstones, Snowdonia) to 7.7% (Antrim) in *B. monticola* (Table 5.3), and from 12.9% (Antrim) to 43.6% (Stiperstones) in *B. pratorum*. *L. buchneri* infections were present in *B. pratorum* at every sample site (Table 5.4; Fig. 5.6). There was a more pronounced difference in levels of *L. buchneri* prevalence between *B. monticola* and *B. pratorum* when compared with the other parasite species studied; 92% of all infections observed occurred in *B. pratorum*. While there was no significant difference in the proportion of infected individuals at the Antrim sample site (Fisher's Exact Test; $p=0.37$), *B. pratorum* had a significantly higher *L. buchneri* infection rate than *B. monticola* at the Dartmoor (Fisher's Exact Test; $p<0.05$), Long Mynd (Fisher's Exact Test; $p<0.01$), Stiperstones (Fisher's Exact Test; $p<0.001$), Snowdonia (Fisher's Exact Test; $p<0.01$), Lake District (Fisher's Exact Test; $p<0.001$), and Dublin (Fisher's Exact Test; $p<0.001$) sample sites (Fig. 5.6).

Syntretus larvae were the least prevalent of all the parasite species screened for. Only 5 infected *B. monticola* workers were found (Table 5.3), and 7 infected *B. pratorum* (Table 5.4). The low levels of infection by this species meant that it

was not possible to analyse these data further.

B. monticola was found to be the more abundant species at the time of sampling at the Lake District and Long Mynd sites, while *B. pratorum* was more abundant at the Antrim and Dartmoor sites. The two study species were equally abundant at the Dublin, Snowdonia and Stiperstones sites (Table 5.2). No connection was found between the relative abundance (as defined in this study) of a species and the prevalence of any of the microparasites studied (Fisher's exact tests used in all cases. *A. bombi*: p=0.756; *C. bombi*: p=0.371; *N. bombi*: p=0.371). Comparisons were not made for the prevalence of *L. buchneri*, as >90% of infections were in *B. pratorum*.

The maximum parasite species richness observed overall was 3 parasite species infecting one individual; the minimum parasite species richness observed overall was 0. Significant differences in parasite species richness were observed between *B. pratorum* and *B. monticola* at the Dartmoor (W=1194, p<0.05), Dublin (W=598.5, p<0.05), and Lake District (W=73, p<0.001) sample sites (Fig. 5.7). No significant differences in parasite species richness were observed at the Antrim (W=654.5, p=0.671), Long Mynd (W=806.5, p=0.775), Snowdonia (W=585, p=0.094), or Stiperstones (W=954, p=0.068) sample sites.

5.3.4 Factors influencing individual infection risk and intensity

For *C. bombi* there was no significant effect of age (as approximated by wing wear, see Chapter 3) in either species *B. monticola*: χ^2=1.45, df=1, p=0.229; *B. pratorum*: χ^2=0.75, df=1, p=0.388. Thorax weight did not vary significantly with prevalence in *B. monticola* (χ^2=1.35, df=1, p=0.246), but was significant in *B. pratorum* (χ^2=4.57, df=1, p<0.05), with smaller individuals showing a higher prevalence of *C. bombi* infection. In both species there was strongly significant variation in prevalence between sample sites (*B. monticola*: χ^2=83.74, df=8, p<0.001; *B.pratorum*: χ^2=36.30, df=7, p<0.001). For *A. bombi*, there was no significant effect of age (*B. monticola*: χ^2=0.76, df=1, p=0.383; *B. pratorum*: χ^2=0.13, df=1, p=0.721) or thorax weight (*B. monticola*: χ^2=0.02, df=1, p=0.895). Sample site was significant for both species (*B. monticola*: χ^2=41.27, df=8, p<0.001; *B. pratorum*: χ^2=22.54, df=7, p<0.01). For *N. bombi* there

Table 5.2. Relationship of parasite prevalence to the relative abundance of *B. monticola* and *B. pratorum*. Sampling time indicates the total number of hours required to collect a full sample of 40 individuals. Where a full sample could not be collected, the species is marked with † in the abundance column; in these cases, the incompletely sampled species was classed as least abundant by default, so no sampling time is given. No sampling times were available for the Dartmoor sample site (marked by †† in the abundance column). However, at the time of sampling *B. pratorum* is known to be more abundant (M. Knight, pers. comm.). Asterisks in the parasite species columns indicate whether the highest prevalence of the parasite was found in *B. monticola* or *B. pratorum*.

Site	Species	Relative abundance	Sampling time (hrs:mins)	*A. bombi*	*C. bombi*	*N. bombi*
Antrim	*B. monticola*	Least †	-		*	
	B. pratorum	Most	-	*		*
Dartmoor	*B. monticola*	Least ††	-	*	*	*
	B. pratorum	Most	-			
Dublin	*B. monticola*	Equal	9h 30mins			*
	B. pratorum	Equal	9h 30mins	*	*	
Lake District	*B. monticola*	Most	-			
	B. pratorum	Least †	-	*	*	*
Long Mynd	*B. monticola*	Most	7h 40mins	*	*	
	B. pratorum	Least	10h 15mins	*		*
Snowdonia	*B. monticola*	Equal	9h			
	B. pratorum	Equal	9h	*	*	*
Stiperstones	*B. monticola*	Least	5h 30mins	*	*	
	B. pratorum	Most	5h			*

Table 5.3. Frequency of parasitic infection in *B. monticola* samples. Number of samples screened indicates the number of individual bees which were analysed for parasite load at each site. The subsequent columns indicate the number of infected individuals that were detected. Only the results from the most effective detection methods are shown. Results shown for *A. bombi*, *C. bombi*, and *N. bombi* are from molecular screening. Results shown for *L. buchneri* and *Syntretus* spp. are from dissection.

Species	Sample site	No. of samples screened	*A. bombi*	*C. bombi*	*L. buchneri*	*N. bombi*	*Syntretus* spp.
B. monticola	Antrim	39	1	24	3	9	0
	Ben Lawers	30	2	16	1	1	0
	Dartmoor	40	1	5	1	11	0
	Dublin	37	0	12	0	23	0
	Glen Shee	36	0	1	1	0	0
	Lake District	40	6	2	0	2	1
	Long Mynd	39	1	17	1	0	0
	Peak District	-	-	-	-	-	-
	Stiperstones	40	13	27	0	0	0
	Snowdonia	40	10	15	0	0	4
	Total:	341	34	119	7	46	5

Table 5.4. Frequency of parasitic infection in *B. pratorum* samples. Number of samples screened indicates the number of individual bees which were analysed for parasite load at each site. The subsequent columns indicate the number of infected individuals that were detected. Only the results from the most effective detection methods are shown. Results shown for *A. bombi*, *C. bombi*, and *N. bombi* are from molecular screening. Results shown for *L. buchneri* and *Syntretus* spp. are from dissection.

Species	Sample site	No. of samples screened	*A. bombi*	*C. bombi*	*L. buchneri*	*N. bombi*	*Syntretus* spp.
B. pratorum	Antrim	31	2	21	4	14	2
	Ben Lawers	-	-	-	-	-	-
	Dartmoor	40	0	1	9	2	0
	Dublin	40	5	23	17	0	0
	Glen Shee	-	-	-	-	-	-
	Lake District	19	8	13	10	1	1
	Long Mynd	36	1	4	9	7	1
	Peak District	40	5	8	8	6	0
	Stiperstones	39	6	3	17	1	0
	Snowdonia	36	11	15	8	3	3
	Total:	281	38	88	82	34	7

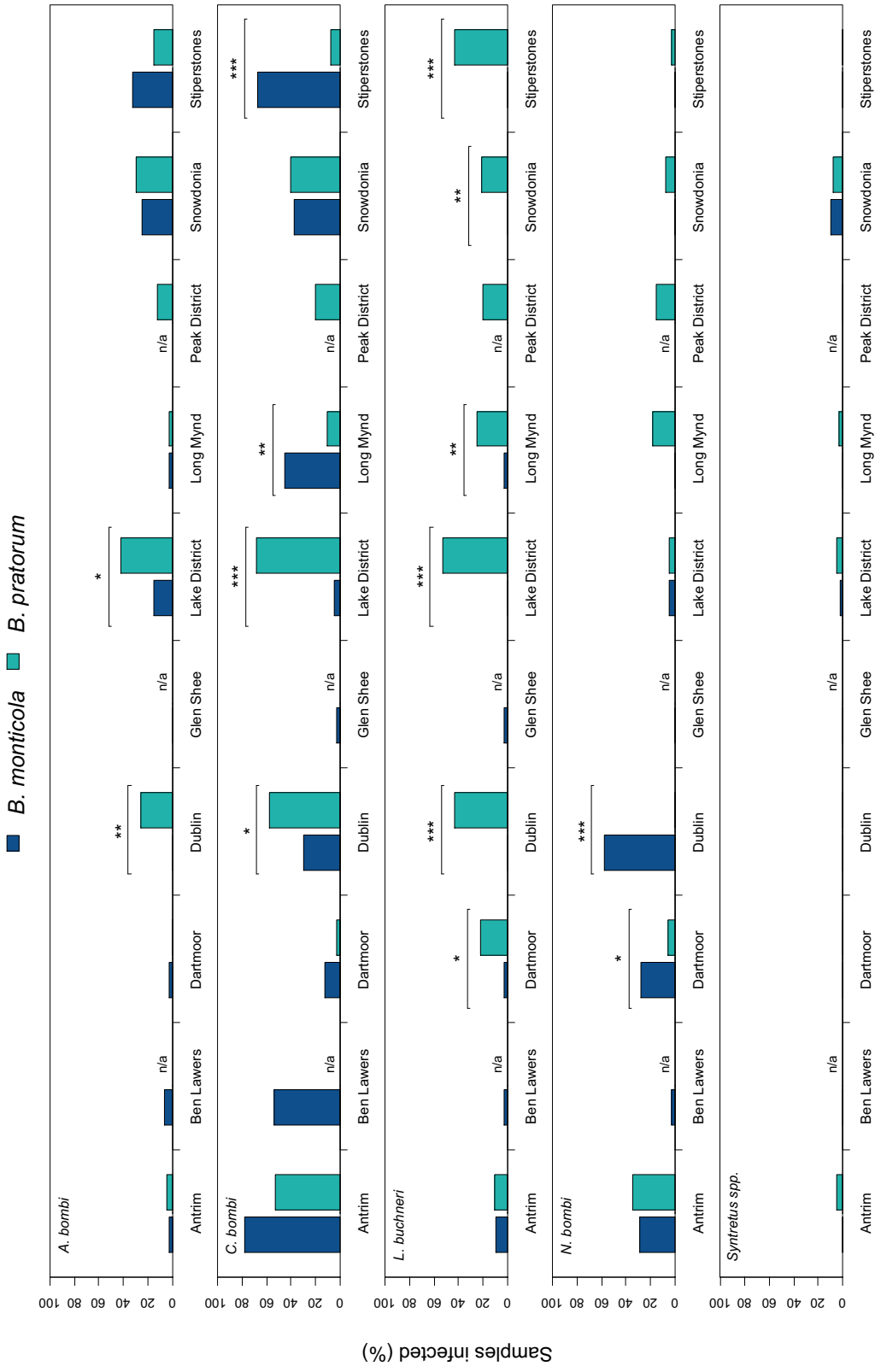

Figure 5.6. Comparison of the prevalence (i.e. the presence or absence of infection) of five internal, bumblebee-specific, parasite species. The sample site is indicated in each panel of the figure. Significant differences are indicated with asterisks over the relevant bars (*= $p<0.05$; **= $p<0.01$; ***= $p<0.001$).

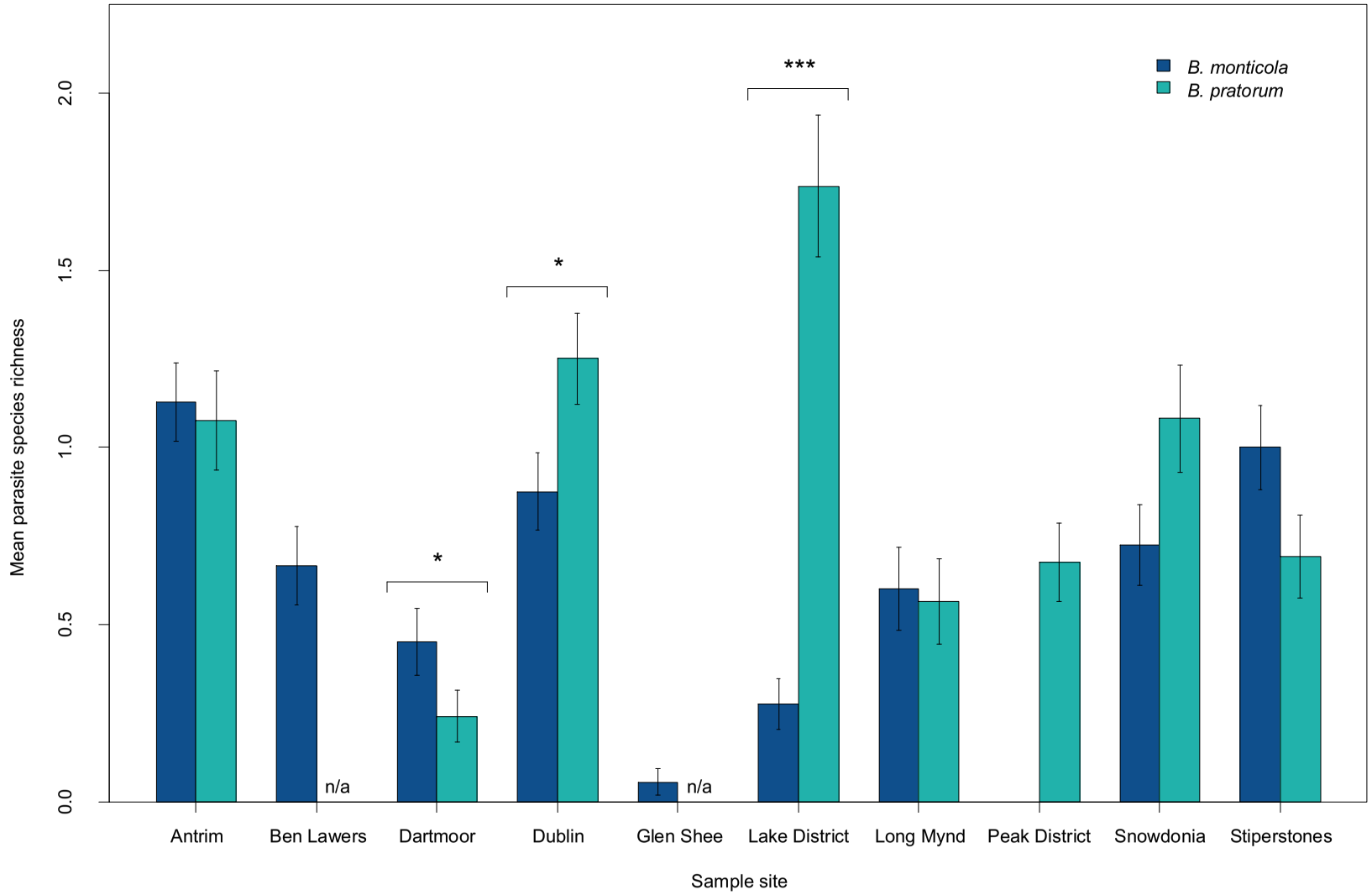

Figure 5.7. Comparison of parasite species richness (defined as the number of parasite species infecting the individual being measured) between *B. monticola* and *B. pratorum*. Bars indicate mean values ± standard error. Significant results are indicated with asterisks over the relevant bars (*= $p<0.05$; **= $p<0.01$; ***= $p<0.001$).

was no significant effect of age in either species (*B. monticola*: χ^2=1.43, df=1, p=0.231; *B. pratorum*: χ^2=0.02, df=1, p=0.885). Thorax weight was significant in *B. monticola* (χ^2=4.50, df=1, p<0.05), with smaller individuals showing a higher prevalence of *N. bombi* infection. Thorax weight was not significant in *B. pratorum* (χ^2=0.17, df=1, p=0.678). Sample site was again significant in both species (*B. monticola*: χ^2=68.48, df=8, p<0.001; *B. pratorum*: χ^2=37.61, df=7, p<0.001). The *L. buchneri* data could only be analysed for *B. pratorum*. There was no significant effect of age (χ^2=<0.01, df=1, p>0.99) or thorax weight (χ^2=0.28, df=1, p=0.594), but sample site was significant (χ^2=20.39, df=7, p<0.01). There were no significant interaction terms in the results for any of the analyses.

For parasite species richness in *B. monticola*, sample site was significant (dev=64.74, df=8, p=<0.001), and age was close to significant (dev=3.75, df=1, p=0.053) with slightly higher parasite species richness seen in older individuals. Thorax weight was not significant (dev=2.55, df=1, p=0.110). For *B. pratorum*, sample site was again significant (dev=48.68, df=7, p<0.001). There was no significant effect of age (dev=0.82, df=1, p=0.366) or thorax weight (dev=0.01, df=1, p=0.919). There were no significant interaction terms for either species.

5.3.5 Assessment of annual variation in parasite load

In addition to the samples collected in 2011, samples of both *B. monticola* and *B. pratorum* were also collected on Dartmoor in 2012 (a total of 22 *B. pratorum* and 16 *B. monticola*). Samples were collected between May-June in both years. The prevalence of parasites was compared between years for both species (Fig. 5.8). For *B. monticola* only *N. bombi* prevalence was significantly different between years (Fisher's Exact Test, p<0.05). Variation in the prevalence of all other parasite species was non-significant (*A. bombi*: Fisher's Exact Test, p>0.99; *C. bombi*: Fisher's Exact Test, p=0.31; *L. buchneri*: Fisher's Exact Test, p>0.99). For *B. pratorum*, *A. bombi* prevalence was significantly different (Fisher's Exact Test, p<0.01). While there was variation in the prevalence of the other parasite species, it was not statistically significant (*C. bombi*, Fisher's Exact Test, p>0.99; *L. buchneri*, χ^2=1.53, df=1, p=0.22; *N. bombi*, Fisher's Exact Test, p=0.53).

The prevalence of parasites was also compared between *B. monticola* and *B. pratorum* for the samples collected in 2012. As with the 2011 samples, there was

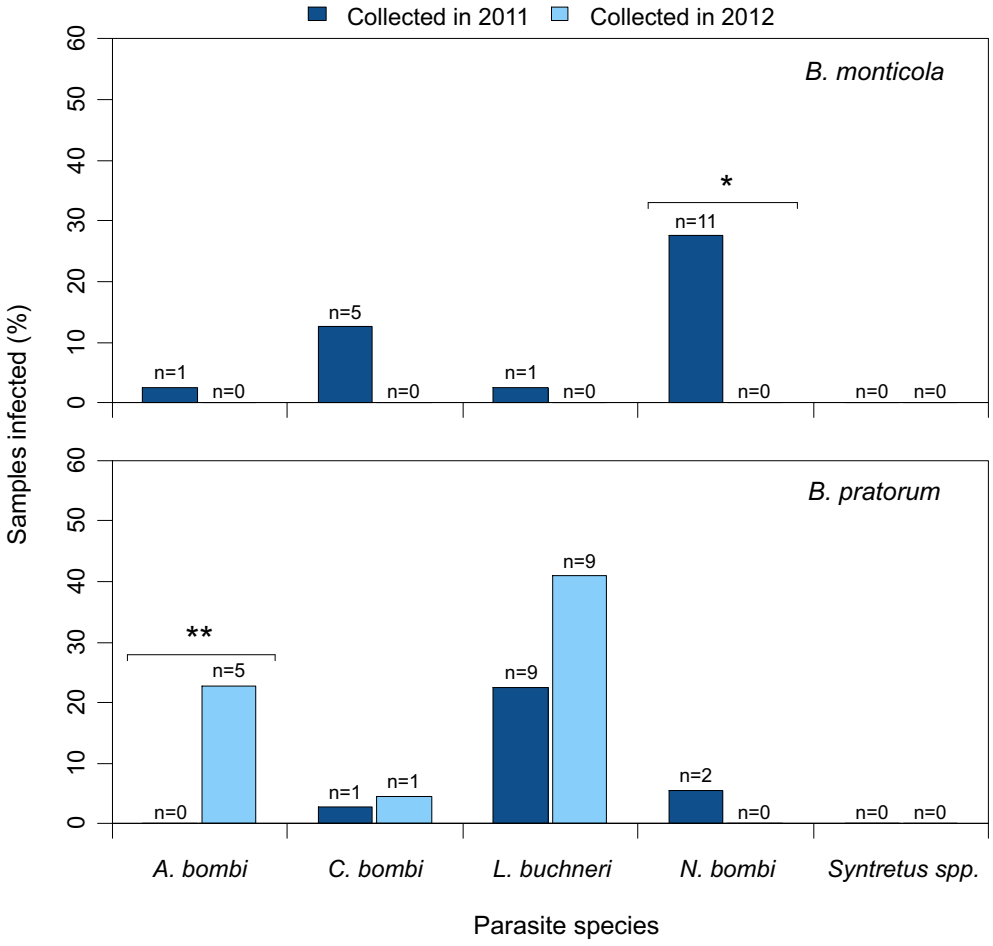

Figure 5.8. Comparison of parasite prevalence in samples collected on Dartmoor in two consecutive years. Results are expressed as a percentage, with the number of infected individuals indicated above each bar. Significant results are indicated with asterisks over the relevant bars (*= $p<0.05$; **= $p<0.01$).

a significant difference in the level of *L. buchneri* infection (Fisher's exact test, $p<0.01$), with 9 infections observed in *B. pratorum* and none in *B. monticola*. No significant differences were observed in the occurrence of *A. bombi* (Fisher's exact test, $p=0.061$) or *C. bombi* (Fisher's exact test, $p>0.99$). No *N. bombi* or *Syntretus* infections were found in either study species.

5.4 Discussion

5.4.1 Molecular markers as a tool for parasite screening

Screening for parasites in *Bombus* has chiefly been achieved using light microscopy to detect and quantify parasite cells (such as Brown et al. 2003b, Whitehorn et al. 2010, Goulson et al. 2012). However, this method can be unreliable for some parasites such as *Nosema* spp. especially when an individual carries only a light infection (Rinder et al. 1998, Klee et al. 2006). In these cases, molecular methods are significantly more accurate at detecting the presence of an infection (Klee et al. 2006), and can also be used to identify cryptic subspecies (such as the two subspecies of *Crithidia*) which cannot be distinguished by eye (Schmid-Hempel & Tognazzo 2010). Results from this study support these findings; the only parasite species which could be reliably detected using light microscopy were *L. buchneri* and *A. bombi* (Fig. 5.5); in the case of *A. bombi*, more infections were detected using molecular screening than by microscopy, which again indicates that PCR-based screening may be a more reliable option. The failure to detect certain species using microscopy may have resulted from the use of frozen specimens; the process of freezing and thawing the samples may have disrupted the parasite cells sufficiently to make them undetectable under a microscope. The same issues my not be encountered using live specimens; however, the training required for identification of parasites, and a complex dissection protocol, make light microscopy a much more time-consuming method when compared with molecular screening. While this study used entire abdomens for DNA extraction (requiring lethal sampling) it would be possible to perform DNA extractions on faecal samples, allowing the use of live specimens (although faecal samples may lead to underestimation of infection rates, see Goulson et al. 2012). Additionally, PCR-based screening can be used for samples preserved in ethanol, which cannot be dissected due to disruption of the internal organs (K. Lloyd, pers. comm.).

Light microscopy was unsuitable for samples preserved by freezing as in this study (although samples freeze-killed using liquid nitrogen have been used successfully by others; Whitehorn et al. 2010, Goulson et al. 2012), or for samples preserved in ethanol, two of the most frequently used and convenient methods for preserving wild-caught specimens. There is also limited sensitivity for the detection of some parasites such as *Nosema* (Rinder et al. 1998, Klee et al. 2006). These factors, combined with the time-consuming dissection protocol and extensive training required, make molecular markers an attractive option for identifying parasitic infection in bumblebees. In addition to much faster sample processing times, molecular screening is much more sensitive than traditional microscopy in some cases (Rinder et al. 1998, Klee et al. 2006, Arbetman et al. 2013), and can help with identification of cryptic subspecies (Schmid-Hempel & Tognazzo 2010). However, while the use of PCR-based methods may provide a quick and sensitive way to detect infection by various parasite species, it can only provide presence/absence data. This lack of resolution may be a disadvantage in comparison to light microscopy, which allows the intensity of an infection to be quantified. However, this could be remedied with the use of qPCR. This would allow both the identification and quantification of parasitic infection, avoiding the need for time-consuming training and dissections. While there has been some use of qPCR in this context (Klee et al. 2006, Bourgeois et al. 2010), it has not yet been widely explored.

Although many more infections were detected using PCR, non-specific products were produced when using the *N. bombi* primers (Klee et al. 2006; Fig. 5.4). Negative control reactions that were run using host thorax tissue showed no amplification (Fig. 5.1), indicating that amplification of host DNA was not the cause of the issue. The bands may have resulted from external contamination; there are many types of tissue present in the abdomen, including matter which has been ingested such as pollen and any contaminants therein. It is therefore possible that the primers were amplifying plant material, other fungal spores or bacteria that had been ingested during foraging, which would explain why non-specific banding patterns were not observed during the negative control stage of primer testing. However, a BLAST search of the primer sequences showed no matches with any listed sequences. The variability of the bands between runs also contradicts the hypothesis of contaminant DNA being amplified. It therefore seems most likely that the bands resulted from non-specific primer binding, and suggests that the *N. bombi* primers need to be carefully checked and possibly re-designed. Ideally, the bands could be excised from the sample gels and sequenced to determine their

source. While this process is out of the scope of the current project, this would certainly be a relevant question to ask in the future. The primers used are not described as suitable for use only in a specific *Bombus* species, or under specific aseptic DNA extraction conditions (Klee et al. 2006). The possibility of their being ineffective under some circumstances therefore needs to be addressed, as they could be a very useful tool.

5.4.2 Variation in parasite prevalence between species and sites

Differences in parasite prevalence between *Bombus* species have been widely reported (Gillespie 2010, Cameron et al. 2011, Cordes et al. 2012, Whitehorn et al. 2013). Species with more isolated populations are likely to have lower genetic diversity, and this has been amply demonstrated in the Hymenoptera (Packer et al. 2005, Zayed et al. 2005, Ellis et al. 2006, Whitehorn et al. 2010). This may be linked with an increased risk of extinction, particularly in haplo-diploid species (such as *Bombus* spp.) which experience a lower effective population size (N_e) than diploid species (Zayed & Packer 2005, Zayed 2009). Traditional population genetic theory would suggest an association between loss of genetic diversity and a reduction in fitness (such as reduced immunocompetence and increased susceptibility to disease and parasitism) as a consequence of this reduction in genetic diversity and adaptive potential (Keller & Waller 2002, Frankham 2005). Such correlations have been observed in many species groups including mammals (Da Silva et al. 2006, 2009), birds (Markert et al. 2004, Brouwer et al. 2007), fish (Lieutenant-Gosselin & Bernatchez 2006), insects (Spielman et al. 2004) and social insects (Tarpy 2003, Hughes & Boomsma 2004, Calleri et al. 2006), including *Bombus* (Herrmann et al. 2007, Whitehorn et al. 2010), lending support to the theory that lower genetic diversity measured at neutral loci will reflect the diversity, and potential loss thereof, at functional loci. Inter-species differences in parasite load may therefore be explained by differences in the distribution and continuity of populations. In the context of this study, it would therefore be expected that *B. monticola*, having a more patchy distribution across the UK and Ireland (BWARS 2014; Fitzpatrick & Murray 2014), would show higher levels of parasite load when compared to *B. pratorum* at the same sample sites. Alternatively, it may be expected that wider ranging species will show increased tolerance to parasitism, as an adaptation to the wider variety of pathogens across its range; this

trend has also been demonstrated in *Bombus* across Europe (Durrer & Schmid-Hempel 1995). In the context of this study, this would lead to the expectation of higher parasite loads in *B. pratorum*, being the wider-ranging species across the UK and Ireland, and inhabiting a wider variety of habitats (Edwards & Jenner 2005). *B. pratorum* also inhabits more urban areas, due to its generalist habitat and dietary requirements (Edwards & Jenner 2005). Population densities in urban areas can be higher than in rural areas, which may result in higher parasite prevalence (Goulson et al. 2012).

This study supports neither of the aforementioned hypotheses. *L. buchneri* was the only parasite to be found almost exclusively in one species, with 92% of infections occurring in *B. pratorum*. The reasons for such a pronounced difference are unclear, as little is known regarding the biology and method of transmission of *L. buchneri* (Yoneda et al. 2008). Unlike the microparasites, the larval stage of *L. buchneri* is mobile; it may therefore be possible that the parasite species is able to exert a degree of preference as to its host. Alternatively, some aspect of the behaviour of *B. pratorum*, possibly foraging or nesting preferences, may allow more frequent contact with *L. buchneri* larvae in comparison to *B. monticola* - further research into the biology of *L. buchneri* is required to shed light on this issue. With the exception of *L. buchneri*, which due to its mobile larval stage may be transmitted differently to the microparasites, no consistent trends were seen in terms of parasite prevalence or parasite species richness between *B. monticola* and *B. pratorum*. However, parasite prevalence varied significantly between sample sites in both study species. This study does not therefore suggest a link between parasite susceptibility and population isolation (and by extension genetic diversity at neutral markers), in contrast to previous work in *Bombus* and other groups (Spielman et al. 2004, Hughes & Boomsma 2004, Calleri et al. 2006, Whitehorn et al. 2010).

While the results presented here do not support a correlation with the degree of population isolation, parasite susceptibility certainly has a genetic component. Host genotypes vary in their susceptibility to parasitism (Imhoof & Schmid-Hempel 1999, Mallon et al. 2003b), while different strains of parasites vary in their ability to infect a host (Yourth & Schmid-Hempel 2006). The infection outcome is generally the result of interactions between the genotypes of the parasite and host. This may be expected to exert strong selective pressures for variation in both parties (Salathé et al. 2008). Parasites in *Bombus* tend to be generalists, infecting multiple species within the genus (Baer & Schmid-Hempel 2001, Colla

et al. 2006, Rutrecht & Brown 2008a,b, Meeus et al. 2011). This would encourage balancing or diversifying selection, consequently increasing the variability in parasite genotypes and thus increase the chances of successfully interacting with a host to cause infection. There is evidence to show that this is the case; *C. bombi* for example is highly genetically variable (Schmid-Hempel & Reber Funk 2004, Schmid-Hempel & Tognazzo 2010) and shows rapid evolution after serial passage through hosts (Schmid-Hempel et al. 2011), and depending on the method of transmission (Schmid-Hempel et al. 1999). It may therefore be expected that an "arms race" would begin, with the host species under selection for high variability in immune gene loci (as is the case for the MHC in vertebrates for example; Wegner et al. 2003, Aguilar et al. 2004).

Contrary to this expectation some immune genes in *Bombus* have been found to show very low levels of polymorphism (Ellis et al. 2012). This may be because some genes are pleiotropic, i.e. perform roles in other processes, and are therefore constrained in their sequences and function; an example of this is the Toll pathway, which is involved in both the innate immune response and embryonic development (Barribeau et al. 2014). Variability at such loci would be detrimental to fitness, and so these loci may be expected to be under purifying selection. This leads to the apparently paradoxical situation in which genes that would be expected to be highly diverse are in fact actively maintained in a constant state. Despite this, the innate immune response in *Bombus* is still capable of highly specific responses to infection depending on the type of pathogen (Schmid-Hempel 2005, Schulenburg et al. 2007). It is believed that this specificity may result from strong selection for variability in the regulatory genes controlling the effector immune loci (Barribeau et al. 2014). AMP expression is generally upregulated in response to infection, including infection by parasites (Schlüns et al. 2010, Deshwal & Mallon 2014). However, the expression of genes linked with immune processes, including the production AMPs, is highly variable between colonies irrespective of parasitic infection (Schlüns et al. 2010, Brunner et al. 2013). This variability in gene expression, rather than in the sequences of genes, may underpin the specificity of the innate immune response, and explain differential responses of host genotypes to infection (Barribeau et al. 2014).

There is an increasing body of evidence to suggest that, while the specific receptor and effector genes involved in innate immunity may be under purifying selection to preserve their various functions (Ellis et al. 2012), regulatory genes may be under strong balancing selection for diversity. There is also the possibility of

frequency-dependent selection on both hosts and parasites, as genotype interactions (and subsequent variation in gene expression) will determine which strains of parasite are most successful in a given season, as well as which host genotypes. Variation in gene expression, resulting from strong selection on the regulatory regions responsible for the immune response, combined with genotype interactions between hosts and parasites, could explain the highly variable nature of parasite load between sample sites for *B. monticola* and *B. pratorum* in this study. AMPs are known to play a vital role in the innate immune response to parasites (Schlüns et al. 2010, Deshwal & Mallon 2014), and their expression varies depending on the host genotype, parasite genotype, and interactions between them (Barribeau & Schmid-Hempel 2013, Barribeau et al. 2014). AMP activity was observed to vary significantly between sample sites for both *B. monticola* and *B. pratorum* in this study (see Chapter 4), and could therefore explain the variation in parasite prevalence with site. The lack of variability between *B. pratorum* and *B. monticola* in terms of parasite load at any given site may also be explained in this way (discussed further in Chapter 7). As *B. monticola* exists in fragmented populations, it is reasonable to assume that its genetic diversity at neutral loci may be reduced compared with the ubiquitously distributed *B. pratorum*, in accordance with studies of other *Bombus* species (Darvill et al. 2006, Ellis et al. 2006, Whitehorn et al. 2010). However, given that genes involved in immunity (and the genes which regulate them) are likely to be under strong selection, it is questionable to what extent traits such as immunity and parasite resistance would correlate simply with diversity measured at neutral loci. In light of this, the lack of an obvious and consistent difference in the response of a species relating to its level of population isolation may not be unexpected, and is reflected in the lack of a consistent difference between species in parasite load or AMP activity (see Chapter 4).

While population isolation may not be correlated with immunocompetence in this case, smaller and more isolated populations may still be at risk of extinction. *B. monticola* populations in the UK and Ireland are unevenly distributed and restricted due to the habitat specialisation of the species (BWARS 2014; Fitzpatrick & Murray 2014). Selection is known to act less efficiently in smaller populations (Campbell et al. 1999), and it may therefore be expected that a species such as *B. monticola* may experience relatively lower fitness due to a lack of selection to promote diversity and/or directional change at crucial loci. However, this was not observed in this study. While there are significant differences in parasite load

between species at some sample sites there are no consistent trends (with the exception of *L. buchneri*, as described before), strongly suggesting that *B. monticola* is not suffering genetic impoverishment relative to *B. pratorum* (or at least not of a nature that affects parasite load and resistance). The quantification of relative abundance of *B. monticola* and *B. pratorum* at the sample sites in this study showed that, locally, the two species were generally of very similar or equal abundance. Hence, while *B. monticola* populations may be fragmented, the N_e of *B. monticola* may still be sufficient to allow the maintenance of efficient selection to promote immunocompetence. While these data are not currently available, it highlights the need for such basic information to be gathered when assessing the risks faced by any particular species. The level of variability in parasite load and AMP activity observed in *B. pratorum* may also be unexpected. As a widely distributed species across the UK and Ireland (BWARS 2014), a high level of gene flow and interbreeding would be expected (Frankham 2005), promoting functional genetic diversity and high parasite resistance across all populations. However, AMP expression may be variable between populations of *B. pratorum*, even though it is almost universally distributed. Populations of *B. terrestris* in mainland Europe show significant differences in the expression of immune genes in different areas, which may be indicative of local adaptation (Brunner et al. 2013, 2014), despite *B. terrestris* being a panmictic species (Estoup et al. 1996). Such fine scale adaptation in a widely distributed species may therefore explain the variability in AMP production and parasite load observed in *B. pratorum* in this study.

Host genotype, parasite genotype, and the interactions between them, form a complex network which can influence host gene expression and the infection success of parasites. However, these interactions are in turn influenced by a myriad of external factors, such as gut microbiota (Koch & Schmid-Hempel 2011, 2012, Koch et al. 2013), food availability and quality of nutrition (Alaux et al. 2010, Sadd 2011, Brunner et al. 2014), and environmental variables (Fig. 5.9).

As discussed, AMPs are crucial for immunity and parasite resistance in *Bombus* (Schlüns et al. 2010, Deshwal & Mallon 2014). A lack of dietary protein has been shown to cause reduced expression of genes responsible for AMP production, and hence a greater susceptibility to infection (Brunner et al. 2014). Dietary breadth has also been linked with immunocompetence in honeybees (Alaux et al. 2010). In the case of *C. bombi*, the infection intensity in *Bombus* workers may be reduced under starvation conditions, possibly due to reduced resource availability for the

parasite (Logan et al. 2005). However, the parasite also shows greater virulence when workers are exposed to starvation stress (Brown et al. 2000). Parasite prevalence may therefore be linked to floral resources at a given site. Assuming that bees are well provisioned, making any infections relatively less virulent, individuals may survive longer and increase levels of horizontal transmission. This may lead to the detection of higher parasite prevalence. Conversely, if forage resources are scarce in a given year, the added stress of starvation may mean that individual bees succumb more quickly to infection, and die more quickly once infected. The summer of 2011 was exceptionally wet, especially in Scotland. This is likely to have affected the foraging capabilities of bees in that region, and may account to some extent for the low levels of parasite prevalence observed at the Ben Lawers and Glen Shee sample sites. It may simply have been that infected individuals died earlier, so were not found during sampling. This is particularly true for a parasite such as *A. bombi*, which affects the adipose tissues; an individual infected with *A. bombi* would experience degradation of adipose tissues (Durrer & Schmid-Hempel 1994), which may speed up the effects of starvation caused by a lack of opportunity to forage. A similar effect may be observed in individuals infected with *C. bombi*; a reduction in learning capacity (Gegear et al. 2005) and subsequent reduction in foraging efficiency could also exacerbate the effects of forage shortage. Data from this study indicated considerable variation in dietary breadth and forage plant choice between sample sites for both *B. monticola* and *B. pratorum* (see Chapter 2); in light of evidence from other studies, it is likely that the observed patterns of parasite prevalence between sites may be linked to foraging behaviour. However, given the overlap in foraging behaviour between the study species (see Chapter 2) this does not fully explain the variability in parasite load between them at individual sites.

Foraging behaviour may in turn be linked with the gut microbiota, which has received much recent attention as a possible extension to the innate immune response (Koch & Schmid-Hempel 2011, 2012, Koch et al. 2013). Species-specific communities of gut microbiota have been described in honeybees and bumblebees (Martinson et al. 2011), which appear to be vertically transmitted between nest mates and therefore crucially linked to eusociality (Koch & Schmid-Hempel 2011); this concept is supported by the lack of such microbial communities in solitary bees (Martinson et al. 2011). The presence of a specific gut microbiota appears to play a role in reducing the likelihood and intensity of infection by *C. bombi*, and is effective against a number of parasite genotypes (Koch & Schmid-Hempel 2011,

2012). Being a gut parasite, *C. bombi* may be particularly affected, but similar effects are not seen against other common parasites such as *N. bombi* (Koch & Schmid-Hempel 2011). Studies in this area have so far been confined to *B. terrestris*, so it is unclear whether gut microbiota would vary significantly between *Bombus* species, and whether the protective effects would be similar. The data reported so far suggest that the microbiota in *B. terrestris* are similar between colonies (Koch & Schmid-Hempel 2011); gut microbiota alone therefore seem an unlikely driver of inter-population variability in parasite susceptibility, although data regarding the possible effects on parasite species other than *C. bombi* are thus far lacking.

The role of abiotic environmental conditions must also be considered. Day-to-day weather conditions may influence foraging behaviour, and hence nutrition, dietary protein and immunocompetence (Brunner et al. 2014). Variation in weather between seasons may cause annual variation in foraging patterns and success, and possibly influence seasonal of immunocompetence and parasite load. Longer-term climatic trends will also influence the vegetation assemblage at a site, which may have knock-on effects on foraging and nutrition. Variation in abiotic factors may therefore contribute to inter-population variability in AMP activity and expression, and parasite load, and may therefore have contributed to the variability reported in this study for both *B. monticola* and *B. pratorum*. However, as there were significant differences between the two study species at some sites, it is unlikely that abiotic factors alone are the driving force behind the patterns observed.

5.4.3 Factors influencing individual infection risk and intensity

Although *B. monticola* and *B. pratorum* did not differ consistently in parasite load, and the variation observed was not related to differing levels of abundance. However, there was some suggestion that individual characteristics may influence the likelihood of infection. Although a relatively weak trend, infection with *C. bombi* was more likely in *B. pratorum* individuals with a lower thorax weight, with the same trend seen for *N. bombi* in *B. monticola*. This may be because smaller bodied individuals have fewer reserves (i.e. fat reserves) to allow an effective immune response to be mounted. Alternatively, the pattern may be linked to behavioural differences linked with size; larger bodied individuals forage more

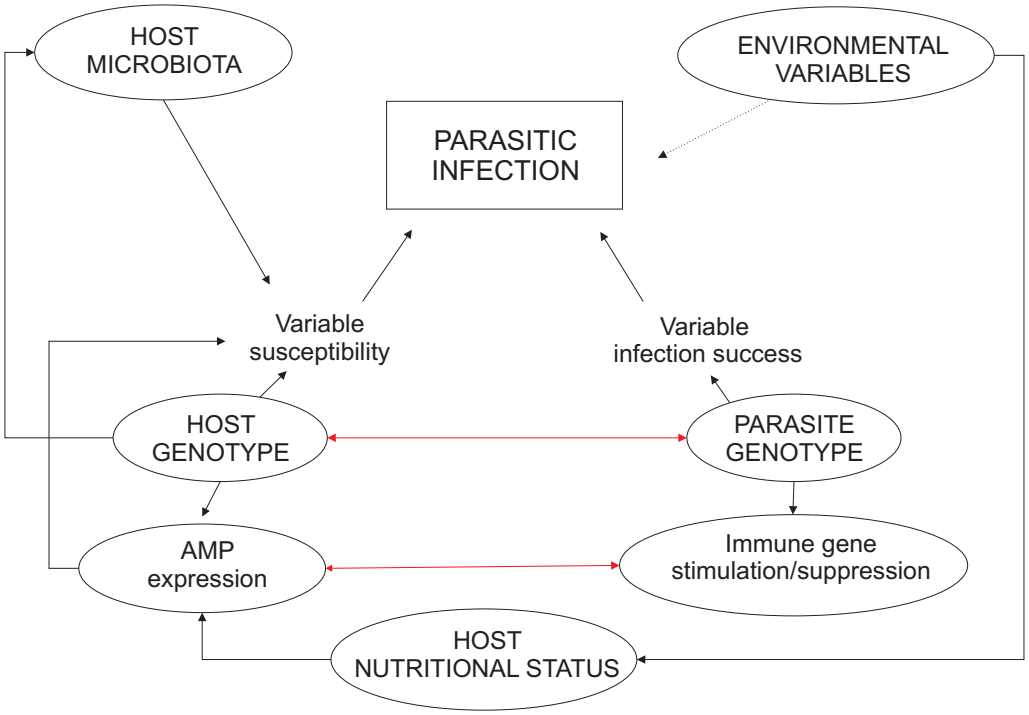

Figure 5.9. Proposed influences on parasitic infection risk in *Bombus*. Interactions between factors are shown with red arrows. Speculative links are shown with dotted lines.

frequently, therefore spending more time outside the hive than smaller bodied individuals (Goulson et al. 2002). *N. bombi* is transmitted via ingestion of infectious spores, so all castes of *Bombus* can be affected (Schmid-Hempel & Loosli 1998, Otti & Schmid-Hempel 2007). However, it may be that larger individuals, being exposed more frequently to the parasite outside the nest during foraging, are more likely to accumulate a higher intensity and more lethal infection than those individuals which are infected secondarily. If this was the case, larger individuals may die sooner, and therefore be collected less frequently during sampling.

For both *B. monticola* and *B. pratorum*, sample site was found to be by far the most significant factor. As discussed, there are many possible reasons for this, which are likely to be strongly inter-linked (Fig. 5.9). However, with data from only one field season, it is difficult to interpret the implications of the data for long-term trends, and thus in an evolutionary context.

5.4.4 Inter-annual variation in parasite prevalence

The prevalence of parasites in *Bombus* species varies between years (Durrer & Schmid-Hempel 1995, Ruiz-González et al. 2012), and the results of this study

concur with previous findings. Significant variation was found in the prevalence of some parasites at the Dartmoor sample site between samples collected in 2011 and those collected in 2012. Estimates of parasite prevalence would therefore differ drastically depending on when samples were collected; this is particularly true for *B. monticola* in the case of this study, as no infections were detected in the 2012 samples. The second sample collected on Dartmoor was smaller, and it could therefore be argued that there were insufficient samples to detect parasites. However, three parasite species were detected in *B. pratorum* in the smaller sample (Fig. 5.8), suggesting that the presence or absence of parasites at the site could be estimated, even if estimating their overall prevalence would require a larger sample size.

Variation in parasite prevalence would be expected in a "Red Queen" scenario (frequency-dependent selection on host and parasite species; Salathé et al. 2008). The interaction between various host and parasite genotypes over the course of the season will influence infection success; this will in turn influence which strains of parasite and which host genotypes are most likely to predominate in the following season. The variability in susceptibility through variation in immune gene expression (Barribeau et al. 2014), combined with future host-parasite genotype interactions, changing forage availability and environmental conditions, means that parasite prevalence will always be dynamic between, and within, years (Durrer & Schmid-Hempel 1995, Ruiz-González et al. 2012, Whitehorn et al. 2013).

These results highlight the challenges of interpreting parasite prevalence data based on samples from one field season. In addition to the potential issues already discussed with measuring parasite prevalence at just one time point, it is difficult to ascertain exactly which evolutionary processes may be acting on a population, when only a snapshot in time can be studied.

5.4.5 Limitations of the study and possible future work

The quantification of the relative abundance of the two study species in this study was necessarily crude, as each site could only be sampled at one time point. It is entirely possible that the relative abundance of the two study species would change over the course of the season, especially given the difference in emergence time between the two species. *B. pratorum* has a shorter colony cycle than *B. monticola* and emerges earlier in the spring; it is therefore possible that where *B.*

pratorum was found to be most abundant, this was simply because *B. monticola* had not yet reached the peak of its colony cycle. Conversely, in areas where *B. monticola* was found to be most abundant, this could simply be because *B. pratorum* colonies had already begun to decline. However, as male *B. pratorum* were not observed at any sites during sampling, this second option is less likely. In addition, a consistent pattern would be expected within species between sites if abundance and emergence time alone were responsible for determining parasite prevalence.

The prevalence of parasites increases over the course of a season (Goulson et al. 2012, Whitehorn et al. 2013). The two species in this study were sampled at roughly the same time at each sample site, so should have been exposed to the same environmental levels of parasites; however the difference in emergence time between the two species means that *B. pratorum* workers may in fact have been exposed for longer than *B. monticola* workers at the time of sampling. A possible increase in exposure time may result in higher parasite prevalence in *B. pratorum* workers relative to *B. monticola* workers. While this was seen at some sample sites, it was not a universal trend. Later emerging species may become infected at a faster rate as a result of increased background parasite levels (Goulson et al. 2012, Whitehorn et al. 2013), so any discrepancies in infection rate caused by emergence time may be counteracted in this way. However, the only way to be certain would again be to sample at multiple time points across the season, which would make it possible to track changes in parasite prevalence, and any difference in the rate of change between *Bombus* species.

The data presented here represent only a snapshot of parasite prevalence and infection dynamics in UK populations of *B. monticola* and *B. pratorum*. As discussed, it is difficult to interpret the processes which may be acting on populations with data from only one time point. Future studies could measure the same variables at multiple points throughout a season (as in Whitehorn et al. 2013) to determine variability of parasite prevalence within years. This would also address the potential problems of variable emergence times in different species; given the varied ecology of *Bombus* as a group, such issues are inherent to any comparative study. Surveys across multiple years would be useful for determining which evolutionary processes are likely to be acting on host-parasite systems in the field, and for mapping the population dynamics of species.

5.5 Conclusions

Although potentially limiting in that only presence/absence data can be obtained, molecular screening was found in this study to be a preferable method for detecting parasitic infection in *Bombus* samples, both in terms of the number of infections detected and the time required to process samples. The development of effective qPCR protocols would also be useful for this purpose, as this would allow relative infection intensity to be determined in addition to presence/absence.

There was no evidence to support any consistent patterns of parasite load in *B. monticola* or *B. pratorum*, although significant differences were occasionally observed. This indicates a lack of correlation between population fragmentation and parasite load in this system, indicating that differences are more likely due to inter-population variability in host genotypes and gene expression, combined with variable parasite genotypes, nutritional status and possible environmental effects. It also appears that while *B. monticola* populations in the UK and Ireland are fragmented and isolated, the level of local abundance is comparable to that of a universally distributed congener, *B. pratorum*. The lack of a consistently higher parasite load may suggest that the N_e of *B. monticola* is larger than would initially be expected, allowing maintenance of selection for parasite resistance. This possibility highlights the need for research into the fundamental properties of populations, particularly those believed to be of conservation concern, as levels of fragmentation may not reflect population viability and functionality.

The lack of consistent differences in parasite prevalence between the two study species, combined with significant inter-site variability within species, raises intriguing questions as to the long-term evolutionary processes underlying parasitism and host resistance. This certainly merits further investigation, particularly with respect to the maintenance of resistance in fragmented populations.

The data presented here highlight the complexity of assessing parasite load in *Bombus* species under field conditions, as well as some key limitations resulting from limited sampling opportunities. More extensive sampling over time is recommended to determine long-term patterns of infection and population dynamics. Further development of quantitative molecular screening methods is also recommended, as this would facilitate the processing of larger sample sizes to a higher level of resolution.

Chapter 6

Troubleshooting and sources of error in AFLP genotyping: a bumblebee case study

6.1 Introduction

Declining and rare species often occur in small, fragmented populations, which are likely to be isolated from each other. Migration between such populations may allow them to act as a larger metapopulation, maintaining a higher effective population (N_e) and reducing the risk of total extinction (Hanski 1998). In situations where migration is not possible or is restricted, small populations will experience higher levels of genetic drift, loss of genetic variation, and possibly an increased risk of extinction (Newman & Pilson 1997, Frankham 2005). This may be particularly true for *Bombus* species, as their eusociality and haplodiploidy mean N_e is lower than in diploid organisms (as the colony, not the individual, is the reproductive unit; Chapman & Bourke 2001, Zayed 2004). Where N_e is low, genetic diversity within the isolated population can become reduced through processes such as inbreeding (an increased probability of matings amongst individuals that share ancestry) and genetic drift (Frankham 2005). Genetic drift is the random sub-sampling of alleles within a population each generation, and through drift individual alleles can become fixed in the population (for example one allele at one locus present at both chromosomes in all individuals in a diploid organism), or can disappear entirely. Thus diversity is lost through successive generations and as a direct result heterozygosity is reduced. Genetic drift can alter allele frequencies even for non-neutral alleles subject to selection, as the process of selection becomes weaker while drift becomes stronger in smaller populations (Campbell et al. 1999). Due to the relaxation of selection, mutations which are mildly deleterious may persist and even become fixed by drift. Over time the accumulation of mildly deleterious alleles, or genetic load, may lead to a reduction in the fitness of the population as a whole when compared with ancestral populations (Hedrick & Kalinowski 2000).

Knowledge of the genetic structuring of populations is an important component in conservation, and can show levels of past and present migration (Bergl & Vigilant 2007), levels of inbreeding (Keller & Fournier 2002, Zayed et al. 2004, Darvill et al. 2006), highlight those populations which may be isolated (Ellis et al. 2006), and define populations for the purposes of management (Coates 2000, Lara-Ruiz et al. 2008). This information may be particularly important for the conservation of rare and endangered species. Genetic diversity and heterozygosity have been linked with fitness-related traits such as immunocompetence and parasite resistance (Baer & Schmid-Hempel 2003, Hughes & Boomsma 2004, Spielman et al.

2004). Consequently a loss of genetic diversity may act to exacerbate the effects of existing problems, such as habitat loss, increasing the rate of population decline and potentially leading to an "extinction vortex" (Gilpin & Soulé 1986).

The relevance of neutral genetic markers for studying fitness-related traits has been questioned (DeWoody & DeWoody 2005, Kohn et al. 2006). Functionally important loci are likely to be under balancing or purifying selection, and may therefore not follow the same patterns as neutral loci in situations where populations are small, isolated, or experiencing reduced migration. An example of this is the multi-histocompatability complex (MHC) locus, which can sometimes retain high levels of diversity in mammals even in populations which are very small and monomorphic at almost all other loci (Aguilar et al. 2004); however, the MHC locus may lose diversity faster than neutral loci after a bottleneck event through a combination of genetic drift and ongoing frequency dependent selection (Sutton et al. 2011). The loss of diversity at this locus has significant fitness implications, as seen in the spread of Tasmanian devil facial tumour disease (Siddle et al. 2007; reviewed by McCallum 2008). The fitness costs of losing diversity or allowing the persistence of deleterious mutations at functionally important loci are likely to create a strong selective pressure to maintain diversity at these sites. Thus, genetic impoverishment when assessed using neutral markers such as microsatellites may not translate into a loss of function or fitness.

B. monticola has shown large declines in range across the UK over the last two decades, while populations of *B. pratorum* have remained largely unchanged, with the species still widespread throughout the country (BWARS 2010-2014). The precise reason for this difference remains unclear, although it may be linked to *B. monticola* having more specific habitat requirements, being restricted to upland and moorland areas (see Chapter 2). Given the increased level of population isolation, it may therefore be expected that *B.monticola* will show a higher level of population genetic differentiation between sites than *B.pratorum*, owing to decreased gene flow. However, the decline of *B. monticola* has not been uniform across the UK; the species is still abundant and widespread in highland regions of Scotland for example, and has begun to colonise parts of Ireland (BWARS 2014; see Chapter 2). The levels of population sub-structuring may therefore vary across the range of the species.

6.1.1 Detecting genetic diversity and population differentiation

Amplified fragment length polymorphisms (AFLPs) were developed in 1995 (Vos et al. 1995). They have a number of applications including estimating genetic diversity (Amsellem et al. 2000, Krauss 2000, Mariette et al. 2001), identifying population structure (Gaudeul et al. 2004), identification of hybrids (Bensch et al. 2002) and speciation (Irwin et al. 2005), and detection of directional selection/adaptation (Campbell & Bernatchez 2004; Svensson et al. 2004, Storz 2005, Bonin et al. 2006). The technique relies on variation in the location and number of restriction sites across the genome in a similar fashion to restriction fragment length polymorphisms (RFLPs). Once DNA is extracted, it is digested using a combination of restriction enzymes, usually one "frequent" and one "rare" cutter (referring to the frequency with which they cut DNA). Using pairs of enzymes increases the specificity of amplification, as subsequent PCR only amplifies fragments containing restriction sequences for both enzymes (Vos et al. 1995). The most common combination of enzymes is *MseI* and *EcoRI*, but there are many different enzymes and combinations which can be used. After digestion, adapters are ligated onto the fragments to allow subsequent addition of primers for PCR. Amplification occurs in two stages. The initial "pre-selective" PCR consistently amplifies only a subset of the total fragments. The second "selective" stage uses primers with additional nucleotides to further reduce the selection of fragments that are amplified. Fluorescent tags can be incorporated at the selective PCR stage.

While initially used mainly in studies of plants and fungi, AFLPs increased in popularity in the early 2000s for studies in other taxa (Bensch & Akesson 2005). Dominant markers (which detect only the dominant allele at a locus) like AFLPs have a number of advantages; they require no prior genome knowledge, making them ideal for studying non-model organisms, and are relatively cheap to develop and use in comparison to codominant marker systems such as microsatellites. AFLPs are particularly useful as they produce more repeatable results than other dominant marker systems such as RAPDs (Jones et al. 1997, Mueller & Wolfenbarger 1999, Nybom 2004; but see Skroch & Nienhuis 1995). A drawback of a dominant marker system is that each individual locus is less informative when compared with a codominant locus (heterozygosity cannot be directly determined for example). It is possible to estimate heterozygosity in a population from an

AFLP data set (Piepho & Koch 2000, Jansen et al. 2001), but the reliability of such estimates has not been fully determined (Bensch & Akesson 2005). However, what dominant loci such as AFLPs lack individually, they make up for in numbers. One of the main advantages cited for AFLPs over codominant, well characterised, and targeted markers such as microsatellites is their much higher frequency across the genome (Meudt & Clarke 2007). While a microsatellite study may screen around 10-20 loci, it is not uncommon for studies using AFLPs to screen up to 500 loci. The larger numbers of loci are effectively a greater sampling effort, meaning that unusual variants are more likely to be detected, as well as signatures of selection. This may be particularly relevant for studies investigating local adaptation (Bonin et al. 2006). AFLP loci are suggested to be more randomly distributed throughout the genome than microsatellites, giving more thorough genome coverage (Meudt & Clarke 2007), although this is not universally accepted (Saliba-Colombani et al. 2000). While dominant markers in general have the drawback that population parameters such as allele frequencies and expected heterozygosity must be estimated (for example by assuming Hardy-Weinberg equilibrium), increasingly sophisticated and specific analysis methods mean that results produced using dominant marker systems, especially AFLPs, can equal or surpass those produced using markers such as microsatellites (McGregor et al. 2000, Belaj et al. 2003, Campbell et al. 2003, Sønstebø et al. 2007).

This study aimed to address the following questions:

1. Does genetic diversity differ between *B. monticola* and *B. pratorum* within sample sites?

2. Is there evidence of population structuring in *B. monticola* and/or *B. pratorum*?

6.2 General methods

6.2.1 Restriction-ligation

Restriction-ligation reactions were performed using 3μl of DNA extract. DNA concentrations were between 50-250ng μl^{-1}. PCR success and subsequent scoring of AFLP loci were unaffected by the quantity of DNA added to the initial restriction reaction, in agreement with previous studies (Trybush et al. 2006). A variety of enzyme combinations were tested (Fig. 6.1). The final reaction conditions used

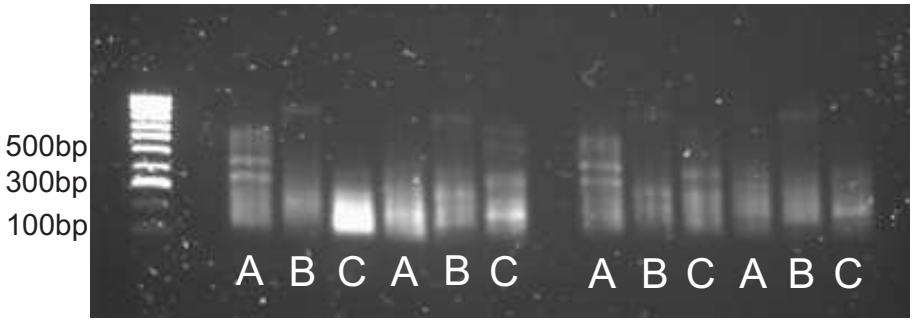

Figure 6.1. Pre-selective PCR products of restriction digests performed using a variety of restriction enzyme combinations. The products were checked at the pre-selective PCR stage, as restriction products were often not bright enough to visualise on an agarose gel. A = *MseI-PstI*; B = *TaqaI-Eco-RI*; C = *TaqaI-PstI*. The combination *MseI-PstI* produced a larger range of fragments (100-600bp) than the initial restriction combination of *EcoRI-MseI* (100-370bp). It was therefore decided to use a *MseI-PstI* restriction digest for subsequent genotyping of the samples.

PstI as the rare cutter and *MseI* as the common cutter, which produced a smear of fragments between 100-600bp (Fig. 6.1). Reactions were allowed to proceed at 56°C for 3 hours according to the manufacturer's instructions; the restriction enzymes were then denatured by heating to 80°C for 20 minutes.

6.2.2 Pre-selective PCR

For the pre-selective PCR reaction, 2μl of the restriction-ligation product was combined with 2X BioMix (Bioline) and 2μM of each pre-selective primer (Table 6.1). The total reaction volume was 10μl. The PCR program was: 94°C for 2mins; 32 cycles of 94°C for 30secs, 56°C for 1min, 72°C for 1min; 72°C for 5mins. The reactions were checked by running 5μl of the PCR product for 1 hour at 100V using a 1% agarose gel stained with SYBRsafe (Life Technologies).

6.2.3 Selective PCR

The pre-selective PCR product was diluted 1:20 using molecular grade H_2O. 2μl of the diluted product was then combined with 2X Biomix and 2μM of each selective primer (Table 6.1) in a total reaction volume of 10μl. Initial PCR runs were performed using the same program as described for the pre-selective PCR step. However, this produced mainly small fragment sizes, so the annealing temperature was lowered and extension times were increased to aid amplification of larger

fragments. Final PCR conditions were as follows: 94°C for 2mins; 11 cycles of 94°C for 30mins, 65°C for 30secs (reducing by 1°C each cycle), 72°C for 5mins; 15 cycles of 94°C for 30secs, 56°C for 30secs, 72°C for 2mins; 72°C for 10mins.

Table 6.1. Sequences of primers used for AFLP screening

Primer name	Fluorescent tag	Primer sequence (5' - 3')
EcoRI Adapter1	N/A	CTC GTA GAC TGC GTA CC
EcoRI Adapter2	N/A	AAT TGG TAC GCA GTC TAC
MseI Adapter1	N/A	GAC GAT GAG TCC TGA G
MseI Adapter2	N/A	TAC TCA GGA CTC AT
PstI Adapter1	N/A	CTC GTA GAC TGC GTA CAT GC
PstI Adapter2	N/A	TGT ACG CAG TCT AC
TaqaI Adapter1	N/A	GAC GAT GAG TCC TGA C
TaqaI Adapter2	N/A	CGG TCA GGA CTC AT
EcoRI Pre-Sel	N/A	GAC TGC GTA CCA ATT CC
MseI Pre-Sel	N/A	GAT GAG TCC TGA GTA AC
PstI Pre-Sel	N/A	GAC TGC GTA CAT GCA GA
TaqaI Pre-Sel	N/A	GAT GAG TCC TGA GCG AAT
MseI Sel1	N/A	GAT GAG TCC TGA GTA ACT T
PstI Sel1	6FAM	GAC TGC GTA CAT GCA GAC G
PstI Sel2	NED	GAC TGC GTA CAT GCA GAT A
PstI Sel3	PET	GAC TGC GTA CAT GCA GAA T
PstI Sel4	VIC	GAC TGC GTA CAT GCA GAG A

Sequences of primers used for AFLP screening (based on Benefer et al, unpublished). Combinations of four restriction enzymes were tested (*EcoRI*, *MseI*, *PstI*, and *TaqaI*). The final protocol used *PstI* as a rare cutter and *MseI* as a common cutter. Selective PCR reactions used the *MseI* Sel1 primer combined with one of the four *PstI* selective primers.

6.2.4 AFLP Genotyping

Samples were processed using a 3130 Genetic Analyser (Applied Biosystems). The selective PCR products with different dyes (Table 6.1, column 2) were combined for each sample and diluted with molecular grade H_2O, then multiplexed in the genetic analyser. FAM, NED and PET labelled products were prepared at a final dilution of 1:20. VIC labelled products were at a final dilution of 1:30. 1μl of the mixed PCR product was added to 15μl of Hi-Di formamide (Applied Biosystems) and 0.3μl of GeneScan 500-LIZ size standard (Applied Biosystems). Samples were heated at 98°C for 5mins, then cooled quickly on ice, before being loaded into the genetic analyser. The run protocol used was the default for AFLP fragment analysis.

6.2.5 Analysis methods

Samples were visualised and sized using PeakScanner v.2 (Applied Biosystems). Samples where no products had been amplified, or where amplification was very weak, were removed. Data were then exported into TinyFLP (v.0.41; Arthofer 2010) for the loci to be scored; using an automated scoring method greatly reduces error rates when using AFLP by removing the opportunity for human error (Bonin et al. 2004). Each primer pair was analysed separately. The settings were optimised in each case to score the maximum number of loci while avoiding artefacts and background noise (Table 6.2). Loci which were present in only one individual were discounted to avoid artefacts of the restriction digest process. Monomorphic peaks were excluded from further analysis, as were any peaks present in more than 97% of samples; these peaks were considered likely to be monomorphic, appearing at less than 100% frequency due to variation in amplification efficiency. All absences were assumed to be true absences. After scoring, all data were combined using TinyCAT (Arthofer 2010). A total of 190 polymorphic loci were scored for *B. pratorum* samples, and 188 for *B. monticola*. To determine the error rate for AFLP genotyping, 10% of samples were re-processed from the restriction digest stage onwards. The allele calls from the repeats were then compared to the original runs of those samples using the same settings in TinyFLP and TinyCAT; the error rate was calculated as the number of differences in allele calls, expressed as a percentage of the total number of comparisons (Bonin et al. 2004).

To determine the genetic diversity of populations, data for each species were pro-

Table 6.2. Settings used for the scoring of AFLP data using TinyFLP and TinyCAT (Arthofer 2010)

Primer name	Dye	Minimum peak height	Maximum peak width	Minimum size (bp)	Maximum size (bp)	Size ance range	tolerMinimum peak-peak distance	Peak difference	height	Minimum frequency	Maximum frequency
PstI Sel 1	B	100	2	50	500	2	2	0		0.4	97
PstI Sel 2	Y	50	2	50	500	2	2	0		0.4	97
PstI Sel 3	R	50	2	50	500	2	2	0		0.4	97
PstI Sel 4	G	50	2	50	500	2	2	0		0.4	97

cessed separately using AFLP-SURV (Vekemans et al. 2002). Genetic diversity was quantified using percentage of polymorphic loci (PLP) and estimated heterozygosity (H_j), using Bayesian methods to estimate allele frequencies, and assuming Hardy-Weinberg equilibrium (Lynch & Milligan 1994). Population structuring was initially investigated using pairwise F_{ST} calculated in AFLP-SURV (Vekemans et al. 2002); neighbour joining trees based on the pairwise F_{ST} data were drawn using PHYLIP (Felsenstein 1993). Population structuring was investigated futher using STUCTURE (v2.3.4; Pritchard et al. 2000, Falush et al. 2007, Hubisz et al. 2009). Optimal settings for STRUCTURE analysis were chosen based on the plateau point of the Alpha plot. The conditions used were as follows: Burnin reps = 60,000 for *B. monticola*, 100,000 for *B. pratorum* data; MCMC reps = 100,000; admixture allowed; LOCPRIOR = 1; K = 1-12; 3 iterations per level of K. The results from STRUCTURE were run through STRUCTURE Harvester (Earl & von Holdt 2012), and the optimal level of K was chosen according to the method of Evanno et al. (2005), based on the log probability of each level of K (LnP(D)), and rate of change in log probability between levels of K (ΔK). Results files for the optimal K value were then collated in CLUMPP using 1000 runs of the LargeK Greedy algorithm (Jakobsson & Rosenberg 2007), and visualised using DISTRUCT (Rosenberg 2004).

6.3 Results

6.3.1 Assessment of AFLP error rate

As many measures as possible were taken to ensure minimal introduction of error (see Analysis Methods). Error rates were calculated as the number of differences observed (presence/absence of bands at any given locus) as a percentage of the total number of comparisons (Bonin et al. 2004). Overall error rates were calculated as 16.7% for *B. pratorum* and 16.6% for *B. monticola*. It is difficult to gauge what an acceptable error rate may be, due to the lack of reported error rates in many papers (Crawford et al. 2012; discussed later). While the error rates in this study seem high compared with some suggested acceptable rates of 2-5% (Bonin et al. 2004), error rates of up to 20% have also been reported in published literature (Holland et al. 2008). The total frequency of genotyping errors will increase with the size of the dataset (Sobel et al. 2002); given the large number of samples

processed for this study, a slightly elevated error rate may be expected.

Table 6.3. Percent of polymorphic loci and expected heterozygosity values for *B. pratorum* and *B. monticola*. Data were processed in AFLP-SURV (Vekemans 2002). Values were calculated following the method of Lynch & Milligan (1994), using a Bayesian framework and assuming Hardy-Weinberg equilibrium. N = sample size; PLP = polymorphic loci (expressed as %); H_j = expected heterozygosity; S.E. (H_j) = standard error of H_j.

Species	Sample Site	N	PLP	H_j	S.E. (H_j)
B. pratorum	Dartmoor	45	33.7	0.116	0.0120
	Long Mynd	37	31.6	0.110	0.0120
	Stiperstones	37	35.3	0.124	0.0127
	Snowdonia	33	44.2	0.152	0.0121
	Peak District	39	34.2	0.109	0.0118
	Lake District	17	50.5	0.156	0.0122
	Ben Lawers	-	-	-	-
	Glen Shee	-	-	-	-
	Dublin	27	38.9	0.126	0.0119
	Antrim	26	35.8	0.126	0.0127
B. monticola	Dartmoor	39	35.6	0.115	0.0119
	Long Mynd	38	30.3	0.108	0.0122
	Stiperstones	34	30.3	0.113	0.0126
	Snowdonia	16	53.7	0.183	0.0126
	Peak District	-	-	-	-
	Lake District	40	37.2	0.137	0.0130
	Ben Lawers	27	50.0	0.156	0.0110
	Glen Shee	32	37.2	0.114	0.0103
	Dublin	40	36.2	0.106	0.0111
	Antrim	24	35.1	0.123	0.0125

6.3.2 Comparison of genetic diversity between *B. monticola* and *B. pratorum*

A higher percentage of polymorphic loci (PLP) is indicative of higher levels of genetic diversity within a population. It was expected in this study that populations of *B. monticola* may show lower levels of PLP relative to *B. pratorum* populations

at the same sites. The maximum differences in PLP between *B. monticola* and *B. pratorum* were seen at the Snowdonia and Lake District sample sites (11.5 and 13.3 percentage points difference respectively; Table 6.3). However, this is likely to be an artefact of the small sample sizes processed in these cases (N = 16 *B. monticola* at the Snowdonia site; N = 17 *B. pratorum* at the Lake District site). This was due to unsuccessful PCR reactions being discounted in the case of the Snowdonia samples, and due to problems collecting *B. pratorum* samples in the Lake District. Aside from these two comparisons, the largest difference in PLP between species was seen at the Stiperstones sample site (5 percentage points), with the value for *B. pratorum* being slightly higher than *B. monticola* (35.3% and 30.3% respectively; Table 6.3). PLP values for *B. pratorum* were very slightly higher overall than those for *B. monticola*, but not considerably so. Expected heterozygosity (H_j) values varied little between the two study species, and were also consistent within species at different sample sites (Table 6.3).

Both measures of genetic diversity were fairly consistent both between and within species. While the PLP may be fractionally higher in *B. pratorum* for some sample sites, the data do not support the hypothesis that *B. monticola* is genetically impoverished relative to *B. pratorum* as a result of its restricted distribution. However, serious questions regarding the reliability of the data (discussed later) mean that very little weight should be given to this interpretation.

6.3.3 Comparison of population structuring between *B. monticola* and *B. pratorum*

The extent of population structuring was analysed separately for each species using the program STRUCTURE (Pritchard et al. 2000, Falush et al. 2007, Hubisz et al. 2009). Based on the LnP(D) and ΔK values (Evanno et al. 2005, see Methods), the optimal value of K for *B. monticola* was 10 (Fig. 6.2). The results indicate strong substructuring of populations, with clustering generally following the geographical proximity of populations (Fig. 6.3). For example, sample sites in Ireland (Dublin and Antrim) are more similar to each other than to other samples sites; the same is true for the sample sites located in Scotland (Ben Lawers and Glen Shee). Given the localised nature of populations of *B. monticola* (BWARS 2014, see Chapter 2), and the geographical separation of the majority of sites, this is perhaps not surprising. However, notable exceptions to this trend are the sample

Table 6.4. Pairwise F_{ST} values for *B. monticola* and *B. pratorum* populations calculated in AFLP-SURV. Calculations used estimated allele frequencies and assumed Hardy-Weinberg equilibrium (according to Lynch & Milligan 1994). Larger values indicate a higher degree of population differentiation.

B. monticola		Antrim	Ben Lawers	Dartmoor	Dublin	Glen Shee	Lake District	Long Mynd	Peak District	Snowdonia
	Ben Lawers	0.2660	-	-	-	-	-	-	-	-
	Dartmoor	0.2037	0.2730	-	-	-	-	-	-	-
	Dublin	0.0547	0.3059	0.2560	-	-	-	-	-	-
	Glen Shee	0.2740	0.0190	0.2868	0.3201	-	-	-	-	-
	Lake District	0.1381	0.2905	0.1276	0.2234	0.3057	-	-	-	-
	Long Mynd	0.1194	0.3592	0.2460	0.1661	0.4016	-	-	-	-
	Peak District	-	-	-	-	-	-	-	-	-
	Snowdonia	0.1488	0.2121	0.2327	0.1679	0.2397	0.2100	0.1620	-	-
	Stiperstones	0.1746	0.3495	0.1802	0.2524	0.3749	0.1275	0.1468	-	0.1921

B. pratorum		Antrim	Ben Lawers	Dartmoor	Dublin	Glen Shee	Lake District	Long Mynd	Peak District	Snowdonia
	Ben Lawers	-	-	-	-	-	-	-	-	-
	Dartmoor	0.2429	-	-	-	-	-	-	-	-
	Dublin	0.0757	-	0.2623	-	-	-	-	-	-
	Glen Shee	-	-	-	-	-	-	-	-	-
	Lake District	0.3764	-	0.4017	0.3940	-	-	-	-	-
	Long Mynd	0.0966	-	0.2269	0.0978	-	0.4138	-	-	-
	Peak District	0.1940	-	0.1412	0.2167	-	0.4221	0.2341	-	-
	Snowdonia	0.2061	-	0.3733	0.2161	-	0.3935	0.2323	0.3580	-
	Stiperstones	0.1714	-	0.1313	0.2178	-	0.4077	0.2153	0.1059	0.3254

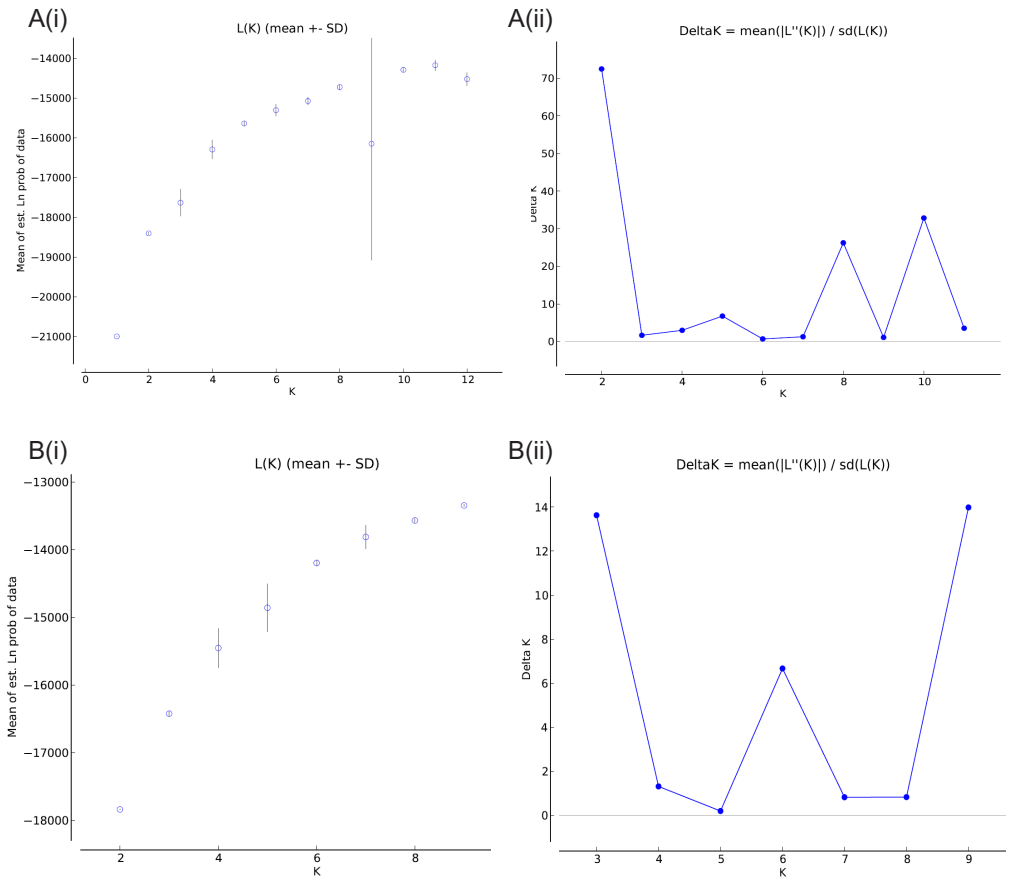

Figure 6.2. Output from STRUCTURE Harvester for A: *B. monticola* and B: *B. pratorum*. Panels A(i) and B(i) = LnP(D); panels A(ii) and B(ii) = ΔK. The optimal K values were K=10 for *B. monticola* and K=9 for *B. pratorum*. Optimal K values were chosen according to Evanno et al. (2005).

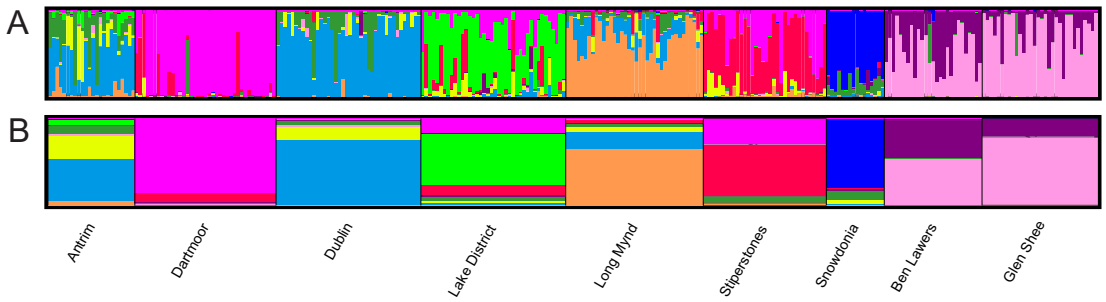

Figure 6.3. DISTRUCT graphic illustrating the population clustering of *B. monticola*. Data were processed using STRUCTURE, and collated using CLUMPP; results shown are for K=10. Panel A = assignment of individual samples to clusters; panel B = overall proportions of each population belonging to each cluster.

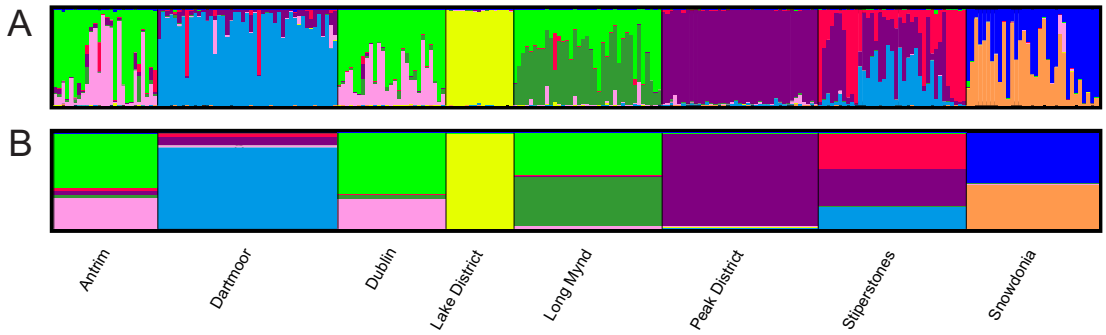

Figure 6.4. DISTRUCT graphic illustrating the population clustering of *B. pratorum*. Panel A = assignment of individual samples to clusters; panel B = overall proportions of each population belonging to each cluster. Data were processed using STRUCTURE, and collated using CLUMPP; results shown are for K=9.

sites at Long Mynd and Stiperstones. Despite being located less than 10km apart, samples from these sites are assigned to different clusters, suggesting that there is very limited, if any, interaction between the two populations (Fig. 6.3). Samples collected from Dartmoor and the Lake District show a high degree of dissimilarity to those collected from other sites. The same is also true for the samples collected in Snowdonia; however, in this case the small sample size analysed (Table 6.3) may have prevented structuring being detected. There are also some similarities apparent between the population of *B. monticola* at Long Mynd and those at Dublin and Antrim (Fig. 6.3), despite the geographical separation of those sites.

The optimal K value (number of clusters) for *B. pratorum* was 9 (Fig. 6.2). The results again indicated strong population structure (Fig. 6.4), which was unexpected given the widespread distribution of the species. The patterns shown were very similar to those observed for the *B. monticola* samples. Similar grouping of the samples collected at Dublin and Antrim was apparent, as was a similarity between samples from Long Mynd and those from Ireland. Interestingly, the

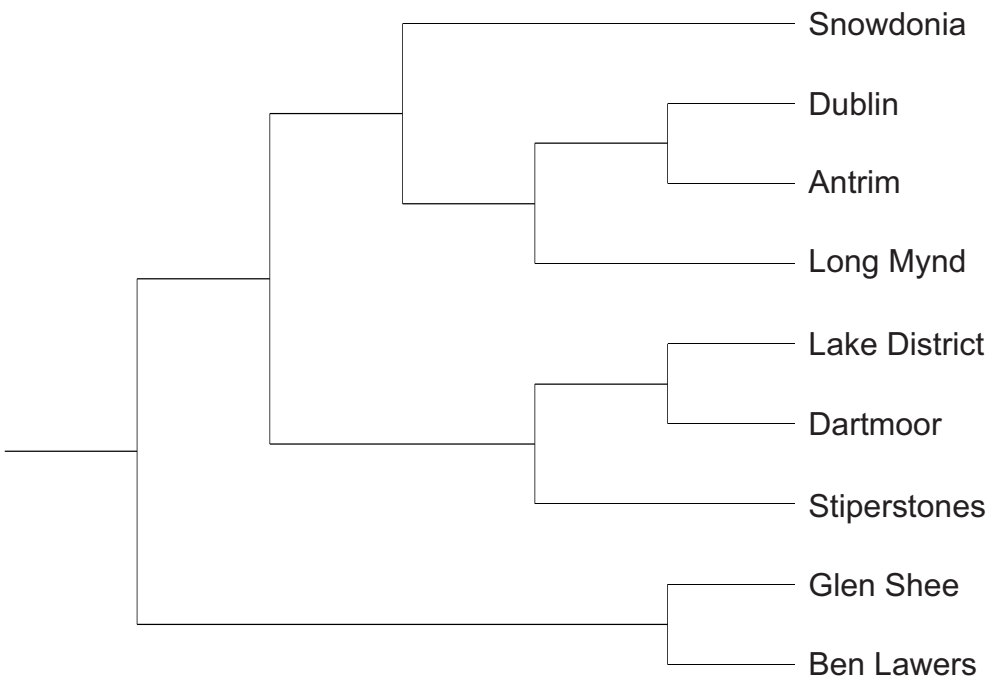

Figure 6.5. Neighbour-joining tree based on F_{ST} values for *B. monticola*. Trees were generated using PHYLIP. The structuring shown matches closely with the results obtained from the analysis using STRUCTURE.

analysis also showed the same apparent lack of interaction between the Long Mynd and Stiperstones sample sites; there was however an indication of interaction between the Stiperstones and Peak District sites. Once again the Dartmoor and Lake District populations were very distinct from the other sites; while this would be easily explained by distance in the Dartmoor population, in the case of the Lake District this may be due to a small sample size (see Table 6.3). The population at the Snowdonia sample site appears to form two distinct clusters; this was not a pattern evident in *B. monticola*, although this may have been due to the small sample size that was analysed.

Population differentiation was also assessed using pairwise F_{ST} (Table 6.4). Results from this test match up well with those predicted by STRUCTURE for both species (Fig. 6.5; Fig. 6.6). For *B. monticola*, sample sites in Scotland show little differentiation from each other, but a large degree of differentiation from other samples sites included in the analysis. For both *B. monticola* and *B. pratorum*, the sites in Ireland showed a high degree of similarity with each other but not with the other sites, with the exception of the Long Mynd (Fig. 6.5; Fig. 6.6). F_{ST} values were generally high in both species. However, such statistics generally tend to be higher when calculated from AFLP data in comparison to markers such as

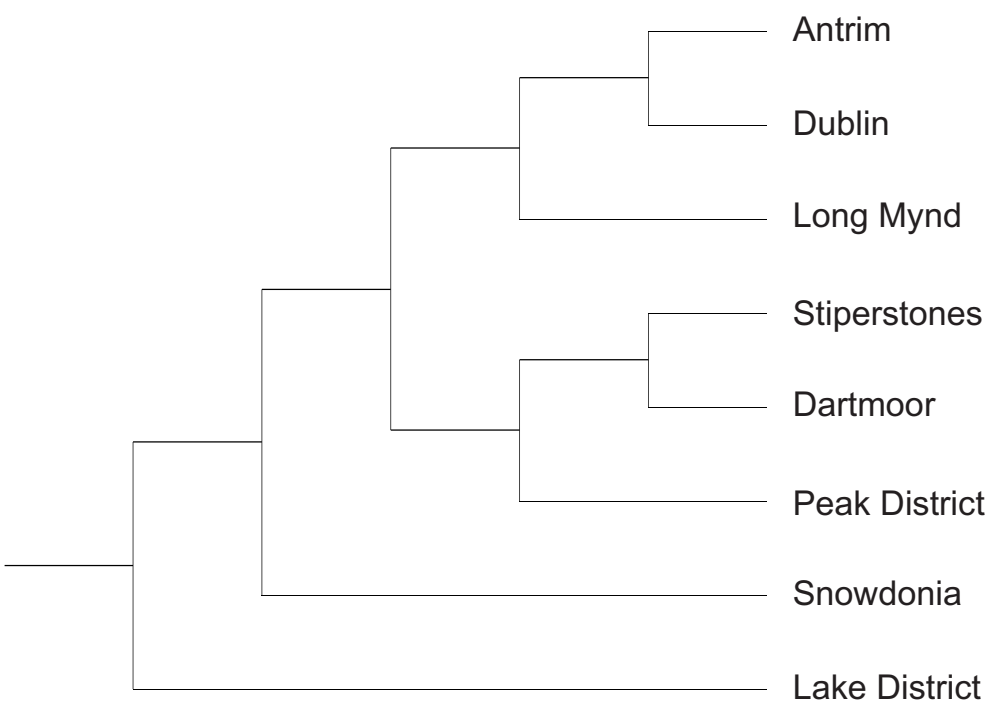

Figure 6.6. Neighbour-joining tree based on F_{ST} values for *B. pratorum*. Trees were generated using PHYLIP. The structuring shown matches closely with the results obtained from the analysis using STRUCTURE.

microsatellites (Woodhead et al. 2005).

6.4 Troubleshooting AFLP analysis

Population structuring was expected in *B. monticola* due to the fragmented nature of its populations. However, it was expected that *B. pratorum* would have far less defined structuring given that this species is more widespread and less confined to particular habitat types (BWARS 2014; Edwards & Jenner 2005). Widespread *Bombus* species have previously been shown to have very little population structuring (Estoup et al. 1996). This was not what the data indicated. Instead, *B. pratorum* showed as much structuring as *B. monticola*, with the same patterns observed in both species.

While population structuring in *B. pratorum* is possible, perhaps due to isolation by distance between the sample sites, some of the groupings of populations were very counter-intuitive. For example, the Long Mynd sample site was grouped with those in Ireland, while the Stiperstones site was grouped with the Dartmoor site for both *B. pratorum* and *B. monticola*. These groupings were surprising as the

sites concerned are widely separated geographically, particularly the Long Mynd and the sample sites in Ireland. By contrast the Long Mynd and Stiperstones sites are very close geographically (<10km apart), and would therefore be expected to show a higher degree of genetic similarity. Instead, samples from these sites were places into separate distinct clusters in both study species.

These groupings, combined with the high degree of similarity in the structuring of populations between *B. monticola* and *B. pratorum*, raised some concerns regarding the reliability of the data. These concerns were compounded by the high error rates observed in the data (although such rates are not unprecedented; Holland et al. 2008, N. Foster pers. comm.). It was subsequently observed that the suggested clusters correlated with the order in which samples were processed using the genetic analyser; unfortunately this also coincided with a breakdown of the genetic analyser, and the subsequent need for long storage periods of samples before processing (Table 6.5). Further investigation into the reliability of the data was therefore conducted to determine whether the observed structure could be an artefact of the processing order.

6.4.1 Additional analysis methods

Checking for artefacts of STRUCTURE settings
Data were initially re-analysed in STRUCTURE with LOCPRIOR=0 (all other conditions were unchanged) to ensure that the clustering patterns observed were not simply an artefact of the labelling of sample sites.

Investigating effects of sample processing order
To determine whether the order of processing had artificially introduced structuring, the data were re-analysed again, using the original STRUCTURE conditions with LOCPRIOR=1. For this run, populations were re-labelled according to the order in which they were processed rather than based on their geographic location (Table 6.5).

Repeating a subset of samples (Repeat 1)
To test whether the observed patterns had resulted from one aberrant run on the genetic analyser, a random subset of *B. monticola* samples from the Long Mynd, Stiperstones, and Snowdonia sites were re-processed from the restriction stage onwards (see Methods). These sites were selected as they represented each

Table 6.5. Length of sample storage time, and order and dates of sample processing for AFLP fragment analysis. Due to an equipment malfunction, some batches of samples had to be stored for longer periods of time before being processed using the genetic analyser. Site number corresponds to the LOCPRIOR number used in subsequent STRUCTURE analysis.Order of sample processing for AFLP fragment analysis

Species	Sample site	Date processed	Site number	Notes
B. pratorum	Dartmoor	22/09/2013	8	Processed after genetic analyser repair using fresh product.
	Long Mynd	18/07/2013	3	Processed after genetic analyser repair using product stored for approx. 8 weeks.
	Stiperstones	21/09/2013	7	Processed after genetic analyser repair using fresh product.
	Snowdonia	08/05/2013	2	Processed before genetic analyser breakdown.
	Lake District	26/04/2013	1	Processed before genetic analyser breakdown.
	Peak District	17/09/2013	6	Processed after genetic analyser repair using fresh product.
	Antrim	25/07/2013	5	Processed after genetic analyser repair using product stored for approx. 8 weeks.
	Dublin	23/07/2013	4	Processed after genetic analyser repair using product stored for approx. 8 weeks.
B. monticola	Dartmoor	22/09/2013	9	Processed after genetic analyser repair using fresh product.
	Long Mynd	18/07/2013	4	Processed after genetic analyser repair using product stored for approx. 8 weeks.
	Stiperstones	21/09/2013	8	Processed after genetic analyser repair using fresh product.
	Snowdonia	08/05/2013	3	Processed before genetic analyser breakdown.
	Lake District	17/09/2013	7	Processed after genetic analyser repair using fresh product.
	Glen Shee	26/04/2013	1	Processed before genetic analyser breakdown.
	Ben Lawers	26/04/2013	2	Processed before genetic analyser breakdown.
	Antrim	25/07/2013	6	Processed after genetic analyser repair using product stored for approx. 8 weeks.
	Dublin	23/07/2013	5	Processed after genetic analyser repair using product stored for approx. 8 weeks.

of the main clusters highlighted from the previous STRUCTURE analysis based on processing order (Fig. 6.3). The Long Mynd and Stiperstones sites were also expected to be more genetically similar than was observed, due to their close geographical proximity. If the observed patterns were caused by one aberrant run, or were a true representation of the population structure of the samples, the repeats would be grouped with one or all of the clusters from previous runs. The repeats were added to the previously analysed data, and the whole dataset was re-analysed in STRUCTURE under the same settings, with populations numbered according to processing order.

Checking genetic analyser run consistency (Repeat 1b)
To determine whether the results were simply due to the genetic analyser causing random variation between runs, the PCR products from the repeated samples were run for a second time. If the genetic analyser was creating random inter-run variation, the re-run would be expected to form a separate cluster distinct from the original runs and the repeat samples. The data were added to the total dataset (containing the original samples and repeat run), and processed using the same STRUCTURE conditions as the repeat run, with samples numbered according to processing order.

Checking sample preparation consistency (Repeat 2)
To test whether the variation was caused during sample preparation rather than by the genetic analyser, the same subset of samples was processed again from the restriction stage onwards (see Methods). The samples were then run through the genetic analyser, added to the total dataset, and analysed in STRUCTURE using the same conditions as described before. In the event of significant variation being caused during sample preparation, this run would be expected to create a separate cluster distinct from the original and repeat runs of the samples.

6.4.2 Results of additional analysis

When the STRUCTURE analysis was repeated with LOCPRIOR=0 to check for artefacts of the settings used, fewer clusters were observed in both species (*B. monticola*: K=10 with LOCPRIOR, K=4 without LOCPRIOR; *B. pratorum*: K=9 with LOCPRIOR, K=6 without LOCPRIOR; Fig. 6.7). However, the grouping of populations was the same as the initial analyses for both species (Fig. 6.8; Fig. 6.9), which indicates that the observed patterns of clustering did not result from

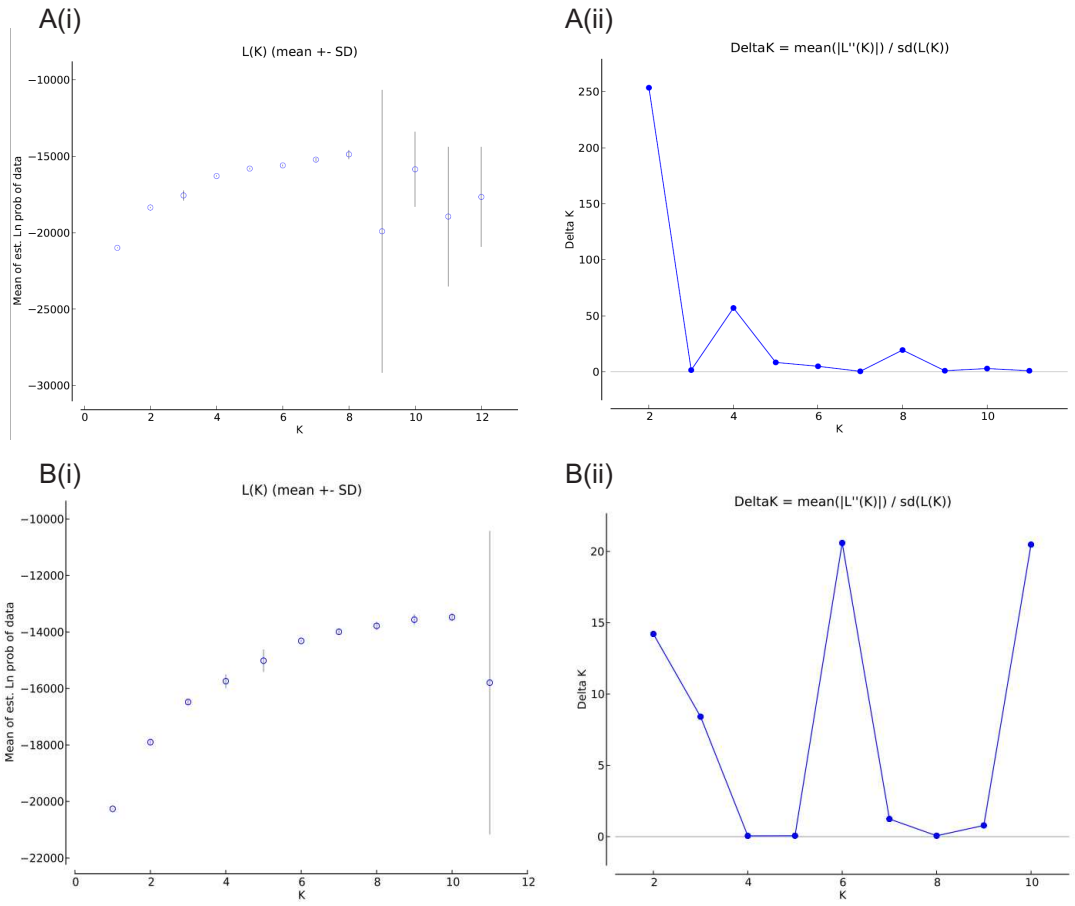

Figure 6.7. Output from STRUCTURE Harvester for A: *B. monticola* and B: *B. pratorum*, when LOCPRIOR=0. Panels A(i) and B(i) = LnP(D); panels A(ii) and B (ii) = ΔK. The optimal K values were K=4 for *B. monticola* and K=6 for *B. pratorum*. Optimal K values were chosen according to Evanno et al. (2005).

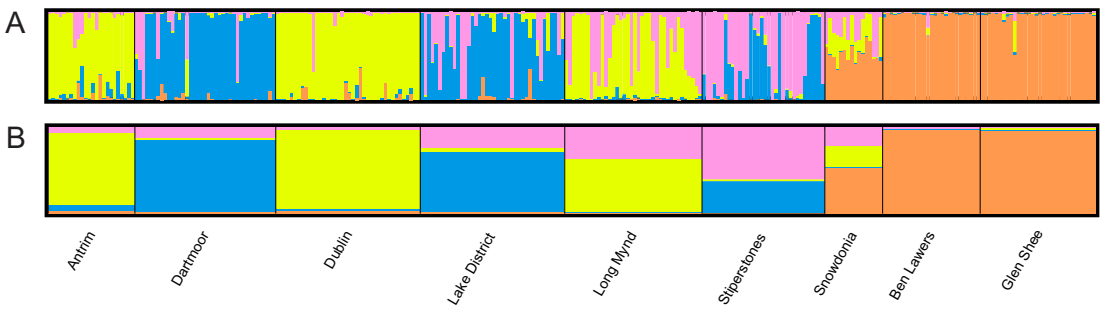

Figure 6.8. Results of STRUCTURE analysis for *B. monticola* when LOCPRIOR=0, to test whether the observed clustering was an artefact of the population numbering system. Panel A = assignment of individual samples to clusters; panel B = overall proportions of each population belonging to each cluster. Although the number of clusters is lower than when LOCPRIOR=1 (Fig. 6.3), the main groupings of populations are the same.

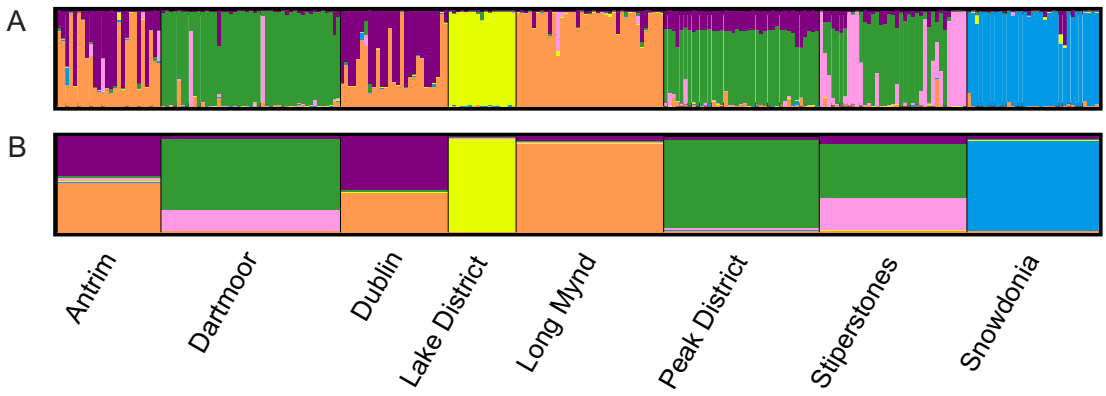

Figure 6.9. Results of STRUCTURE analysis for *B. pratorum* when LOCPRIOR=0, to test whether the observed clustering was an artefact of the population numbering system. Panel A = assignment of individual samples to clusters; panel B = overall proportions of each population belonging to each cluster. Although the number of clusters is lower than when LOCPRIOR=1 (Fig. 6.4), the main groupings of populations are the same.

the population numbering system that was used. However, when the data were re-analysed with LOCPRIOR=1 based on the order of processing, four distinct groups were observed in *B. pratorum*, and three in *B. monticola* (Fig. 6.10). These groups matched closely with the processing order, and hence with the technical problems with the genetic analyser (Table 6.5). This strongly suggested that the observed population structuring was an artefact rather than representative of the "real" structuring of the populations. This would also explain why the same patterns of structuring were observed in both *B. monticola* and *B. pratorum*, contrary to the original hypothesis.

The repeated subset, containing samples from the Snowdonia, Long Mynd and Stiperstones sample sites (Repeat 1), initially supported this explanation as they formed a separate cluster which showed no variability (Fig. 6.11). This pattern appeared to support the hypothesis that a technical fault may have introduced random inter-run variability into the data, resulting in the appearance of strong population structuring that correlated with processing order. However, when the same PCR products were run for a second time (Repeat 1b), the results clustered exactly with the previous run, showing no variability (Fig. 6.12). This suggests that the genetic analyser was not introducing random error at this stage, and made it seem most likely that error was introduced during sample preparation. When the sample subset was processed for a second time (Repeat 2), the results clustered with those from Repeat 1 (Fig. 6.13).

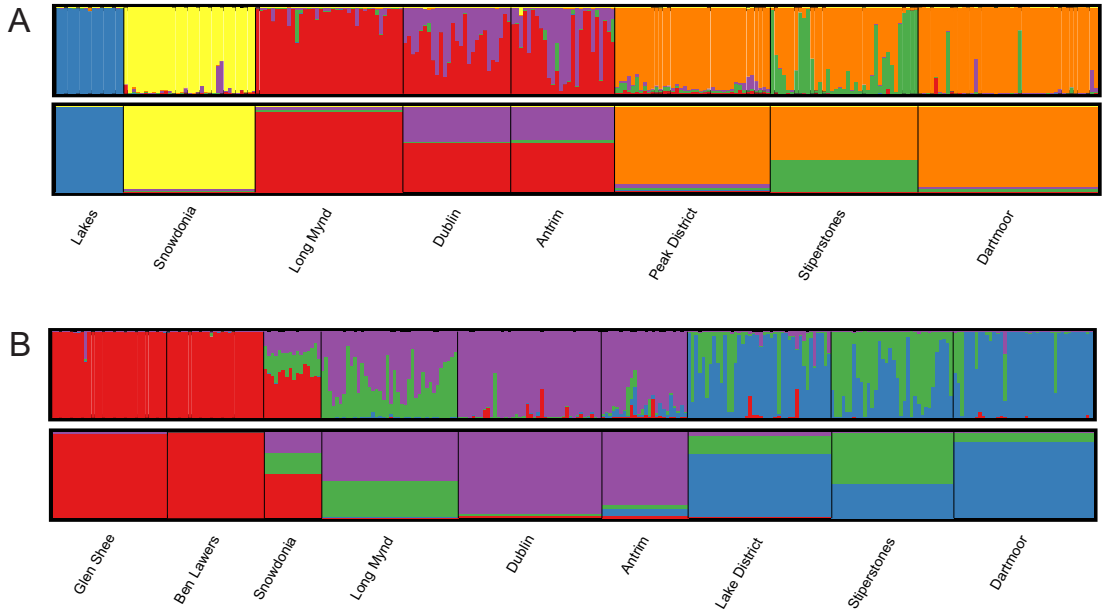

Figure 6.10. Results from STRUCTURE analysis of A)*B. pratorum*, and B) *B. monticola*, where samples were coded according to the order they were processed. Based on the ΔK value (as described in Evanno et al. 2005), an optimal K value of 6 was chosen for *B. monticola*, and 4 for *B. monticola*. For both species the top panel indicates assignment of individual samples to different clusters; the bottom panel indicates assignment of varying proportions of populations to different clusters. Clusters closely match the order in which samples were processed, and correspond to a breakdown and repair of the genetic analyser (Table 6.5).

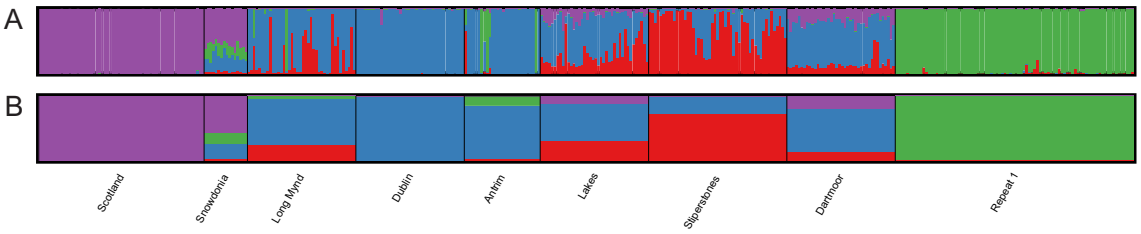

Figure 6.11. DISTRUCT image showing the clustering of original runs, and the repeated subset of *B. monticola* samples (labelled as Repeat 1). Panel A = assignment of individual samples to clusters; panel B = overall proportions of each population belonging to each cluster. The repeats contained samples from the Snowdonia, Long Mynd and Stiperstones sample sites, but do not cluster with any of the previous runs of samples from these sites.

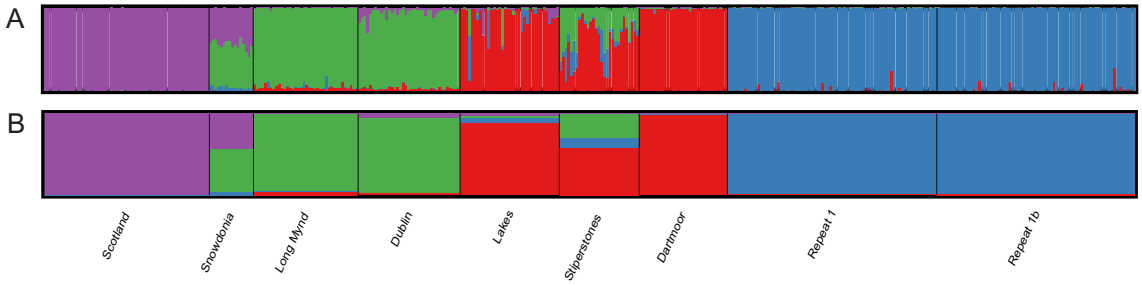

Figure 6.12. DISTRUCT image showing the clustering of original runs, the repeated subset of *B. monticola* samples (labelled as Repeat 1), and the re-run of the PCR products used for Repeat 1 (labelled as Repeat 1b). Panel A = assignment of individual samples to clusters; panel B = overall proportions of each population belonging to each cluster.

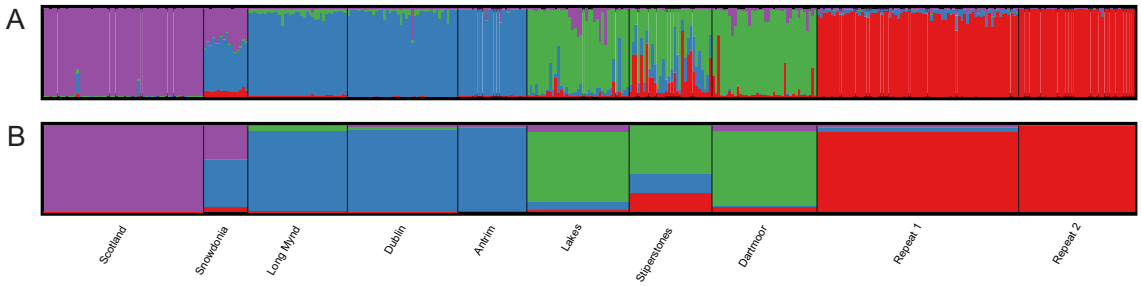

Figure 6.13. DISTRUCT image showing the clustering of original runs, the repeated subset of *B. monticola* samples (labelled as Repeat 1), and a subsequent repeat of the same subset (labelled as Repeat 2). Panel A = assignment of individual samples to clusters; panel B = overall proportions of each population belonging to each cluster.

The results of these investigations have so far failed to explain the patters observed in the AFLP data. While appearing to be linked to the order in which samples were processed, subsequent testing does not support the hypotheses that significant random variation is being introduced by either the genetic analyser or the sample preparation method. However, the fact that the repeated subset formed one distinct cluster with no internal variation, suggests that the initial results may have over-estimated the degree of population structuring.

6.5 Discussion

This study initially aimed to determine whether *B. monticola*, a species with a fragmented distribution in the UK and Ireland, showed a higher level of population structuring than *B. pratorum*, a species with a contiguous population across the same area. In addition, this study, aimed to identify loci under selection using

AFLP genome scanning, an approach which has previously been used to identify adaptive marker loci associated with fitness-related traits (Beaumont & Balding 2004, Bonin et al. 2006, Nunes et al. 2012). While these initial aims could not be addressed, this study highlights possible sources of error for AFLP analysis which have not been previously discussed. This information may be useful for the planning and execution of future studies using AFLP markers.

6.5.1 Assessment of genetic diversity and population structuring

Initial results suggested that levels of genetic diversity were very similar between *B. monticola* and *B. pratorum*, and indicated a high degree of population structuring in both species that generally matched the geographic locations of the sample sites. This was expected for *B. monticola*, as populations of the species are isolated from each other, being restricted to upland areas (Rasmont & Iserbyt 2013, BWARS 2010-2014; see Chapter 2). The isolation of the populations would be expected to reduce migration and inter-breeding, leading to an increased susceptibility to genetic diversity loss through genetic drift and inbreeding. However, the indicated level of population structuring was unexpected for *B. pratorum* given the contiguous nature of its populations (BWARS 2014, see Chapter 2). It was expected that the *B. pratorum* samples would indicate one panmictic population across the UK, as the wide distribution (BWARS 2014) and generalist life history (Goulson et al. 2005) of the species would favour migration and inter-breeding between populations. High values for population differentiation may be due to some extent to the marker system used; estimates of population differentiation such as F_{ST} are often higher when calculated from AFLP data as opposed to data from other marker systems such as microsatellites (Woodhead et al. 2005). However, anomalies in the initial results led to additional analysis of the data and raised questions regarding its reliability, as well as questions of how best to process samples and data when using AFLP.

6.5.2 Assessment of error rates

While there are advantages to using AFLPs, there can also be issues with their use as a marker system. Error can be introduced to any marker system through

low-quality DNA, sample contamination, and human error. However, AFLPs specifically are prone to the introduction of error through incomplete restriction digests, allele homoplasy, and misinterpretation of AFLP profiles during scoring (reviewed by Bonin et al. 2004, Vekemans et al. 2002, Pompanon et al. 2005, Bonin et al. 2007, Holland et al. 2008, Zhang & Hare 2012). They can also be technically challenging to optimise without previous experience (Jones et al. 1997), which may contribute to human error. Error rates can vary between loci, and less reliable loci are often removed from datasets before analysis; there are objective methods proposed for identifying such loci (Whitlock et al. 2008, Herrmann et al. 2010). The number of loci used, and the reliability of a dataset, can therefore vary depending on the protocols used at the sample preparation and scoring stages, and also on the error rate that is considered acceptable in a given study. This inherent variability makes the reporting of error rates and detailed protocols vital (Bonin et al. 2004, Pompanon et al. 2005).

The overall error rates in this study were found to be around 17% for each species. This rate seems high when compared to the error rates of 2-5% suggested by some studies (Bonin et al. 2004, Pompanon et al. 2005), although error rates of around 20% have been classed as acceptable in some instances (Holland et al. 2008). However, it is difficult to be certain how these error rates compare to those generally found in studies using AFLPs. The reporting of error rates in AFLP studies is notoriously rare, despite recommended methods for evaluating and reporting the reliability of data (Bonin et al. 2004, Pompanon et al. 2005). A review of 470 AFLP studies published between 2010-2011 found that 54% of papers made no mention of assessing error rates (Crawford et al. 2012). Of those that did mention assessing reliability of their data, around 90% did not report a specific error rate, or provide sufficient details of how this was achieved (Crawford et al. 2012). While this does not necessarily mean that the results reported are unreliable or of bad quality, it is difficult to evaluate how robust a study is without this information. It also makes comparisons between studies questionable, as variation in sample preparation and scoring methods can influence the outcomes in terms of genetic diversity estimates and population structuring estimates. This means that, while many studies report successful use of AFLPs, it is difficult to assess the quality and reliability of the data used. It is also difficult to know, when optimising protocols for AFLP, whether the error rates experienced are within the bounds expected. It is therefore relevant at this stage to reiterate calls for more consistent error reporting (Bonin et al. 2004, Pompanon et al. 2005, Crawford

et al. 2012).

6.5.3 Possible sources of error in this study

Error can be introduced to AFLP-based studies in a number of ways, and it can be difficult to identify the main sources of error in many cases (Bonin et al. 2004).

Contamination/low quality DNA
Like all universal marker systems, AFLPs are sensitive to contamination with foreign DNA. However, it is unlikely that contamination was the main source of error in this study. The tarsal samples were cleaned using sterile water and ethanol to remove any pollen and other material before DNA extraction. Extractions were performed in a clean laboratory environment using sterile equipment and molecular grade reagents, to exclude as far as possible the risk of contamination. During sample scoring there were no peaks present to indicate contamination i.e. strong peaks present in certain batches of samples. Results of AFLP analysis can also be affected by the use of low-quality DNA (Bonin et al. 2004). Samples in this study were processed using ammonium acetate extraction with multiple clean-up steps (see Methods). All samples were tested using a NanoDrop after extraction to check for salt and/or protein contamination. In the event of possible contamination, samples were re-cleaned and re-tested. The minimum DNA concentration used was 50ng μl^{-1}. While sample concentrations varied (see Methods), AFLP analysis has been found to be relatively insensitive to DNA concentration (Trybush et al. 2006), so this variation is unlikely to be the cause of the elevated error rates observed. Variation in DNA concentration would also not explain the high variability seen between re-runs of the same samples.

Incomplete digestion of DNA
Incomplete digestion of DNA is a common source of error in AFLP studies (Bonin et al. 2004). For this study, all samples were incubated under the same conditions (see Methods), as recommended by previous studies using AFLP in *Bombus* (Wilfert et al. 2006). An excess of restriction enzyme was used to ensure efficient digestion, as incubating samples for longer time periods risked star activity of the restriction enzymes (non-specific cleaving of DNA). It may therefore be assumed that the digestion of DNA was as complete as could be achieved with the methods and materials available. This supposition is supported by the fact that most traces showed a similar range of size fragments.

Variation in PCR efficiency

It is possible that variation in PCR efficiency could have influenced the reliability of the data. However, this should have been countered in this study by the use of automated scoring with very conservative parameters (discussed later). Samples where the PCR had failed or amplification was particularly weak were removed from the overall dataset before loci were scored, so would not have contributed to the overall error rates. As seen with the restriction digest, all samples produced a similar size range of fragments which suggests a reasonable degree of consistency (although there is still the risk of fragment homoplasy; discussed later). Comparisons of peak profiles between repeated samples did not indicate a high degree of variability in the strength of peaks. Instead, peaks appeared and disappeared between runs and varied greatly in frequency, with very few peaks being conserved. It therefore seems unlikely that the efficiency of the PCR reactions was the main source of error in this study.

Fragment homoplasy

Like all dominant markers, AFLPs are vulnerable to size homoplasy. This can result from either the co-migration of non-homologous fragments of the same size, or the loss of an allele due to different mutations (Meudt & Clarke 2007, Simmons et al. 2007). Fragment homoplasy is more common in smaller fragments (Vekemans et al. 2002), but is generally uncommon (van der Voort et al. 1997, Mendelson & Shaw 2005), especially in intraspecific comparisons (Vekemans et al. 2002, reviewed by Bonin et al. 2007). However if unidentified, fragment homoplasy can result in an underestimation of population differentiation (Hansen et al. 1999, Caballero et al. 2008).

Given that error rates were calculated within species in this study, and that population differentiation was indicated to be very high, fragment homoplasy is unlikely to be the cause of the high rates of error observed in samples of both *B. monticola* and *B. pratorum* in this study.

Scoring error

Discussions of introduction of error into AFLP analysis often cite scoring errors, particularly as the result of human error, as a major contributor (Bonin et al. 2004, Pompanon et al. 2005). For this reason, data collected in this study were scored using an automated system (TinyFLP; Arthofer 2010) which can greatly reduce the introduction of error, provided that the parameters are carefully set (Bonin et al. 2004, Pompanon et al. 2005, Whitlock et al. 2008, Herrmann et al.

2010). In addition, the parameters applied when scoring loci in this study were very conservative (Arthofer 2010). Failed PCR runs or those which had amplified poorly were removed before loci were scored. Peaks were only scored when present in more than one individual, to reduce the effect of artefacts from variability in digestion and amplification. Monomorphic peaks were excluded from the dataset, as were peaks present in more than 97% of samples. This was to reduce the risk of inadvertently including monomorphic loci (variability in amplification may result in loci being scored as polymorphic, when in fact they would be monomorphic under "perfect" conditions). The conservative nature of the data scoring in this project, when combined with the use of an automated scoring system to remove any subjectivity from scoring, means that scoring error alone is unlikely to be the root cause of the high error rates seen in this study. Automated scoring may still be vulnerable to "shifting" peaks, i.e. when the size of a peak appears to vary by a slight margin between runs; however, the bin widths set should account for this variation (Table 6.2). Visual inspection of peak profiles between runs did not indicate that shifting of peaks was occurring; rather, it seemed that peak profiles between runs of the same samples were entirely different, with very few peaks being preserved.

Order of sample processing

Sources of error in AFLP studies have been investigated in some detail (Bonin et al. 2004, Pompanon et al. 2005). However, the potential sources of error discussed so far would be expected to cause variability between individual samples, rather than between batches.

In this study the samples from each site were processed in batches for ease of organisation. In the author's experience processing samples in a logical order is commonplace, particularly in studies with very large sample sizes. However, the results reported here suggest that this approach may run the risk of masking errors resulting from events surrounding the order in which samples are processed. The unexpected results observed for the Long Mynd and Stiperstones sample sites would not have been detected without running samples from those sites out of sequence. Further investigation showed that the results were not an artefact of the population numbering system used in STRUCTURE (Fig. 6.8; Fig 6.9), indicating that the observed population structuring correlated exactly with the order in which samples were processed (Table 6.5).

It was initially thought that the genetic analyser could be introducing random

errors due to sub-optimal functioning. However, generally in the event of a serious problem no reads can be obtained at all (M. Knight, pers. comm.). It is also worth noting that the use of other marker systems, such as microsatellites, on the same machine did not seem to be affected during this time, and equipment inconsistency was not indicated by the repeat samples run at a later date (Fig. 6.12). While a technical problem cannot be entirely ruled out, it is more likely that the variability in runs is an indirect result of the breakdown of the genetic analyser, possibly because some samples needed to be stored for a considerable time before being processed. Samples were stored under standard conditions for PCR products (4°C) and DNA extracts (-20°C), but it is possible that sample degradation may have occurred during this time. Slight shortening of DNA fragments, or the loss of the fluorescent tags, could significantly affect the traces produced from AFLP, and thus increase inter-run variability. This explanation has merit, as the repeat samples to calculate the overall error rates were performed towards the end of the study. If sample degradation was the root of the observed variability, repeat samples processed closer together in time would be more similar than those processed with a delay. This is precisely what was observed - the first set of repeats was very different to the output from the initial runs (Fig. 6.11), but subsequent repeats were much more similar to each other (Fig. 6.12; Fig. 6.13). The possibility of sample storage introducing such a degree of variability merits further exploration. While the possible sources of error for individual samples has been well documented (Bonin et al. 2004, Pompanon et al. 2005), the possible effects of sample storage times have not been discussed. Studies using large sample sets, or with limited facilities for processing samples, may store PCR products and/or DNA extractions for some time before they are read using a genetic analyser. This may often be the case when using AFLP, as large sample sizes are recommended for population genetics applications of these markers (Lynch & Milligan 1994, Mariette et al. 2002, Nybom 2004).

6.6 Future work

The data presented here indicate serious issues regarding the reliability of data collected in this study using AFLP. Given the link between the order in which samples were processed and the apparent structuring of populations, it is likely that the inconsistencies are due to extended sample storage times, made necessary

by an equipment malfunction. However, further tests would be required to support this hypothesis further.

To determine the potential impact of sample storage either as a raw DNA extract or as a PCR product, it would be useful to repeatedly process samples which have been stored for variable lengths of time, and compare the peak profiles produced back to when the sample was freshly extracted from the source tissue. This would allow quantification of the degree of variation introduced by possible sample degradation over time. If significant variability is observed, recommendations may be to process PCR products immediately, to store samples for the minimum time possible, and to store source material at a lower temperature (such as -80°C) to maximise stability. Such a finding could have implications for studies using very large sample sizes, as any time lag between the first and last samples processed could introduce artificial variability into the data. It would also be recommended that error rates are estimated as soon as possible after the original sample runs, so that samples do not have time to degrade and inflate error estimates. Given the lack of consistent error reporting from AFLP studies (Crawford et al. 2012), and a general reluctance to publish negative or unsuccessful results, it is difficult to assess whether sample storage times may have been an issue previously. Future work could include a survey of laboratories which routinely use AFLPs, which would give an indication of how widespread an issue this may be.

It may also be beneficial to compare the results from markers such as AFLP with those from marker systems more well-established in the target organism, such as microsatellites (should such markers already exist). As these are species targeted, this would provide further support for any population structuring observed and confirm a lack of interference from other sources such as cross contamination. It may also give an indication of the relative sensitivity of different marker systems to sample storage and degradation; if AFLP are shown to be particularly sensitive, requirements for sample storage may be an important additional consideration when selecting marker systems for future work.

6.7 Conclusions and recommendations

The initial aims of this study were to assess the level of population structuring in a species with a fragmented distribution, *B. monticola*, relative to a ubiquitously

distributed species, *B. pratorum*. However, the level of variability between runs, combined with the strong effect of the order of sample processing and high error rates, means that reliable conclusions cannot be reached to this end. It is difficult to know whether such issues have been experienced in other studies, due to a lack of consistent reporting of error (Crawford et al. 2012). The principle recommendation of this study would therefore be to reiterate previous calls for thorough and transparent reporting of error rates, and if possible identifying the sources of error, in AFLP studies (Bonin et al. 2004, Pompanon et al. 2005, Crawford et al. 2012).

Processing samples in a logical order, particularly when working with a large data set, is commonplace. However, it may run the risk of masking errors resulting from equipment faults. In this study samples were processed in geographical order (southernmost to northernmost), with the exception of the Stiperstones and Dartmoor sites which had to be re-run as the initial runs failed. If this had not been necessary the effect of processing order would not have been identified, as the suggested clustering of populations would have been geographically plausible (if unexpected in the case of *B. pratorum*). This would have led to erroneous conclusions being drawn from the data. It would be advisable for future studies to ensure that samples are randomised before and during processing, as this would make obvious groupings by processing order easier to identify. However, this may be logistically very difficult in studies using a large sample size (which is generally necessary for effective results to be obtained from AFLP data; Lynch & Milligan 1994, Mariette et al. 2002, Nybom 2004).

Another recommendation resulting from this study is to run all samples and repeats in as short a time as possible. The patterns of population structuring indicated by this analysis corresponded strongly with the order of sample processing, and with the breakdown of the genetic analyser. Failure of equipment is inevitable, but this study highlights the potential knock-on effects on data and the interpretation thereof. Re-runs of samples did not indicate significant inconsistencies being introduced by the genetic analyser, instead suggesting the possibility of DNA degradation over time during the study. This was unexpected, as PCR products are considered stable when stored at 4°C. However, given that AFLPs are entirely reliant on small variations in fragment size to distinguish loci, it may be that as a marker system they are particularly sensitive to sample degradation. This highlights the need for repeat runs to be conducted as soon as possible after the initial samples are processed. This is particularly true for repeats being used

to calculate overall error rates, which should reflect both sample preparation and storage time. Again, randomising samples within plates for processing may make it easier to detect artefacts introduced as a result of sample degradation during storage. Storage of samples and/or PCR products may be necessary when working with large sample sizes. The data presented here highlight the need for this to be explicitly stated, and accounted for if possible, when interpreting results.

Chapter 7

General discussion and a comparative analysis of factors affecting bumblebee population viability

7.1 Introduction

Innate immunity in insects is highly complex, consisting of various pathways which can elicit a highly specific response to infection (Schmid-Hempel 2005, Schulenburg et al. 2007). The anti-microbial peptide (AMP) and phenoloxidase (PO) activity of *B. monticola* and *B. pratorum* were investigated (Chapter 4), along with parasite load (Chapter 5). However, thus far these various aspects of immunity, and their link with the relative level of population isolation, have been considered singly. In reality, these factors are fundamentally inter-linked, and act in concert to create the overall immune phenotype (Cotter et al. 2004, Lambrechts et al. 2004). It is therefore necessary to consider the interactions between the aspects of immunity already measured, together with the possible influence of abiotic environmental variation, to obtain a clearer picture of the overall immunocompetence of the study species.

7.1.1 Trade-offs in immunity

The immune response is costly to an individual (reviewed by Lawniczak et al. 2007), both in terms of the resources required to mount the response, and in terms of the potential self-harming effects that the response can have (Nappi & Ottaviani 2000, Cerenius et al. 2008, Zhao et al. 2011). This is particularly true of the melanisation response, which produces toxic quinone species and various other products which are damaging to the host (Cerenius & Söderhäll 2004, Sadd & Siva-Jothy 2006, Cerenius et al. 2008, Zhao et al. 2011). For these reasons, i.e. the costs of mounting a response and the need to minimise self-harm, many studies have reported trade-offs in the immune response of invertebrates (Cotter et al. 2004, 2008). High PO activity may be maintained at the expense of AMP activity, and vice-versa. The costs of immunity may also affect other fitness related traits such as feeding and foraging efficiency (Kraaijeveld et al. 2001, Adamo et al. 2010), reproduction (Adamo 2004, Stahlschmidt et al. 2013), growth (Rantala & Roff 2005), learning (Mallon et al. 2003a) and longevity (Armitage et al. 2003). Dietary breadth has been linked with immunocompetence in honeybees (Alaux et al. 2010), while dietary protein is crucial for the production of AMPs in *Bombus* species (Brunner et al. 2014); some insects may be able to compensate somewhat for the costs of immunity by modifying their behaviour, for example by foraging more selectively on high-protein foods (Povey et al. 2009). Senescence of the im-

mune system has been shown in invertebrates, including *Bombus*, under both lab (Doums et al. 2002, Moret & Schmid-Hempel 2009) and field conditions (Whitehorn et al. 2010), possibly due to increasing trade-offs between behaviours such as foraging and investment in immunity (König & Schmid-Hempel 1995, Doums & Schmid-Hempel 2000).

Conversely, it has been observed in some species that different aspects of the immune response can act together to fight infection, resulting in a positive relationship between different aspects of the innate immune response (Lambrechts et al. 2004). This may be an indication that certain populations, individuals, or genotypes are generally superior in terms of immunocompetence. If this is the case, it would have different implications for the underlying evolutionary processes in a population; instead of selection acting separately on different immune traits, possibly at the expense of others, it may act on the entire immune system as a unit, providing strong directional pressure for a strong overall immune response.

Based on the data already presented, and informed by previous studies in *Bombus* and other closely-related species such as *Apis mellifera*, it may be expected that the measurements of PO and AMP activity in this study will be inter-dependent (Cotter et al. 2004, Lambrechts et al. 2004). These responses may also relate to dietary breadth (Alaux et al. 2010, Brunner et al. 2014). The responses may also be related to individual age (Doums et al. 2002, Moret & Schmid-Hempel 2009).

7.1.2 Parasitic infection in *Bombus*

The abundance of individual parasite species in *B. monticola* and *B. pratorum* was compared (Chapter 5). However, in reality hosts are constantly challenged with a community of pathogens (Rutrecht & Brown 2008b), and this is evident from the data presented previously in this thesis (see Chapter 5). In the case of parasites it is possible that infecting species may interact, either mutualistically or antagonistically, thus influencing the likelihood of multiple species infections within the same host (Janovy Jr et al. 1995, Poulin 1997, Rutrecht & Brown 2008b). The majority of studies of parasite dynamics in *Bombus* have focused on single parasite systems (for example Brown et al. 2003c, Otterstatter & Thomson 2008, Ruiz-González et al. 2012); to date only one study has investigated the independence of multiple infections in *Bombus*, and that suggests that parasites in queens are likely to infect a host independently of one another (Rutrecht &

Brown 2008b). It is important to determine the processes underlying host infection, as interactions between parasite species may have important implications for evolutionary processes, such as the development of virulence (reviewed by Long & Boots 2011) and maintenance of genetic diversity in both parasites and hosts (Schmid-Hempel & Crozier 1999).

The successful infection of a host by a parasite is highly influenced by interactions between host and parasite genotypes, and subsequent variation in host gene expression (Barribeau et al. 2014; also see Discussion, Chapter 5). AMPs are known to play an important role in parasite defence, as well as in general antimicrobial defence (Schlüns et al. 2010, Deshwal & Mallon 2014). Variability in AMP gene expression was suggested as an explanation for the variable levels of parasite infection observed in different populations of *B. monticola* and *B. pratorum* in this study; however, the link between these two factors was not formally tested. Likewise, PO activity and the melanisation response also play an important role in defence against parasitic infection (Cerenius et al. 2010,b), and a negative correlation between PO activity and the occurrence of some parasites has previously been observed in *Bombus* (Whitehorn et al. 2010). However, this correlation has yet to be tested in *B. monticola* and *B. pratorum* in the context of this study.

This final analysis aimed to bring together the datasets from the previous five chapters to address the following questions:

1. Is there evidence for correlations and/or trade-offs between PO and AMP activity?

2. Do parasite communities vary between sample sites?

3. Do parasite species infect their host independently of each other?

4. What association is there between parasite load and immunocompetence?

7.2 Methods

Foraging data were collected as described in Chapter 2. Dietary breadth at a sample site is defined as the number of plant species on which worker bees were sampled, and is defined separately for *B. monticola* and *B. pratorum*. Individual age and thorax weight were assessed as described in Chapter 3. PO and AMP

activity data were collected as described in Chapter 3. Data regarding parasite load were collected as described in Chapter 4.

7.2.1 Statistical analysis

Given the high levels of variability already observed between sample sites in all variables (see Chapters 2-5), sample site was included as a random factor in all analyses to allow testing for universal trends. *B. monticola* and *B. pratorum* were treated separately for all analyses.

Correlations between PO and AMP activity
The possibility of a correlation/trade-off between the PO and AMP responses was tested in two ways. Initially, the PO activity in samples with and without zones of inhibition (i.e. with/without AMP activity) was tested using a GLMM with a binomial distribution. Thorax weight, age, and dietary breadth (defined as the diversity of forage plant species used, see Chapter 2) were also included as covariates, and all two-way interactions were considered. The possible correlation of PO activity with zone of inhibition diameter was also tested, using a GLMM performed on the subset of samples which had produced zones of inhibition. As before, thorax weight, age, and dietary breadth were included as explanatory factors, as well as PO activity and all two-way interactions.

Variation in parasite communities between sample sites
Presence-absence data for *A. bombi*, *C. bombi*, *L. buchneri*, and *N. bombi* were analysed using Primer-E software (Plymouth Marine Laboratory; Clarke & Warwick 2001) for variation in parasite assemblage between sample sites. Data were analysed separately for *B. monticola* and *B. pratorum*. Comparisons were made using Bray-Curtis similarity due to the binary nature of the data. Comparisons were not explicitly made between *B. monticola* and *B. pratorum* as no consistent differences were observed in parasite prevalence in previous analyses (see Chapter 5). Data were represented using an MDS plot, and formally tested for similarity between sites using ANOSIM analysis in Primer-E (v. 6).

Independence of infection by each parasite species
The independence of infection by each parasite species was assessed separately for *B. monticola* and *B. pratorum*. Data were pooled from all sample sites, and the total number of infections for each parasite species were analysed using pairwise

G-tests (as in Rutrecht & Brown 2008b) adjusted using the Bonferroni correction for multiple testing (MacDonald & Gardner 2000). After adjustment, significance was set at $p<0.008$.

Association between parasite load and immunocompetence
The prevalence of each parasite species was tested separately for a correlation with AMP and PO activity. The presence/absence data for each parasite species were pooled across all sites, and tested for independence from the presence/absence of zones of inhibition (again across all sites) using G-tests. Using the subset of data from samples which had produced zones of inhibition, binomial GLMMs were run for each parasite species, with zone of inhibition diameter, thorax weight, age, and PO activity as explanatory factors, along with all two-way interactions. Parasite species richness was analysed separately for *B. monticola* and *B. pratorum*, using GLMMs with a Poisson distribution for count data. Thorax weight, PO activity, zone of inhibition diameter, dietary breadth and age were included as explanatory factors.

Significance levels for all GLMMs were determined using likelihood ratio testing on the AIC value. All statistics were performed using R (v. 2.15.2) and Primer-E (v. 6).

7.3 Results

7.3.1 Interactions between PO and AMP activity

In *B. monticola*, samples which had produced a zone of inhibition had a higher level of PO activity ($\chi^2(1)=15.03$, $p<0.001$), suggesting a positive correlation between the two immune mechanisms. While site was treated as a random factor, the increase in PO activity with AMP zone of inhibition appears variable between sites (Fig. 7.1), which corresponds with the high levels of variability in both AMP and PO activity that have already been observed between sample sites (see Chapter 4). By contrast, no significant difference in PO activity was seen in *B. pratorum* samples with or without zones of inhibition ($\chi^2(1)=0.20$, $p=0.656$; Fig. 7.1). Dietary breadth was significantly higher in *B. monticola* samples with a zone of inhibition present ($\chi^2(1)=5.69$, $p=0.017$), but there was no significant effect of diet in *B. pratorum* ($\chi^2(1)=0.23$, $p=0.631$). As in previous analyses there

were no significant effects of age or thorax weight(see Chapter 4). There were no significant interaction terms for either species.

In *B. monticola* there was a significant positive correlation between PO activity and zone of inhibition diameter ($\chi^2(1)=25.43$, $p<0.01$), but no effect of dietary breadth ($\chi^2(1)=<0.01$, $p>0.99$). In previous analyses age and thorax weight were found to be significantly correlated with zone diameter in *B. monticola* (see Chapter 4); however, they were not found to be significant in this analysis (thorax weight: $\chi^2(1)=2.33$, $p=0.127$; age: $\chi^2(1)=1.82$, $p=0.177$). This may be due to the treatment of site as a random factor. Both age and thorax weight varied significantly between sites in *B. monticola* (see Chapter 3); accounting for this site-by-site variation may have removed the significant effect of these variables. For *B. pratorum* thorax weight was not significant, as found in previous analysis (see Chapter 4). There was also no significant effect of dietary breadth on zone size ($\chi^2(1)=2.86$, $p=0.091$). However, there was a significant interaction of zone diameter with PO activity and age ($\chi^2(1)=5.93$, $p<0.05$), with zone diameter being higher in older individuals with lower PO activity. This correlates with previous findings that PO activity decreased in older individuals while AMP activity appeared to increase, and may provide support for the theory of an age-related trade-off in immunity (see Chapter 4).

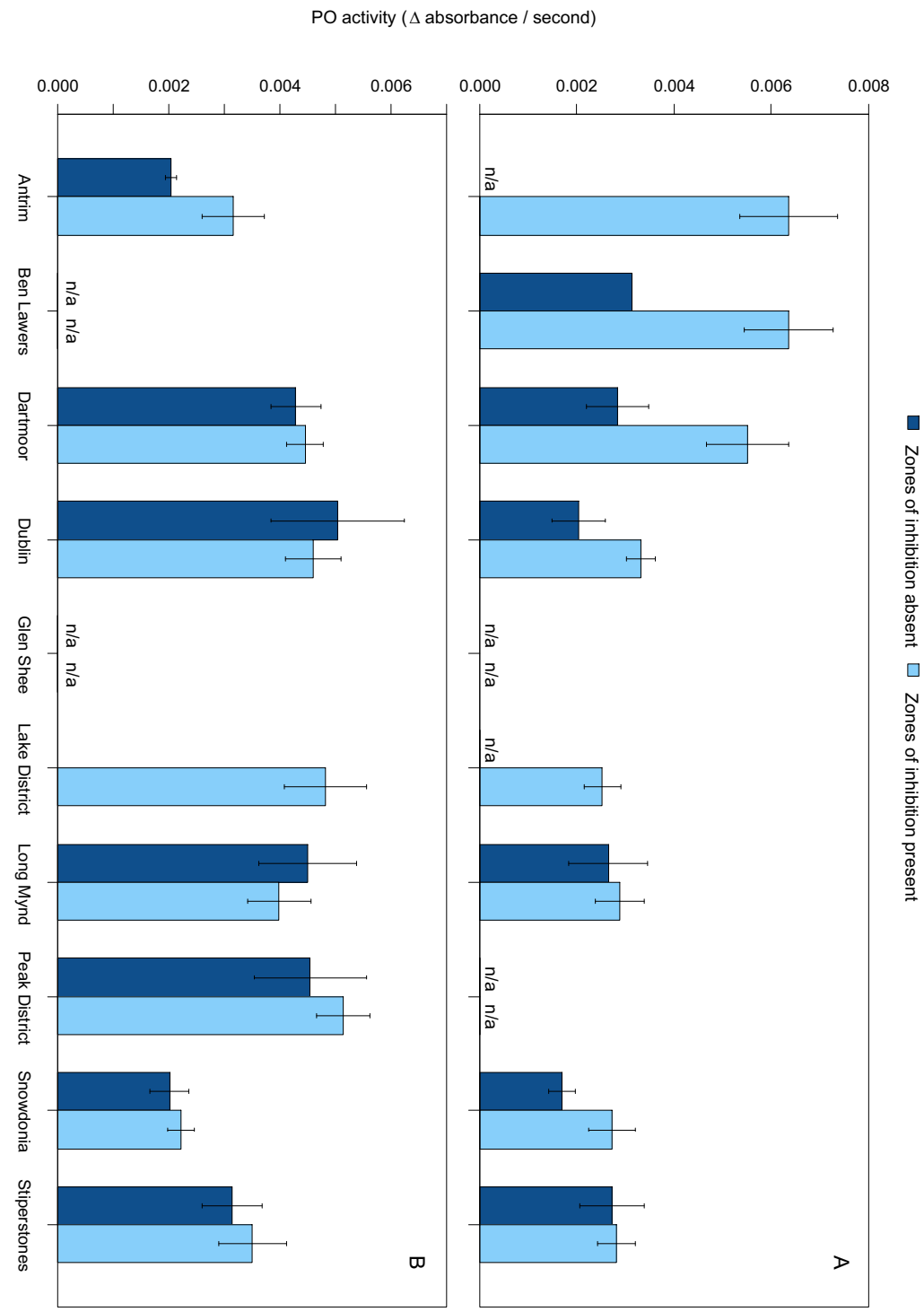

Figure 7.1. PO activity in samples where zones of inhibition were present/absent. Bars show means ± standard error. Panel A = *B. monticola*; panel B = *B. pratorum*.

7.3.2 Variation in parasite community between sites

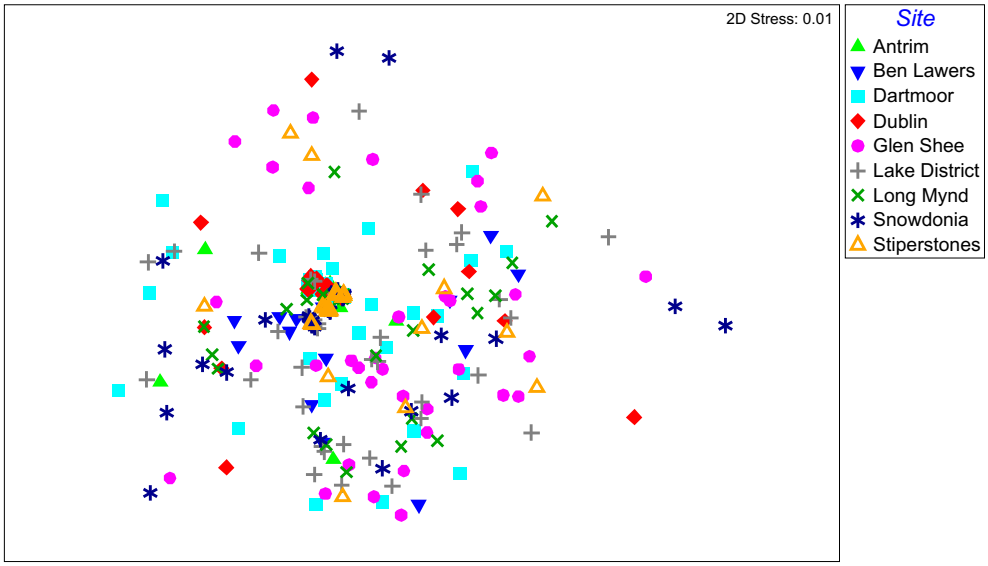

Figure 7.2. Initial MDS plot showing variation in parasite assemblage in *B. monticola* according to sample site. Comparisons were made based on the presence/absence of *A. bombi*, *C. bombi*, and *N. bombi* in each individual, using Bray-Curtis measures of similarity.

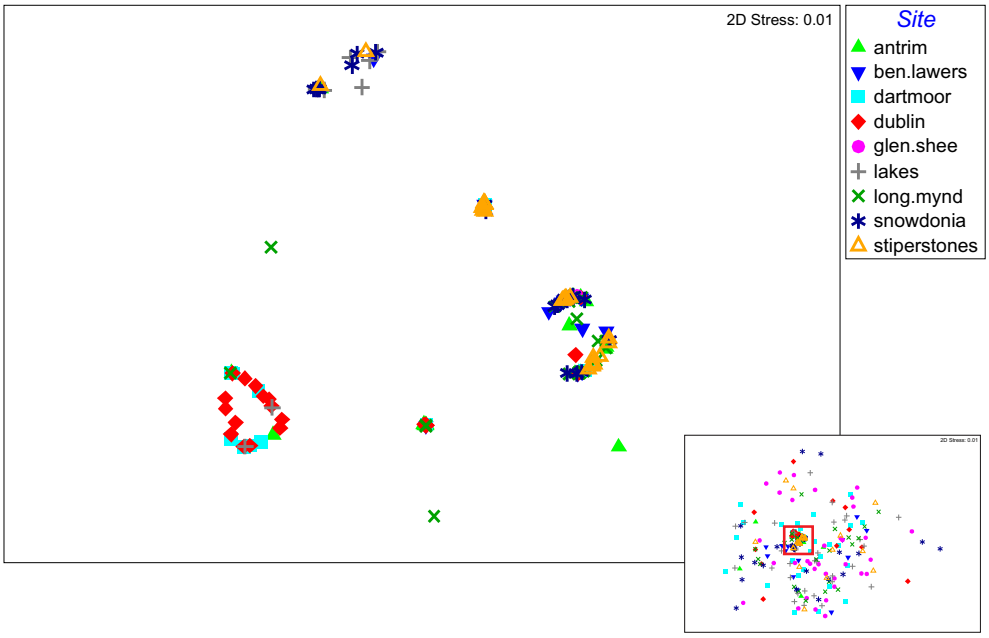

Figure 7.3. Magnification from initial MDS plot of parasite assemblage in *B. monticola*. Thumbnail image indicates the section of the original MDS plot that has been expended (outlined in red). Comparisons were made based on the presence/absence of *A. bombi*, *C. bombi*, and *N. bombi* in each individual, using Bray-Curtis measures of similarity.

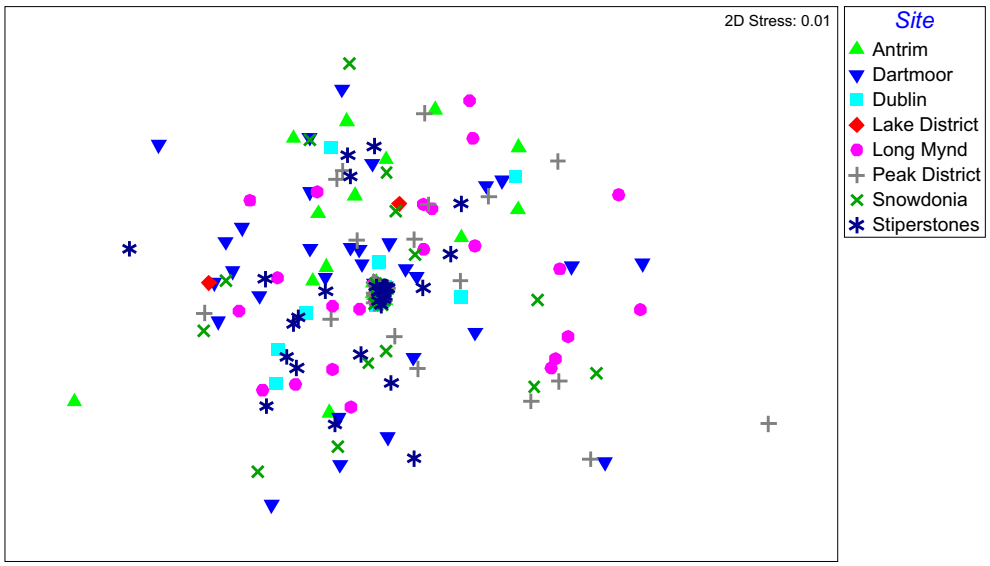

Figure 7.4. Initial MDS plot showing variation in parasite assemblage in *B. pratorum* according to sample site. Comparisons were made based on the presence/absence of *A. bombi*, *C. bombi*, *L. buchneri* and *N. bombi* in each individual, using Bray-Curtis measures of similarity.

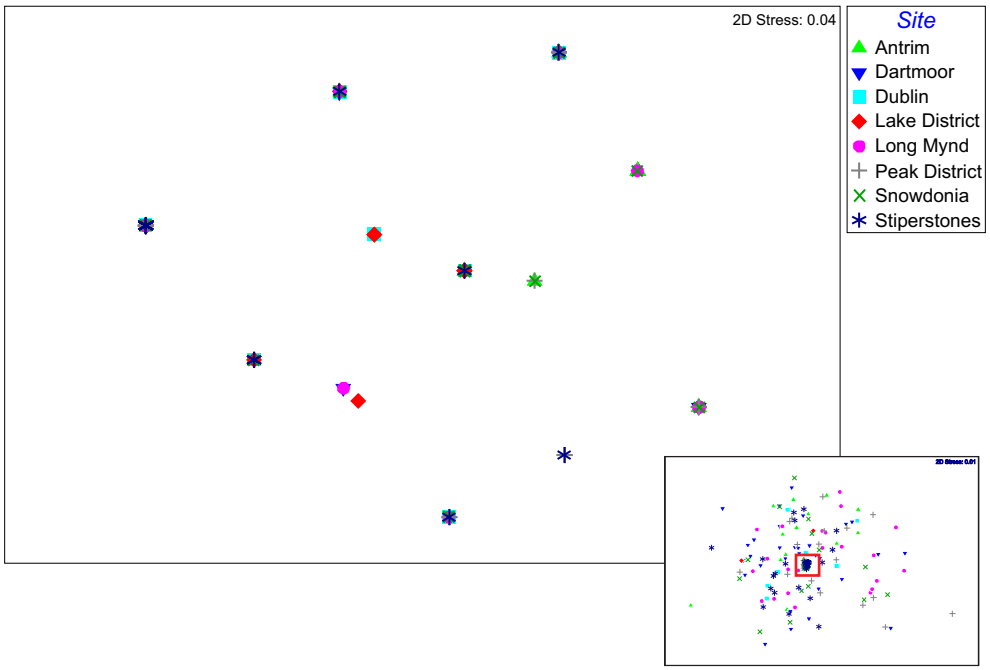

Figure 7.5. Magnification from initial MDS plot of parasite assemblage in *B. pratorum*. Thumbnail image indicates the section of the original MDS plot that has been expended (outlined in red). Comparisons were made based on the presence/absence of *A. bombi*, *C. bombi*, *L. buchneri* and *N. bombi* in each individual, using Bray-Curtis measures of similarity.

For both *B. monticola* and *B. pratorum* the initial MDS plots suggested no differences in parasite assemblage between sample sites, as there was no obvious

clustering of points by site (Fig. 7.2; Fig. 7.4). However, in both species there was a small group of points which clustered together. This area of the plot was magnified for clarity (Fig. 7.3; Fig. 7.5). Once magnified, it was possible to see some clustering of points by site in *B. monticola*, although there were still no obvious groupings in *B. pratorum*; this may be an indication of the presence of groups of closely related individuals, which would be expected to have a similar degree of susceptibility to certain pathogens (see Discussion, section 7.4.3). The MDS plots showed that as well as variation in parasite assemblage between sites, the degree of similarity in parasite assemblage within sites was also variable. This was illustrated by the dispersed nature of points for some sites (such as Glen Shee in *B. monticola*), versus the clustered nature of points from other sites (such as Stiperstones in *B. monticola*). ANOSIM analysis confirmed a significant degree of variation in the parasite assemblage between sample sites in both study species (*B. monticola* global R: 0.261, p<0.001; *B. pratorum* global R: 0.149, p<0.001). This is likely to be due to the absence of detection of some parasites at some sample sites (see Chapter 5).

7.3.3 Independence of parasitic infection

Table 7.1. Pairwise comparisons of parasite prevalence to test for independence of infection. Results were adjusted for multiple testing using the Bonferroni correction. Results significant after correction for multiple testing are indicated with asterisks. Significant results indicate deviation from independence.

B. monticola		*A. bombi*	*C. bombi*	*L. buchneri*
	C. bombi	G=2.162, p=0.142	-	-
	L. buchneri	G=0.117, p=0.732	G=0.043, p=0.836	-
	N. bombi	G=5.808, p=0.016	G=0.100, p=0.757	G=0.043, p=0.836
B. pratorum		*A. bombi*	*C. bombi*	*L. buchneri*
	C. bombi	G=17.630, p<0.001***	-	-
	L. buchneri	G=0.117, p=0.732	G=0.284, p=0.594	-
	N. bombi	G=0.955, p=0.329	G=3.537, p=0.060	G=8.167, p=0.004*

For *B. monticola* no significant interactions were observed between the infection rates of different parasite species, indicating that infections by different parasites are independent events (Table 7.1). For *B. pratorum* a significant interaction was

observed in infections by *C. bombi* and *A. bombi* (G=17.633, χ^2 df=1, p<0.001), and between infection by *L. buchneri* and *N. bombi*; no other significant results were observed (Table 7.1).

7.3.4 Association between parasite load and immunocompetence

In *B. monticola* the presence/absence of a zone of inhibition was not correlated with infection by *C. bombi* (G=0.01, df=1, p=0.926) or *A. bombi* (G=0.32, df=1, p=0.570). However, there was a significant relationship between the presence of *N. bombi* and zones of inhibition (G=5.49, df=1, p<0.05). Almost all cases of *N. bombi* infection coincided with the production of zones of inhibition (Fig. 7.6), but the total number of *N. bombi* infections detected was low (46 out of 341 individuals) so the biological significance of this result may be limited. There were insufficient *L. buchneri* infections in *B. monticola* to allow further analysis (see Chapter 5). For *B. pratorum* the presence/absence of a zone of inhibition was not correlated with the occurrence of *C. bombi* (G=2.46, df=1, p=0.117), *N. bombi* (G=0.83, df=1, p=0.360), or *L. buchneri* (G=0.16, df=1, p=0.691). However, there was evidence of a relationship between the occurrence of *A. bombi* and the presence of zones of inhibition (G=4.54, df=1, p<0.05). Almost all individuals infected with *A. bombi* also produced a zone of inhibition (Fig. 7.7), but as with *N. bombi* infection in *B. monticola* the number of *A. bombi* infections detected was low overall (only 38 out of 281 individuals).

There were no significant interaction terms in any of the analyses of parasite prevalence in *B. monticola*. There was a significant effect of PO activity on the occurrence of both *C. bombi* ($\chi^2(1)$=9.02, p<0.01) and *A. bombi* ($\chi^2(1)$=11.1, p<0.001), with uninfected individuals showing higher levels of PO activity. *N. bombi* occurrence was also significantly affected by PO activity ($\chi^2(1)$=9.47, p<0.01), but in this case PO activity was higher in infected individuals. Parasite occurrence was not significantly affected by diet breadth (*A. bombi*: $\chi^2(1)$=1.78, p=0.182; *C. bombi*: $\chi^2(1)$=1.43, p=0.232; *N. bombi*: $\chi^2(1)$=2.21, p=0.137), or zone of inhibition size (*A. bombi*: $\chi^2(1)$=1.80, p=0.180; *C. bombi*: $\chi^2(1)$=0.15, p=0.701; *N. bombi*: $\chi^2(1)$=0.35, p=0.557).

As with *B. monticola*, there were no significant interaction terms in any of the analyses of parasite prevalence in *B. pratorum*. There was a significant effect

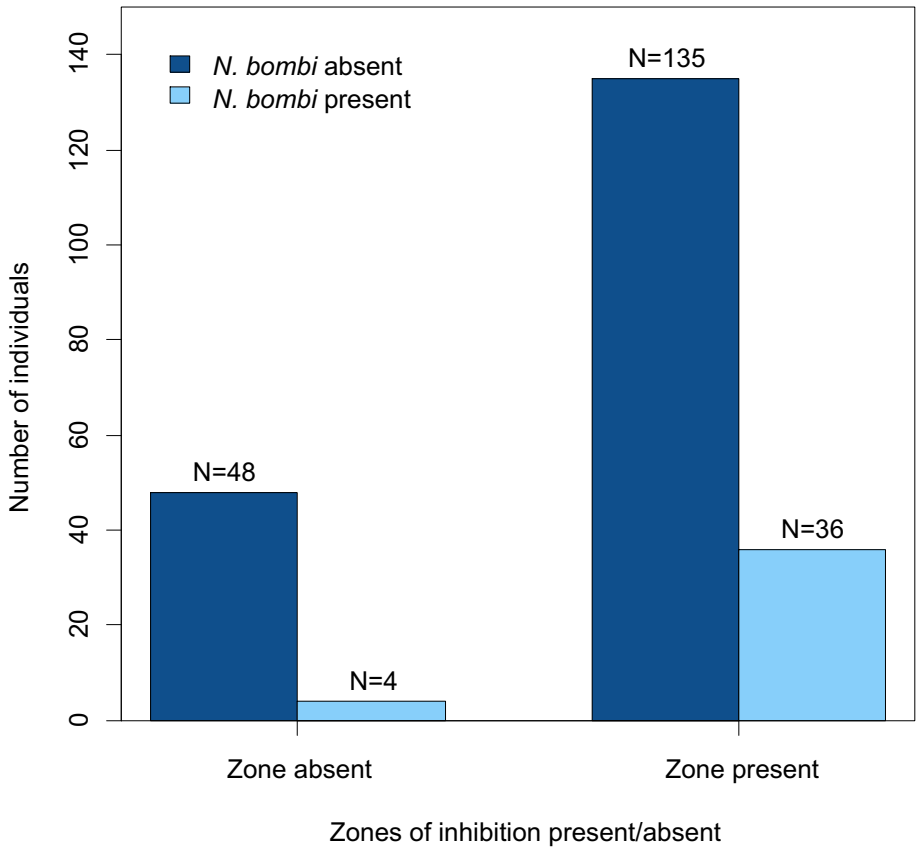

Figure 7.6. Occurrence of *N. bombi* in relation to the presence/absence of zones of inhibition in *B. monticola*.

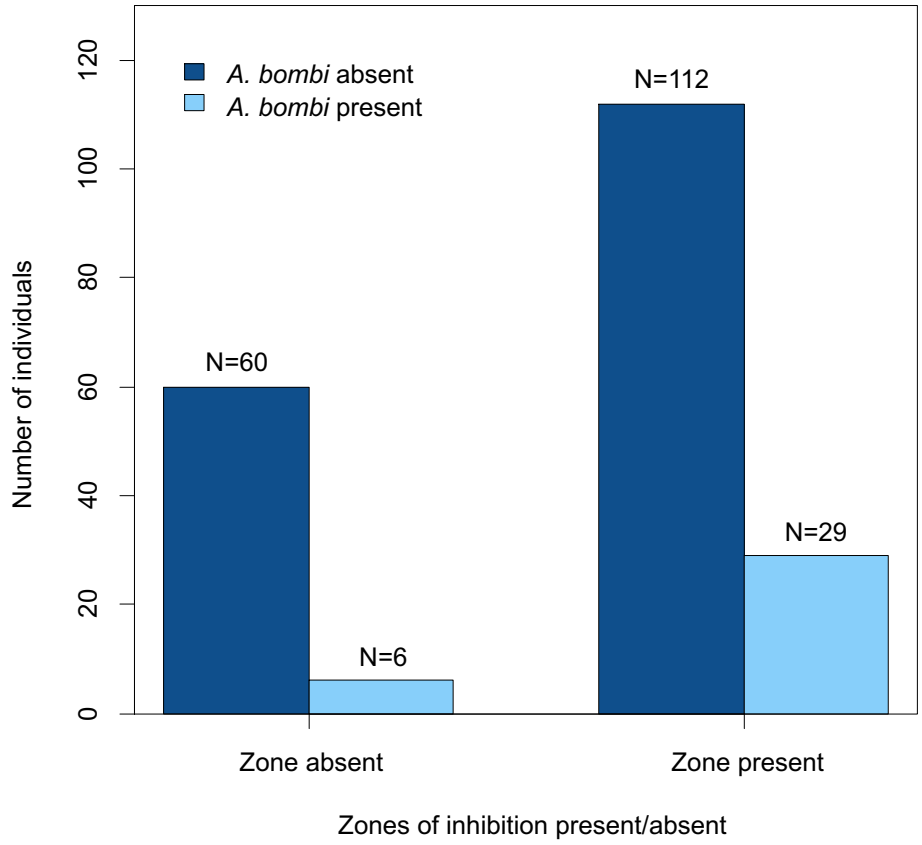

Figure 7.7. Occurrence of *A. bombi* in relation to the presence/absence of zones of inhibition in *B. pratorum*.

of PO activity on the presence/absence of both *A. bombi* ($\chi^2(1)$=6.96, p<0.01) and *L. buchneri* ($\chi^2(1)$=4.27, p<0.05), with higher PO activity observed in uninfected individuals in both cases. There was no significant effect of PO on *C. bombi* ($\chi^2(1)$=0.30, p=0.586) or *N. bombi* ($\chi^2(1)$=0.44, p=0.507). As with *B. monticola*, parasite occurrence in *B. pratorum* was not significantly associated with diet breadth (*A. bombi*: $\chi^2(1)$=3.29, p=0.07; *C. bombi*: $\chi^2(1)$<0.01, p>0.99; *N. bombi*: $\chi^2(1)$=3.33, p=0.068; *L. buchneri*: $\chi^2(1)$=0.04, p=0.850) or zone of inhibition size (*A. bombi*: $\chi^2(1)$=1.06, p=0.302; *C. bombi*: $\chi^2(1)$<0.01, p>0.99; *N. bombi*: $\chi^2(1)$=1.51, p=0.219; *L. buchneri*: $\chi^2(1)$=2.24, p=0.134).

As well as being negatively correlated with some individual parasite species, PO activity was significantly negatively correlated with parasite species richness in both *B. monticola* ($\chi^2(1)$=4.87, p<0.05) and *B. pratorum* ($\chi^2(1)$=6.61, p<0.05). Parasite species richness was not correlated with zone of inhibition presence/absence in either species (*B. monticola*: $\chi^2(1)$=0.29, p=0.589; *B. pratorum*: $\chi^2(1)$=0.66, p=0.0.418). Parasite species richness was also not found to be correlated with zone of inhibition diameter in either species (*B. monticola*:$\chi^2(1)$=0.09, p=0.768; *B. pratorum*: $\chi^2(1)$=0.16, p=0.693). There were no significant interaction terms in any of the analyses.

7.4 Discussion

7.4.1 Trade-offs in immunity

Producing an immune response is costly. The costs may be in terms of resource allocation, possibly a reduction in reproduction or life expectancy as a result of immune challenge. Alternatively, the costs may be direct harm to the host caused by the toxic by-products of an immune response; this is particularly true in the case of the PO response. Balancing these costs is the basis of observed trade-offs in many invertebrates (Cotter et al. 2004, 2008). However, the data presented here are mixed.

PO activity was lower in older *B. pratorum* workers (see Chapter 4). The melanisation reaction (of which PO is a crucial component) is very costly to the host in terms of self-harm from toxic by-products (Nappi & Ottaviani 2000, Cerenius et al. 2008, Zhao et al. 2011), and has previously been observed to senesce with

age in *Bombus* (Doums et al. 2002, Moret & Schmid-Hempel 2009, Whitehorn et al. 2010). In addition to the decrease of PO activity with age, a significant increase in the activity of certain AMPs was also observed in *B. pratorum* (although there are limitations to the interpretation of the zone of inhibition assay used, see Chapter 4). It was postulated that this could be indicative of an age-mediated switch from one immune mechanism to another; the analysis presented in this chapter provides further support for this hypothesis, by highlighting a significant interaction between PO activity, AMP activity, and age, showing AMP activity to be highest in older individuals with low PO activity. It therefore appears that in *B. pratorum*, there is a trade-off between PO activity and the activity of certain AMPs depending on the age of an individual. This may be because the costs associated with AMP production can be mediated to some extent by a change in foraging behaviour to higher protein food sources (Povey et al. 2009), while the self-harming effects of the toxic by-products of melanisation (Nappi & Ottaviani 2000, Cerenius et al. 2008, Zhao et al. 2011) response cannot be so easily avoided and may accumulate with time (see Chapter 4 discussion).

No such trend was observed in *B. monticola*. Age was unrelated to both AMP and PO activity once the effect of sample site was removed as a random factor. However, there was still evidence of interactions between the two mechanisms. It was observed that individuals which had produced a zone of inhibition showed significantly higher PO activity than those which had not (Fig. 7.1). This was initially surprising, given the costs of immunity already discussed (Nappi & Ottaviani 2000, Brunner et al. 2014). However, there is evidence from other invertebrate species that certain lineages/genotypes may be superior in terms of overall immune performance, showing higher levels of activity in all immune pathways rather than trade-offs between systems (Lambrechts et al. 2004). This may be due to additional protective effects of synergistic action of products from both the melanisation and AMP cascades (Lambrechts et al. 2004). Given the complex communities of pathogens experienced by wild populations (Rutrecht & Brown 2008b), it may be expected that maintaining a strong overall immune response would benefit survival, rather than preferentially investing in one mechanism over another. However, this theory may be contradicted by evidence for trans-generational immune priming, which prepares offspring to resist certain groups of pathogens (Moret 2006, Sadd & Schmid-Hempel 2006) at the expense of others (Sadd & Schmid-Hempel 2009). This strategy would seem maladaptive in a complex pathogenic environment, unless the costs of maintaining immunity were more detrimental to

fitness (and therefore colony success in the case of social insects) than the potential costs of infection (see Chapter 4 discussion). Alternatively, it is possible that trade-offs in immunity are present in *B. monticola*, but between PO activity and different groups of AMPs than those measured in this study. Specific AMPs are produced depending on the pathogen encountered (Schulenburg et al. 2007, Choe et al. 2002, Bulet et al. 1999, Khush et al. 2002); it may therefore be the case that PO activity in *B. monticola* is balanced against AMP activity, but against a group of AMPs not detected by an assay against gram-positive bacteria. To test this more fully would require further assays using a variety of pathogens, and is therefore outside the scope of this study. However, further testing would provide a more complete picture of possible trade-offs between the components of the innate immune system in *Bombus*. There is also the possibility that *B. monticola* and *B. pratorum* may be experiencing trade-offs in immunity, but in a fitness trait which has not been measured in this study. For example, there are many instances in invertebrates where the costs of immunity are offset against traits such as reproducton (Stahlschmidt et al. 2013), foraging (Adamo et al. 2010), learning ability (Mallon et al. 2003a), and longevity (Armitage et al. 2003). Worker bees in *Bombus* are generally sterile, but trade-offs of immunity with reproduction may be observed over longer time periods by comparing differnt aspects of immunity with the founding success of new queens produced by the colony for example, or with the number of sexuals produced. This approach would give a better indication of the implications of variable immunocompetence for long-term colony and population viability.

Different *Bombus* species are likely to experience different costs in various aspects of their life histories. For example, it is known that *Bombus* species vary in their nesting preferences (Edwards & Jenner 2005), with some suggestion of differential foraging behaviours (Edwards & Jenner 2005, Goulson et al. 2005). *Bombus* species can also vary greatly in their average size (Peat et al. 2005a,b). Data presented in Chapter 2 showed that *B. monticola* workers had a larger mean thorax weight than *B. pratorum*, and also that the relationship of body size to wing size and wing wear was considerably different between the two species (see Chapter 2 results and discussion). This suggests that the costs associated with flight and foraging are likely to differ between the two species, even within the same sample sites. This being the case, it is likely that trade-offs and energy budgets would also differ between the species. The costs of immunity may include an increased requirement for dietary protein (Brunner et al. 2014), and may therefore be me-

diated by preferential foraging on plant species such as Fabaecae which produce protein-rich pollen (Carvell et al. 2001, Goulson et al. 2005, 2008a, Hanley et al. 2008). While there were no consistent effects of dietary breadth observed in this study, only limited foraging data could be collected (see Chapter 2). Changes in behaviour to compensate for the costs of immunity may therefore have occurred, but been undetected due to the limited sampling time available. Further observation of the foraging preferences of different *Bombus* across the season would be relevant, as this may indicate potential dietary sources of immune variation between populations, as well as different compensation behaviours between species.

7.4.2 Parasitism and immunity in *B. monticola* and *B. pratorum*

Parasitism can have major fitness implications, and may influence the risk of population extinction. Resistance to parasitism is therefore very relevant to consider when assessing the long-term viability of populations (Cameron et al. 2011, De Castro & Bolker 2005, Bonsall 2004).

The majority of studies of parasitism in *Bombus* consider only single parasite systems, but wild populations are exposed to a complex parasite community, and are often infected with multiple species (Rutrecht & Brown 2008b, Whitehorn et al. 2010, Graystock et al. 2013). Determining the potential interactions between parasite species is important, as it could have implications for the evolution of virulence and transmission strategies in different parasite species. Parasites in *Bombus* are believed to infect their hosts independently (Rutrecht & Brown 2008b), meaning that the evolutionary processes determining their prevalence and adaptation to their hosts are also likely to be independent. This is largely supported by the data presented here, as no correlations were observed between infections by different parasite species. However, there were some exceptions, namely the co-occurrence of *C. bombi* and *A. bombi*, and *N. bombi* and *L. buchneri*, in *B. pratorum*. It has been suggsted that *C. bombi* may act to suppress the immune response of its host, which may subsequently make the host more vulnerable to infection by other species of parasite. However, if this were the case, *C. bombi* infection would be expected to positively correlate with infections by all other parasite species. As *A. bombi* is a high-impact parasite (Rutrecht & Brown 2008b), it seems more likely that infection with *A. bombi* may increase susceptibility to other infections.

However, both *A. bombi* and *N. bombi* were detected at relatively low frequencies in both study species (see Chapter 5); the observed correlations may therefore be an artefact of a small sample size, and would require further testing to support them.

Both the melanisation reaction and the AMP response play a vital role in defence against parasites in invertebrates (Deshwal & Mallon 2014), so it would be expected that levels of parasite prevalence would correlate with the activity of these two immune mechanisms. This was true of PO activity in *B. pratorum*, which was lower in individuals infected with *C. bombi*, *A. bombi* and *L. buchneri*, although not *N. bombi*. Parasite species richness was also lower in individuals showing higher PO activity. This corroborates previous findings in *Bombus*, which have shown negative correlations between PO activity and parasitic infection (Whitehorn et al. 2010). The melanisation cascade (measured here by proxy using PO activity) is sometimes described as the first line of defence against infection, so higher levels of PO activity would therefore be expected to correlate with a lower incidence of successful infection by parasites (Whitehorn et al. 2010). AMP production is induced by an immune challenge, and also plays an important role in parasite defence (Deshwal & Mallon 2014, Barribeau et al. 2014). However in this study parasitic infection was not correlated with the presence/absence of zones of inhibition or with zone of inhibition diameter in *B. monticola* or *B. pratorum*. This was surprising; it was hypothesised that infected individuals may show higher levels of AMP activity as a response to infection. However, this may be explained by the specificity of AMPs (Schulenburg et al. 2007, Choe et al. 2002); as this study used a gram-positive bacteria to test for AMP activity, it may simply be the case that AMPs used for parasite defence were not detected (see Chapter 4 discussion). As described above, it would be useful to expand the study to include tests of haemolymph samples against other types of pathogen, to gain a more complete understanding of AMP activity and its link with parasitic infection.

7.4.3 Immunocompetence in *B. monticola* and *B. pratorum*

A recurring trait throughout this study has been the surprising lack of a consistent difference between *B. monticola* and *B. pratorum* in the components of immunocompetence measured. As a species undergoing a contraction in range and existing

in patches of isolated habitat, it was expected that *B. monticola* would experience reduced migration, lower genetic diversity, and a subsequent reduction in fitness, as demonstrated in other species (Ellis et al. 2006, Zayed et al. 2005, Baer & Schmid-Hempel 2001).

The evidence presented provides little support for this original hypothesis. There were very few consistent significant differences between *B. monticola* and *B. pratorum* in PO or AMP activity (see Chapter 4). While it appears that the various components of immunocompetence may interact differently in *B. monticola* and *B. pratorum* (see discussion above), this does not seem to translate to consistently higher susceptibility to parasitism in either species. Based purely on the data from this study it therefore appears that a species existing in fragmented populations (*B. monticola*) is not less immunocompetent than a panmictic congener (*B. pratorum*). Rather, the data highlight the complexity of making such a comparison. While certain aspects of the innate immune response may vary, interactions with other mechanisms and potentially with behavioural changes (such as variable foraging behaviour) may mean that these changes do not translate to variability in the overall immune phenotype.

The most striking feature of this study overall is the high degree of variability between sample sites for both species in terms of foraging behaviour (see Chapter 2), immune responses (see Chapter 4), and parasite load (see Chapter 5). The variability of susceptibility between genotypes has been well established, so may be expected to vary between colonies and populations (see Chapter 4, Chapter 5). However, the inadvertent sampling of sisters, which are highly genetically similar (Estoup et al. 1995, Schmid-Hempel & Schmid-Hempel 2000), may bias results and inflate the apparent differentiation of populations in terms of susceptibility to infection. Related individuals are more similar in their susceptibility and response to infection than unrelated individuals, and such similarity within sample sites would explain both the variability of responses between rather than within sample sites, and may also explain the clustering of some points in the analysis of parasite communities. While there was little obvious clustering of points by sample site (Fig. 7.2; Fig. 7.4), the clustering of subgroups of points may indicate groups of sisters with a high level of genetic similarity, and therefore a similar degree of susceptibility to infection (Fig. 7.3). Without genetic data, it is not possible to confirm the presence of sisters in the overall data set, and therefore not possible to quantify the potential impact of relatedness on the results (see section 7.5.4). This must be considered as an important caveat when interpreting the results;

however, given that all possible measures were taken to avoid the over-sampling of any one colony (see Chapter 2), it may reasonably be assumed that the sampling of sisters is unlikely to be the main factor driving the observed trends. Infection is known to cause upregulation of immune genes (Labbé & Little 2009, Barribeau & Schmid-Hempel 2013, Johnston & Rolff 2013, Barribeau et al. 2014), with immune responses being highly specific to the threat being faced (Shulenberg et al. 2007). Significant variability was shown in parasite communities between sample sites (see Results of this chapter) for both *B. monticola* and *B. pratorum*, and there was also suggestion that the bacterial pathogen communities may vary between sites, accounting for the high degree of similarity in AMP activity between species at the same sample sites (see Chapter 4). Variable pathogen communities may in part explain the high degree of site-by-site variability in immune functions and parasite load, a hypothesis supported by the degree of interaction shown between immune functions and parasite load.

Additionally, susceptibility to infection is known to vary with host genotype, and also to be intimately linked with pathogen genotypes and the interactions between hosts and pathogens in terms of genotypes and gene expression (Barribeau et al. 2014). While inter-site variability would be expected for isolated populations, such as those of *B. monticola*, populations of *B. pratorum* were expected to show much less differentiation. Instead, similar levels of variability were observed in both species. This raises two intriguing possibilities. Firstly, it is possible for local adaptation to occur even in a panmictic species (as has also been observed in *B. terrestris* populations; Estoup et al. 1996), though this adaptation may not be evident when using neutral markers. Secondly, a species existing in fragmented populations may retain a sufficiently high N_e to allow the maintenance of selection and diversity of functional immune loci (see Chapter 5 discussion). These questions highlight the need for research into fundamental population processes, such as migration, population structuring and N_e; without these data, it is difficult to interpret what evolutionary processes are underpinning the observed trends. For example, is immune function maintained by purifying or diversifying selection? Are populations of *B. monticola* sufficiently large to maintain efficient selection for immunity? Is there evidence for frequency-dependent selection on host and parasite genotypes? While this study was unfortunately not able to compare empirical immunocompetence data with genetic data to answer these questions, it has reinforced the requirements for thorough and transparent reporting of results, and potentially highlighted sources of error to be considered which have not previ-

ously been discussed (see Chapter 6). The complexity of understanding variation in immunocompetence with relation to population fragmentation highlights the need for fundamental genetic data to be obtained.

7.5 Future work

7.5.1 Foraging preferences

This study was only able to estimate the foraging preferences of *B. monticola* and *B. pratorum* due to time constraints on sampling. However, it was acknowledged that foraging preferences are changeable over the course of a season, and are likely to change between sample sites (see Chapter 2). A better understanding of the foraging preferences of different *Bombus* species, and specifically how those preferences may differ between sites and species, would be highly relevant to know for studies of immunocompetence, particularly in light of recent research regarding the importance of diet and gut microbiota for immunity (Koch & Schmid-Hempel 2011, 2012, Koch et al. 2013, Brunner et al. 2014).

7.5.2 AMP and PO activity

This study showed minimal differences between *B. monticola* and *B. pratorum* in terms of PO and AMP activity, but a high degree of inter-site variability. However, it is difficult to interpret such data when only one timepoint is available, particularly given the complex interactions between different aspects of the immune response, and the interaction with pathogens at the point of infection. As discussed in Chapter 4, AMP activity is difficult to quantify *in vitro*, being an induced response. Extensions could include testing haemolymph samples using more types of pathogen (such as gram negative bacteria and fungal spores), to gain a better overview of AMP expression in the host at the time of sampling. Additionally, given the increasing number of gene sequences available for *Bombus* (reviewed in Barribeau et al. 2014) it may also be possible to conduct gene expression comparisons between sites and species. This may give an insight into any inter-species differences in trade-offs and general immune functioning, as well as indications of the specific effects induced by infection.

7.5.3 Parasitic infection

As with AMP and PO activity, it is difficult to interpret parasite dynamics from data collected at one timepoint. Data presented in this study suggest high levels of inter-annual variability in parasite prevalence within sample sites, so monitoring prevalence over a longer time period would provide an indication of the underlying evolutionary processes (such as a Red Queen scenario, negative frequency dependent selection and so on). Additionally, development of more qPCR methods for parasite screening would greatly facilitate studies into this area, reducing sample processing times and thus allowing for greater sample sizes to be processed, with higher resolution data being collected. Such techniques could be combined with gene expression data as a natural extension into host-parasite gene expression interactions (described by Barribeau et al. 2014; also see Chapter 5 discussion).

7.5.4 Genetic data

The original aim of this study was to use AFLP genome scans to determine levels of population structuring in *B. monticola* and *B. pratorum*, and potentially identify regions under directional selection. Unfortunately this aim could not be achieved due to technical difficulties; however, the study did highlight important sources of error, namely the long-term storage of samples, which have not been discussed prior to this as important potential sources of error for AFLP analysis (Bonin et al. 2004, Pompanon et al. 2005; see Chapter 6). Extensions for further testing the effects of sample storage were outlined in Chapter 6.

As discussed, variable susceptibility to infection is likely to result from interactions between different genotypes, and the differential expression of immune genes between populations (see Chapters 4-5). Strong inter-site variability in immunocompetence and parasite load in both *B. monticola* and *B. pratorum* may indicate highly variable pathogen communities, immune challenges and subsequent selection pressures. However, within-site similarity may also be an artefact of inadvertently sampling sisters; genetic analysis would be required to determine the likelihood of this (although all possible efforts were made to avoid sampling bias, see Chapter 2).

Given the lack of consistent species differences in immunocompetence, it was inferred that *B. monticola* populations may have a sufficiently large N_e to maintain

selection for immunity. However, these data are currently lacking, as is the case for many *Bombus* species in the UK. Questions raised by this study have reinforced the need for research into fundamental questions, to inform further research and interpret existing data.

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