The ecology and population genetics of a complex of cryptic bumblebee species

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2016

A thesis submitted for the degree of

Doctor of Philosophy

School of Natural Sciences

The University of Stirling

SUMMARY ABSTRACT

Bumblebees are ecologically and economically important as pollinators, but some species are suffering severe declines and range contractions. In this thesis, three cryptic bumblebee species are studied to elucidate differences in their distribution, ecology and population genetics.

As a result of their high morphological similarity, very little is known about the *lucorum* complex species: *B. lucorum, B, cryptarum* and *B. magnus*. In this study, their distributions across Great Britain were assessed using molecular methods, revealing that *B. lucorum* was the most abundant and most generalist of the three species, whereas *B. magnus* was the rarest and most specialised, occurring almost exclusively on heathland. Additionally, both *B. magnus* and *B. cryptarum* were more likely to be present at sites with cooler summer temperatures.

Cryptic species represent interesting models to investigate the levels of niche differentiation required to avoid competitive exclusion. Characterising the niches of these species at a single site across the flight season revealed differences along three niche dimensions: temporal activity, weather sensitivity and forage-resource use. These species exhibited asymmetric niche overlap; a combination of ecological divergence and spatio-temporal heterogeneity may contribute to maintaining them in sympatry.

Population genetic studies can be highly informative for understanding species ecology and for conservation management. The differences in habitat specialisation exhibited by these bumblebee species provide the opportunity to test conflicting hypotheses about links between dispersal and ecological specialisation: are habitat specialists selected to have low or high dispersal ability? Based on microsatellite analysis, the generalist *B. lucorum* had high levels of genetic diversity and little population structure across large spatial scales. The

habitat specialist *B. magnus* had the lowest genetic diversity but similar levels of population differentiation to the moderate generalist, *B. cryptarum*. However, unlike *B. cryptarum*, *B. magnus* population differentiation was not affected by geographic distance, suggesting that this specialist species may maintain effective dispersal across large scales despite being restricted to a fragmented habitat.

Bergmann's rule is a well-known ecogeographic rule describing geographical patterns of body size variation, whereby larger endothermic species are found more commonly at higher latitudes. Ectotherms, including insects, have been suggested to follow converse Bergmann's gradients, but the facultatively endothermic nature of bumblebees makes it unclear which pattern they should adhere to. This thesis reports caste-specific differences in body size between the three *lucorum* complex species in agreement with Bergmann's rule: queens and males of *B. cryptarum* and *B. magnus*, which were found more commonly at higher latitudes and at sites with cooler temperatures, were larger than those of *B. lucorum*.

Population genetic studies of invertebrates generally require the destruction of large numbers of individuals, which is often undesirable. Testing a variety of faecal collection and DNA extraction methods demonstrated that it is possible to obtain DNA of sufficient quality for genotyping from bumblebee faeces, without harming the individuals. This method would be valuable for studies of rare or declining bee species, for queens in reintroduction projects, and may be applicable to other arthropods.

Overall this thesis contributes substantially to our knowledge of the ecology and population genetics of three important pollinator species. It provides data to inform species conservation, as well as understanding of ecosystem functioning and population dynamics. Furthermore, it successfully uses these cryptic species as a model to test several fundamental ecological theories.

DECLARATION OF AUTHORSHIP

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

Signed …….

Date ……

ACKNOWLEDGEMENTS

I thank the University of Stirling for providing funding for this work. A great many people have contributed to the completion of this project and I would like to express my sincere gratitude to everyone who has been involved, provided advice, assistance or support. In particular, I would like to thank my supervisors, Matt Tinsley, Dave Goulson and Penelope Whitehorn. Dave gave me the opportunity to carry out this research and to take part in some interesting fieldwork. However, in my third year Dave moved to Sussex and Matt was left to supervise me for the remaining three years. Matt, I thank you wholeheartedly for taking on this role: the successful completion of this thesis is largely down to all your guidance, optimism and encouragement.

I would like to give a special mention to Steph O'Connor, who was a brilliant and valuable office mate, full of stories, as well as lots of practical advice and ideas particularly in the early stages of lab and fieldwork. I am very happy we are still in touch and always have as much fun as ever.

Having been at Stirling for a long time now, there are people to thank who have been gone a long-while; these include Lucy Woodall, who introduced me to the molecular lab at Stirling, and Andreia Penado, who drove us round Scotland, England and Wales, shared a tent with me for six weeks and with whom I experienced Scottish midges for the very first time.

I am also extremely grateful to my wonderful office mates, Katie Murray, Kirstie Hazelwood, Anwen Bill and Anna Doeser, as well as Hannah Feltham, Penelope Whitehorn, Emma Bush, Izzy Jones and Zarah Patterson, who have all made my time as a PhD student so enjoyable, put up with complaints and provided much needed support and friendship.

I am very appreciative to my family, Mum, Dad, Eloise and Tom (and newer arrivals, Bella and Zachary), who have all been encouraging and supportive. Rosie Ashworth also

deserves a special mention, having been a lovely flatmate and always there for a coffee break or, more recently, a pint.

Finally, my most heartfelt thanks go to Chris Wood. Chris now knows a lot about bumblebees and understands the love-hate relationship so many PhD students have with R. I am enormously grateful for your unwavering support, patience, endless good humour and for distracting me from PhD worries with all of the amazing adventures we have shared. With this thesis complete, I look forward to many, many more.

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1.1.1 Species diversity

It is very difficult to quantify the extent of biodiversity on earth; even the number of recognised species is difficult to calculate. Groombridge and Jenkins (2002) estimate that 1.75 million species have been described so far, whereas Mora *et al.* (2011) calculate it to be 1,438,769 species. Predictions for the number of species yet to be discovered are even more variable and highly speculative: estimates include approximately 8,961,000 (Mora *et al.* 2011) and 12,240,000 eukaryotes (Groombridge & Jenkins 2002), suggesting that despite 250 years of taxonomic study, only a small proportion of the terrestrial species (~14%; Mora *et al.* 2011) and oceanic species (~9%; Mora *et al.* 2011) have been classified. This means that we lack a reference point against which to measure past and future biodiversity losses.

Human interference in natural ecosystems is one of the major drivers of biodiversity loss, with anthropogenic habitat destruction and degradation representing one of the most important causes of species declines and extinctions (Tilman *et al.* 1994). Biodiversity provides vital resources and ecosystem services, which include food, timber, nutrient cycling, water purification and pollination (Daily 1997; Cardinale *et al.* 2012). As a result, maintaining biodiversity in the face of serious declines is of vital importance; furthermore, understanding the mechanisms driving biodiversity declines and the factors that can interfere with its persistence are major challenges for biologists.

1.1.2 Species concepts

Despite the immense importance of species determination in biology, the delimitation of species is not simple. There are many different concepts for species delimitation, advocated by different biologists, many of which are at least partially incompatible, creating disagreement about the theoretical concept of the species and difficulties in defining the

boundaries and numbers of species (de Quiroz 2007). Three of the best known species concepts are the "biological species concept", which is based on interbreeding and defines species as collections of interbreeding natural populations that are reproductively isolated from other such collections (Mayr 1942, 1995; Coyne & Orr 2004); the "ecological species concept", which is based on the operational criteria of the same niche or adaptive zone (Van Valen 1976; de Quiroz 2007) and "typological" concepts or those based on morphological differences (Coyne & Orr 2004), although these latter concepts are generally no longer applied in isolation. A large range of other more minor concepts also exist (reviewed in Coyne & Orr 2004).

These alternative concepts are based on different important biological properties, depending on the focus of the biologists studying them. The problems occur because the properties used by these concepts to define species, arise at different times, and in a different order, during the process of speciation. Definitions based on a single one of these different properties can therefore come to different conclusions concerning which lineages deserve to be recognised as species (de Quiroz 2007).

1.1.3 Speciation

Speciation is a major feature of evolution, yet understanding it remains difficult. This is because the evolution of reproductive isolation can result from a variety of different mechanisms, which can be very difficult to identify (Schluter 2001). Speciation can be classified based on the mechanisms that drive the evolution of reproductive isolation, i.e. ecological speciation, speciation by divergence under uniform selection, speciation by genetic drift and polyploid speciation, or more traditionally by the geographical arrangement of populations undergoing speciation (allopatric, sympatric or parapatric) (Schluter 2001).

Ecological speciation occurs as a result of divergent selection on traits between populations in contrasting environments, which leads directly or indirectly to the evolution of barriers to gene flow or reproductive isolation (Mayr 1942; Dobzhansky 1951; Schluter 2001). Ecologically-based selection can arise as a consequence of the interaction of individuals with their environment and other species, including biotic or abiotic elements of habitat, such as climate, resources, resource competition, predation, etc. (Schluter 2001; Rundle & Nosil 2005). A classic scenario of ecological speciation involves reproductive isolation between two populations developing initially in allopatry, as they adapt to their unique environments. This is followed by the secondary establishment of sympatry, after which premating isolation evolves to completion via reinforcement (Schluter 2001). However, the timing of secondary contact is flexible, and the initial allopatric phase is not essential, making it possible for ecological speciation to occur in both sympatry and allopatry (Schluter 2001).

Non-ecological speciation includes models that also involve selection, but where it is either non-ecological and/or not divergent between environments, such as some examples of speciation by sexual selection (Chapman *et al.* 2003a) and the fixation of incompatible alleles in allopatric populations occupying similar environments (Schluter 2001). Likewise, speciation by polyploidy and genetic drift, in which chance events feature strongly, also represent non-ecological mechanisms of speciation (Rundle & Nosil 2005; Schluter 2001; reviewed in Coyne & Orr 2004).

Determining the evolutionary forces and genetic changes at the source of speciation is difficult (Schluter 2001; Coyne & Orr 2004); the biogeographic mode of speciation has the potential to be more easily resolved (Coyne & Orr 2004). The biogeographic mode of speciation can be determined by establishing whether the proportion of breeding individuals that are immigrants within a population, is initially reduced via barriers that are external to

the organisms or by biological features of the organisms themselves. Allopatric speciation occurs when there are no breeding immigrants from the outset (Futuyma & Mayer 1980; Coyne & Orr 2004), whereas for sympatric speciation the initial restriction of gene flow is caused by the organism's biological characteristics (Futuyma & Mayer 1980) and therefore reproductive isolation evolves within the organism's average dispersal distance (Mayr 1963).

1.2 Cryptic species

Cryptic species further complicate the issue of species recognition and identification. Cryptic species are those that are recognised as a species based on an accepted concept but are at least superficially indistinguishable based on their morphology; this means that they have often been previously classified as a single nominal species (Bickford *et al.* 2007; Williams *et al.* 2012b). The development of molecular tools has led to the detection of numerous cryptic species in most types of organisms and habitat (Pfenninger & Schwenk 2007), including arctic flora (Grundt *et al.* 2006), insects (Maingon *et al.* 2003; McBride *et al.* 2009; Williams *et al.* 2012b; Adler *et al.* 2014; Kenyon *et al.* 2015; Vodă *et al.* 2015a), fish (Feulner *et al.* 2006; García-Dávila *et al.* 2013; Puckridge *et al.* 2013) and mammals (Ravaoarimanana *et al.* 2004; Racey *et al.* 2007; Esselstyn *et al.* 2013).

The presence of cryptic species adds considerable difficulties to estimating biodiversity, particularly in some taxonomic groups. They also pose problems for our understanding of ecology and for conservation management. For example, incorrectly identifying species, by overlooking cryptic species, can lead to erroneous interpretations of the ecological characteristics of large groups of species (Bickford *et al.* 2007). The discovery of complexes of cryptic species within previously recognised species can alter hypotheses about levels of specialism and generalism: the detection of many cryptic species within species of parasitoid fly, changed their categorisation from relative generalist to groups of

host-specific specialists (Smith *et al.* 2006, 2007, 2008). Similarly, a study of the butterfly *Astraptes fulgerator* in Costa Rica found that this supposed widespread, generalist species actually represents a complex of food plant specialists with differing ecological characteristics (Hebert *et al.* 2004). Likewise, the nominal species *Mordellistena convicta*, a tumbling flower beetle, and *Cotesia melitaeartum*, a parasitoid of lepidopteran larvae, have been found to comprise complexes of cryptic species with greater host-specificity than previously thought (Blair *et al.* 2005; Kankare *et al.* 2005). On the other hand, the presence of cryptic species within species forming mutualistic interactions leads to less ecological specialisation than previously assumed (Bickford *et al.* 2007). For example, figs and their pollinating fig wasps have historically been considered to exhibit one-to-one host-symbiont relationships, but the detection of cryptic species among fig wasps has led to the conclusion that these interactions can involve more pollinator species per host fig species (Molbo *et al.* 2003; Darwell *et al.* 2014). A similar result was found for the mutualism between the Australian butterfly, *Jalmenus evagoras*, and its attendant ants, which were significantly more diverse than formerly supposed (Eastwood *et al.* 2006).

Inaccurately identifying species also hampers our ability to conserve them. Measuring and mapping biodiversity is one of the first steps for conservation prioritisation: without recognising cryptic diversity we cannot determine levels of vulnerability. Moreover, candidates for cryptic species are often concealed within widespread species (Angulo & Icochea 2010), which are actually complexes of morphologically similar but geographically restricted species. In an area of Southeast Asia with the highest relative rate of deforestation in any tropical region, studies of forest dwelling frogs have revealed at least 14 species within two nominal species. These were both thought to be geographically widespread, but instead represent multiple species with smaller geographic ranges, and therefore greater vulnerability to extinction (Stuart *et al.* 2006). Also in this region, in the central highlands of Vietnam and

north-eastern Cambodia, Rowley *et al.* (2015) estimate that two thirds of diversity in a group of small, micro-endemic frogs, within the genus *Leptolax*, still remains hidden. These frogs are evergreen forest specialists, but the forests on which they rely, are being destroyed at such a rate that some species may become extinct before they are described (Rowley *et al.* 2015).

Cryptic species complexes in already endangered nominal species consequently pose more problems for conservation, as species that are already considered endangered may consist of multiple species with smaller distributions. Such cryptic species will be even rarer than the nominal species and may require different conservation strategies (Bickford *et al.* 2007). These findings illustrate the importance of accurate assessments of diversity and distributions to enable appropriate management and thereby reduce the risk of extinctions of evolutionary lineages.

1.3 Generalists and specialists

Species vary in their niche breadth; some "specialist" species have very narrow environmental tolerances, which restrict them to particular habitats, whereas other "generalist" species exhibit broader tolerances, allowing them to exploit a larger diversity of habitat types. Quantitatively measuring the breadth of a species' niche can be difficult, hence specialists and generalists are often defined based on nonquantitative contrasts between them (Futuyma & Moreno 1988).

The evolution of either specialism or generalism can depend on the environment. For example, habitat heterogeneity, in both space and time, generates diversifying selection and thus tends to favour the evolution of ecological generalists, whereas specialists are favoured in more homogeneous and stable environments (Futuyma & Moreno 1988; Kassen 2002; Richmond *et al.* 2005). In spatially heterogeneous but temporally stable habitats, selection may increase fidelity of habitat selection or decrease dispersal propensity, which can result in

a correlation between dispersal and niche breadth (Futuyma & Moreno 1988). It has also been suggested that the narrow tolerances of specialised species result in them having more fragmented distributions, thus making them more susceptible to allopatric speciation than generalist species.

Natural selection creates an evolutionary trade-off between generalising to perform many tasks reasonably well and specialising to perform a few tasks very well (Levins 1968). Thus it is assumed that specialists perform better than generalists in their optimal habitat at the expense of their performance in other habitats (Levins 1968; Futuyma & Moreno 1988; Pianka 1994). The coexistence and relative success of specialist and generalist species within communities is linked to levels of dispersal, environmental disturbance and inter-specific competition (Richmond *et al.* 2005; Devictor *et al.* 2008; Büchi & Vuilleumier 2014).

1.4 Molecular tools for identifying cryptic species

Since phenotypic characteristics have historically been the basis for most taxonomic studies (Wilson 1995; Bickford *et al.* 2007), it has been the development of molecular tools that has led to the discovery of large numbers of cryptic species. Genomic approaches were established for identifying organisms through diversity among DNA sequences (Wilson 1995). In 2003, Hebert *et al.* proposed DNA "barcoding" as a method of species identification; this involves species discrimination based on the analysis of a small segment of the genome. The mitochondrial cytochrome c oxidase I gene (COI) was proposed as a single gene barcode locus to identify species across the whole animal kingdom (Hebert *et al.* 2003a; b). Mitochondrial DNA (mtDNA) was selected because it can be easily amplified from a wide range of taxa, it has a haploid mode of inheritance so the sequence can be obtained without cloning, unlike nuclear DNA it does not contain introns, and exhibits low

recombination (Hebert *et al.* 2003a; Hurst & Jiggins 2005). Within the mitochondrial genome, the COI gene was suggested as the best candidate due to the availability of robust universal primers, its relatively high rate of molecular evolution and lack of insertions or deletions relative to ribosomal genes (Hebert *et al.* 2003a).

DNA barcoding can be used for two purposes: distinguishing between species and also for identifying new species (DeSalle *et al.* 2005). However, since its proposal DNA barcoding has come under considerable criticism, particularly for this second purpose (Will & Rubinoff 2004; DeSalle *et al.* 2005; Taylor & Harris 2012; Krishna Krishnamurthy & Francis 2012). Much of the controversy appears to have stemmed from confusion about what DNA barcoding is and its taxonomic objectives. DeSalle *et al.* (2005), Will & Rubinoff (2004) and Krishnamurthy & Francis (2012) all review the problems associated with DNA barcoding as proposed by Hebert *et al.* (2003a). Some of their main issues include a lack of a common distance threshold for species delimitation, as levels of variation vary between groups of taxa, and concern about whether the COI gene is an appropriate tool to use. Furthermore, it is difficult to argue that a barcode alone justifies recognising species that do not exhibit any ecological or morphological differences (Rubinoff & Sperling 2004). Ultimately, caution is urged in the use of a single standardised region for delimitating new species; barcodes should be used as part of an integrative approach to taxonomy that incorporates phenotypic and ecological information, as well as other sources of genetic data (DeSalle *et al.* 2005; Krishna Krishnamurthy & Francis 2012).

Nonetheless, DNA barcoding has been used successfully. As previously discussed, the traditional use of phenotypic traits for species identification has various limitations: phenotypic plasticity or cryptic taxa can lead to misidentification and morphological keys may only be suitable for particular life stages or genders (Valentini *et al.* 2009; Krishna Krishnamurthy & Francis 2012). In cases such as these DNA barcoding has made significant

contributions, for example in classifying butterflies and aphids that have different life stages, e.g. adult, larval, winged or wingless (Hebert *et al.* 2004; Footit 2009). They also complement morphological studies where taxa are easily misidentified due to cryptic species (Rubinoff 2006; Carolan *et al.* 2012; García-Dávila *et al.* 2013). Other areas of research where DNA barcoding can be particularly useful are when whole organisms are not available for morphological examination and identification (Valentini *et al.* 2009). In this respect, DNA barcoding is especially valuable when using hair, faeces or urine to monitor rare or elusive species, such as following the wolf expansion in France (Valière *et al.* 2003), studying European wildcats (Velli *et al.* 2015) and distinguishing sympatric large cat species in the Russian Far East (Sugimoto *et al.* 2006).

1.5 Non-lethal genetic sampling

1.5.1 Non-invasive genetic sampling

When used appropriately DNA barcoding can be a very valuable tool for biologists and ecologists. However, there are many other molecular tools that are similarly useful for ecology and conservation and can be applied to investigate a whole plethora of questions concerning genetic issues in conservation biology. Some have already been discussed, such as resolving taxonomic uncertainties, but others include identifying populations at risk of extinction, determining population genetic structure, choosing populations and sites for reintroduction purposes, as well as understanding species biology (Beebee & Rowe 2004; Frankham 2005). Whatever the objective, genetic studies require DNA; in the past, this required relatively large amounts of fresh tissue, which often involved killing the animal. The development of the polymerase chain reaction (PCR) meant that DNA could be amplified from tiny quantities of tissue (Mullis & Faloona 1987; Saiki *et al.* 1988), which allowed

samples to be obtained non-destructively and noninvasively and was a huge break-through for conservation biology (Morin & Woodruff 1996; Taberlet & Luikart 1999; Taberlet *et al.* 1999). The animals being studied no longer had to be killed, captured, disturbed or even observed, which is essential in behavioural studies or when working with rare or endangered species. Non-invasive genetic sampling using a wide variety of DNA sources is now applied to a whole range of questions, including: rare species detection; population size estimation; understanding social structure, genetic diversity and gene flow; and determining diet (Waits & Paetkau 2005; Beja-Pereira *et al.* 2009). These studies use both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) for different purposes: mtDNA is often used for species identification e.g. Lefort *et al.* 2012, whereas nDNA is preferred for identifying individuals and gender e.g. McCarthy *et al.* 2015 and Norman & Spong 2015 (Waits & Paetkau 2005).

1.5.2 Non-destructive genetic sampling of insects

These non-invasive genetic techniques are now commonly used for studies of many vertebrate species (Beja-Pereira *et al.* 2009). Nonetheless, recent work has shown that optimal sampling methods still depend both on the study species and study site, making it difficult to generalise across systems (Mumma *et al.* 2015). The focus of non-invasive sampling on vertebrates represents a bias in conservation efforts towards these species and the challenges present in applying these methods to invertebrates (Monroe *et al.* 2010). Most genetic studies of insects, have consequently been based on destructive methods that involve sacrificing the individuals (Donald *et al.* 2012). Population genetic studies often require the sampling of large numbers of individuals, which if performed lethally, may reduce subsequent populations sizes or alter the population structure (Starks & Peters 2002). This is particularly undesirable when studying rare or endangered species and small or declining populations; it also makes behavioural studies very difficult (Starks & Peters 2002). In social

insect species with large colonies, workers may be sampled with little impact on colonies; but for species such as bumblebees with small colony sizes, the removal of workers may reduce colony performance (Schmid-Hempel *et al.* 1993; Chaline *et al.* 2004), particularly if large numbers of workers are removed from the same colony. Furthermore, lethal sampling is undesirable for genotyping queens of eusocial insect species that are destined to found colonies (Chaline *et al.* 2004).

Some non-lethal methods of DNA sampling have been established for insects, such as wing (Vila *et al.* 2009; Hamm *et al.* 2010) and leg clipping (Fincke & Hadrys 2001; Starks & Peters 2002; Holehouse *et al.* 2003) but it is not entirely clear what impact these techniques have on the fitness of the individuals (Vila *et al.* 2009; Marschalek *et al.* 2013). In the wild, butterflies experience wing wear resembling the removal of tissue that occurs from wing clipping, and insects also naturally lose legs with little impact on their fitness (Fincke & Hadrys 2001). This has led researchers to assume that these non-lethal methods are unlikely to harm the fitness of the insects (Fincke & Hadrys 2001; Koscinski *et al.* 2011). Indeed, Marschalek *et al.* (2003) found that removing a prothoracic leg for DNA extraction from males of the butterfly *Lycaena hermes* did not affect their behaviour, longevity or survival. Similarly, in two other butterfly species, *Pieris rapae* and *Coenonympha tullia*, neither wing clipping nor hind leg removal affected post-release flight behaviour or survival (Koscinski *et al.* 2011). On the other hand, Starks & Peters (2002) successfully amplified a high percentage of microsatellites using tibial samples from eusocial wasps, but the procedure reduced the survival of the individuals, although it did not appear to prevent them from carrying out tasks within the colony (Starks & Peters 2002). In addition, Vila *et al.* (2009) used wing clippings from the moth *Graellsia isabelae* to amplify both mtDNA and nuclear DNA; this had no effect on mating success of males, nor on survival of either sex, but decreased mating success of females. Butterflies have very large wing areas relative to their body mass (Dudley 1991),

which may allow them to suffer considerable loss to wing tissue with little negative effects on flight performance (Koscinski *et al.* 2011), thus explaining why some studies find little effect of wing clipping. However, when wing sizes are smaller, the area clipped represents a larger proportion of the total area, which could lead to greater wing loading. Flight then involves higher energetic requirements and may be slowed, which could negatively impact activities such as predator avoidance or territorial defence (Marschalek *et al.* 2013). This may be particularly relevant for insects with much smaller wings for their body size, such as bees. However, wing clipping has been used as a method of non-lethal DNA sampling in the honeybee, *Apis mellifera* (Chaline *et al.* 2004).

Worker bumblebees with experimentally reduced wing area have been shown to experience reduced survival compared to controls, and natural wing wear also correlated with increased mortality (Cartar 1992). A study investigating the mechanism behind these results did not find that reducing wing size resulted in increased metabolic costs, suggesting that higher mortality is not due to flight being more energetically expensive in bees with reduced wing area (Hedenström *et al.* 2001). Instead, Hedenström *et al.* (2001) suggest that it may be due to reduced maneouvrability affecting an individual's capacity to escape predation. Despite this, Châline *et al.* (2004) recommend wing clipping rather than removal of legs when sampling honeybees because legs and tarsi are vital for performing many tasks within the colony. Honeybee queens do lose legs naturally but they may have a reduced capacity to move around the colony and they lay eggs more slowly (Chaline *et al.* 2004). In contrast, Holehouse *et al.* (2003) recommend using tarsal samples for non-lethally obtaining DNA from bumblebees; no effect of tarsal sampling was found on survivorship, body mass, frequency or duration of foraging trips or mass of pollen or nectar collected. However, they only investigated the effects on workers and they had low power for their analyses. It is

probable that tarsal clipping may have a greater impact on bumblebee queens, since, unlike honeybee queens, they have to raise the first brood of workers alone (see Section 1.6.3).

Shed exuvaie from the larvae of butterflies (Frye & Robbins 2015), damselflies (Watts *et al.* 2005) and diving beetles (Inoda *et al.* 2015) have also been shown to be suitable as a non-invasive source of DNA. This could therefore be a method applied for the study of bumblebees, but obtaining it on a large enough scale for population studies would be difficult, as it would require finding bumblebee nests, which is notoriously difficult (O'Connor *et al.* 2012).

Faeces are also a valuable source of DNA used in genetic studies of many vertebrates (Taberlet *et al.* 1997; Goossens *et al.* 2000; Jones *et al.* 2008; Caballero *et al.* 2015; Skevington *et al.* 2015), but few methods exist for using this approach with invertebrates. The low number of examples of studies using methods such as these may be a result of difficulties obtaining enough DNA of sufficient quality for genetic analyses. For example Monroe *et al.* (2010) found that DNA obtained from feacal pellets and shed exuvaie from larvae of the endangered dragonfly, was not of high enough quality to permit reliable microsatellite analysis. However, Fumanal *et al.* (2005) successfully used direct PCR of fecal secretions to amplify a 288bp fragment of the COI gene permitting identification of morphocryptic entities within the phyotphagous weevil, *Ceutorhynchus assimilis*. Lefort *et al.* (2012) and Feinstein *et al.* (2004) extracted DNA from the frass of two species of scarab beetle larvae and two species of butterfly larvae respectively. Faecal material therefore represents an untested non-lethal source of bumblebee DNA that could be extremely valuable for the study of bumblebee ecology and population genetics.

1.6 Bumblebee ecology and evolution

1.6.1 Ecosystem services and bumblebees as keystone pollinators

Ecosystem services can be defined as benefits to people provided by ecosystems (Daily 1997), and one of the most important of these is pollination by wild animals (Klein *et al.* 2007). Pollination is necessary for seed set in the majority of plants and can occur via a variety of mechanisms, including via animal vectors. Insects are particularly well suited to this role, with the vast majority of the world's pollinators consisting of wasps, bees, flies, beetles, butterflies and moths (Vanbergen 2013). Of these, bees are particularly important to the pollination of many agricultural crops (Klein *et al.* 2007).

Honeybees, *Apis mellifera*, are the most widely managed pollinators of crops and have a long history of domestication, with the result that they have been introduced to almost every country in the world (Goulson 2003). Nevertheless, they are not necessarily the most effective, and wild bees are also very important ecosystem service providers (Willmer *et al.* 1994; Garibaldi *et al.* 2013). In Europe and North America some of the most important pollinators of crops are bumblebees (Corbet *et al.* 1991). Bumblebees are often considered 'keystone' species in plant-pollinator systems for a variety of reasons. They are hardy pollinators that are able to forage in much poorer weather conditions than honeybees (Corbet *et al.* 1991; Willmer *et al.* 1994). They are also efficient, carrying more pollen on their bodies and visiting more flowers per minute than honeybees (Willmer *et al.* 1994). Furthermore, bumblebees can exploit many different flowers due to variation in body size and tongue lengths (Sladen 1912; Peat *et al.* 2005; Goulson *et al.* 2005, 2008b). They are able to buzz pollinate (by rapidly vibrating their flight muscles to shake the anthers of a plant to release pollen), a characteristic that makes them essential for many crops such as tomatoes, cranberries, blueberries and kiwi fruit, which they can pollinate more efficiently than honeybees (reviewed in Goulson 2010). In much of their range they are also the most

abundant native pollinators of both crops and wild flowers (Goulson 2010). Bumblebees are therefore economically important as pollinators: they are used worldwide for the pollination of high value crops (Velthuis & Doorn 2006). More than one million nests are commercially reared and distributed for use in more than 60 different countries (Goulson 2010). Furthermore, by pollinating both rare and abundant plant species, they maintain diversity in plant communities (Stubbs & Drummond 2001; Kremen *et al.* 2002; Memmott *et al.* 2004; Goulson *et al.* 2008a).

1.6.2 Bumblebee evolution and diversification

Approximately 250 species of bumblebee exist worldwide, distributed across the temperate, alpine and arctic regions of the northern hemisphere and also South America. Recent bumblebee classifications place all known living species in a single genus, *Bombus*, which includes the 'cuckoo' bumblebees that were previously classified as a separate genus, *Psithyrus* (Williams 1994; Cameron *et al.* 2007). Early classification depended heavily on colour patterns, but the variation in coat colour within and between populations, along with the apparent convergent evolution of colour patterns driven by Müllerian mimicry, means that bumblebee taxonomy has always been difficult (Plowright & Owen 1980; Williams 1994, 2007). Since phylogenetic relationships may correspond to ecological characteristics (Blomberg & Garland Jr. 2002; reviewed in Losos 2008), establishing an accurate phylogeny is important not only for taxonomists but also for ecologists and conservationists. Using a recent comprehensive phylogeny including 218 of the ~250 described species of bumblebee, based on five genes (Cameron *et al.* 2007), Hines (2008) provides a detailed assessment of the historical biogeography, divergence times and diversification patterns of *Bombus* species, and estimates the early divergence of extant bumblebee lineages to have occurred from 25-40 million years ago (Ma).

Bombus species richness peaks in the mountains of central China and is high throughout montane regions and cool temperate latitudes of the Orient and Palearctic (~175 species). North America is home to a smaller number of species (~60 species) and the smallest number are found in South America (~22 species) (Williams 1994, 1998; Hines 2008). Both Hines (2008) and Williams (1985) find that the initial *Bombus* diversification was likely to have occurred in the mountains of Asia. Dispersal into North America is inferred to have occurred in the late Miocene, between 10 and 20 Ma, via the Bering continental connection (Fig. 1.1, Williams 1985; Hines 2008). In the past five million years, most intercontinental movements have involved widespread, cold-adapted Old World sister taxa. Around 3.5 Ma the Bering Strait separated the two landmasses, resulting in vicariance events splitting species with a boreal distribution (Hines 2008). Evidence suggests bumblebees spread into South America, first in the late Miocene (6-8 Ma) with more additional taxa arriving around 3.5 Ma following the formation of the Panamanian isthmus (Hines 2008).

Large-scale environmental changes, such as the Plio-Pleistocene glaciations, have therefore strongly influenced bumblebee diversification (Hines 2008). For 20 million years, *Bombus* diversification remained relatively constant, but with the climatic cooling in the late Miocene, the rate of speciation decreased (Condamine & Hines 2015). This is likely due to the creation of mid-latitude cool temperate habitat favourable to the cool-temperate bees, which allowed increased gene flow in this expanding niche (Condamine & Hines 2015). In the late Pliocene, speciation increased again; the climatic instability of this period potentially split continuous populations into new species (Condamine & Hines 2015). During glacial periods populations may have become isolated in refugia, resulting in allopatric differentiation, followed by recolonisations during the post-glacial periods (Condamine & Hines 2015).

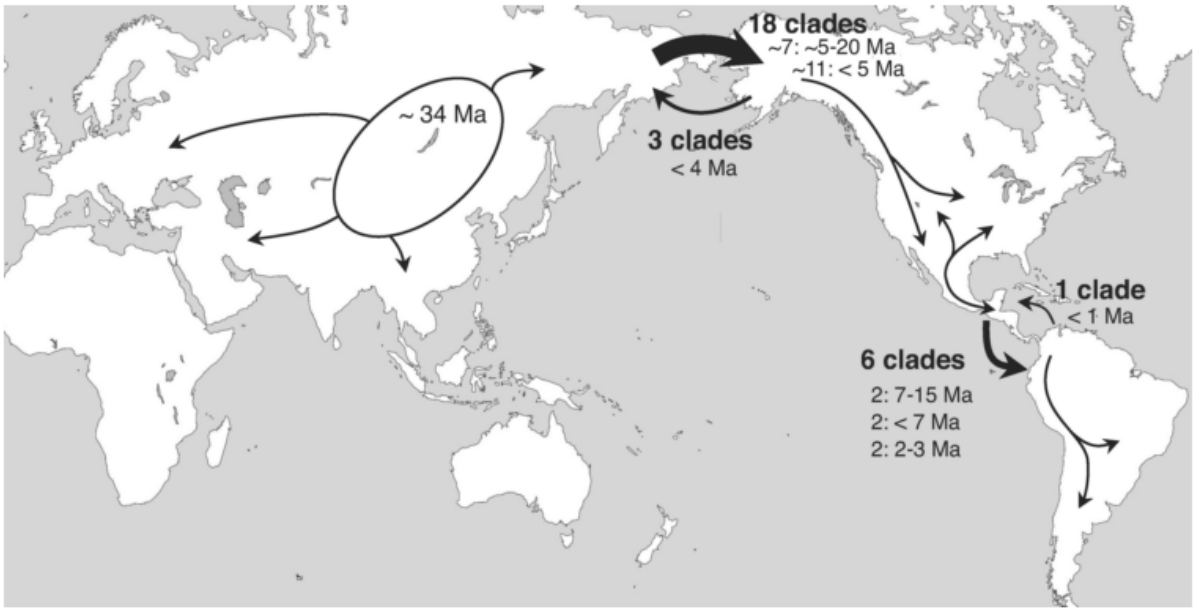


Figure 1.1. A summary of the historical dispersal events inferred across bumble bee lineages with approximate timings. The thickness of arrows is scaled relative to the number of dispersing lineages. (Taken from Hines 2008).

1.6.3 Bumblebee life-cycle

Most bumblebees are primitively eusocial; the female sex is differentiated into two castes, queens and smaller, usually non-reproductive workers. Each queen single-handedly founds a colony, where she raises her daughters, the workers, then later new queens and males. The exceptions are the cuckoo bees (subgenus *Psithyrus*), which are social parasites of other bumblebee species. *Psithyrus* do not have a worker caste: queens invade their hosts nest, attempt to usurp the resident queen and use the resident bumblebee workers to rear her own offspring, all of which are males or future breeding females. Excluding *Psithyrus*, most other bumblebee species generally have an annual life-cycle, which is characterised by colony founding, colony growth, production of new queens and males, and colony expiration (Figure 1.21.2).

Mated queens emerge from hibernation in late winter or spring, forage and search for suitable nest sites, which vary between species. *Bombus lucorum* and *B. terrestris* use pre-existing holes underground, such as the disused burrows of rodents; whereas bumblebees

belonging to the subgenus *Thoracobombus* nest within tussocks of grass or dense vegetation, on or just above the surface of the ground (Alford 1975; Goulson 2010). Disused nests of small mammals are commonly used because they provide a supply of insulating material, which the queens use to form their nest. The queen provisions her nest with pollen and nectar and lays the first batch of eggs (Alford 1975). The first batch of offspring are workers and the queen ceases to forage soon after they emerge. During colony development, the number of workers increases rapidly, although potential nest size is highly variable between species and the failure rate can be high (Goulson 2010). At some point the nest switches to rearing males and queens rather than workers. Queens can only be produced if there is sufficient food available and sufficient workers to provide it, as they require more food and take a longer time to develop than worker larvae. Therefore only the largest nests produce both queens and males, with smaller nests producing only males and some small nests failing to produce either (Schmid-Hempel & Schmid-Hempel 1998). The new queens feed and build up large fat reserves, necessary for surviving hibernation. Males leave the nest to search for mates and do not return. Once they have mated, young queens begin to search for suitable hibernation sites, where they may spend potentially six to nine months, depending on species and spring temperatures (Alford 1975). As some queens enter this phase well-before the start of winter, "diapause" is potentially a more accurate term for this period of inactivity. However, as it has evolved to allow survival throughout the winter, the term "hibernation" is retained to describe the whole length of diapause (Alford 1975). During this time, the queens rely on the fat reserves stored in their abdomen to survive; small queens that have insufficient reserves are less likely to survive diapause (Beekman *et al.* 1998). With the departure of the young queens and males, the nest rapidly degenerates.

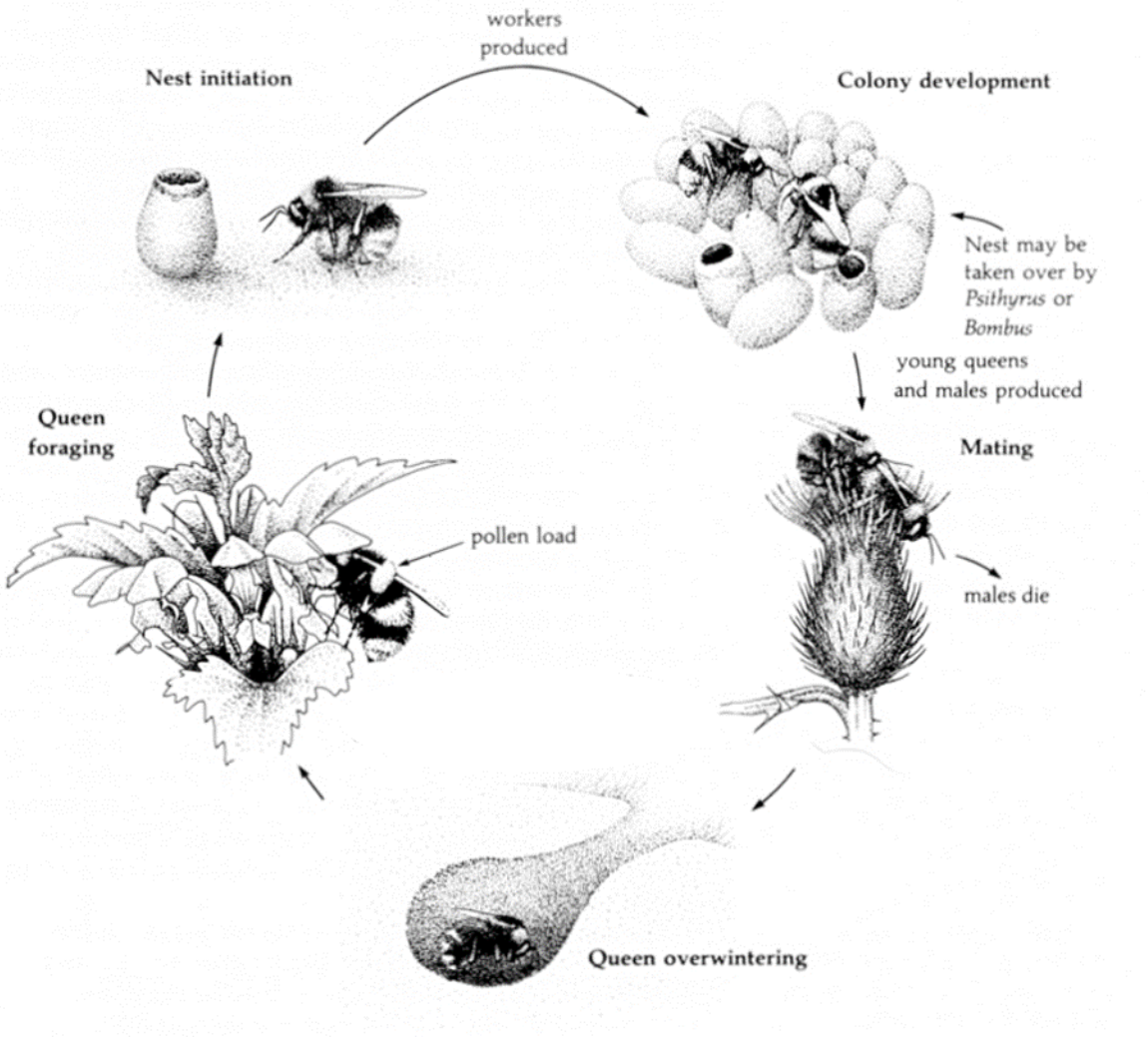


Figure 1.2. Bumblebee colony life-cycle (taken from Prys-Jones & Corbet 1991)

1.6.4 Thermoregulation

Unlike many smaller insects, bumblebees, as well as some other large flying insects, such as dragonflies and sphingid moths, are able to elevate their thoracic temperatures well above the ambient temperature by generating considerable amounts of metabolic heat (Heinrich 1974, 1979). This is essential for bumblebee flight because in order to generate enough power to fly, bumblebees require a thoracic temperature above 30°C (Heinrich 1974, 1979; Goulson 2010), while ambient temperatures in the temperate regions where most bumblebee species live, are usually in the range of 5-25 °C. Indeed, large queens can

maintain a thoracic temperature of 36 °C, and therefore fly, with ambient temperature as low as 0-3°C (Heinrich 1974, 1979). Small individuals cannot fly at such low temperatures, as their smaller size and greater surface area to volume ratio means they cannot generate or retain sufficient heat; conversely, this means that small bees can fly at higher ambient temperatures than large bees (Heinrich 1979).

Flight activity itself generates heat, but bumblebees are also able to warm themselves up before initiating flight. This is achieved by activating the flight muscles whilst stationary: they "shiver", contracting the flight muscles, which are decoupled from the wings. The metabolic rate of this muscular activity during heat production in stationary bees, can be almost equivalent to that of flight (Heinrich 1974, 1979). Substrate cycling may also contribute to the production of heat in the resting flight muscles of bumblebees (Newsholme *et al.* 1972; Clark *et al.* 1973) but this theory has been challenged (e.g. Staples *et al.* 2004).

Bumblebees are covered in thick insulating hair, which in combination with high metabolism and relatively large body size, causes a build-up of body heat that could result in overheating; bumblebees therefore also exhibit cooling mechanisms. These involve regulated 'countercurrent exchange' of heat between the thorax and abdomen, which is usually 10-15 °C cooler than the thorax in flying individuals (Heinrich 1979)

For bumblebee colonies, the production of a large number of queens and males at the end of the season requires large numbers of workers. In order to produce many workers rapidly, energy input needs to be high and continuous. Nectar and pollen are very concentrated food resources, but they are only available for a limited period of time, which means that the colony cycle is under time constraints. The evolution of thermoregulation means that bumblebees can fly, forage and care for their brood even when weather conditions are harsh and where all other bees are excluded. Both in and out of the nest, elevation of adult body temperature accelerates larval growth rates and the speed of resource harvesting

(Heinrich 1979). Indeed, established bumblebee colonies maintain a stable nest temperature of approximately 30 °C, despite large changes in air temperatures (Heinrich 1979; Goulson 2010). The temperature generated in large colonies can get too high, causing individuals to start fanning the nest to circulate the air (Heinrich 1979; Vogt 1983). Queens also incubate their eggs; once she has founded her nest and laid the first batch of eggs, whenever she is not foraging, the queen spends her time incubating her eggs. She builds the brood clump in such a way as to ensure close contact between the brood and the ventral surface of her abdomen, which is almost hairless, allowing more efficient heat transfer (Heinrich 1979; Goulson 2010). This is energetically costly, but if the queen runs low on food and stops incubating, egg, larval and pupal development stops or slows greatly (Heinrich 1979).

Bumblebees are found from the subarctic to the tropics (Williams 1998), therefore the different species inhabiting these different environments will have different thermoregulation requirements. Similarly bumblebees vary in size between species, within species (between castes) and within castes (Alford 1975; Peat *et al.* 2005; Goulson 2010), which should influence their thermoregulatory capacity; although the precise details of how these capabilities vary with species body size, geography, environment and climate are not fully understood.

1.7 Bumblebee declines, conservation and population genetics

1.7.1 Bumblebee declines

Bumblebees have recently experienced severe declines across much of their range, particularly in developed regions such as Western Europe and North America (Williams 1982; Fitzpatrick *et al.* 2007a; Goulson *et al.* 2008a; Williams & Osborne 2009; Cameron *et al.* 2011). In the UK, three of the 25 native species have become extinct since the 1960s and

an additional eight species have undergone major range declines (Goulson 2010). The pattern is similar in Europe: between 1950 and 2000, 13 species went extinct in at least one European country and four species went extinct in 11 of the central and western European countries (Kosior *et al.* 2007). Alarming declines have also been observed in North America; in the past 20 years, the relative abundances of four species have declined by up to 96% and they have contracted their range by 23-87% (Cameron *et al.* 2011).

In western Europe, the primary cause of these declines is the intensification of farming practices that has led to the loss and fragmentation of suitable habitat and necessary floral resources (Goulson *et al.* 2006, 2008a; Goulson 2010). Flowering crops such as oilseed rape seem to contribute to supporting bumblebee populations in arable landscapes (Westphal *et al.* 2003; Herrmann *et al.* 2007), but they are unlikely to provide the continuous succession of floral resources throughout the spring and summer that bumblebee colonies require. Other factors contributing to declines include loss of suitable nesting sites (Goulson *et al.* 2008a; Goulson 2010), pesticides (Gill *et al.* 2012; Whitehorn *et al.* 2012) and impacts of non-native bees (Meeus *et al.* 2011; Schmid-Hempel *et al.* 2013).

1.7.2 Conservation genetics of bumblebees

Habitat loss can result in populations of species becoming smaller and more isolated from each other. Small populations suffer more from environmental and demographic stochasticity, which can make them more vulnerable to extinction (Frankham *et al.* 2002). If populations are part of a functioning metapopulation, then local extinctions can be balanced by re-colonisation, and dispersal will maintain genetic cohesion. However, if populations become isolated, their extinction risk is augmented by inbreeding, and suitable patches may not be recolonised. Furthermore, without gene flow resulting from immigration, small populations may lose genetic variation via genetic drift, which can reduce long-term viability

of populations through inbreeding depression and a reduced ability to adapt in response to environmental change (Frankham *et al.* 2002, 2014; Keller & Waller 2002; Reed & Frankham 2003).

Neutral genetic diversity should be positively correlated with effective population size (N_e), which is distinct from the census population size (N_c). N_e relates to the number of successful breeding individuals in each generation, is a more important measure of population viability, and is also often lower than N_c (Beebee & Rowe 2004). Bumblebees may be particularly badly affected by fragmentation, as they are predisposed to low levels of heterozygosity and to inbreeding. Their social structure means that the majority of the population is made up of workers, who rarely reproduce successfully, greatly reducing the effective population size (Chapman & Bourke 2001). Moreover, unlike many other hymenopterans, bumblebee queens of most species are also monoandrous, which further increases their susceptibility to inbreeding (Estoup *et al.* 1995; Schmid-Hempel & Schmid-Hempel 2000).

Bumblebees are also haplodiploids, meaning there are only 75% as many gene copies in any one generation compared to diploid organisms, further reducing the effective population size. As a result the effective population size is approximately 1.5 times the number of successful colonies, which is potentially orders of magnitude lower than the observed number of workers (Goulson 2010). Small effective population size may render bumblebees susceptible to inbreeding. However, haplodiploidy may present a mechanism by which recessive deleterious mutations can be purged from populations through haploid males, and thus reduce the impacts of inbreeding on population fitness (Werren 1993). Conversely, the single-locus complementary sex determination (sl-CSD) system found in haplodiploid Hymenoptera may contribute additional genetic costs of inbreeding. In this system, individuals that are heterozygous at the polyallelic sex determining locus develop into

females, whereas hemizygotes develop into haploid males. However, in small populations, genetic drift results in a reduction in allelic richness at the CSD locus (Cook & Crozier 1995). This increases the likelihood of a queen mating with a male with a matching allele. When this occurs, diploid individuals are produced that are homozygous at this locus and these develop into diploid males. These males generally have low viability, and are usually sterile or have low reproductive success (reviewed in Cowan & Stahlhut 2004) and therefore represent a significant fitness cost. In a few species, diploid males can mate successfully and produce triploid offspring, which are themselves unable to reproduce and therefore the cost is simply delayed (Darvill *et al.* 2012). In social Hymenoptera, such as bumblebees, additional costs result from diploid males being produced instead of workers, which decreases the size of the workforce, colony growth and the potential success of the colony (Whitehorn *et al.* 2009).

Molecular studies of *Bombus* species have shown that rare and declining species often tend to have reduced genetic variation (Ellis *et al.* 2006b; Charman *et al.* 2010; Darvill *et al.* 2010; Cameron *et al.* 2011; Lozier *et al.* 2011): two very rare species in the UK are *B. distinguendus* and *B. muscuorum*, which exhibit low expected heterozygosity (H_E) of 0.39 (Charman *et al.* 2010) and 0.43-0.51 (Darvill *et al.* 2006, 2010) respectively. More common species generally show higher levels of diversity (Darvill *et al.* 2010; Lozier *et al.* 2011; Moreira *et al.* 2015). For example, Dreier *et al.* (2014) found some of the highest levels of H_E in two very common UK species, *B. hortorum* (H_E = 0.84) and *B. terrestris* (H_E = 0.84) in southern England.

1.7.3 Bumblebee dispersal

Another factor that influences the long term viability of metapopulations is dispersal ability. Low dispersal ability can make organisms more prone to inbreeding and local extinction, less capable of colonising and recolonising suitable unoccupied habitat patches,

and can increase population structuring as a result of reduced gene flow (Slatkin 1985; Frankham *et al.* 2002; Ronce 2007; Clobert *et al.* 2012). In bumblebees, dispersal is mediated by queens and males when they first disperse to mate, and by mated queens before and after over-wintering (Alford 1975; Goulson *et al.* 2008a). Young mated queens have occasionally been observed to travel long distances following hibernation (see Goulson 2010), but it is very difficult to determine dispersal distances based on random observations. The spread of species that have been introduced to new areas where they are not native can also be informative about dispersal ability. *Bombus terrestris* was introduced to Tasmania in 1992, where it was estimated to spread initially at approximately 10 km per year (Stout & Goulson 2000), however, within 12 years this species had reached the northern coast (~200 km) (Hingston 2006). In South America, *B. terrestris* has been shown to spread even more rapidly: in the order of 200 km per year (Schmid-Hempel *et al.* 2013).

An alternative method of measuring gene flow between populations and thus inferring whether movement of individuals occurs frequently uses neutral genetic markers. Microsatellites have frequently been used for this purpose and have proven very informative. Such studies focussed first on two abundant and widespread European species, *B. terrestris* and *B. pascuorum*. In mainland Europe, *Bombus terrestris* appears to exhibit very little population structuring, however island populations are more strongly differentiated, suggesting that stretches of sea represent barriers to movement and gene flow (Estoup *et al.* 1996; Widmer *et al.* 1998; Moreira *et al.* 2015). *Bombus pascuorum* was shown to exhibit higher levels of population differentiation, with the Alps representing a partial barrier to gene flow (Widmer & Schmid-Hempel 1999). More recent studies in the UK, showed no genetic structuring between populations of *B. pascuorum* spanning the whole of the UK (Ellis *et al.* 2006b). Similarly in America several species, including *B. vosnesenskii*, showed very low population differentiation over large spatial scales (>1500 km; Lozier *et al.* 2011). These

studies suggest that for some species dispersal must be common and potentially occur over relatively long distances in order to maintain genetic cohesion over such large scales.

Much stronger population structuring has been detected in fragmented populations of rare species in the UK, such as *B. sylvarum* (Ellis *et al.* 2006b), *B. muscorum* (Darvill *et al.* 2006) and to a lesser extent, *B. distinguendus* (Charman *et al.* 2010). Nevertheless, not all abundant species seem to have strong dispersal abilities: *B. hortorum* is a very widespread species in the UK that shows very strong genetic differentiation between islands in the north-west of Scotland and thus appears to be extremely sedentary (Goulson *et al.* 2011).

It has been suggested that there are links between a species' level of specialisation and it's dispersal ability or propensity: specialists have been proposed to have evolved lower rates of dispersal than generalists (Colas *et al.* 1997; Mathias *et al.* 2001; Bonte *et al.* 2012; Dahirel *et al.* 2014). Very tight dietary or habitat specialisations are unusual in European bumblebees; most species are found in a broad range of biotopes (Goulson *et al.* 2006). Two species that show associations with heathland to varying extents are *B. jonellus* and *B. monticola*. *Bombus jonellus* is a heathland species in the north of the UK (Goulson *et al.* 2006; Darvill *et al.* 2010) and *B. monticola* is described as associated with upland heaths and moors (Edwards & Jenner 2005; Benton 2006). In a study of gene flow on islands in the Hebrides (Scotland), Darvill *et al.* (2010) found that *B. jonellus* had a higher propensity to disperse than *B. muscorum*, a more generalist species, and speculated that this was due to the association of *B. jonellus* with a fragmented habitat. Similarly, *B. monticola* has recently colonised Ireland (Fitzpatrick *et al.* 2007a), an unusual event, as colonisations are rare among bumblebees in the UK, which suggests that *B. monticola* can also disperse over long distances. Determining patterns of population connectivity and gene flow can therefore give us insights into the ecology and dispersal capacity of bumblebee species and is very useful when trying to assess the risks facing bumblebee populations and species.

1.8 Cryptic bumblebee species: the *lucorum* complex

Bumblebees exhibit a variety of distinctive colour patterns that are often used to identify species (for example Edwards & Jenner 2005); however, these patterns can be very similar between species and also highly variable within species (Williams 2007). As a result, the taxonomy of species has been quite uncertain and several examples of cryptic species occur amongst bumblebees (Hines *et al.* 2006; Ellis *et al.* 2006a; Wolf *et al.* 2010; Williams *et al.* 2012b). The European species of the subgenus *Bombus sensu stricto* pose particular difficulties, which is problematic as they are of economic interest due to their commercial exploitation (Velthuis & Doorn 2006; Goulson 2010). There has been much disagreement on the taxonomy of this group in the past decades, which now consists of five species in Europe: *Bombus terrestris* (L., 1758), *B. lucorum* (L., 1761), *B. cryptarum* (Fabricius, 1775), *B. magnus* (Vogt, 1911) and *B. sporadicus* (Nylander, 1848). Until the mid-twentieth century only two species, *B. lucorum* and *B. terrestris*, were widely accepted and these are still difficult to distinguish (Wolf *et al.* 2010). Indeed, over 100 infrasubspecific names have been reported for *B. lucorum* alone (Williams 1998).

Bombus lucorum was described by Linneaus in 1761, *B. magnus* was described almost a century later and most subsequent studies will have been confused by the fact that their samples comprised the unknown species *B. cryptarum* (Bossert 2015), although two forms of *B. lucorum* ('dark' and 'blonde') were recognised (Løken 1973). A potential third species was first recognised by Rasmont (1981), but biochemical methods did not confirm its existence for some time, probably due to a mix of species among samples (Pamilo *et al.* 1997; Bossert 2015). Differences in male labial gland secretions finally confirmed that recognition signals differed between the three taxa, providing evidence for three separate species (Bertsch 1997; Bertsch *et al.* 2005; Bertsch & Schweer 2012). Studies of nucleotide sequences of the mitochondrial COI gene then added further support for the distinct

taxonomic status of the *lucorum* complex species in Europe (Bertsch *et al.* 2005; Murray *et al.* 2008; Williams *et al.* 2012b; Carolan *et al.* 2012). Although they vary slightly between studies, these results show a significantly greater divergence between the three taxa than within, with estimates of inter-specific Tamura-Nei genetic distances of 0.033-0.044 compared to intra-specific distances of 0.001-0.004 (Carolan *et al.* 2012) and 0.023-0.036 compared to 0.002-0.004 (Murray *et al.* 2008).

A number of morphological characteristics have been proposed as useful for distinguishing these species particularly for identifying colony-founding queens in the spring (Fig. 1.3). These characters include the extent that the collar of yellow hairs at the anterior of the thorax extends below the tegula (Alford 1975), the level of melanisation in this collar (Bertsch *et al.* 2005), the presence or absence of an S-shaped band of black hairs on the side of the collar (Prŷs-Jones & Corbet 1991; Edwards & Jenner 2005; Bertsch *et al.* 2005).

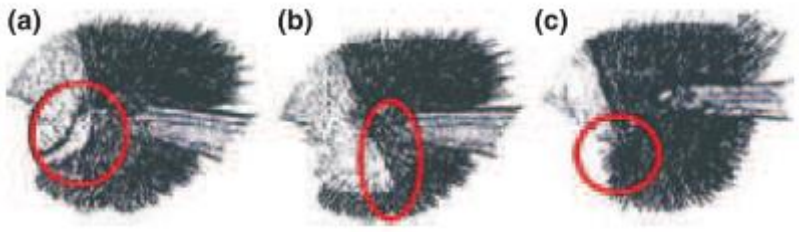


Figure 1.3. Morphological characters suggested for the identification of queens of the lucorum complex. *Bombus cryptarum* queens (a) have been proposed to exhibit an 'S' shaped line of black hairs within the yellow hairs of the thoracic collar, and the yellow collar of *B. cryptarum* and *B. magnus* (b) is supposed to extend further below the wing than that of *B. lucorum* (c) (Alford 1975; Prŷs-Jones & Corbet 1991; Edwards & Jenner 2005; Bertsch *et al.* 2005). Taken from Waters *et al.* 2010.

However, there has been some debate about the reliability of these traits. Bertsch *et al.* (2005) described geographical variation in these traits that made it harder to discriminate these species (Figs. 1.4 & 1.5). Waters *et al.* (2010) demonstrated that the extent and breadth

of the yellow collar is not suitable for distinguishing workers of *B. magnus* and *B. lucorum*. Williams (2000) found that the length and breadth of the yellow collar showed a continuum of variation between *B. magnus* and *B. lucorum* queens in the UK. Similarly, Carolan *et al.* (2012) found that all morphological characters most commonly cited as distinguishing these taxa showed overlap among species at both local and European scales. These studies therefore indicate that these traits cannot be used to reliably and consistently distinguish between the lucorum complex species.

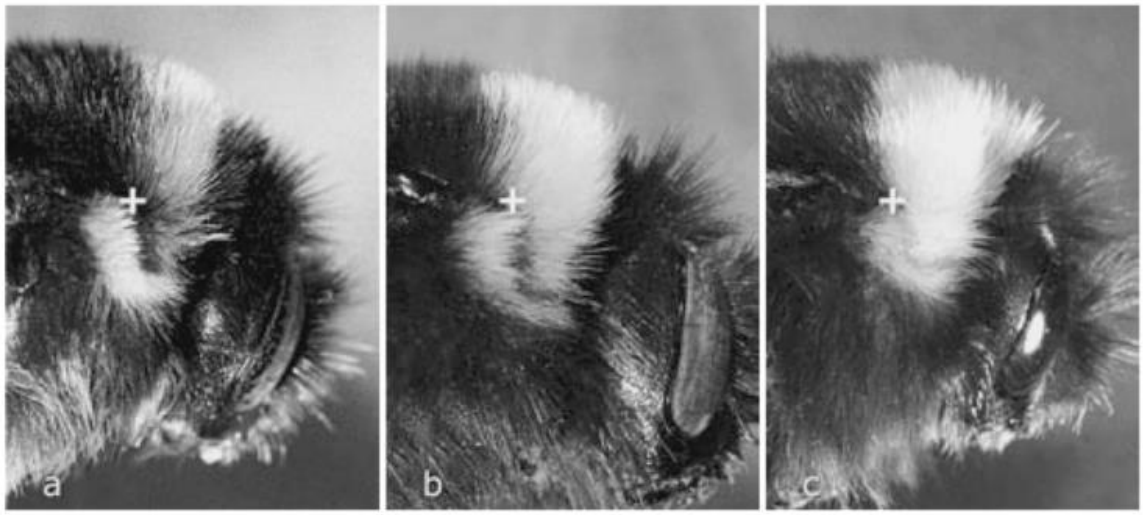


Figure 1.4. S-shaped band of dark hair in the yellow collar of *B. cryptarum* from (a) Menz, Germany, (b) Duncansby Head, Scotland and (c) Dunnet, Scotland. Taken from Bertsch *et al.* 2005

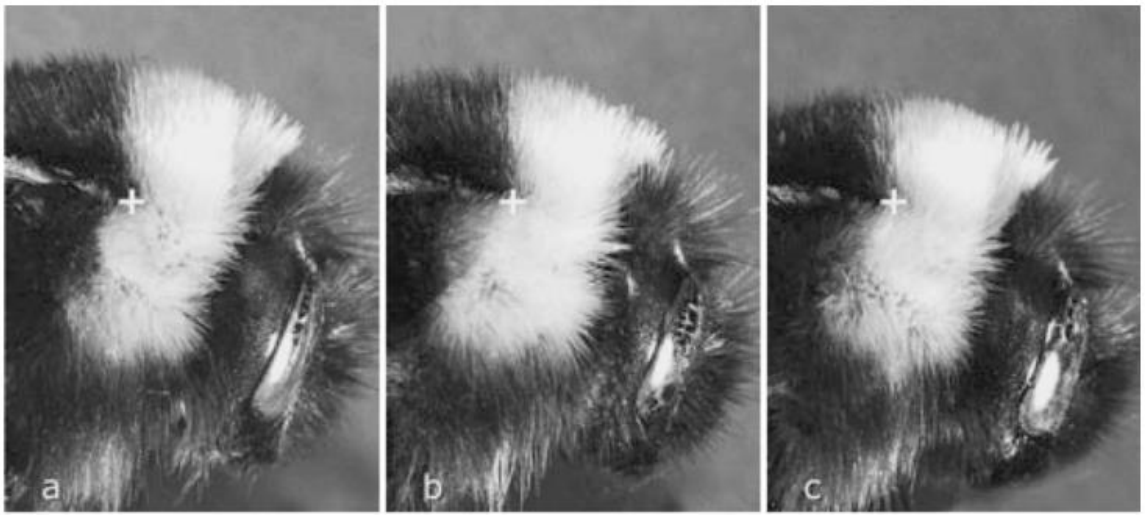


Figure 1.5. The broad bright yellow collar, showing no melanisation in *B. magnus* from (a) Menz, Germany, (b) Duncansby Head, Scotland and (c) Dunnet, Scotland. Taken from Bertsch *et al.* 2005

As a consequence of the difficulties in identifying the *lucorum* complex species, relatively little is known about their ecology and distribution. Descriptions of their ecology and distribution, obtained prior to the utilisation of molecular methods for species identification, are likely to be problematic (see Rasmont, 1984; Rasmont *et al.*, 1986 and Pamilo *et al.*, 1997 for European distributions). In fact, *B. cryptarum* was only discovered to be present in the British Isles in 2005 (Bertsch *et al.* 2005).

The most reliable information available about the worldwide distributions of these species comes from a study by Williams *et al.* (2012) of the subgenus *Bombus s. str* using COI barcode variation. They find *B. lucorum* to be present from Iceland in the west, across Europe to the mountains of Central Asia and in Mongolia. *Bombus cryptarum* appears to have the broadest distribution of all *Bombus s. str.* species: it was found from Great Britain, across Europe and central Asia to western North America. *Bombus magnus* is present in Great Britain, Spain, Denmark, Sweden and near Moscow, Russia. In 2008, Murray *et al.* developed a PCR-RFLP method that allowed a relatively cheap, rapid and accurate, discrimination of the *lucorum* complex and *B. terrestris* in Europe. This tool has made it possible to begin to gain a more detailed understanding of the distributions and habitat use of these important pollinators (Murray *et al.* 2008; Anagnostopoulos 2009; Waters *et al.* 2010a; Stanley *et al.* 2013b; Vesterlund *et al.* 2014). Using this method, Murray *et al.* (2008) found all three species of the *lucorum* complex to be widely distributed in Ireland; *B. lucorum* was found at all sampled locations and was the most abundant (56%), *B. magnus* was more common at rural than urban sites and *B. cryptarum* was the least abundant of the three (18.4%). A more detailed, but spatially restricted, study by Waters *et al.* (2010) in the Western Isles of Scotland found that the abundances of the three species were opposite to those in Ireland: *B. lucorum* was the least abundant of the three species (30%) and *B. cryptarum* was the most abundant (43.8%). *Bombus magnus* was previously assumed to be

associated with cool, wet and upland areas (Alford 1975; Benton 2006), whereas these two studies find this species in both upland and lowland sites, but absent from urban areas. Waters *et al.* (2010) also suggest that *B. magnus* appears to be associated with heathland and the food plant *Calluna vulgaris*.

The patchy and fragmented coverage of these studies means that further work is clearly necessary. Although, the *lucorum* complex species have been shown to occur sympatrically, the species composition varies considerably at a regional level (Murray *et al.* 2008; Waters *et al.* 2010a). So far we still lack knowledge on what drives variation in species abundances and distribution; very little is known about the ecology of these three species.

1.9 Aims and objectives

Bumblebees are vitally important both ecologically and economically as pollinators. However, in recent decades many species have suffered severe declines and range contractions across much of North America and Europe. In order to assess the risk to each of these species and the ecosystem services they provide, a thorough understanding of their ecology is required. Effective population sizes of bumblebees also appear to be low compared to those of many solitary insects, and potentially compared to other social insects too, due to their monogamous nature (Goulson *et al.* 2008a; Goulson 2010). Given the potentially serious consequences of population fragmentation and inbreeding for bumblebees, it is essential that we understand the genetic structure of wild populations. However, cryptic species are common among bumblebees, making basic ecological studies and conservation management difficult. The overall aim of this thesis is to further our knowledge of the ecology and population genetics of the three cryptic bumblebee species belonging to the *lucorum* complex, which forms part of the economically exploited subgenus *Bombus sensu stricto*. The *lucorum* complex, as a whole is very widespread across the Palearctic and

Nearctic, but little is known about the individual species. To overcome these problems, genetic methods for distinguishing these species were applied in order to:

(i) determine the geographic distribution and abundance of each of three *lucorum* complex species in Great Britain (Chapter 2)

(ii) assess niche differentiation across multiple sites by comparing geographic range, forage use and sensitivity to summer temperatures (Chapter 2)

(iii) compare interspecific variation along different niche dimensions across a whole flight season at a single location (Chapter 3)

(iv) estimate niche region and niche overlap for each species (Chapter 3)

(v) assess genetic diversity and population structuring for each species across Great Britain (Chapter 4)

(vi) identify differences in body size between the three species and compare these to their ecological characteristics (Chapter 5)

Focussing this research on cryptic species also permits the exploration of more general ecological questions, as they represent an ideal model for comparative studies. This thesis therefore additionally considers:

(a) how cryptic species partition niches to avoid competitive exclusion (Chapters 2 & 3)

(b) levels of population structure in species that differ in their level of dietary and habitat specialism (Chapter 4)

(c) patterns of body size in facultatively endothermic insects in relation to Bergmann's rule (Chapter 5)

In addition, a further study develops a novel and valuable genetic tool for non-destructive DNA sampling and extraction, permitting population genetic studies of bumblebees without harming the individuals (Chapter 6).

Chapter 2

Revealing the hidden niches of cryptic bumblebees in Great Britain: implications for conservation

A version of this chapter has been published as:

Scriven, J.J., Woodall, L.C., Tinsley, M.C., Knight, M.E., Williams, P.H., Carolan, J.C., Brown, M.J.F., and Goulson, D. (2015) Revealing the hidden niches of cryptic bumblebees in Great Britain: Implications for conservation. *Biological Conservation*, **182**, 126-133.

D. Goulson and M. Tinsley supervised the project, L. Woodall assisted with lab work, M. Knight contributed some samples and D. Gouslon, P.Williams, J. Carolan and M. Brown collaborated on a grant to fund the first stages of this project. All authors commented on draft versions of this manuscript. The published version is presented here.

2.1 Abstract

Bumblebees are ecologically and economically important, and some species have suffered dramatic population declines. The absence of morphological diagnostic characters for the identification of some species creates difficulties for basic ecological studies, and for conservation management. The widespread and commercially exploited bumblebee subgenus *Bombus sensu stricto* contains a cryptic species complex, known as the *lucorum* complex, which in Europe comprises *B. lucorum, B. cryptarum* and *B. magnus*. Little is known about these species and much of what has been reported is likely to have suffered from incorrect identification. Although the *lucorum* complex as a whole is common in Great Britain, we aimed to determine whether the populations of the individual species are vulnerable and require conservation action. Using genetic methods to distinguish them, we determined the geographic distribution and abundance of the *lucorum* complex species in Great Britain, and assessed the extent of niche differentiation between these species. We detected major differences in the geographic range, forage use and sensitivity to summer temperatures of the three species. *Bombus lucorum* was found to have the broadest distribution and diet, being present throughout mainland Great Britain, whereas *B. cryptarum* and *B. magnus* were absent from large areas of central and southern England. *Bombus cryptarum* and *B. magnus* were more likely to be found at sites with lower summer temperatures. *Bombus magnus*, the least abundant species, was found to exhibit an unusually tight biotope association with heathland habitat. This has conservation implications for *B. magnus* given the current threats to this habitat type.

2.2 Introduction

Bumblebees (*Bombus*: Hymenoptera, Apidae) are ecologically and economically important as pollinators (Velthuis & Doorn 2006; Goulson 2010). Some species have recently suffered severe declines and range contractions across much of Western Europe and North America (Williams 1982; Fitzpatrick *et al.* 2007a; Goulson *et al.* 2008a; Williams & Osborne 2009; Goulson 2010; Cameron *et al.* 2011). In the UK, seven out of the 27 species are listed as priority species in the UK post-2010 Biodiversity Framework (previously Biodiversity Action Plan), a higher proportion than known for any other invertebrate group (Goulson 2010). *Bombus* species are also notorious for possessing convergent colour patterns and displaying high intraspecific variation, resulting in cryptic species (Williams 2007). The inability to correctly identify such species creates difficulties for basic ecological and population genetic studies as well as for their conservation management.

Cryptic species can be defined as two or more distinct species that are similar or identical in morphology (Williams *et al.* 2012a). Speciation is not always accompanied by morphological change, and as a result, the true number of biological species is likely to be greater than the current total of nominal species, most of which are delineated on a purely morphological basis (Bickford *et al.* 2007). The development of molecular genetic tools has enabled the detection of numerous cryptic species. Large genetic distances within traditionally recognised species, usually in combination with morphological, geographical, ecological or behavioural differences, have led to the discovery of cryptic species in a diverse range of organisms, from tropical butterflies (Hebert *et al.* 2004), to arctic flora (Grundt *et al.* 2006), fish (Feulner *et al.* 2006; Puckridge *et al.* 2013) and lemurs (Ravaoarimanana *et al.* 2004).

Theories on the ecological specialisation of species can be seriously challenged by the existence of cryptic species complexes. Studies of a range of insects have revealed that

presumed dietary generalists are in fact complexes of dietary specialists (Hebert *et al.* 2004; Smith *et al.* 2007). The occurrence of cryptic species also has important repercussions for conservation; in an area of Southeast Asia with the highest relative rate of deforestation in any tropical region, studies of forest dwelling frogs have revealed at least 14 species within two nominal species. These were both thought to be geographically widespread, but instead represent multiple species with smaller geographic ranges, and therefore greater vulnerability to extinction (Stuart *et al.* 2006). Such findings illustrate the importance of accurate assessments of diversity and distributions to enable appropriate management and thereby reduce the risk of extinctions of evolutionary lineages. Cryptic species complexes in already endangered nominal species consequently pose more problems for conservation, as species that are already considered endangered may consist of multiple species with smaller distributions. Such cryptic species will be even rarer than the nominal species and may require different conservation strategies (Bickford *et al.* 2007).

The subgenus *Bombus sensu stricto* is a widespread and commercially exploited taxon of bumblebee, which contains five species in Europe, *B.* (*Bombus*) *cryptarum,* (Fabricius), *B.* (*B.*) *lucorum* (Linnaeus), *B.* (*B.*) *magnus* (Vogt), *B.* (*B.*) *sporadicus* (Nylander), *B.* (*Bombus*) *terrestris* (Linnaeus). The taxonomic status of the last two species is widely accepted but *B. lucorum, B. magnus* and *B. cryptarum* are morphologically indistinguishable in much of their range, triggering considerable debate about their status. *Bombus magnus* and *B. cryptarum* have been regarded as subspecies of *B. lucorum* and are often referred to collectively as the '*lucorum* complex' or simply synonymized to *B. lucorum* (Edwards & Jenner 2005; Benton 2006). Recent studies using CO1 barcode analysis show discrete differences between the three species (Murray *et al.* 2008; Williams *et al.* 2012b; Carolan *et al.* 2012), in accordance with studies of labial gland secretions (Bertsch *et al.* 2005). Diagnostic morphological characters have also been previously reported for queens, but some of these have now been

demonstrated to overlap considerably, and vary along a continuum, thus making them unreliable and leading to a high potential for misidentification (Carolan *et al.* 2012).

In Ireland, *B. lucorum* is classified as of Least Concern according to the IUCN Red List criteria. *Bombus cryptarum* and *B. magnus* cannot be assigned to a threat category because they are currently Data Deficient (Fitzpatrick *et al.* 2006, 2007b). The situation is no clearer in Great Britain, where the distribution of the three taxa is only known for the Western Isles of Scotland (Waters *et al.* 2010a). The difficulty in identifying these species means that little is known about their ecological attributes; much of what can be found in standard texts will actually be referring to data for multiple species and is therefore of limited value. Consequently, the only reliable information we have on the ecology of these three species comes from Murray *et al.* (2008) and Stanley *et al.* (2013) who used molecular methods to study the *lucorum* complex in Ireland and Waters *et al.* (2010) who studied them in the Western Isles of Scotland. Niche-partitioning might be expected between these species (Goulson *et al.* 2008b) and indeed some ecological differences have been suggested. Specifically, Waters *et al.* (2010) found that *B. magnus* appeared to be strongly associated with the heathland forage plant *Calluna vulgaris*. These studies suggest that the three taxa are widespread throughout Ireland and the Western Isles of Scotland but have differing patterns of geographic distributions. These studies have suggested some differences in the ecology, abundance and distribution of the three taxa, which, given the ongoing concerns over bumblebee declines, indicates the need for further work to reveal the biology of these species and reassess their conservation status.

The aim of this study was to assess the distribution and abundance of the *lucorum* complex species in Scotland, England and Wales and establish whether the populations of the individual species are vulnerable and require conservation action. Genetic methods were used to distinguish the three species. We then tested for niche differentiation between them by

assessing how climatic factors and habitat associations correlate with the distributions of the three species. Further, we assessed foraging behaviour and quantified the differences in diet breadth and forage use between the three species. In particular, we tested the specific hypothesis that *B. magnus* is a heathland specialist, using a paired sampling strategy where heathland and non-heathland sites were sampled at each location.

2.3 Materials and methods

Sampling

Queens, workers and males were sampled across Great Britain from June-September during the summers of 2010 and 2011. In July 2010, 13 locations were sampled along a North-South line through the approximate centre of Scotland and England; during June-August 2011, 14 further locations were sampled focussing on the periphery of the UK. The 2011 fieldwork tested the hypothesis that *B. magnus* is a heathland specialist (Murray *et al.* 2008; Waters *et al.* 2010a) using a paired sampling design: 11 of the 14 locations comprised a pair of sites representing heathland and non-heathland habitats within 15km of one another. All locations sampled in 2010 consisted of non-heathland habitat, although some were close to heathland. We aimed to catch at least 100 bees at each location, but occasionally this was not possible (mean = 89.4 ± 12.9 SE). For bees caught foraging on a flower (as were most), forage plant identity was recorded. Whole bees were stored in absolute ethanol. Thorax width of all individuals sampled in 2011 was measured using callipers to examine size differences between species.

Species identification

DNA extraction from the samples collected in 2010 was performed using a Chelex® 100 protocol (Walsh *et al.* 1991) and from the 2011 samples using a HotShot protocol (Truett

et al. 2000). For species identification we followed a PCR-RFLP method based on amplification of the cytochrome oxidase I (COI) gene developed by Murray *et al.* (2008). The pattern of digested fragments for each individual was compared with the characteristic patterns associated with each of the cryptic species and *B. terrestris* (see Fig. 3 in Murray *et al.* 2008), in order to determine their species identity. To confirm RFLP identification; 108 individuals (46 *B. terrestris*, 55 *B. lucorum*, 2 *B. magnus*, 2 *B. cryptarum*, 2 *B. soroeensis*, 1 *B. sylvestris*), collected from all but one of the 2010 sample sites, were amplified using the PCR-RFLP primers. Resulting PCR amplicons were purified (ExoSAP; Werle *et al.* 1994) and sent for sequencing (DNA Sequencing and Services, Dundee, U.K.). Consensus sequences were aligned (Geneious v 6.1.7) then checked against the RFLP banding pattern. For those samples that did not exhibit a clear RFLP banding pattern after two amplifications (174 of 2 415), we used microsatellite data for species assignment (obtained from a separate study comparing population structure of the three species, Scriven *et al.* in prep.; Chapter 4). In brief, individuals were genotyped at 13 microsatellite loci (Appendix 2.1 & 2.2). Structure v 2.3.4 (Pritchard *et al.* 2000) was used to cluster the samples according to species. The USEPOPINFO model was applied to define "learning samples" that are pre-defined as coming from particular clusters (the known species from RFLP analysis) to assist ancestry estimation for the remaining individuals of unknown origin. The Admixture and Independent Allele Frequency models were also used and the software was run with four clusters (K, for the three *lucorum* complex species and *B. terrestris* using 50 000 burn-in periods followed by 100 000 MCMC repetitions).

Analyses

Differences in habitat use and forage use between the three bumblebee species were examined using χ^2 tests of association on data pooled across all sites in contingency tables.

For habitat use, data from all castes were included; for forage use, only data from queens and workers were used. Males often rest upon flowers when not foraging or searching for queens (Alford 1975), so they were not included in the analysis of forage use. Diet breadth was calculated and compared between bumblebee species using rarefaction: 100 samples were randomly drawn from those recorded for each species, without replacement, and the number of forage plants represented in this subsample recorded; 100 replicates were performed per species to estimate the mean number of plant species each bee species would be expected to visit in the specified number of flower visits.

Other analyses were carried out using R version 3.0.2 (R Core Team 2014). Generalised linear models with a binary error distribution were used to investigate the biogeographical and climatic correlates (UK Meteorological Office 2014) of *lucorum* complex species presence at sites. The response variable was the presence or absence of a species at a site. Explanatory variables tested were habitat type (heathland or non-heathland), mean maximum daily temperature from March to August (the approximate flight period of these species), elevation (m) and all two-way interactions. Associations with average rainfall and the number of days of ground frost from March to August were also investigated; however, they were negatively correlated with mean maximum temperature ($r = -0.55$ and -0.57 respectively). These correlations meant we could not adequately distinguish their effects, hence rainfall and frost were dropped from analyses because mean temperature has greater explanatory power (at least 2 AIC points). These variables were chosen because previous studies have shown them to influence bumblebee species distributions (Williams 2007; Goulson 2010; Lye *et al.* 2010). The preference of each species for the ericaceous plants *Calluna vulgaris* or *Erica spp.* was examined using linear mixed effects models with individual bee as the unit of replication, and whether the bee was recorded on a *Calluna vulgaris* or *Erica spp.* flower or not as the binary response. Linear mixed-effect models were

fit with lmer in the lme4 package (ver. 1.0-5; Bates *et al.* 2015) in R. The fixed effects investigated the influence of the species that an individual bee belonged to and the habitat type in which it was found, with location as a random effect. The most parsimonious combination of fixed effects was determined using maximum likelihood (ML) rather than restricted maximum likelihood (REML).

For all analyses optimal models were selected to minimise AICc after using the function dredge in the MuMIn package (ver. 1.9.5; Barton 2013) to run a complete set of models with all combinations of fixed effects and their two-way interactions. The models presented are the best models with a difference of 2 AICc points. Pairwise differences between factor means were investigated using Tukey's post hoc tests.

2.4 Results

Species identification

Of the 2 415 bees sampled, 20.3% of the samples collected were identified as *B. terrestris.* These were inadvertently collected during sampling as *B. terrestris* workers can be confused with *B. lucorum* workers (Wolf *et al.* 2010) and represented an average of 19.9 ± 3.7% SE (max. 72.5% and min. 0%) of samples taken from each location. All *B. terrestris* samples were excluded from further analyses. We did not include *B. terrestris* in this study because many *B. terrestris* individuals are easily distinguished using morphological traits, so only a proportion of all *B. terrestris* individuals (those that strongly resemble the *lucorum* complex species) were collected in our sampling. Of the remaining 1 924 bees that belonged to the *lucorum* complex, 65.5% were identified as *B. lucorum*, 23.7% were *B. cryptarum*, and 10.8% were *B. magnus* (Appendix 2.3).

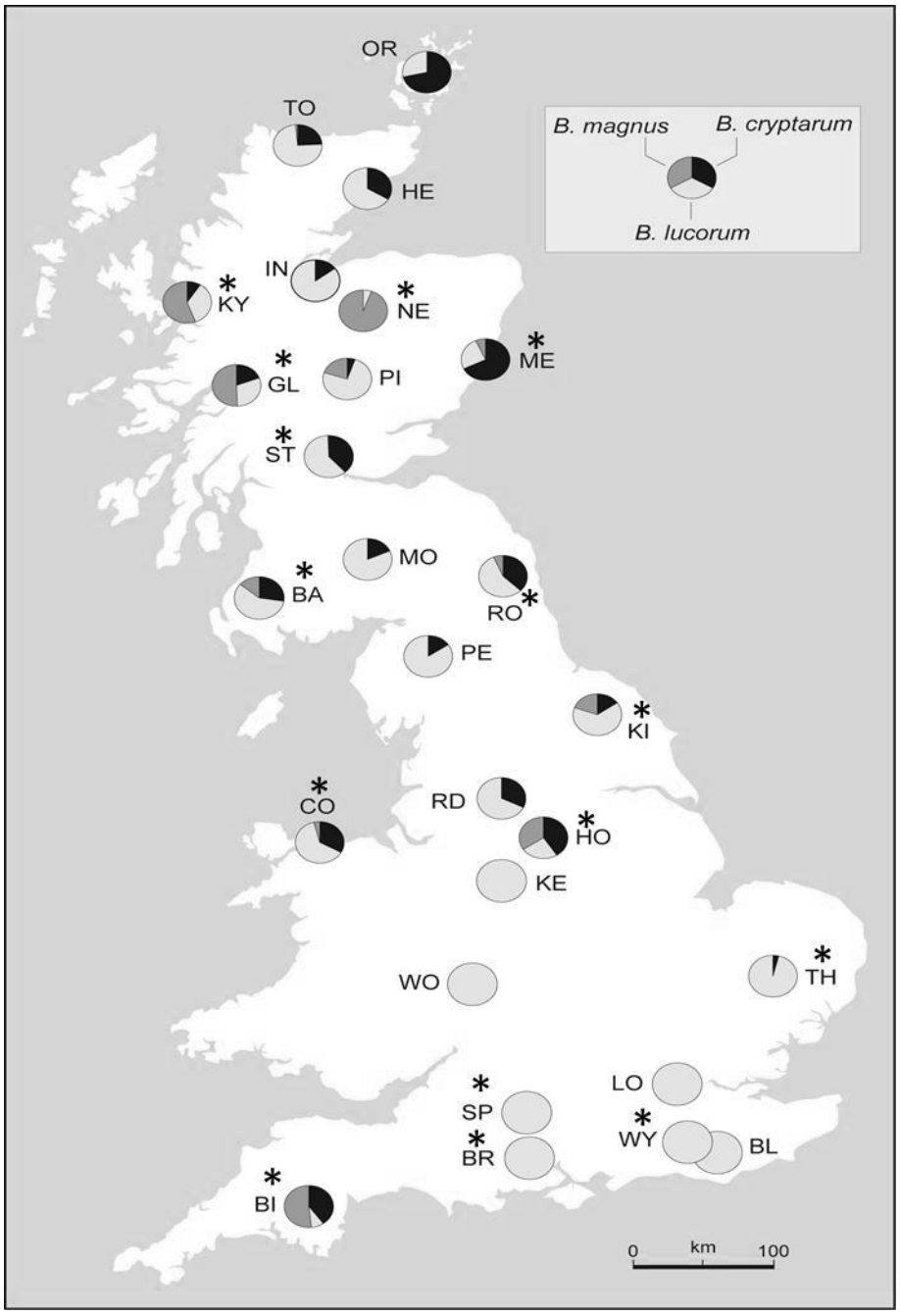


Figure 2.1. The distribution of *Bombus lucorum* complex species across Great Britain. Sites marked with a * were sampled in 2011. The number of specimens identified per site, and habitat types sampled, are shown in Appendix 2.3.

Table 2.1. The probability of *B. cryptarum* and *B. magnus* individuals being found at a site, in relation to multiple independent variables. Summary of the results of a generalised linear model that investigated the effects of habitat type (heathland or non-heathland), mean maximum daily temperature from March to August and elevation. Significant results are shown in italics.

	B. cryptarum				***B. magnus***			
Parameter	**Estimate**	**SE**	**χ^2**	**Prob > χ^2**	**Estimate**	**SE**	**χ^2**	**Prob > χ^2**
Elevation (m)	-0.015	0.009	3.551	0.060				
Average max. daily temperature (°C)	-2.475	0.925	25.204	$5.157x10^{-7}$	-1.694	0.614	16.324	$5.339x10^{-5}$
Habitat: Non-heathland	-1.256	1.458	0.755	0.385	-3.398	1.325	10.169	*0.001*

Table 2.2. Forage use and measures of diet breadth for *B. lucorum* complex queens and workers pooled across sample sites. Diet breadth is measured via rarefaction to estimate the number of plant species each bee species would be expected to visit in a total of 100 flower visits.

	B. lucorum	***B. cryptarum***	***B. magnus***	**All bee species**
Total sample size	689	321	188	1198
No. of plant taxa visited	43	25	6	47
Diet breadth (± SD)	22.57 ± 2.24	15.20 ± 1.88	4.76 ± 0.85	

Geographic distributions and habitat use

The three species exhibit marked differences in their distributions across the UK. *Bombus lucorum* was found at every location sampled, from the Orkney Islands in the north, to Dartmoor in the south west and East Sussex in the south east (Fig. 2.1). *Bombus cryptarum* was found in almost all locations sampled to the north of ~53^{o}N, hence including North Wales, northern England and Scotland; it was the most abundant species present in Orkney and on the east coast of Aberdeenshire. *Bombus cryptarum* was also found in small numbers in East Anglia, and was abundant on Dartmoor in the southwest. *Bombus magnus* was the most restricted of the three species, found at 11 of 27 locations. Its distribution is similar to that of *B. cryptarum,* being largely found north of ~53^{o}N. It was the most abundant species at four locations, three in the highlands and west of Scotland, and also on Dartmoor in the southwest.

There was a marked difference in the strength of association of the three species with heathland habitats (Fig. 2.2, $\chi^2_2 = 435.94$, $P < 0.001$). *Bombus magnus* exhibited striking habitat specialisation, occurring almost exclusively on heathland (Fig. 2.2). When samples were collected from paired heathland and non-heathland habitats, *B. magnus* was almost always found in only the heathland habitat: only at two of 11 locations was *B. magnus* detected in the non-heathland habitat and then either only one or two individuals were found. Both *B. lucorum* and *B. cryptarum* were found more commonly in non-heathland than heathland habitats, but a greater proportion of *B. cryptarum* (46.4 %) than *B. lucorum* (20.1%) were detected on heathland (Fig. 2.2).

For *B. magnus* and *B. cryptarum,* we tested the biological and climatic correlates of species presence or absence at each site (*B. lucorum* was present at all sites, so was excluded from this analysis). For *B. cryptarum,* increasing average maximum daily temperatures significantly decreased the likelihood of presence at a site; the negative effect of elevation was not quite significant (see Table 2.1 & Fig. 2.3a). For *B. magnus*, the likelihood of occurrence similarly declined significantly with increasing average maximum daily temperature, (see Table 2.1 & Fig. 2.3). The likelihood of occurrence for *B. magnus* was also significantly lower on non-heathland habitat: for a standardised summer maximum temperature of 15°C the probability of *B. magnus* occurring at a non-heathland site is approximately 0.1, whereas at a heathland site, it is approximately 0.8 (see Table 2.1 & Fig. 2.3b). Other fixed effects (Table 2.1) and all two way interactions were not significant. The significant effect of average maximum temperature remained when this analysis was performed on heathland (parameter estimate = -1.24 ± 0.63, $\chi^2_1 = 6.48$, P = 0.011) and non-heathland sites separately (parameter estimate = -2.68 ± 0.63, $\chi^2_1 = 11.02$, P < 0.001).

Forage use

Bombus lucorum queens and workers had the largest diet breadth (Table 2.2 & Appendix 2.4), visiting a wide range of species from 20 different plant families. *Bombus cryptarum* workers and queens were found on a more restricted variety of species than *B. lucorum* workers. The majority (90.5%) of *B. magnus* workers and queens were found foraging on *Calluna vulgaris* or *Erica cinerea* and *Erica tetralix* (Table 2.2 & Appendix 2.4) and consequently had the lowest diet breadth of the three species. The number of bees feeding on *Erica spp*. and *Calluna vulgaris* (heather) compared to all other plant species differed significantly across the 3 bumblebee species ($\chi^2_2 = 253$, P < 0.001). *Bombus magnus*

individuals foraged most often on heather (90.5%), followed by *B. cryptarum* (43.9%); *B. lucorum* individuals foraged on these flowers least often (27.3%).

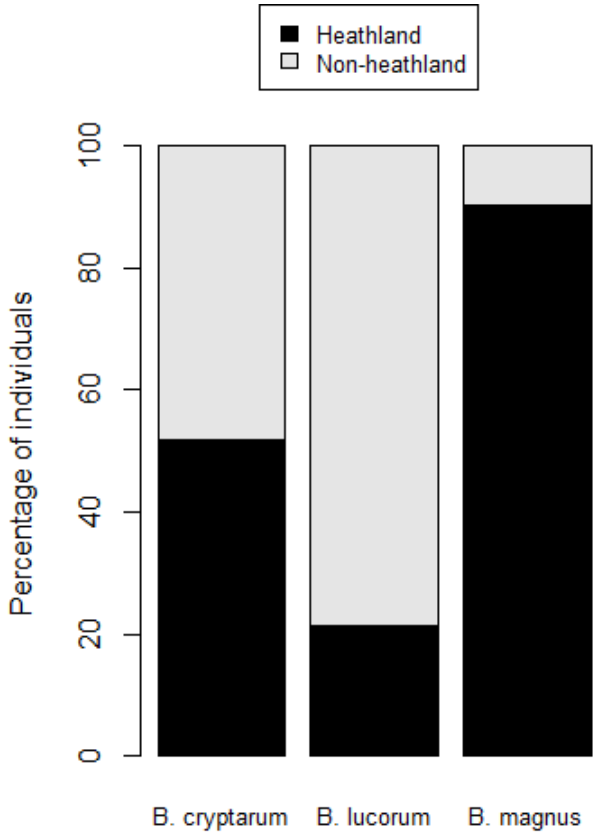


Figure 2.2. Habitat use by all castes of *B. lucorum*, *B. magnus* and *B. cryptarum*, indicated by the percentage of bees caught in each habitat type, pooled for all sample sites.

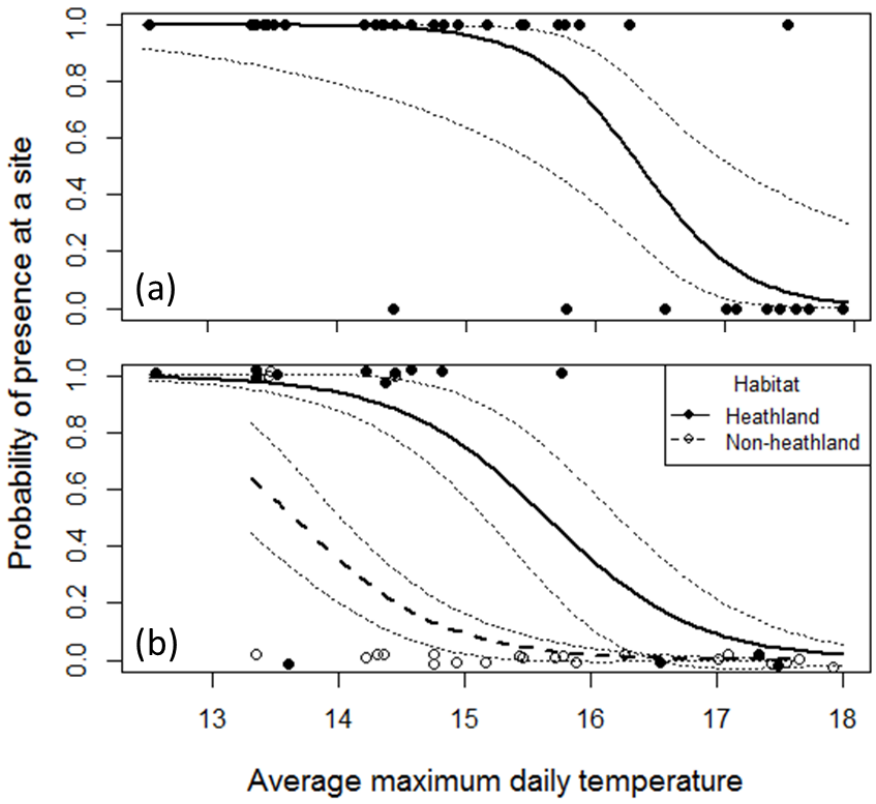


Figure 2.3. (a) Probability of *B. cryptarum* presence at sites as a function of mean maximum daily temperature (°C) from March to August, and (b) of *B. magnus* presence as a function of mean maximum daily temperature (°C) on heathland (filled circles) and non-heathland habitat (non-filled circles). Bold lines represent the relationship between the presence of the species and the mean maximum daily temperature estimated from a generalised linear model. Small dashed lines represent 95% confidence intervals (CI) around this estimated relationship.

We tested whether the apparent preference of *B. magnus* for foraging on *Erica spp.* or *Calluna vulgaris* was simply a consequence of this bee species occurring predominantly in heathland habitats where heather plants are most common. This was done by assessing how the probability of foraging on *Erica spp.* or *Calluna vulgaris* varied between bee species across both habitat types. The likelihood of bees foraging on these flowers was significantly influenced by which bumblebee species they belonged to ($\chi^2_2 = 42.1$, $P < 0.001$) and habitat

type (χ^2_1 = 210, P < 0.001). Furthermore, a significant interaction between species and habitat (χ^2_2 = 10.6, P < 0.01) demonstrated that the differences between species in the extent of their preference for heather varied between the habitats. Whilst *B. magnus* individuals were significantly more likely to forage on heather when on heathland than either *B. cryptarum* (parameter estimate = -4.5 ± 1.18, P < 0.001) or *B. lucorum* (parameter estimate = -4.36 ± 1.18, P < 0.001, Fig. 2.4), on non-heathland habitats, all three were equally likely to be found foraging on *Erica spp*. or *Calluna vulgaris* (Fig. 2.4).There was no significant difference in the likelihood of *B. cryptarum* and *B. lucorum* foraging on these heather flowers when on heathland (parameter estimate = -0.17 ± 0.3, P > 0.1, Fig. 2.4).

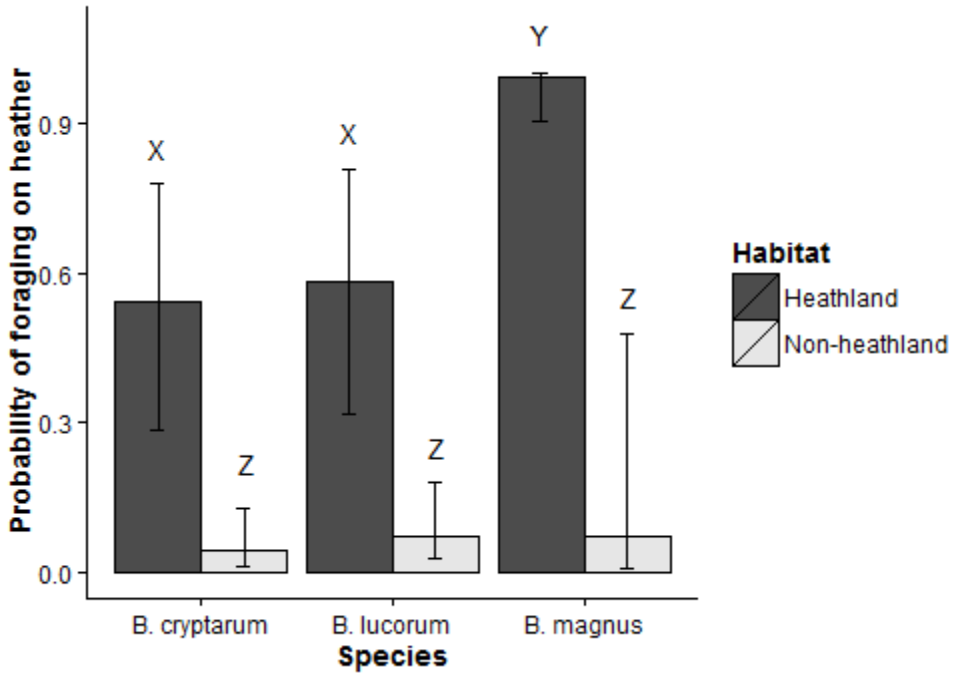


Figure 2.4. The probability of individuals (queens and workers) of each taxa foraging on *Calluna vulgaris, Erica tetralix* or *Erica cinerea* compared to all other plant species, according to habitat type. Porbabilities were estimated from a linear mixed effect model. Error bars show 95% confidence intervals. Probabilities with different letters are significantly different (P < 0.001).

2.5 Discussion

This study has substantially enhanced our understanding of the distribution of the three cryptic members of the *lucorum* species complex in Britain. Previous authors studying more restricted geographic areas in Ireland (Murray *et al.* 2008) and Western Scotland (Waters *et al.* 2010a) concluded that *B. lucorum, B. cryptarum* and *B. magnus* are common, widely distributed and sympatric. By undertaking a more wide-ranging study, we demonstrate that across the UK *B. magnus* and *B. cryptarum* are associated with cooler climates than *B. lucorum*, being found most commonly in northern and western Britain and that they are absent from a large portion of the south and east. Our data also demonstrate that *B. magnus* exhibits a tight association with heathland habitats.

The absence of morphological diagnostic characters leads to a lack of even basic knowledge about the ecology and distribution of cryptic species. Without ecological knowledge of cryptic species, we have no way of discerning whether populations are stable or establishing effective conservation management strategies when necessary. This is particularly true for pollinator groups such as bumblebees, which are important both ecologically and economically, and comprise species that are suffering dramatic declines resulting from habitat loss and fragmentation (Goulson 2010) and agricultural intensification (Williams 1986; Goulson *et al.* 2006). This study therefore contributes vital information for this purpose.

In the Western Isles of Scotland, *B. lucorum* was the least common of the *lucorum* complex species (Waters *et al.* 2010a). In contrast, in this study of mainland Great Britain, and also in Ireland (Murray *et al.* 2008; Stanley *et al.* 2013b), *B. lucorum* was the most common species (double the proportion found in Waters *et al.* 2010a). In the current study, *B. lucorum* was found at all sampled sites, making it the most widespread of the species,

although a greater proportion of individuals were found in non-heathland than heathland habitat. Unlike in the Western Isles, where *B. cryptarum* workers were shown to have the broadest diet (Waters *et al.* 2010a), in our study *B. lucorum* workers (and queens) exhibited the largest diet breadth, exploiting a greater number of plant species than either *B. cryptarum* or *B. magnus*. Such a large diet breadth may be a reflection of the broad range of habitats and locations that this species inhabits. Overall, *B. lucorum* appears to be the most generalised of the three species, occupying the broadest climatic range, feeding on a wide range of flowers, and is the only species of the three to be found in the intensively farmed and urbanized south east of England.

Bombus cryptarum was the second most common species in this current study. However, previous studies show that in Ireland it was the least common of the three (Murray *et al.* 2008), whereas in the Western Isles, it was the most common (almost half of the individuals, Waters *et al.* 2010). It was also found to be the most polylectic in the Western Isles, visiting a wide range of food plants belonging to many families, including non-native garden plants (Waters *et al.* 2010a). In the rest of Scotland, England and Wales, it also appears to be highly polylectic, but less so than *B. lucorum*, possibly because its narrower geographic distribution inevitably means it encounters fewer plant species.

In the Western Isles of Scotland (Waters *et al.* 2010a) and Ireland (Murray *et al.* 2008), *B. magnus* was the second most common of the three species, whereas in this study of mainland Great Britain, *B. magnus* was the least abundant of the three species (approximately three times lower than in the other two studies). It has previously been described as associated with upland, northerly, and westerly areas, and thus the generally cooler, wetter regions in the UK (Alford 1975; Benton 2006). Waters *et al.* (2010a) and Murray *et al.* (2008) found that their data for *B. magnus* in Ireland and the Western Isles of Scotland did not support this. Instead, Murray *et al.* (2008) found that this species was present in both

upland and lowland sites but was absent from urban areas and Stanley *et al.* (2013) found that it was absent from mass flowering crops in Ireland. Our results for Great Britain correspond to the findings of Waters *et al.* (2010a) that *B. magnus* is strongly associated with heathland, but is not restricted to upland areas. Waters *et al.* (2010a) also found that *B. magnus* was particularly associated with the forage plant *Calluna vulgaris*; our results indicate an association with the three Ericaceae, *Calluna vulgaris, Erica cinerea* and *Erica tetralix*. This apparent preference for these Ericaceous flowers leads to *B. magnus* exhibiting the lowest diet breadth.

Tight dietary specializations or biotope associations are unusual in European bumblebees. In a study on the biotope associations of UK bumblebee species, Goulson *et al.* (2006) found that they were all recorded in more than one, most being found in a broad range of different biotopes. Even very rare species such as *B. sylvarum*, which is the second rarest extant species in the UK, do not seem to have tight biotope associations. *Bombus jonellus, B. muscorum* and *B. soroeensis* are also associated with heathland to varying extents, especially in the north of the UK, but all three also have significant populations in non-heathland habitats (Darvill *et al.* 2006, 2010; Goulson *et al.* 2006) and specialisation in habitat and food associations may often be related to the position of a site within a species' global range (Williams *et al.* 2007). In this study, only 9.5% of *B. magnus* individuals were found in habitat other than heathland, or on flowers other than *Erica spp.* or *Calluna vulgaris*; all of these individuals were found very near to large areas of heathland, suggesting that they were probably individuals spilling out from heathland habitat. This apparent tight association exhibited by *B. magnus* could impose a serious disadvantage for a social organism that needs to maintain colonies with high energy demands beyond the flowering season of any one (or two) plant species (Williams 2005) and seems to be quite unusual amongst bumblebees.

In Great Britain there are two types of heathland habitat, lowland and upland heath. The lowland heaths of southern England make up 14% of this habitat type in Europe (Groves *et al.* 2012), yet around 80% has been lost since 1800 due to agriculture, urbanisation and changes in land management (Price 2003). Upland heath is a sub-montane habitat characterised by common or ling heather *Calluna vulgaris*, found mostly in the British Isles, and along parts of the western seaboard of the northwest European mainland. *Calluna vulgaris* occurs much more widely than this but the massive extent of rotationally burned heather is unique to the UK and Ireland (Thompson *et al.* 1995). In the UK, large proportions of upland heath have also been lost to afforestation and over-grazing by sheep (Thompson *et al.* 1995). Consequently, both lowland and upland heathland are listed as UK post-2010 Biodiversity Framework priority habitats, meaning that they have been identified as being the most threatened and requiring conservation action. Habitat degradation can have considerable implications for the species that are associated with it. In fact habitat loss is widely agreed to be the most important factor driving bee declines (Brown & Paxton 2009). A direct result of habitat loss is habitat fragmentation, which impacts surviving populations through genetic isolation and subsequent inbreeding (Zayed 2009; Whitehorn *et al.* 2011) or simply the inability of small remaining habitat fragments to support viable bee populations (e.g. Ellis *et al.* 2006). In this case, *B. magnus* may already have suffered from past losses of heathland and further loss of this habitat is likely to lead to population declines. The apparent dietary specialisation of *B. magnus* could make this especially problematic. Only a small number of bumblebee species (six in the UK) remain common and ubiquitous and do not appear to have exhibited obvious range contractions as a result of changes to the environment in the last 60 years (Goulson *et al.* 2005). These species seem to have more generalised foraging preferences than some of the rare species, which may mean they have a greater ability to adapt to changing forage resources (Goulson *et al.* 2005). In addition, species with narrow

diet breadth have access to fewer resources, so, as biotopes become degraded and floral resources decline, these specialists are likely to be the first to disappear (Goulson *et al.* 2006). Presently, we have no way of knowing whether the populations of the species within the *lucorum* complex are currently stable or if they have experienced population changes in the past.

We acknowledge that our diet breadth estimates are likely to be conservative, since fieldwork targeted flower patches and times of day where bees were abundant enough to collect an adequate sample size to accurately characterise feeding behaviour. This may have led us to miss a small number of bees foraging on some rare flower species. However, it is unlikely to have strongly affected the results; our estimates will be representative of foraging behaviour in the substantial majority of individuals. There was no possibility that this introduced bias into our diet breadth comparisons between the different *lucorum* complex species, as species identity was only determined *post-hoc* by molecular methods. It should be noted that our analysis techniques cannot entirely disentangle effects of habitat preference on observed diet breadth; localized species, or species with specialized habitat preferences, will encounter fewer flower species and thus inevitably tend to have a more restricted diet (see Williams 2005).

Bombus cryptarum and *B. magnus* occurred more commonly where temperatures were lower and were found to be generally more common at northerly latitudes, a preference that was not detected for *B. lucorum*. They were consequently absent from much of the south and east of England. Heathland habitats were sampled in this area but *B. magnus* was not found to be present (though Williams *et al.* 2012 report a specimen from the heathland of Dungeness in the South East of England). It may be that these sites are too warm, or that *B. magnus* used to occur there in the past when the heathland area was larger and less fragmented. The south-east of England is also highly urbanized. Urban areas can support

diverse pollinator assemblages but they can also have negative impacts on pollinator species (Bates *et al.* 2011). One obvious outlier in the distributions of both *B. cryptarum* and *B. magnus* is the Birch Tor site on Dartmoor in the south-west of England (Fig. 2.1), where *B. magnus* and *B. cryptarum* were more abundant than *B. lucorum*. This appears incongruous (Fig. 2.1) but due to the high altitude the temperature at this site is actually much lower than at other sites with similar latitude, meaning the presence of *B. magnus* and *B. cryptarum* at Birch Tor is consistent with their preferences. Further sampling in the southwest of England and in Wales would help reveal whether these are isolated populations of *B. magnus* and *B. cryptarum,* or whether they are actually present in suitable areas throughout the western side of Great Britain.

The lack of diagnostic characteristic traits for these species in Scotland and Ireland (Carolan *et al.* 2012), as well as geographical variation in colour pattern across taxa, means that the potential for misidentification of these species is very high. As a consequence, descriptions of the ecology and distribution of these three species, obtained prior to the utilisation of molecular methods for species identification, are likely to be problematic (see Rasmont 1984; Rasmont *et al.* 1986; Pamilo *et al.* 1997 for European distributions). Therefore, the only reliable information available about the worldwide distributions of these species comes from a study by Williams *et al.* (2012) of the subgenus *Bombus s. str*. They find *B. lucorum* to be present from Iceland in the west, across Europe to the mountains of Central Asia and in Mongolia. *Bombus cryptarum* appears to have the broadest distribution of all *Bombus s. str.* species. It was found from Great Britain, across Europe and central Asia to western North America. *Bombus magnus* is present in Great Britain, Spain, Denmark, Sweden and near Moscow, Russia. Further work would evidently be beneficial.

This study has revealed that while these species have a sympatric distribution across much of northern England, Northern Wales and Scotland, they exhibit clearly discernible

differences in their ecological characteristics. This demonstrates the importance of correctly identifying cryptic species, not just amongst important pollinators such as bumblebees (e.g. Ellis *et al.* 2006a; Williams 2007) but in insects in general, where they are also common (e.g. Hebert *et al.* 2004; Smith *et al.* 2007). Failure to account for cryptic diversity could result in missing the causal link between changes in species distribution and environmental variation, incorrect delineation of units for conservation and consequently, serious repercussions for their management.

Further studies of these three species would be required to determine whether the observed differences are the result of preference or the outcome of inter-specific competition. In addition, it would be interesting to determine what *B. magnus* feeds on during the periods when *Erica spp*. and *Calluna vulgaris* are not in flower on heathland habitats. A long term study would be able to establish whether the populations of these three species are stable or declining, particularly focussing on the response of *B. magnus* populations to past and present heathland loss/ degradation. Our ongoing research is investigating the population genetics of this species complex to provide insight into differences in genetic diversity, and reveal whether the highly specialised *B. magnus* is suffering from population fragmentation as a result of its tight association with a declining and fragmented habitat type.

2.6 Acknowledgements

We thank Andreia Penado, James Morrison & Carolyn Goldie for help collecting samples, as well as Steph O'Connor and Penelope Whitehorn for advice on fieldwork and analysis. The project was partly supported by the UK Research Councils' SynTax programme and the University of Stirling.

2.7 Appendix

Appendix 2.1. Multiplex groups for microsatellite analysis

Multiplex group	Locus	Source
1	B11	Estoup *et al.* 1995 & 1996
	B118	Estoup *et al.* 1995 & 1996
	B121	Estoup *et al.* 1995 & 1996
	B10	Estoup *et al.* 1995 & 1996
	B124	Estoup *et al.* 1995 & 1996
2	BT26	Funk *et al.* 2006
	BT09	Funk *et al.* 2006
	BL11	Funk *et al.* 2006
	BT18	Funk *et al.* 2006
3	BL03	Funk *et al.* 2006
	BL06	Funk *et al.* 2006
	BT10	Funk *et al.* 2006
	BT24	Funk *et al.* 2006

Appendix 2.2. PCR conditions for the three microsatellite multiplex groups

	Multiplex group 1			**Multiplex groups 2 & 3**		
	Time	**Temp. (°C)**	**Cycles**	**Time**	**Temp. (°C)**	**Cycles**
Activation	15 min	95		15 min	95	
3-step cycling:			35			40
Denaturation	30 s	94		30 s	94	
Annealing	90 s	49		90 s	54	
Extension	90 s	72		90 s	72	
Final extension	10 min	72		10 min	72	

Appendix 2.3. Sample sizes from 27 sites in Great Britain identified to species. The codes for site names are used in Figure 2.1.

		Year	Latitude	Longitude	*B. cryptarum*	*B. lucorum*	*B. magnus*	Total
Orkney	OR	2010			**5**	**2**		**7**
Non-heathland			59.05	-3.09	5	2		7
Tongue	TO	2010			**20**	**61**	**1**	**82**
Non-heathland			58.49	-4.43	20	61	1	82
Helmsdale	HE	2010			**25**	**49**		**74**
Non-heathland			58.05	-3.83	25	49		74
Inverness	IN	2010			**9**	**52**		**61**
Non-heathland			57.49	-4.46	9	52		61
Kyle of Lochalsh	KY	2011			**7**	**27**	**42**	**76**
Heathland			57.23	-5.40	1	7	42	50
Non-heathland			57.28	-5.52	6	20		26
Nethy Bridge	NE	2011				**1**	**17**	**18**
Heathland			57.23	-3.68		1	17	18
Mergie	ME	2011			**74**	**28**	**7**	**109**
Heathland			57.00	-2.34	52	6	5	63
Non-heathland			56.99	-2.29	22	22	2	46
Pitlochry	PI	2010			**4**	**56**	**15**	**75**
Non-heathland			56.77	-3.93	4	56	15	75
Glencoe	GL	2011			**20**	**31**	**52**	**103**
Heathland			56.66	-5.05	2	2	51	55
Non-heathland			56.68	-5.12	18	29	1	48
Stirling	ST	2011			**128**	**203**	**1**	**332**
Heathland			56.19	-3.89	93	56		149
Non-heathland			56.14	-3.92	35	147	1	183
Rothbury	RO	2011			**36**	**54**	**6**	**96**
Heathland			55.34	-2.12	25	15	6	46
Non-heathland			55.29	-1.85	11	39		50
Bargrennon	BA	2011			**14**	**30**	**7**	**51**
Heathland			55.11	-4.49	4	1	7	12
Non-heathland			55.01	-4.54	10	29		39
Moffat	MO	2010			**16**	**70**		**86**
Non-heathland			55.06	-3.27	16	70		86
Penrith	PE	2010			**9**	**48**		**57**
Non-heathland			54.69	-2.80	9	48		57
Kirkbymoorside	KI	2011			**12**	**51**	**15**	**78**
Heathland			54.33	-0.94	10	28	15	53
Non-heathland			54.22	-0.88	2	23		25
Rochdale	RD	2010			**11**	**23**		**34**
Non-heathland			53.76	-2.34	11	23		34
Hope	HO	2011			**30**	**18**	**25**	**73**
Heathland			53.39	-1.69	21	3	25	49

Non-heathland			53.35	-1.75	9	15		24
Conwy	CO	2011			**18**	**34**	**2**	**54**
Heathland			53.28	-3.88	11	26	2	39
Non-heathland			53.23	-3.84	7	8		15
Keele	KE	2010				**62**		**62**
Non-heathland			53.02	-2.28		62		62
Thetford	TH	2011			**3**	**71**		**74**
Heathland			52.42	0.71	3	37		40
Non-heathland			52.40	0.92		34		34
Worcester	WO	2010				**22**		**22**
Non-heathland			52.33	-2.26		22		22
London	LO	2010				**107**		**107**
Non-heathland			51.40	0.06		107		107
SalisburyPlain	SP	2011				**28**		**28**
Non-heathland			51.27	-1.71		28		28
Wych cross	WY	2011				**34**		**34**
Heathland			51.07	0.05		34		34
Blackboys	BL	2010				**35**		**35**
Non-heathland			50.96	0.18		35		35
Bramshaw	BR	2011				**61**		**61**
Heathland			50.89	-1.69		49		49
Non-heathland			50.95	-1.78		12		12
Birch Tor	BI	2011			**14**	**3**	**18**	**35**
Heathland			50.61	-3.87	14	3	18	35
Total					**455**	**1261**	**208**	**1924**

Appendix 2.4. Forage use and measures of diet breadth for *B. lucorum* complex queens and workers pooled across sample sites. Values represent the number of individuals of each bee species and, in parenthesis, the percentage of the total number individuals of the corresponding bee species. Diet breadth is measured via rarefaction to estimate the number of plant species each bee species would be expected to visit in a total of 100 flower visits.

	B. lucorum	***B. cryptarum***	***B. magnus***	**All bee species**
Anchisa arvensis	1 (0.15)			1 (0.08)
Anemone spp.	3 (0.4)			3 (0.3)
Brassica napus	1 (0.15)			1 (0.08)
Calluna vulgaris	21 (3)	51 (15.9)	101 (53.7)	173 (14.4)
Centaurea spp.	4 (0.6)	1 (0.3)		5 (0.4)
Chamerion angustifolium	97 (14.1)	42 (13.1)	15 (8)	154 (12.9)
Cirsium spp.	31 (4.5)	7 (2.2)		38 (3.2)
Crataegus spp.	1 (0.15)			1 (0.08)
Echium vulgare	18 (2.6)	1 (0.3)		19 (1.6)
Erica spp.	160 (23.2)	89 (27.7)	69 (36.7)	318 (26.5)
Eryngium giganteum	1 (0.15)			1 (0.08)
Filipendula ulmaria	56	29 (9)	1 (0.5)	86 (7.2)
Geranium pratense	1 (0.15)			1 (0.08)
Geum rivale	1 (0.15)	3 (0.9)		4 (0.3)
Hypericum perforatum	13 (1.9)	1 (0.3)		14 (1.2)
Jacobaea vulgaris	5 (0.7)			5 (0.4)
Knautia arvensis	1 (0.15)			1 (0.08)
Lathyrus pratensis	1 (0.15)			1 (0.08)
Lavandula spp.	7 (1)			7 (0.6)
Ligustrum spp.	9 (1.3)			9 (0.8)
Linaria vulgaris	10 (1.5)			10 (0.8)
Lotus corniculatus	2 (0.3)			2 (0.2)
Lysimachia vulgaris	1 (0.15)			1 (0.08)
Malva sylvestris	1 (0.15)			1 (0.08)
Melilotus officinalis	12 (1.7)			12 (1)
Mentha spicata	5 (0.7)			5 (0.4)
Onobrychis vicifolia	1 (0.15)			1 (0.08)
Phacelia spp.	3 (0.4)	2 (0.6)		5 (0.4)
Plantago spp.		1 (0.3)		1 (0.08)
Potentilla spp.	1 (0.15)			1 (0.08)
Prunus avium	1 (0.15)			1 (0.08)
Rhododendron spp.	25 (3.6)	2 (0.6)		27 (2.3)
Rosa spp.	18 (2.6)	2 (0.6)		20 (1.7)
Rubus spp.	34 (4.9)	19 (5.9)		53 (4.4)
Salix spp.	3 (0.4)	2 (0.6)		5 (0.4)
Saxifraga tridacylites	2 (0.3)	1 (0.3)		3 (0.3)
Scabiosa spp.		1 (0.3)		1 (0.08)
Tanacetum vulgare	2 (0.3)	1 (0.3)		3 (0.3)
Taraxacum spp.	1 (0.15)			1 (0.08)
Teucrium scorodonia	9 (1.3)	1 (0.3)		10 (0.8)

Thymus spp.	12 (1.7)	2 (0.6)	1 (0.5)	15 (1.3)
Tilia spp.	6 (0.9)			6 (0.5)
Trifolium pratense	2 (0.3)	1 (0.3)		3 (0.3)
Trifolium repens	96 (13.9)	36 (11.2)	1 (0.5)	133 (11.1)
Ulex spp.	10 (1.5)	20 (6.2)		30 (2.5)
Vaccinium spp.		1 (0.3)		1 (0.08)
Vicia cracca		5 (1.6)		5 (0.4)
Total sample size	**689**	**321**	**188**	**1198**
No. of plant taxa visited	**43**	**25**	**6**	**47**
Diet breadth (± SD)	**22.57 ± 2.24**	**15.20 ± 1.88**	**4.76 ± 0.85**	

Chapter 3

Niche partitioning in a sympatric cryptic species complex

A version of this chapter has been published as:

Scriven, J.J., Whitehorn, P.R., Goulson, D. and Tinsley, M.C. (2016) Niche partitioning in a sympatric cryptic species complex. *Ecology and Evolution*. doi: 10.1002/ece3.1965

P. Whitehorn, D. Goulson and M. Tinsley all supervised the project and commented on draft versions of this manuscript. The published version is presented here.

3.1 Abstract

Competition theory states that multiple species should not be able to occupy the same niche indefinitely. Morphologically similar species are expected to be ecologically alike and exhibit little niche differentiation, which makes it difficult to explain the co-occurrence of cryptic species. Here, we investigated interspecific niche differentiation within a complex of cryptic bumblebee species that co-occur extensively in the United Kingdom. We compared the interspecific variation along different niche dimensions, to determine how they partition a niche to avoid competitive exclusion. We studied the species *B. cryptarum*, *B. lucorum*, and *B. magnus* at a single location in the northwest of Scotland throughout the flight season. Using mitochondrial DNA for species identification, we investigated differences in phenology, response to weather variables and forage use. We also estimated niche region and niche overlap between different castes of the three species. Our results show varying levels of niche partitioning between the bumblebee species along three niche dimensions. The species had contrasting phenologies: The phenology of *B. magnus* was delayed relative to the other two species, while *B. cryptarum* had a relatively extended phenology, with workers and males more common than *B. lucorum* early and late in the season. We found divergent thermal specialisation: In contrast to *B. cryptarum* and *B. magnus*, *B. lucorum* worker activity was skewed toward warmer, sunnier conditions, leading to interspecific temporal variation. Furthermore, the three species differentially exploited the available forage plants: In particular, unlike the other two species, *B. magnus* fed predominantly on species of heather. The results suggest that ecological divergence in different niche dimensions and spatio-temporal heterogeneity in the environment may contribute to the persistence of cryptic species in sympatry. Furthermore, our study suggests that cryptic species provide distinct and unique ecosystem services, demonstrating that morphological similarity does not necessarily equate to ecological equivalence.

3.2 Introduction

According to competition theory, ecologically similar co-existing species must partition resources (Hardin 1960). Multiple species should not be able to occupy the same niche indefinitely, as the best adapted species should eventually exclude inferior species from a given location (Gause 1932; Holt *et al.* 1994). Closely related species are often morphologically, physiologically and behaviourally similar. Morphologically similar species are expected to be ecologically alike and exhibit little niche differentiation (Cothran *et al.* 2013; Violle *et al.* 2011). This makes it difficult to explain the co-occurrence of cryptic species, which are distinct species with similar or identical morphology, often historically 'hidden' under a single species name and thus wrongly classified (Bickford *et al.* 2007; Williams *et al.* 2012b). Indeed, some studies have found that cryptic species are less likely to co-occur than congeneric non-cryptic species (Vodă *et al.* 2015a, 2015b). Yet, cryptic species are common in nature and often do co-occur at local scales (Feulner *et al.* 2006; Gabaldón *et al.* 2013; Ortells, Gómez, & Serra 2003; Stuart, Inger, & Voris 2006; Van Campenhout *et al.* 2014); this makes them important test cases for studying the mechanisms that facilitate species coexistence. In this study we investigated interspecific niche differentiation within a bumblebee cryptic species complex, comparing the degree to which species vary in different niche dimensions.

Approximately 250 species of bumblebees exist worldwide, distributed across the temperate, alpine and arctic regions of the northern hemisphere and also South America. In much of this range, it is common for multiple species to occur in sympatry despite high niche overlap. Morphologically, most bumblebee species are very similar, with obvious differences only in size, tongue length and coloration (Goulson & Darvill 2004; Goulson 2010). Since they also all rely exclusively on pollen and nectar for food, theory would predict that

bumblebee communities should be shaped by high levels of interspecific competition for these resources (Heinrich 1976; Inouye 1978).

Bombus species are notorious for possessing convergent colour patterns between species, but also for displaying high intraspecific variation (Ellis *et al.* 2006a; Williams 2007; Williams *et al.* 2012a). The subgenus *Bombus sensu stricto* is a widespread and commercially exploited taxon of bumblebee, comprising 17 species worldwide (Williams *et al.* 2012b), of which five are found in Europe: *Bombus* (*Bombus*) *cryptarum,* (Fabricius), *B.* (*B.*) *lucorum* (Linnaeus), *B.* (*B.*) *magnus* (Vogt), *B.* (*B.*) *sporadicus* (Nylander) and *B. (B.) terrestris* (Linnaeus). The taxonomic status of the latter two species is well established but *B. lucorum*, *B. magnus* and *B. cryptarum* are morphologically indistinguishable in much of their range (Fig. 3.1), which has triggered considerable debate about their species-status. *Bombus magnus* and *B. cryptarum* have previously been regarded by some as subspecies of *B. lucorum* and are often referred to collectively as the '*lucorum* complex', or simply synonymized to *B. lucorum* (Benton 2006; Edwards & Jenner 2005). However, these three species are now recognised as a cryptic species complex: studies on labial gland secretions have shown discrete genetic differences between the three species (Bertsch *et al.* 2005), as have studies of the CO1gene (Murray *et al.* 2008; Williams *et al.* 2012b; Carolan *et al.* 2012), which suggest that *B. magnus* and *B. cryptarum* are more closely related to each other than to *B. lucorum* (Bertsch *et al.* 2005; Murray *et al.* 2008; Williams *et al.* 2012b). Morphological diagnostic characters have been proposed for queens, but some of these vary along a continuum, overlapping considerably between species, making them unreliable for identification (Carolan *et al.* 2012).

With a history of identification difficulties, relatively little is known about the field ecology of these cryptic *lucorum* complex species; in particular, the details of how they differentially exploit their general niche remain unclear. The three species are

morphologically and ecologically similar; they are all short-tongued species (Stanley *et al.* 2013b), meaning they have potential foraging access to the same floral resources. They occur sympatrically with broadly overlapping ranges in the UK and Ireland (Murray *et al.* 2008; Waters *et al.* 2010a; Williams *et al.* 2012b; Stanley *et al.* 2013b; Scriven *et al.* 2015; Chapter 2). All three species co-occur at many locations in Great Britain and Ireland; additionally, Chapter 2 (Scriven *et al.* 2015), Murray *et al.* (2007) and Stanley *et al.* (2013) found *B. lucorum* to be present at every site surveyed, suggesting that it is a relative ecological generalist. The only study in which *B. lucorum* was found to be absent from some locations was carried out in the far north west of Great Britain (Waters *et al.* 2010a). Studies of geographic distributions have suggested that the *lucorum* complex species may be adapted to exploit different climatic conditions (Waters *et al.* 2010a; Scriven *et al.* 2015; Chapter 2): unlike *B. lucorum*, both *B. cryptarum* and *B. magnus* occurred most commonly at sites with lower summer temperatures (Scriven *et al.* 2015; Chapter 2). Furthermore, Chapter 2 (Scriven *et al.* 2015) and Waters *et al.* (2011) found that *B. magnus* was strongly associated with the forage plants *Calluna vulgaris* and *Erica spp*. and consequently with heathland habitats where these ericaceous plants were present.

Bumblebees and some other pollinators have recently suffered declines in abundance and range contractions across much of Western Europe and North America (Cameron *et al.* 2011; Goulson, Lye, & Darvill 2008; Goulson 2010; Williams 2005; Williams 1982). In the UK, seven out of the 27 species are listed as priority species in the UK post-2010 Biodiversity Framework (previously Biodiversity Action Plan), a higher proportion than for any other invertebrate group (Goulson 2010). Thus, as well as enabling us to test fundamental ecological theories, a thorough understanding of niche use in bumblebees has important conservation implications. This is especially critical for *B. magnus*, which is the rarest of the

lucorum complex species and is tightly associated with threatened heathland habitat (Waters *et al.* 2010a; Scriven *et al.* 2015; Chapter 2)

Figure 3.1. One of the *lucorum* complex species, which are morphologically indistinguishable in the field. This indivivdual was feeding on heather in Glencoe. Photo credit: Jessica Scriven.

In this study we determine how these three cryptic species, *B. lucorum*, *B. cryptarum* and *B. magnus*, partition a niche to avoid competitive exclusion. For the first time, we characterise the niches of these species at a single site across the duration of their flight season. We assess niche differentiation in three different ecological dimensions: patterns of temporal activity, weather sensitivity and forage resource use. In doing so, we aim to test which of these niche-use phenotypes has most flexibly responded to the selection pressures

generated by interspecific competition to facilitate niche differentiation and species co-occurrence.

3.3 Methods

Sampling

The study site was the area in and around Glencoe village in the Highlands of Scotland, UK. A previous study found all three *lucorum* complex species in good numbers at this site (Scriven *et al.* 2015; Chapter 2). Sampling was carried out below 150m altitude within a 3km radius of 56.68° N and -5.09° W, which included two villages and the bottom of Glencoe valley. The site was visited repeatedly between April 30th and October 2nd 2014, on average every 11 days (interval: max. 13 days, min. 9 days). Sampling was carried out over approximately two days per visit (max. three days, min. one day). Road verges, paths and any other accessible areas were searched continuously on foot throughout the day, from early morning until the evening; the exact times changed according to daylight hours throughout the season. Routes walked were varied so that all areas were visited at different times of day. Bumblebees resembling the *lucorum* complex species were caught and placed in a queen marking cage. For each individual captured we recorded: date, time of day, forage plant, temperature (°C) using a TES Dual K-type Thermometer (model 1312A), wind speed (m/s) using an Airflow Developments anemometer (model LCA6000), amount of sun (scale 0-4, Appendix 3.1) and amount of rain (scale 0-5, Appendix 3.2). A single tarsus was removed from each individual and stored in absolute ethanol for subsequent DNA extraction, after which bees were released. All bees were checked for missing tarsi to prevent sampling the same individuals twice. Bees were predominantly captured when foraging on flowers. Early in the season queens were observed foraging high in the canopy of *Acer*

pseudoplatanus and *Salix sp.* trees, it was not possible to catch these individuals; therefore they are not included in this study.

Species identification

DNA was extracted from tarsal samples using Chelex® 100 (Walsh *et al.* 1991). For species identification we used a PCR-RFLP method, digesting an amplified fragment of the cytochrome oxidase I (COI) gene following Murray *et al.* (2008): this yields a diagnostic digestion pattern for each of the cryptic *lucorum* complex species and *B. terrestris*. Samples that did not produce unambiguous results after two attempts were discarded. Of the 519 bees sampled, 4.2% were identified as *B. terrestris*, some workers of which are morphologically similar to *B. lucorum* workers (Wolf *et al.* 2010). These *B. terrestris* individuals were excluded from further analyses.

Analyses

All analyses were carried out using R version 3.0.2 (R Core Team 2014). To reduce the number of weather-related explanatory variables, we employed principal component analysis (PCA) using the FactoMineR package (ver. 1.28, Lê *et al.* 2008). All variables were scaled to unit variance prior to analysis. PCA scores for axis 1 were associated with the level of sunshine and temperature (Appendix 3.4); this PCA variable (PCA 1) was used in some subsequent analyses.

To compare the seasonal and daily activity of the three bumblebee species and determine if they were differentially affected by weather variables, we performed pairwise analyses between the species. We used generalised linear models with binary error distributions to test the association between these variables and the relative probability a sampled bumblebee belonged to a particular species within each pair. This analysis was

performed separately for each caste (overwintered queens, workers, males); *B. magnus* was excluded from the analysis of males due to low sample size. Optimal models were selected to minimise AICc using the function dredge in the MuMIn package (ver. 1.9.5; Barton 2013) to run a complete set of models with all combinations of fixed effects and their two-way interactions.

To define the niche of each species we used the nicheROVER package (ver. 1.0; Swanson *et al.* 2015); we calculated the niche region (N_R) for each of the three bumblebee species and the degree of niche overlap, based on phenology, time of day and sensitivity to weather variables. N_R is defined as a specific region of parameter space in which a randomly chosen individual has the probability α of being found (Swanson *et al.* 2015). For these analyses α = 95%. Niche overlap was calculated as the probability that a sampled individual from species *A* was found in the N_R of species *B* (Swanson *et al.* 2015). Analyses were carried out separately for queens and workers; sample sizes for males were too small.

We determined diet differences between bumblebee species in three separate comparisons, for queens, workers and males. We took the records of the flower species that each captured bee was visiting and used rarefaction to account for differences in sample size in our calculations of diet breadth (Williams, Araújo, & Rasmont 2007). We noted the number of observations for the bee species with the fewest samples, rounded this down to the nearest multiple of five, and then drew this number of random samples of foraging observations without replacement for each bumblebee species. We drew 100 replicate subsamples per bee species to estimate the mean number of plant taxa each bee species would be expected to visit during a comparable number of flower visits, then determined the number of forage plant species in these subsamples. The preference of each bumblebee species for the ericaceous plants *Calluna vulgaris* or *Erica spp.* was examined using generalised linear models with individual bee as the unit of replication. The binary response represented

whether the bee was recorded foraging on a *C. vulgaris* /*Erica spp.* flower (1) or any other plant species (0) and bumblebee species was used as the explanatory variable. This analysis was performed separately for queens and workers. We also calculated dissimilarity between the diets of the three species as Bray-Curtis distance measures (Bray & Curtis 1957) employing the Vegan package (ver. 2.3-0, Oksanen *et al.* 2015) using relative abundances.

3.4 Results

Species identification

Of the 497 bees that belonged to the *lucorum* complex, 51.7% were *B. cryptarum*, 40.4% were *B. lucorum*, and 7.8% were *B. magnus*. Queens of the three species were similarly abundant; however, in comparison to *B. lucorum* and *B. cryptarum*, workers and males of *B. magnus* were relatively rare (Table 3.1).

Table 3.1. The total number of queens, workers and males sampled for each species

Species	Queens	Workers	Males	Total
B. lucorum	21	153	27	201
B. cryptarum	26	174	57	257
B. magnus	23	14	2	39
Total	70	341	86	497

Interspecific differences in phenology and diurnal activity

We found interspecific phenological differences for each of the separate castes. For over-wintered queens, *B. magnus* was scarcer than the other two species early in the season but became relatively more common as the season progressed (Figs 3.2b-d & Appendix 3.3).

In contrast, there was no significant difference in the dates when *B. cryptarum* and *B. lucorum* queens were on the wing (Figs 3.2a, d & Appendix 3.3).

The relative abundance of foraging workers of the three species also varied throughout the season, reflecting distinct phenologies. Relative to either *B. lucorum* or *B. cryptarum*, *B. magnus* workers were significantly more common later in the season than they were at the beginning (Figs 3.2f-h & Appendix 3.5). This meant that the period during which *B. magnus* workers were active coincided with the flowering of *Erica cinerea* and *C. vulgaris*. All *B. magnus* workers (n = 14) were on the wing when heather was flowering, whereas a lower percentage of all *B. cryptarum* (81%, n = 174) and *B. lucorum* (95%, n = 153) workers were flying at this time; nevertheless, this interspecific difference in the degree of activity-bias towards the heather flowering season was not significant (Fisher Exact test $p > 0.1$). Comparing *B. cryptarum* and *B. lucorum* phenology, we found that at the beginning and end of the season, *B. cryptarum* workers were more common than *B. lucorum*, but in between, both species were equally abundant (Fig. 3.2e & Appendix 3.5). The strength of this seasonal shift in relative abundance varied according to the weather conditions (see below; Appendices 3.5 & 3.6).

Considering new reproductives, when males were first encountered (21st July), they were mostly *B. lucorum* but this trend reversed significantly later in the season so that *B. cryptarum* males became more common (Fig. 3.3 & Appendix 3.7). Only two *B. magnus* males were found in the entire study, but the first of these was found over a month later (27th August) than when the first *B. cryptarum* and *B. lucorum* males appeared (Fig. 3.3b). Only five new queens were captured (two *B. cryptarum*, two *B. lucorum* and one *B. magnus*), too few to draw conclusions about their phenology.

Effects of weather on activity of the three species

We used PCA to reduce the number of weather variables. The first PCA axis, which accounted for 40.28% of the total variation, exhibited a positive correlation with temperature and amount of sunshine: increasingly positive values represent generally warmer and sunnier conditions (Appendix 3.4b). The three species showed variation along this axis: the average observations for *B. magnus* were lower (negative values) than for *B. lucorum* (positive values), observations for *B. cryptarum* were intermediate (Appendix 3.4a). The second PCA axis was negatively associated to the wind speed; however, there was little variation between the species in this metric (Appendix 3.4a). We substituted values from the first PCA axis (PCA 1) for the explanatory variables 'temperature' and 'level of sunshine' in subsequent analyses for worker and males. For over-wintered queens, we found that using PCA 1, which represents a combined measure of warmth and sunniness, instead of the separate sunshine and temperature variables did not improve the best model; therefore we retained both weather variables. Controlling for phenological variation (by retaining date as a fixed effect explanatory variable), *B. magnus* queens were relatively less active than *B. lucorum* in sunny conditions and relatively more active when it was overcast (Fig. 3.4a & Appendix 3.3). We did not detect any significant effect of weather on the relative abundance of *B. cryptarum* queens in comparison to queens of either of the other species (Appendix 3.3).

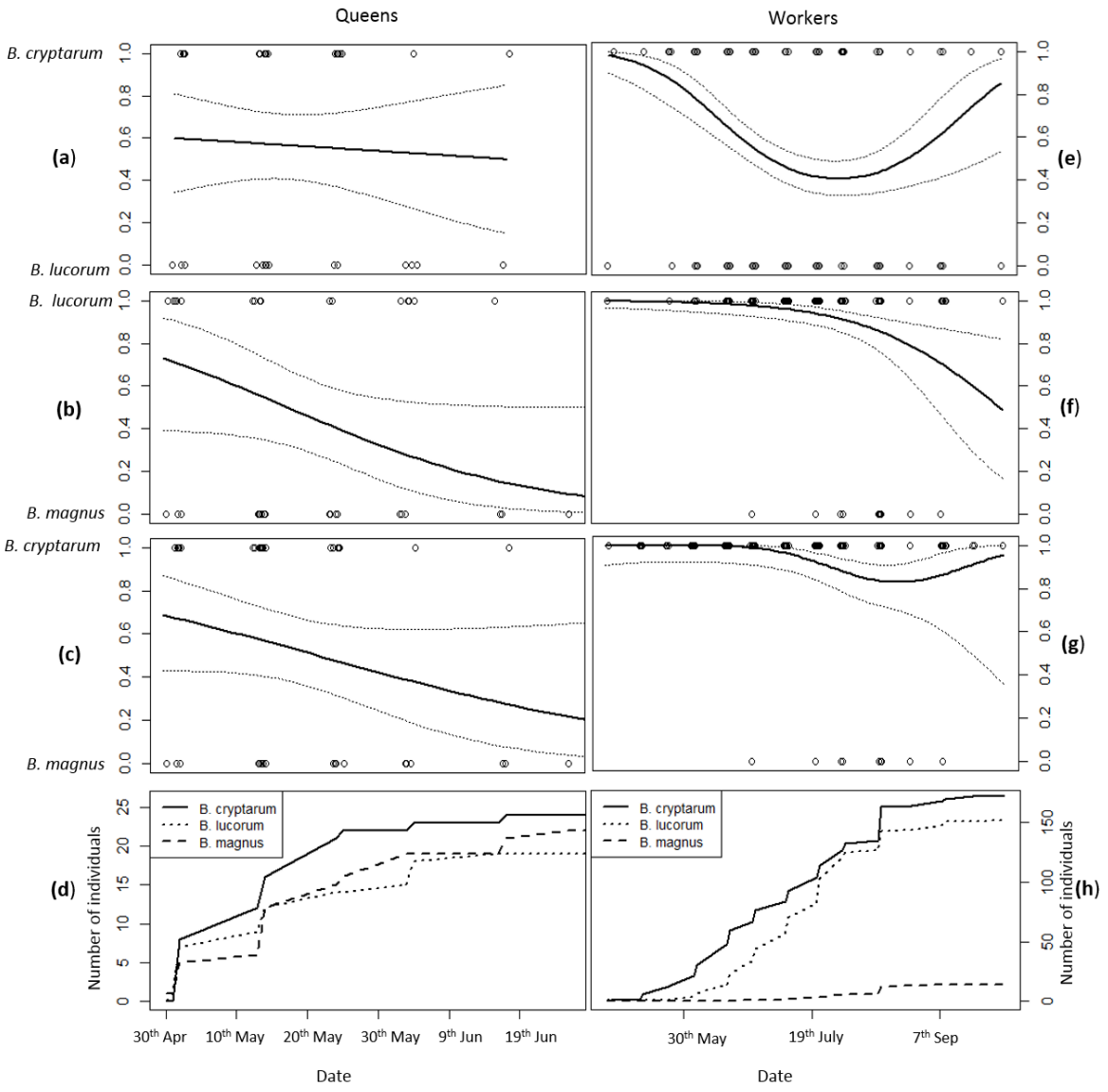


Figure 3.2. Interspecific variation in phenology of queens (a-d) and workers (e-h) of the *lucorum* complex species. Pannels show the changes in the probability of an individuals belonging to: *B. cryptarum* compared to *B. lucorum* (a & e), *B. lucorum* compared to *B. magnus* (b & f) and *B. cryptarum* compared to *B. magnus* (c & g) as a function of date. The relative abundance of species-pairs changed significantly through the season for all comparisons except for between *B. cryptarum* and *B. lucorum* queens (a; see S. 7). Trend lines are model-fits from generalised linear models representing quadratic relationships in (e & g) and linear relationships in (b, c & f); 95% confidence intervals are shown around these relationships. Pannels d & h show how the cumulative abundance of over-wintered queens (d) and workers (h) shifted through the season for each bumblebee species.

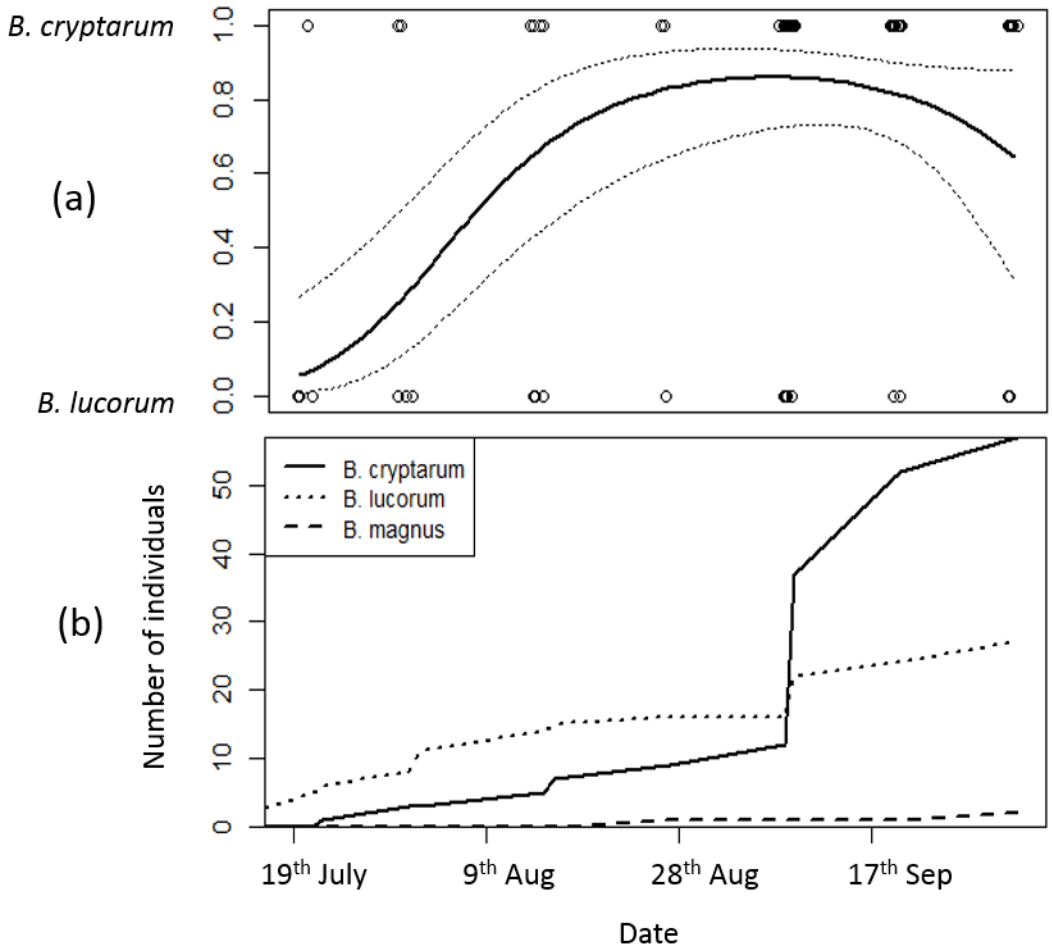


Figure 3.3. Interspecific variation in phenology of males of *B. cryptarum, B. lucorum* and *B. magnus*. (a) the probability that a male belonged to either *B. cryptarum* or *B. lucorum* changed significantly through the season. The trend line shows a quadratic relationship fit from a generalised linear model with 95% confidence intervals. The numbers of *B. magnus* males were too low to perform this analysis. (b) Changes in the cumulative number of males of each of the three bumblebee species over the season.

Weather differentially affected worker activity for *B. cryptarum* and *B. lucorum.* Averaging across the whole season, *B. cryptarum* workers were relatively more common than *B. lucorum* workers when conditions were cooler and cloudier (although this effect was not significant: Fig. 3.4b & Appendix 3.5). Nevertheless, there was a significant effect of the interaction between the date quadratic term and PCA 1 ($\chi^2_2 = 7.57$, p = 0.02, Appendix 3.5). This interaction demonstrated that whilst *B. lucorum* workers were rare early and late in the season, becoming relatively more common around midsummer, the midsummer-increase in

relative abundance was more pronounced when it was warm and sunny, compared to cooler cloudy conditions (Appendix 3.6). Weather did not significantly affect the relative abundance of *B. magnus* workers compared to either of the other species. Considering males, there were fewer *B. cryptarum* males encountered compared to *B. lucorum* in the coolest, cloudiest conditions (Fig. 3.4c & Appendix 3.7).

Temperature varies throughout the day, so we tested whether the differential temperature effects on the probability of activity in *B. cryptarum* and *B. lucorum* led to temporal separation of foraging across the day. Model selection using AICc incorporating time of day instead of PCA 1, favoured models incorporating time (436.7 vs. 432.8 AICc points), but the pattern was the same. The significant interaction between the quadratic terms, date and time of day ($\chi^2_4 = 16.1$, $p < 0.005$, Appendix 3.8), showed that the mid-season peak in the relative abundance of *B. lucorum* workers was strongest early in the morning compared to later in the day. We did not detect differences in temporal activity in any other interspecific comparisons.

Niche overlap between the bumblebee species

We defined the niche region (N_R) exploited by each bumblebee species based on the date, weather conditions and time of day when each individual was out foraging. Amongst queens, there was little difference in N_R and the probabilities of overlap were quite similar for all interspecific comparisons (overlap probability: 0.76-0.88; Fig. 3.5a & Appendix 3.10). Amongst workers, *B. magnus* had the smallest N_R (Fig. 3.5b), which is due in large part to a narrower range of dates (later in the season) when they were on the wing. *Bombus cryptarum* workers displayed the largest N_R, partly driven by the fact they were on the wing for the longest period (Fig. 3.5b).

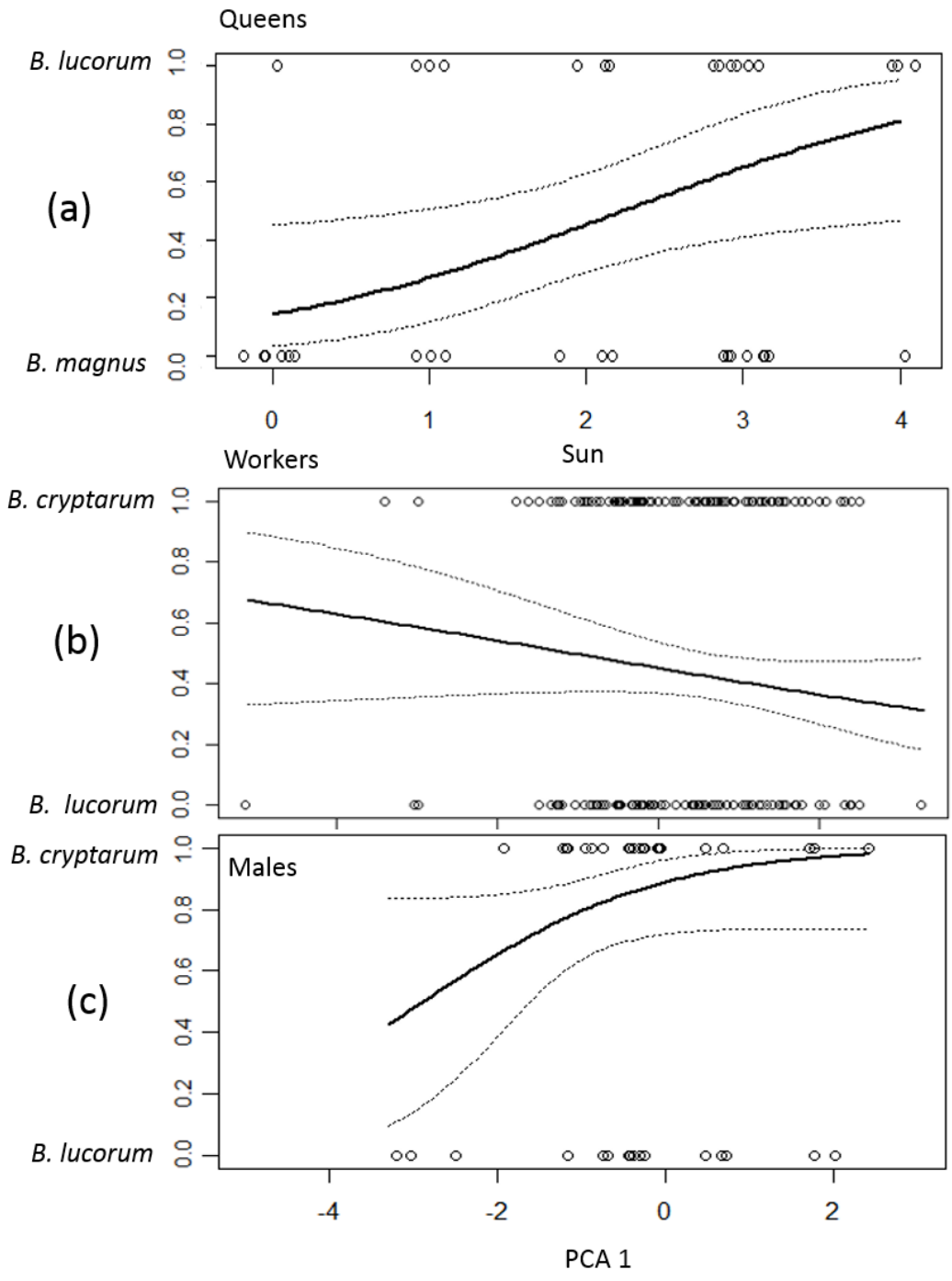


Figure 3.4. Differences in the effects of weather variables on the *lucorum*-complex species. (a) the level of sunshine differentially affected the relative proportions of queens of *B. lucorum* and *B. magnus*: the sun axis represents a scale ranging from 0 (heavy complete cloud cover) to 4 (< 25% cloud cover; see Table S 1). In (b & c) PCA 1 represents a scale where low values indicate cool cloudy conditions and higher values indicate warmer, sunnier conditions (see Fig. S 4): figures display the relative impact of changes in this weather axis on the probablity of workers (b) and males (c) of being *B. cryptarum* compared to *B. lucorum.* Trend lines are model fits from a generalised linear model with 95% confidence intervals.

We therefore found strong asymmetry in the degree of interspecific niche overlap for workers. Whilst *B. magnus* workers were highly likely to exploit the niche of *B. cryptarum* and *B. lucorum* workers (overlap probability: 0.94 and 0.95 respectively), the probability that *B. cryptarum* and *B. lucorum* workers were found in the niche of *B. magnus* workers was much lower (overlap probability: 0.47 and 0.50 respectively, Appendix 3.10).

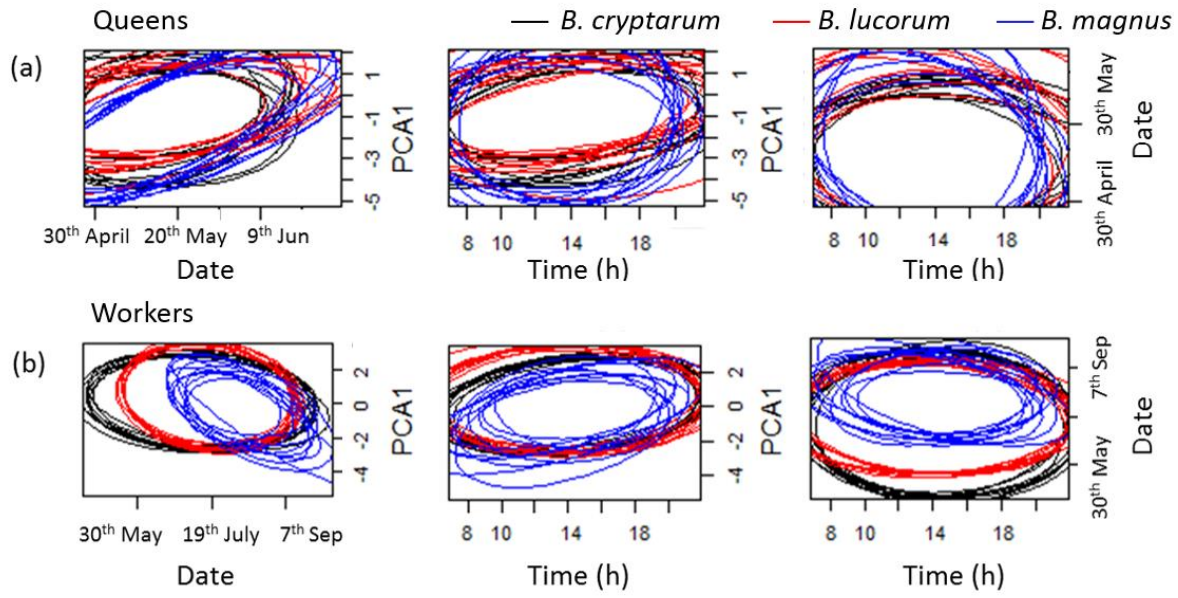


Figure 3.5. Comparisons of the niche size for the three *lucorum* complex species. Panels show ten random elliptical projections of niche region (N_R) for each bumblebee species defined by pairs of variables for (a) queens and (b) workers. Niche regions were estimated based on the modelled influence of seasonal, weather and daily activity variables on occurrence. Each plot illustrates the projected niche region for a different combination of the three variables. PCA 1 represents a weather axis where low values indicate cool cloudy conditions and higher values indicate warmer, sunnier conditions (Appendix 3.4). Black lines represent *B. cryptarum*, red represent *B. lucorum* and blue represent *B. magnus*.

Forage use

Queens of all three bumblebee species were found feeding on a similar number of plant taxa (range 7-8). However, 52.7% of *B. magnus* queens (n = 14) were recorded visiting *Erica spp.*, whereas only a single individual of both *B. cryptarum* (n = 13) and *B. lucorum* (n= 10) foraged on heather plants (Appendix 3.11). Previous studies (Waters *et al.* 2010a;

Scriven *et al.* 2015; Chapter 2), suggest a tight association between *B. magnus* and heather (*C. vulgaris* and *Erica spp*). We tested this explicitly and found that queens of the three bumblebee species differed significantly in their probability of foraging on heather, compared to all other plant species ($\chi^2_2 = 6.79$, $p < 0.05$); parameter estimates show this was due to *B. magnus* queens foraging on this taxon more often than *B. cryptarum*, but post hoc tests did not detect any individually significant differences between pairs of species ($p > 0.05$).

Concerning workers, the plants most commonly visited by *B. cryptarum* were *C. vulgaris* (17.1%) and *Erica spp*. (15.3%). However, the proportional representation of these species in the *B. cryptarum* diet was markedly lower than for *B. magnus*, of which 84.6% foraged on *C. vulgaris* (61.5%) or *Erica spp*. (23.1%, Appendix 3.12). Only 26% of *B. lucorum* workers were foraging on these plant taxa. We found that, like queens, workers of the three bumblebee species also differed significantly in their probability of foraging on heather compared to all other plant species ($\chi^2_2 = 15.15$, $p < 0.001$): *Erica spp*. and *C. vulgaris* made a significantly larger contribution to the diet of *B. magnus* workers than to the diets of *B. cryptarum* or *B. lucorum* ($p < 0.01$). This difference remained when the analysis was restricted to only the period when *Erica spp*. and *C. vulgaris* were in flower ($\chi^2_2 = 16.92$, $p < 0.001$): comparisons were individually significant for both *B. magnus* - *B. cryptarum* ($p < 0.05$) and for *B. magnus* - *B. lucorum* ($p < 0.005$). Moreover, during this heather flowering period, the probability of *B. cryptarum* foraging on *Erica spp*. and *C. vulgaris* was significantly greater than for *B. lucorum* ($p < 0.05$). The forage plant that made the greatest contribution to the diet of *B. lucorum* workers was *Rubus sp.* (27.6%), a species that contributed significantly less (12.9%) to the diet of *B. cryptarum* ($\chi^2_1 = 9.97$, $p = 0.002$, Appendix 3.12).

Males of *B. cryptarum* and *B. lucorum* had a similar diet breadth; however their diets only overlapped on four plant taxa (total taxa = 9 & 7 respectively, Appendix 3.13). The plant

most frequently visited by *B. cryptarum* males (66.1%) was *Succisa pratensis*, which contributed significantly less (29.6%) to the diet of *B. lucorum* males ($\chi^2_1 = 8.3$, $p = 0.003$). The other most often utilised forage plant for *B. lucorum* males was *Chamerion angustifolium* (29.6%), which was not used at all by *B. cryptarum* (Fisher's exact test $p < 0.001$). Only two *B. magnus* males were found preventing assessment of diet breadth for males of this species.

We calculated between-species dissimilarity in diet composition using the Bray-Curtis coefficient (Bray & Curtis 1957). A Bray-Curtis distance value of zero indicates that the two forage assemblages are identical for both species, whereas a value of one means they are completely dissimilar. The degree of dietary dissimilarity between *B. cryptarum* and *B. lucorum* was almost identical for queens and workers (0.30 & 0.31 respectively), whereas for males it was greater (0.69, Table 3.2). Amongst queens, the greatest difference in diet composition was between *B. cryptarum* and *B. magnus*. For workers, the degree of dissimilarity was greatest between *B. magnus* and the other two species (> 0.6); the diets of *B. lucorum* and *B. cryptarum* workers were more similar (0.31, Table 3.2).

Table 3.2. Differences in the plant species assemblages used as forage resources by the three *lucorum* complex species. Bray-Curtis distance measures showing the dissimilarity between the diets of each caste of each of the three bumblebee species. A value of 0 indicates that the two assemblages were identical, whereas a value of 1 indicates that they were completely different. *Bombus magnus* males are not included because the sample size was too low: only over-wintered queens are included.

	Caste	***B. cryptarum***	***B. lucorum***
B. lucorum	Queens	0.30	-
	Workers	0.31	-
	Males	0.69	-
B. magnus	Queens	0.51	0.35
	Workers	0.64	0.72

3.5 Discussion

The sympatric occurrence of cryptic species challenges ecological theory because their strong biological similarity should generate intense interspecific competition and potential competitive exclusion (Gause 1932; Hardin 1960; Cothran *et al.* 2013; Van Campenhout *et al.* 2014). Nevertheless, the *lucorum* complex contains three cryptic bumblebee species with near-identical morphology, which co-occur across large parts of the UK (Bertsch *et al.* 2005; Carolan *et al.* 2012; Scriven *et al.* 2015; Waters *et al.* 2010a) and elsewhere (Murray *et al.* 2008; Stanley, Knight & Stout 2013; Williams *et al.* 2012). In this study of *B. lucorum*, *B. cryptarum* and *B. magnus* we demonstrate clearly that although the niches of these three cryptic species overlap considerably, they do have distinct ecologies. We reveal niche utilisation differences that may be sufficient to prevent competitive exclusion by reducing the intensity of interspecific competition. We also provide the first reliable evidence for differences in their phenology.

We focussed on interspecific variation in three fundamental biotic and abiotic niche-use dimensions at a single site: differences in responses to weather conditions, different forage use and different temporal activity patterns. *Bombus magnus* had the most distinct niche. It has a narrow, highly specialised diet, feeding predominantly on species of heather plant (Ericacae). The phenology of all three *B. magnus* castes was delayed relative to the other two species; furthermore queens of *B. magnus* were more active in overcast conditions, compared to those of *B. lucorum*. In contrast, although all castes of *B. cryptarum* and *B. lucorum* were on the wing for the same period of time, *B. lucorum* workers showed a strong peak in abundance around mid-summer, followed by an earlier peak in the production of male reproductives. *Bombus lucorum* worker activity was skewed towards warmer, sunnier conditions compared to *B. cryptarum* and, the elevated abundance of *B. lucorum* in mid-summer was strongest in warm conditions. *Bombus cryptarum* had a different phenology:

worker numbers increased faster, and decreased more slowly as the season progressed, compared to *B. lucorum*. This may in part be because *B. cryptarum* is better adapted for activity in colder conditions: its workers foraged more in cooler cloudier conditions than did *B. lucorum* (though, conversely, we captured fewer *B. cryptarum* males in cool cloudy conditions). There was some subtle diurnal temporal separation of foraging behaviour between *B. lucorum* and *B. cryptarum*: in mid-summer, when *B. lucorum* was relatively most common, its workers were more numerous early in the morning and least active in the afternoon.

The broad conclusions that *B. lucorum* is adapted for activity in warmer sunnier conditions, whereas *B. magnus* and *B. cryptarum* are adapted to forage in cooler cloudier conditions, recapitulates previous species distribution analysis. Chapter 2 (Scriven *et al.* 2015) showed that across Great Britain, *B. magnus* and *B. cryptarum* were more commonly found at sites with lower summer temperatures. Our current findings may help explain why *B. lucorum* is ecologically dominant throughout much of lowland southern and eastern England where it is warmer and sunnier, whereas *B. cryptarum* and *B. magnus* tend to occur in upland and northerly locations (Scriven *et al.* 2015; Chapter 2). Similarly, our demonstration that the colony cycle of *B. magnus* is delayed in the season relative to the other two species is supported by previous work at our fieldwork location in August 2013, when both workers and males of *B. lucorum* and *B. cryptarum* were present, whereas only *B. magnus* workers were detected (Scriven *et al.* 2015; Chapter 2). Such variation in the timing of male production in these three species may reduce the likelihood of hybridisation, thereby reinforcing reproductive isolation.

The observation that *B. magnus* foraged more often on heather plants supports observations that *B. magnus* is more commonly found on heather moorland (Scriven *et al.* 2015; Chapter 2). Previous studies also revealed differences in the diet of these three species;

however, these studies have combined data from multiple sites. For example, Chapter 2 (Scriven *et al.* 2015) found that across the UK, *B. lucorum* had the broadest diet and *B. magnus* had the narrowest diet breadth. However, it is not clear whether this resulted from different forage preferences, because bee species with restricted geographic ranges may have access to a restricted range of forage plants. In the present study, all the bumblebees had the same forage plants to choose from; despite this, clear differences in their diets remained.

Cryptic species provide important case studies to investigate the types of niche-utilisation traits that diverge most readily after speciation events. The *lucorum* complex species seem to have diverged relatively recently (< 100,000 years ago; based on COI divergence and diversity reported by Carolan *et al.* 2012 and Murray *et al.* 2008, using the approach of Jiggins and Tinsley 2005, and the standard insect molecular clock of Brower 1994) and previous work suggests that *B. magnus* and *B. cryptarum* are the most closely related of the three (Bertsch *et al.* 2005; Murray *et al.* 2008; Williams *et al.* 2012). The interspecific niche differentiation we have observed may have underlain this speciation process. Evolution in metabolic pathways or morphology may be responsible for the thermal specialisation for activity in cooler conditions exhibited by *B. cryptarum* and *B. magnus*. Bumblebees are facultatively endothermic, requiring pre-flight metabolic warm-up, large body size and thoracic insulation for flight. Thermal specialisation is an important mechanism that may reduce the strength of interspecific competition; it may also mean that the members of a community of bee species can offer complementary pollination services to plants (Herrera 1997; Peat *et al.* 2005; Lye *et al.* 2010; Frund *et al.* 2013). However, in our dataset, air temperature and time of day covaried (after accounting for seasonal changes), therefore it is not possible to definitively rule out divergent circadian rhythms as an explanation for interspecific differences in the association between activity and temperature. The most dramatic aspect of niche divergence within this cryptic species complex is the

strong likelihood of *B. magnus* to forage on the heathers, *C. vulgaris*, *E. cinerea* and *E. tetralix* to the exclusion of other potentially suitable species that were common in the area.

We have shown significant differences along three niche dimensions of three cryptic species that are likely to facilitate their coexistence. However, there is also considerable niche overlap, which must lead to competition. Direct interference competition between these bumblebee species is unlikely, but there is the potential for exploitative competition for resources. An important resource for which both inter and intra-specific competition may occur is pollen and nectar. Therefore, since all three *lucorum* complex species are present at this site and draw on similar resources, the differences found in their use of forage plants may possibly be driven by competition and reflect differences in their realised niches, rather than fundamental niches. In contrast, the other niche dimensions investigated, phenology and response to weather, are less likely to be influenced by competition, and may thus represent interspecific differences in the fundamental niche. Patterns of bumblebee visitation to the same plant species can vary through space and time, potentially as a response to variation in pollen abundance and quality (Vaudo *et al.* 2014) and also to avoid interspecific competition (Lye *et al.* 2010). We found that interspecific niche overlap was higher for queens than it was for either workers or males. However, seasonal changes in the abundance of forage plants relative to bumblebees means it is hard to determine the impact of this shift in niche overlap on the strength of interspecific competition acting on the different castes. In terms of the temporal and weather niche, *B. magnus* workers were most differentiated, with worker production delayed compared to the other species, potentially in order to coincide with the flowering of their principal forage plants. Consequently, despite being most differentiated, the niche of *B. magnus* workers was situated mostly within the niche region of the other two species. This creates an asymmetry in niche overlap, with *B. magnus* potentially suffering more strongly from competition than either of the species it interacts with. However, more

specialised species are presumed to be more efficient in their preferred conditions than generalists (Pianka 1994). In the UK, *C. vulgaris* forms an important, and often dominant, component of both upland and lowland heaths (Groves *et al.* 2012; Thompson *et al.* 1995). As a specialist on heather species, *B. magnus* may be an optimal forager on this resource, exploiting it more efficiently than the other species. Furthermore, by delaying worker production until *C. vulgaris* and *Erica spp*. flower, *B. magnus* could be able to profit from this extremely abundant resource, limiting the impact of overlap along the other niche dimensions, whilst avoiding worker competition with the other two bumblebee species earlier in the season when resources are more limited.

The niche differences that we have observed in this study may assist co-occurrence of these cryptic species by causing variation in the responses of each species to spatio-temporal heterogeneity in seasonally changing foraging sites. When the resources available for colony growth are continuously changing, the competitive relations between colonies of different species can be reversed, leading to the maintenance of a larger number of species in a region (Westphal, Steffan-Dewenter & Tscharntke 2006). The composition and abundance of bee populations have been shown to undergo considerable variation between years (Iserbyt & Rasmont 2012; Minckley *et al.* 1999; Oertli, Müller, & Dorn 2005). Iserbyt & Rasmont (2012) found that in one mountainous region, the dominant bumblebee species one year was seldom dominant another year, some species disappeared totally for several years and the proportion of permanent species was low. We observed clear abundance differences in the bumblebee species: *B. cryptarum* was the most common species and *B. magnus* was by far the least abundant. Yet previous sampling in 2011 found the most common species to be *B. magnus* (50.5%), whereas *B. cryptarum* was the least common (19.4%, Chapter 2; Scriven *et al.* 2015). Clearly, the relative proportions of *B. magnus* and *B. cryptarum* can vary strongly between years, suggesting that the two species do not respond to environmental fluctuations

in the same way. This could therefore represent a system where ecological divergence, niche partitioning and spatio-temporal heterogeneity in the environment mean that none of the three species is able to consistently exclude another to the point of local extinction. This has considerable implications for conservation, as small alterations to any of these dimensions could modify inter-specific interactions putting one or more species at risk. *Bombus magnus* relies heavily on threatened and declining heathland habitat; further losses could therefore shift the balance and seriously affect populations of this species. Studying these species over several consecutive years may reveal trends in the population composition linked to annual climatic variations and allow us to understand in more detail what climatic factors affect the success of these three species. Similarly, broadening the study to include other sites would demonstrate whether these patterns are consistent across areas.

The discovery of co-occurring cryptic species presents problems for several areas of ecological theory: the limits of ecological differentiation required for species coexistence, phylogenetic limiting similarity and competitive exclusion (Violle *et al.* 2011; Gabaldón *et al.* 2013; Van Campenhout *et al.* 2014). We show that a combination of varying levels of ecological divergence in different niche dimensions and spatio-temporal heterogeneity in the environment may contribute to the persistence of cryptic species in sympatry. Furthermore, our study suggests that cryptic species provide distinct and unique ecosystem services, clearly demonstrating that morphological similarity between species does not necessarily equate to ecological equivalence.

3.6 Acknowledgements

We thank the National Trust for Scotland for allowing us to use their land and the Glencoe Visitor Centre staff and rangers in particular, for their help and advice. The project was funded by the University of Stirling.

3.7 Appendix

Appendix 3.1. Scale used for categorising amount of sun.

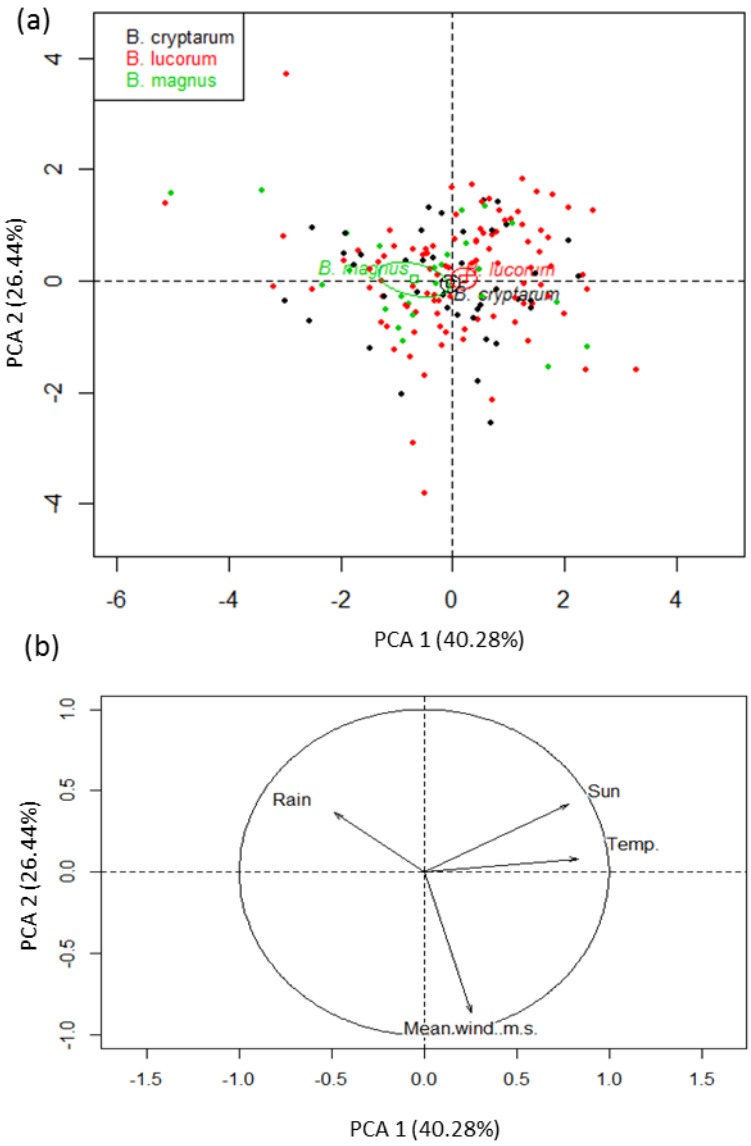
Scale value	Description
0	Heavy complete cloud cover
1	Light complete cloud cover
2	Some breaks in cloud, > 75% cloud cover
3	25-75% cloud cover
4	< 25% cloud cover

Appendix 3.2. Scale used for categorising rainfall.

Scale value	Description
0	Completely dry, no precipitation
1	Light mist
2	Drizzle
3	Light rain
4	Rain
5	Heavy, torrential rain

Appendix 3.3. Phenological variation and differences in responses to weather conditions between over-wintered queens of each bumblebee species. Results from pairwise analyses between the species, using generalised linear models with binary error distributions, testing the effects of date and the amount of sun on the relative abundance of over-wintered queens. Significant results are shown in italics. Values for the best model are shown in bold, other values are included for comparisons. Negative parameter estimates indicate a decrease in the probability of individuals belonging to the first species in the comparison whereas a positive estimate indicates an increase in the probability of individuals belonging to the first species.

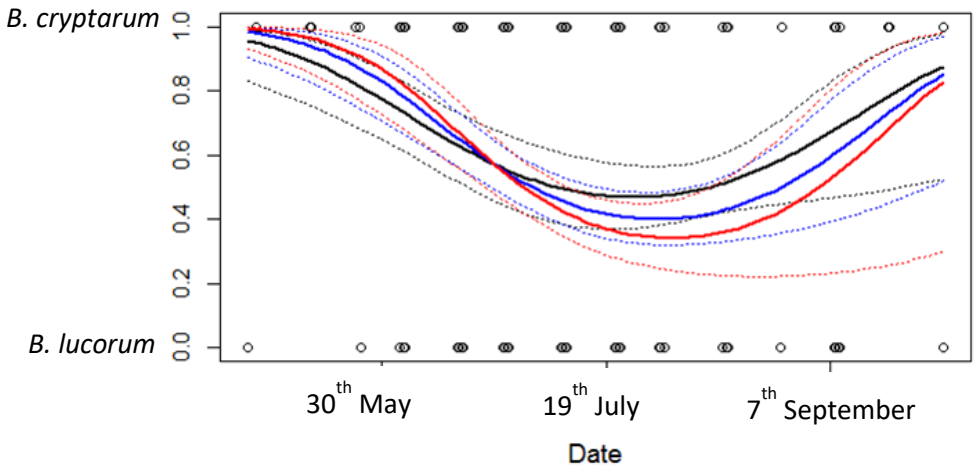
	B. cryptarum - B. lucorum				***B. lucorum - B. magnus***				***B. cryptarum - B. magnus***			
Parameter	**Estimate**	**SE**	**χ^2**	**P**	**Estimate**	**SE**	**χ^2**	**P**	**Estimate**	**SE**	**χ^2**	**P**
Date	-0.01	0.03	0.10	0.75	**-0.06**	**0.03**	4.16	*0.04*	**-0.05**	**0.03**	4.17	*0.04*
Sun	-0.22	0.31	0.52	0.47	**0.81**	**0.36**	6.19	*0.01*	0.45	0.29	2.66	0.10



Appendix 3.4. Results of principal component analysis (PCA) on the variation in weather condition metrics (sun, wind speed, rain and air temperature) when each individual bumblebee was encountered. Axis 1 (PCA 1) and Axis 2 (PCA 2) describe 40.3% and 26.4% of the total variation respectively. (a) Each point represents an individual bee. The square boxes show the average observations for each species and the ellipses show the 95% confidence levels around these values. (b) Vector plot showing the contribution of different weather variables to PCA 1 and 2.

Appendix 3.5. Differences in phenology and responses to weather conditions of workers for each bumblebee species. Results from pairwise analyses between the species, using generalised linear models with binary error distributions, testing the effects of date (with linear and quadratic terms) and PCA 1on the relative abundance of workers. PCA 1 represents a scale where low values indicate cool cloudy conditions and higher values indicate warmer, sunnier conditions (Appendix 3.4). Significant results are shown in italics. Values for the best model are shown in bold, other values are included for comparisons. Negative parameter estimates indicate a decrease in the probability of individuals belonging to the first species in the comparison whereas a positive estimate indicates an increase in the probability of individuals belonging to the first species.

	B. cryptarum - B. lucorum				***B. lucorum - B. magnus***				***B. cryptarum - B. magnus***			
Parameter	**Estimate**	**SE**	**χ^2**	**P**	**Estimate**	**SE**	**χ^2**	**P**	**Estimate**	**SE**	**χ^2**	**P**
Date	**-6.80**	**2.40**			**-0.04**	**0.01**	9.90	*0.002*	**-29.90**	**13.47**		
Date^2	**9.56**	**2.68**	14.20	*< 0.001*	11.89	8.03	3.29	0.07	**13.44**	**8.48**	4.14	*0.04*
PCA 1	**-0.11**	**0.10**	0.63	0.429	-0.86	0.87	0.45	0.50	-0.94	1.27	0.05	0.82
Date: PCA 1	**-3.66**	**2.26**			16.72	11.76			16.48	18.45		
Date^2: PCA 1	**1.73**	**1.71**	7.57	*0.02*	-15.43	11.80	2.41	0.30	-10.15	11.08	0.99	0.61



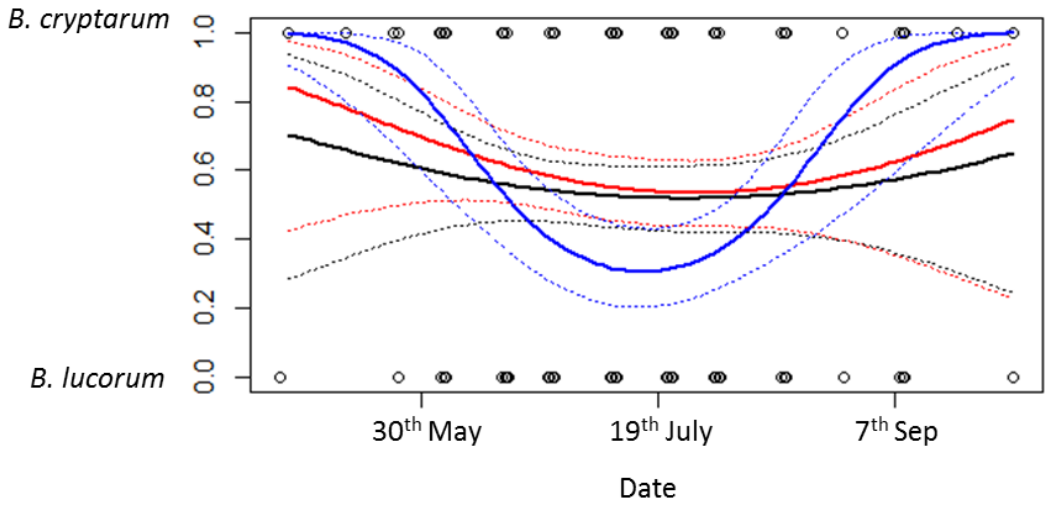
Appendix 3.6. The effect of seasonality and changing weather conditions on the abundance of *B. cryptarum* and *B. lucorum* workers on the wing. Trend lines are model fits from generalised linear models with 95% confidence intervals. Black lines represent a low PCA 1 score (1st quartile) hence cool, cloudy conditions; blue lines represent a mid value for PCA 1 (median) and red lines indicate a high PCA 1 score (3rd quartile) hence warm, sunny conditions (see Appendix 3.4 for details of PCA 1).

Appendix 3.7. Differences in phenology and responses to weather conditions between males of *B. cryptarum* and *B. lucorum*. Results from pairwise analyses between the species, using generalised linear models with binary error distributions, testing the effects of date (with linear and quadratic terms) and PCA 1on the relative abundance of males. PCA 1 represents a scale where low values indicate cool cloudy conditions and higher values indicate warmer, sunnier conditions (Appendix 3.4). Significant results are shown in italics. Negative parameter estimates indicate a decrease in the probability of individuals belonging to the first species in the comparison whereas a positive estimate indicates an increase in the probability of individuals belonging to the first species. *Bombus magnus* males were not included because the sample size was very low.

	B. cryptarum* - *B. lucorum			
Parameter	**Estimate**	**SE**	**χ^2**	**Prob > χ^2**
Date	**9.69**	**2.56**		
Date^2	**-7.83**	**2.98**	4.31	*0.04*
PCA 1	**0.72**	**0.40**	4.15	*0.04*

Appendix 3.8 . Changes in seasonal and daily activity in workers of *B. cryptarum* and *B. lucorum*. Results from pairwise analyses between the species, using generalised linear models with binary error distributions, testing the effects of date and time of day (with linear and quadratic terms) on the relative abundance of workers. Significant results are shown in italics. Negative parameter estimates indicate a decrease in the probability of individuals belonging to the first species in the comparison whereas a positive estimate indicates an increase in the probability of individuals belonging to the first species.

	B. cryptarum - B. lucorum			
Parameter	**Estimate**	**SE**	χ^2	**P**
Intercept	0.26	0.14		
Date	-2.02	2.76		
Date^2	13.27	3.58	17.86	*<0.001*
Time	3.07	2.69		
Time^2	0.75	3.25	2.21	0.33
Date x Time	-24.27	55.37		
Date^2 x Time	-27.79	67.55		
Date x Time^2	-12.53	64.81		
Date^2 x Time^2	274.19	89.31	16.12	*0.003*



Appendix 3.9. The effect of date and time of day on the abundance of *B. cryptarum* and *B. lucorum* workers on the wing. Trend lines are model fits from generalised linear models with 95% confidence intervals. Blue lines represent early in the day (10am); black lines represent the middle of the day (1pm) and red lines represent later in the day (4pm).

Appendix 3.10. Differences in niche overlap between queens and workers of each of the three *lucorum* complex species**.** Mean overlap probability between each bumblebee species at the niche region size α = 0.95 with 95% credible intervals. α represents the probability of an individual being found in the estimated niche region (Swanson *et al.* 2015). The niche regions were defined based on the date, weather and time of day when individuals were active. The overlap probability represents the likelihood that an individual from Species A will be found in the niche of Species B.

		Species B					
		Queens			**Workers**		
Species A	Parameter	***B. cryptarum***	***B. lucorum***	***B. magnus***	***B. cryptarum***	***B. lucorum***	***B. magnus***
B. cryptarum	**Mean**	**-**	**0.88**	**0.78**	**-**	**0.90**	**0.47**
	2.5%	-	0.71	0.56	-	0.84	0.29
	97.5%	-	0.98	0.94	-	0.95	0.67
B. lucorum	**Mean**	**0.82**	**-**	**0.76**	**0.91**	**-**	**0.50**
	2.5%	0.62	-	0.54	0.86	-	0.31
	97.5%	0.96	-	0.94	0.96	-	0.71
B. magnus	**Mean**	**0.76**	**0.81**	**-**	**0.94**	**0.93**	**-**
	2.5%	0.56	0.61	-	0.81	0.78	-
	97.5%	0.93	0.96	-	1.00	0.99	-

Appendix 3.11. Forage use and measures of diet breadth for *lucorum* complex over-wintered queens. Values represent the number of individuals of each bee species and, in parentheses, the percentage of the total number individuals of the corresponding bee species. Diet breadth is measured via rarefaction to estimate the number of plant species each bee species would be expected to visit in a total of 5 flower visits. Garden plant 1 was not a native wildflower, found in a garden that was not identified.

	B. cryptarum	***B. lucorum***	***B. magnus***	***Total***
Acer pseudoplatanus	2 (15.4)	3 (30)		5
Erica spp.	1 (7.7)	1 (10)	6 (42.9)	8
Cotoneaster horizontalis	2 (15.4)	1 (10)	1 (7.1)	4
Cytisus scoparius	2 (15.4)	1 (10)	2 (14.3)	5
Erica cinerea	1 (7.7)	2 (20)	2 (14.3)	5
Garden plant 1	1 (7.7)			1
Lotus corniculatus		1 (10)	1 (7.1)	2
Rhododendron spp.		1 (10)	1 (7.1)	2
Salix spp.	3 (23.1)			3
Taraxacum spp.	1 (7.7)			1
Thymus polytrichus			1 (7.1)	1
Total sample size	**13**	**10**	**14**	**37**
No. of plant taxa visited	**8**	**7**	**7**	**11**
Diet breadth (± S.D.)	**4.3 ± 0.65**	**4.2 ± 0.68**	**3.6 ± 0.83**	

Appendix 3.12. Forage use and measures of diet breadth for *B. lucorum* complex workers. Values represent the number of individuals of each bee species and, in parentheses, the percentage of the total number individuals of the corresponding bee species. Diet breadth is measured via rarefaction to estimate the number of plant species each bee species would be expected to visit in a total of 10 flower visits. Garden plants 2-4 were exotic taxa found in gardens and not identified.

	B. cryptarum	***B. lucorum***	***B. magnus***	***Total***
Papaveroideae spp.	1 (0.6)			1
Acer pseudoplatanus	4 (2.4)			4
Aegopodium podagraria	8 (4.7)	3 (2)		11
Calluna vulgaris	29 (17.1)	22 (14.5)	8 (61.5)	59
Centaura nigra	2 (1.2)	6 (3.9)		8
Chamerion angustifolium	13 (7.6)	19 (12.5)		32
Cirsium arvense		4 (2.6)		4
Cotoneaster horizontalis	2 (1.2)			2
Cytisus scoparius	5 (2.9)	1 (0.7)		6
Erica cinerea	26 (15.3)	18 (11.8)	3 (23.1)	47
Erica tetralix	1 (0.6)			1
Filipendula ulmaria	2 (1.2)			2
Garden plant 2	1 (0.6)			1
Garden plant 3		1 (0.7)		1
Garden plant 4		1 (0.7)		1
Hydrangea spp.	6 (3.5)	3 (2)		9
Lavandula spp.		1 (0.7)		1
Lotus corniculatus	13 (7.6)	4 (2.6)		17
Lotus pedunculatus	4 (2.4)	6 (3.9)		10
Lupinus	1 (0.6)			1
Narthecium ossifragum	1 (0.6)			1
Potenilla erecta	3 (1.8)			3
Rhododendron spp.		2 (1.3)		2
Rubus spp.	22 (12.9)	42 (27.6)		64
Salix spp.	1 (0.6)			1
Sanguisorba spp.	1 (0.6)			1
Succisa pratensis	2 (1.2)	2 (1.3)	1 (7.7)	5
Thymus polytrichus	4 (2.4)		1 (7.7)	5
Trifolium repens	17 (10)	17 (11.2)		34
Ulex europaeus	1 (0.6)			1
Total sample size	**170**	**152**	**13**	**335**
No. of plant taxa visited	**25**	**17**	**4**	**30**
Diet breadth (± S.D.)	**6.9 ± 1.2**	**6 ± 1.1**	**3.5 ± 0.6**	

Appendix 3.13. Forage use and measures of diet breadth for *B. lucorum* complex males. Values represent the number of individuals of each bee species and, in parentheses, the percentage of the total number individuals of the corresponding bee species. Diet breadth is measured via rarefaction to estimate the number of plant species each bee species would be expected to visit in a total of 25 flower visits. Diet breadth was not calculated for *B. magnus* as the sample size was too low.

	B. cryptarum	***B. lucorum***	***B. magnus***	**Total**
Calluna vulgaris	4 (7.1)	4 (14.8)		8
Centaura nigra	1 (1.8)	1 (3.7)		2
Chamerion angustifolium		8 (29.6)		8
Erica cinerea	3 (5.4)		1 (50)	4
Hylotelephium telephium	6 (10.7)			6
Lavandula spp.	2 (3.6)			2
Lotus pedunculatus	1 (1.8)			1
Nepeta racemosa	1 (1.8)			1
Rubus spp.		2 (7.4)		2
Senecio jacobaea		1 (3.7)		1
Succisa pratensis	37 (66.1)	8 (29.6)		45
Symparicarpos albus	1 (1.8)	3 (11.1)	1 (50)	5
Total sample size	**56**	**27**	**2**	**85**
No. of plant taxa visited	**9**	**7**	**2**	**12**
Diet breadth (± S.D.)	**6.2 ± 1.1**	**6.8 ± 0.4**		

Chapter 4

Specialist species exhibit low population structure across fragmented landscapes: evidence for dispersal adaptation from a complex of cryptic bumblebee species

A version of this chapter is being prepared for resubmission to Molecular Ecology as:

Scriven, J.J., Abdelaziz, M. Whitehorn, P.R., Goulson, D. and Tinsley, M.C. Specialist species exhibit low population structure across fragmented landscapes: evidence for dispersal adaptation from a complex of cryptic bumblebee species.

P.Whitehorn, D. Goulson and M. Tinsley supervised the project, M. Abdelaziz provided advice on analysis methods used, and all authors commented on draft versions of the manuscript.

4.1 Abstract

Two conflicting hypotheses predict habitat-specialist species to either have high or low dispersal capabilities. Specialists that exploit discontinuous patchy habitats may incur high emigration costs if they frequently fail to reach suitable environments, possibly leading to selection of decreased dispersal propensity. Alternatively, specialists may be selected for enhanced dispersal abilities, enabling them to traverse ecologically unsuitable areas to exploit distant habitat patches for which they are well adapted. We investigated these alternatives using the '*lucorum* complex': three cryptic bumblebee species with near-identical morphology that vary along a generalist-specialist continuum. For the first time, we assessed genetic diversity and population structuring in these species, using individuals from multiple sites across Great Britain and 13 microsatellite loci. As expected, the most abundant and strongly generalist species, *B. lucorum*, exhibited the highest genetic variation and very little population differentiation across large spatial scales (F_{ST} = 0.025), and *B. magnus*, a tight specialist on heathland habitat patches and the least abundant species had the lowest levels of genetic diversity. However, the specialist, *B. magnus*, exhibited similar levels of population differentiation as the moderate generalist *B. cryptarum* (F_{ST} = 0.048 and 0.052 respectively), and unlike *B. cryptarum*, did not exhibit strong evidence for isolation by distance. Our data demonstrate that specialist species may be strong dispersers and may be well adapted to persist within habitat patches in fragmented landscapes. This study also confirms the species status of *B. cryptarum*, *B. lucorum* and *B. magnus* using nuclear DNA for the first time.

4.2 Introduction

Species vary in their habitat requirements: specialist species exhibit narrow environmental tolerances, constraining them to particular habitats, whereas generalist species have much broader environmental tolerances and are able to exploit a wide range of habitats. As a consequence, specialist species can be restricted to patches of suitable habitat within a matrix of unsuitable habitat. Such discontinuous distributions of suitable habitat can increase the cost of emigration for specialists if migrants frequently disperse to habitat patches for which they are poorly adapted (Bonte *et al.* 2012). These high emigration costs are hypothesized to have favoured the evolution of decreased dispersal propensity in specialist species (Colas *et al.* 1997; Mathias *et al.* 2001; Bonte *et al.* 2012; Dahirel *et al.* 2014).

Dispersal is an important strategy for avoiding inbreeding, as well as resource and kin competition (Frankham *et al.* 2002; Clobert *et al.* 2012). Effective dispersal can lead to gene flow, counteracting the population structuring that can be caused by genetic drift (Slatkin 1985; Ronce 2007) but also potentially hampering local adaptation (Bonte *et al.* 2012). Given that generalist species tend to be widespread with high gene flow, populations of generalists often exhibit high genetic diversities (Li *et al.* 2014, but see Engler *et al.* 2014) and low genetic differentiation (Packer *et al.* 2005; Habel *et al.* 2009, 2010). If populations lack gene flow, small isolated populations can suffer from reduced genetic diversity, elevated disease and increased risk of extinction (Wright 1943; Frankham *et al.* 2002; Whitehorn *et al.* 2011). Thus specialist species with patchy spatial distributions may be selected to have strong dispersal abilities, enabling them to move across areas of unsuitable habitat; individuals may gain enhanced fitness by colonising unoccupied habitat patches or by mating with unrelated individuals, thereby reducing genetic isolation of fragmented populations (Zavodna *et al.* 2005; Sallé *et al.* 2007; Exeler *et al.* 2008, 2010; Centeno-Cuadros *et al.* 2011; Ginson *et al.* 2015). This argument runs counter to the hypothesis that the costs of emigration for

specialists should select for reduced dispersal propensity. Whether we should expect specialist species to be good or poor dispersers therefore remains an open question.

Whilst some ecological specialists have tight associations with food plants (or other niche elements) that are widespread and therefore have no spatial restrictions, other specialists can be associated with food plants that are only found in certain habitats, which effectively makes them habitat specialists. Among bees, specialist species that collect pollen from just one or a few plant species may have their distribution limited by that of their floral host(s). If the distribution of these floral species is patchy, specialist bees may have a fragmented spatial distribution, potentially reducing gene flow between populations, making them prone to inbreeding and loss of genetic variation (Futuyma & Moreno 1988; Frankham *et al.* 2002; Packer *et al.* 2005; Zayed & Packer 2007).

Generalist bee species should be able to maintain gene flow through areas where specialist species are absent. Indeed, habitat fragmentation has been found to have a bigger impact on specialist bees than generalist species (Biesmeijer *et al.* 2006; Cane *et al.* 2006). Furthermore, specialists can exhibit high levels of spatial genetic differentiation (Packer *et al.* 2005; Zayed *et al.* 2005), suggesting that populations of these species may be more isolated than populations of generalist bee species. However, this may not be the case for all specialist species: pioneer species with high dispersal ability can be less susceptible to habitat fragmentation, being adapted for survival in changing and fragmented habitats, which appears to be the case for *Andrena vaga,* a pioneer specialist solitary bee (Exeler *et al.* 2008; Černá *et al.* 2013). Similarly, other factors can also influence population genetic structuring in bees, including nesting behaviour (Exeler *et al.* 2010), natural barriers such as elevation gradients and water bodies (Cameron *et al.* 2011; Lozier *et al.* 2013), and urbanised or agricultural land (Jha & Kremen 2013b; Jha 2015). To help clarify to what extent we might expect habitat specialists to evolve strong dispersal capabilities, we investigated levels of

genetic structure in populations of three cryptic species of bumblebee that co-occur in the UK and differ in their degree of ecological specialism.

Approximately 250 species of bumblebees exist worldwide, distributed across the temperate, alpine and arctic regions of the northern hemisphere and also South America. Bumblebees are important and effective pollinators, often considered 'keystone' species in plant-pollinator systems, as they pollinate both rare and abundant plant species, thus maintaining diversity in plant communities (Stubbs & Drummond 2001; Kremen *et al.* 2002; Memmott *et al.* 2004; Goulson *et al.* 2008a). Like many other pollinators, a number of bumblebee species have suffered major declines in abundance and have undergone range contractions across much of Western Europe and North America (Fitzpatrick *et al.* 2007a; Goulson *et al.* 2008a; Williams & Osborne 2009; Goulson 2010; Cameron *et al.* 2011). Bumblebees exhibit inter-specific variation in dispersal capabilities and population differentiation (Goulson 2010; Darvill *et al.* 2010; Lepais *et al.* 2010). Combined with their ecological and economic importance, this makes them interesting for analysing patterns of gene flow and divergence. Here, we study these patterns in three species of bumblebee and investigate the extent to which sympatric cryptic species that vary along a specialist-generalist continuum, differ in their population genetic structure.

The subgenus *Bombus sensu stricto* is a widespread and commercially exploited taxon of bumblebee, comprising 17 species worldwide (Williams *et al.* 2012b), with five species found in Europe: *B. (B.) lucorum* (Linnaeus), *B. (B.) magnus* (Vogt), *B. (B.) cryptarum* (Fabricius), *B.* (*Bombus*) *terrestris* (Linnaeus) and *B. (B.) sporadicus* (Nylander). The taxonomic status of the latter two species is widely accepted but *B. lucorum*, *B. magnus* and *B. cryptarum* are morphologically indistinguishable in much of their range, which has triggered considerable debate about their species status. *Bombus magnus* and *B. cryptarum* have previously been regarded as subspecies of *B. lucorum* and are often referred to

collectively as the '*lucorum* complex', or simply synonymized to *B. lucorum* (Edwards & Jenner 2005; Benton 2006). However, studies of the COI mitochondrial gene and labial gland secretions have found discrete differences among the three taxa, which are now recognized as a cryptic species complex (Bertsch *et al.* 2005; Murray *et al.* 2008; Williams *et al.* 2012b; Carolan *et al.* 2012).

Due to the difficulties in distinguishing these three species, relatively little is known about their ecology and distribution. Some recent work has demonstrated that in the UK and Ireland, the *lucorum* complex species exhibit broadly overlapping distributions (Murray *et al.* 2008; Waters *et al.* 2010a; Williams *et al.* 2012b; Scriven *et al.* 2015; Chapter 2), with all three species found to co-occur at many locations in Great Britain (Scriven *et al.* 2015; Chapter 2). Significant differences exist in their ecology: *B. lucorum* is a very generalist species with a broad diet that exploits a wide variety of habitats. Indeed, Chapter 2 (Scriven *et al.* 2015), Murray *et al.* (2007) and Stanley *et al.* (2013) found *B. lucorum* at all locations surveyed. *Bombus cryptarum* is also a generalist forager: in the Western Isles and western Scotland it had a broader diet than *B. lucorum* (Waters *et al.* 2010a; Scriven *et al.* 2016; Chapter 3), but across the whole of Great Britain it was found foraging on 25 different plant taxa, which is considerably fewer than the 45 taxa used by *B. lucorum* (Scriven *et al.* 2015; Chapter 2). *Bombus cryptarum* also exhibits a more restricted UK distribution than *B. lucorum* (Scriven *et al.* 2015; Chapter 2). Conversely, *B. magnus* is a specialist species that predominantly forages on *Calluna vulgaris* and *Erica spp*. and therefore is found almost exclusively on heathland (Waters *et al.* 2010a; Scriven *et al.* 2015; Chapter 2). Across Great Britain, it consequently has the most restricted distribution and is also the least abundant of the three *lucorum* complex species (Scriven *et al.* 2015; Chapter 2).

In this study we use 13 microsatellite markers to characterise the genetic diversity and population structure of the three *lucorum* complex species for the first time. Using these data,

we test whether habitat specialism is associated with high or low levels of population structure indicative of differences in dispersal. Basing our study on a cryptic species complex assists the comparative approach we use to study the consequences of habitat specialization on population structure because many other aspects of the biology of these species are near-identical.

4.3 Materials and Methods

Sampling

The specimens included in this study have been used previously to assess the distribution and ecological differences of the *lucorum* complex species (see Chapter 2; Scriven *et al.* 2015). Workers, queens and males were sampled across Great Britain from June to September during the summers of 2010 and 2011 (Fig. 4.1 and Appendix 4.1). Areas were searched and all bumblebees resembling the *lucorum* complex species were caught. The mean number of individuals caught per site was 89.4 ± 12.9. As populations of these species may be continuous and overlapping, sampling sites do not represent discrete populations and there are likely to be unsampled populations between sites. Whole bees were stored in absolute ethanol at ambient temperatures.

DNA extraction and amplification

DNA was extracted from either thorax muscle or single tarsi using a Chelex® 100 protocol (Walsh *et al.* 1991) for the samples collected in 2010, and using a HotShot protocol (Truett *et al.* 2000) for the samples from 2011. In Chapter 2 (Scriven *et al.* 2015) we identified the specimens to species using RFLP data from the cytochrome oxidase 1 (CO1) gene.

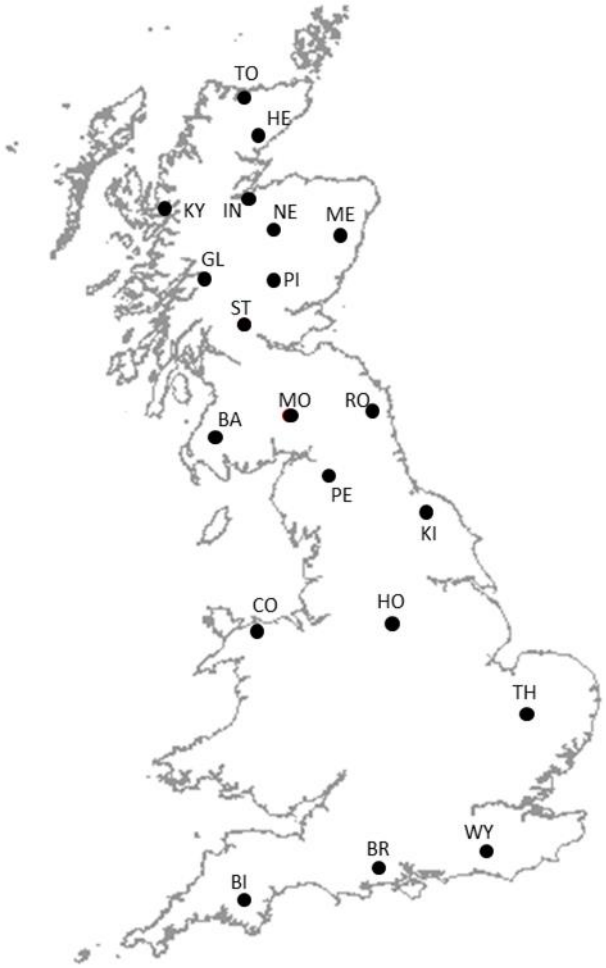


Figure 4.1. The sampling sites from which we obtained genotypes for more than one colony across all three *lucorum* complex species (see Table 4.1). Further information about the sites can be found in Appendix 4.1.

For the present study, samples were genotyped using 13 microsatellite loci in 3 multiplex reactions (Appendix 4.2). Multiplex PCRs were performed using QIAGEN Multiplex PCR Kits. Each 10µl reaction volume contained 5µl QIAGEN Multiplex Master Mix, 1µl Q-solution, 2 µl dH_20, 1µl of undiluted template DNA and 0.2µM of all primers except for B10 and B121 for which 0.4µM were used (all with the forward primer fluorescently labelled). Reactions containing primers BL03, BL06, BT10, BT24, BT09, BT26, BL11, BT18 were initially heated to 95°C for 15 minutes to activate the HotStarTaq DNA polymerase, before 40 cycles of 94°C for 30 s, 54°C for 90 s and 72°C for 90 s followed by a final extension period of 10 min at 72°C. Reactions with B121, B118, B124,

B11 and B10 also included an initial step of heating at 95°C for 15 minutes, followed by 35 cycles of 94°C for 30 s, 49°C for 90 s and 72°C for 90 s and the same final extension step. All PCR reactions were performed using both negative (water) and positive controls (DNA extracted from worker wing muscle using HotShot technique). PCR products were analysed on a 3730 automated capillary DNA sequencer (Applied Biosystems; The Sequencing Service, University of Dundee) with a 1:40 dilution before the run for multiplex group one, and a 1:80 for multiplex groups two and three. They were scored with reference to an internal size-standard (GeneScan500 ROX; Applied Biosystems Inc.) using GeneMarker software version 1.97 (SoftGenetics). Samples for which amplification was not successful were re-run: any that failed to amplify or could not be genotyped at more than five loci after repeated attempts were excluded from the analysis.

Sibling removal, Hardy-Weinberg and linkage equilibrium

To ensure the observed pattern of genetic structure was not confounded by family structure, COLONY v 2.0.4.4 (Jones & Wang 2010) was used to assign workers to colonies and remove all but one representative from each colony for subsequent analyses. This program uses maximum likelihood methods to assign sibship or parent-offspring relationships, and is the most reliable method for assigning sibship in bumblebees (Lepais *et al.* 2010).

We performed replicate genotyping of 40 random individuals and calculated an average scoring error rate of 0.006 (SD = 0.01) per locus (Pompanon *et al.* 2005). The probability of large allele dropout, scoring errors and null alleles was calculated using MICROCHECKER version 2.2.3 (Van Oosterhout *et al.* 2006) and tests for deviation from Hardy-Weinberg equilibrium and linkage disequilibrium were performed in GENEPOP

version 4.2.2. (Raymond & Rousset 1995) using a Markov chain approximation to exact tests and likelihood-ratio tests, respectively.

Inter-specific genetic differentiation

The Bayesian clustering method implemented in STRUCTURE 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003) was used to examine population structure by finding the true number (K) of clusters in our sample of individuals. Analyses were performed using the admixture model and correlated allele frequencies, with 20 000 burn-in iterations and 50 000 samples, with 10 independent runs for each value of K. Two methods were used for the selection of K: plotting (i) the negative log-likelihoods [(ln P(D)] (Pritchard *et al.* 2000) and (ii) ΔK (Evanno *et al.* 2005) vs. K using Structure Harvester (Earl & VonHoldt 2012). Principal component analysis (PCA) was also used to investigate genetic differentiation across all three species using the R package *adegenet* (ver. 3.0.2.: Jombart, 2008).

Intra-specific genetic diversity

Observed heterozygosity (H_O), expected heterozygosity (H_E), number of effective alleles (AE) and mean private allelic richness (R_P) were calculated using GenAIEx 6.5 (Peakall & Smouse 2012) across all species for each locality. These were calculated with various restrictions on the minimum number of samples per site to investigate bias due to small sample sizes. H_E, R_P and AE were compared between species with Kruskal–Wallis rank sum tests, followed by post hoc Wilcoxon rank sum tests using R version 3.0.2 (R Core Team 2014). The relationships of these genetic diversity measures with latitude, longitude and mean daily temperature between March and August were examined using Spearman rank correlation tests in R.

Population structure

Three estimators of population differentiation were calculated: F_{ST} (Weir & Cockerham 1984), G_{ST} (Nei 1973) and D_{est} (Jost 2008) using GenAIEx 6.5 (Peakall & Smouse 2006, 2012). For these measures, only populations containing at least five individuals were used. Geographic distances between populations were calculated based on Euclidean (straight-line) distances. Significance of the relationship between genetic differentiation (F_{ST}) and geographic distance (isolation by distance: IBD) was tested using Mantel tests implemented in IBDWS (Jensen *et al.* 2005). The slope of the IBD relationship indicates how strongly genetic divergence increases with geographical distance. We tested for interspecific variation in the strength of IBD by assessing overlap in the 95% confidence intervals of these slopes.

4.4 Results

Sibling removal, Hardy-Weinberg and linkage equilibrium

A total of 1,135 individual diploid individuals (queens and workers) were genotyped at 13 microsatellite loci. Of these, 13 individuals failed to amplify at more than five loci and 34 from one location consistently failed to amplify at the same five loci; all of these individuals were excluded from further analysis. Following the removal of probable full sibs, 914 individuals belonging to the three *lucorum* complex species, from 20 different sites (Fig. 4.1), were retained, each of which represented a distinct colony.

Microchecker (Van Oosterhout *et al.* 2004) results indicated only very low frequencies of null alleles (<3% per locus). With one exception, populations exhibited deviation from HWE at 1-3 loci (mean = 4.1% loci, SD = 5.8%); for *B. lucorum*, the Bargrennan site (BA) exhibited deviation from HWE at nine of the 13 loci (69%), due to significant heterozygote deficiencies. This site was therefore excluded from further intra-

specific and population structure analyses. Significant linkage disequilibrium was detected for multiple pairs of loci but this occurred in less than 10% of the populations and not consistently for any loci pairs or populations; therefore all loci were retained for the analyses.

Inter-specific genetic differentiation

Examining clustering among all the samples revealed hierarchical genetic structuring which clearly delineated the three species. Analysis of individuals of all three *lucorum* complex species revealed that K = 2 clusters best fit the sampled individuals (Appendix 4.3): one cluster encompassed all individuals previously identified from mitochondrial DNA (see Chapter 2; Scriven *et al.* 2015) as *B. lucorum,* and the second cluster comprised both *B. cryptarum* and *B. magnus* individuals (Fig. 4.2a). When only this second cluster was analysed, K = 2 was again the most appropriate number of clusters (Appendix 4.4), with each cluster representing *B. cryptarum* and *B. magnus* individuals separately (Fig. 4.2b). Similarly, principal components analysis (PCA) based on individual genotypes, grouped the individuals into three distinct clusters according to species along the first two principle coordinates (PC1 & PC2), explaining 21% of the total variation (Fig. 4.3).

Intra-specific genetic diversity

Within-population genetic diversity for each of the three bumblebee species was quantified using a variety of measures. Across populations of all three species with at least five genotyped individuals, the mean number of alleles per locus ranged from 7.7 to 10.4 and the effective number of alleles from 5.1 to 6.3. The overall level of genetic diversity for each of the three *lucorum* complex species was relatively high (Table 4.1): mean observed heterozygosity (H_O) varied from 0.69 to 0.77 and expected heterozygosity (H_E) from 0.66 to 0.77. For almost all genetic diversity estimates the rarer, specialist species, *B. magnus,*

exhibited the lowest values, whereas the most common species, *B. lucorum*, had the highest. The only exception was the number of private alleles, for which the generalist *B. lucorum* had the lowest values and *B. magnus* displayed the highest.

Based on the Kruskal-Wallis rank sum test, the three species exhibit significantly different H_E ($\chi^2_2 = 29.5$, $P < 0.001$), and number of effective alleles (AE) ($\chi^2_2 = 14.9$ $P = 0.001$) but no significant differences in the mean private allelic richness ($\chi^2_2 = 0.7$, $P = 0.7$). Post hoc Wilcoxon rank sum tests showed that H_E was significantly higher in *B. lucorum* than *B. cryptarum* (W = 4.5, $P < 0.001$) and *B. magnus* (W = 0, $P < 0.001$), and *B. cryptarum* also exhibited higher H_E than *B. magnus* (W = 90.5, $P = 0.03$; Fig. 4.4). A similar pattern was revealed for AE (Fig. 4.4) except that there was no significant difference between *B. cryptarum* and *B. magnus* (W = 61, $P = 0.9$), which both exhibit significantly lower AE than *B. lucorum* (W = 37, $P < 0.001$ and W = 19.5, $P = 0.002$ respectively). Small sample sizes at some of the sites could have influenced the patterns observed, however, we found that restricting the analyses to sites with larger numbers of individuals did not alter the patterns detected (Appendices 4.5-4.7 & 4.12).

We did not find any significant relationships for any of the genetic diversity measures with latitude, longitude or mean summer temperature. For none of the species was there significant variation between populations in these genetic diversity indices.

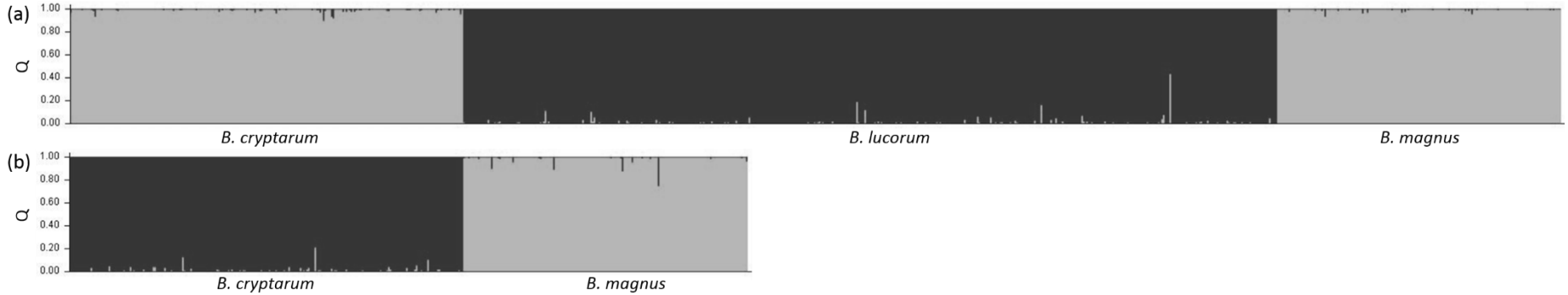


Figure 4.2. Genetic clustering of *lucorum* complex individuals. (a) Clustering using Structure version 2.3.4, assuming two genetic clusters (K = 2) for individuals of all three species of the *lucorum* complex and (b) only individuals previously identified as *B. cryptarum* and *B. magnus* using mtDNA. Each vertical bar represents an individual with the colour indicating the probability of membership to each of the two assigned clusters (Q).

Table 4.1. Population genetic parameters of workers of the three *lucorum* complex species at sampling sites with at least five individuals estimated from the analysis of 13 microsatellite loci. N, number of genotyped diploid individuals, where each individual represents a unique colony; Na, mean number of different alleles; AE, mean effective number of alleles; H_O, mean observed heterozygosity; H_E, mean expected heterozygosity; R_P, mean private allelic richness.

Species	Population	N	Na ± SE	AE ± SE	H_O ± SE	H_E ± SE	Rp ± SE
B. cryptarum		**230**	**8.21 ± 0.40**	**5.1 ± 0.26**	**0.71 ± 0.02**	**0.70 ± 0.02**	**0.2 ± 0.05**
	Tongue (TO)	19	9.54 ± 1.52	5.72 ± 1.00	0.7 ± 0.06	0.72 ± 0.05	0.23 ± 0.12
	Helmsdale (HE)	16	8.15 ± 1.21	5.18 ± 0.85	0.71 ± 0.06	0.71 ± 0.06	0.23 ± 0.12
	Kyle of Lochalsh (KY)	6	5.00 ± 0.70	3.86 ± 0.66	0.69 ± 0.08	0.62 ± 0.07	0
	Mergie (ME)	62	13.38 ± 2.48	6.42 ± 1.36	0.69 ± 0.06	0.72 ± 0.06	0.85 ± 0.25
	Glencoe (GL)	10	7.15 ± 0.86	4.53 ± 0.70	0.74 ± 0.06	0.70 ± 0.05	0.08 ± 0.08
	Stirling (ST)	16	8.85 ± 1.34	5.89 ± 1.07	0.74 ± 0.05	0.73 ± 0.05	0
	Rothbury (RO)	21	9.92 ± 1.57	5.94 ± 1.18	0.71 ± 0.06	0.72 ± 0.06	0.15 ± 0.10
	Bargrennon (BA)	8	6.46 ± 1.03	4.97 ± 0.96	0.71 ± 0.08	0.65 ± 0.07	0.08 ± 0.08
	Moffat (MO)	6	5.85 ± 0.15	4.14 ± 0.56	0.83 ± 0.04	0.70 ± 0.04	0.15 ± 0.10
	Kirkbymoorside (KI)	8	6.00 ± 0.88	4.00 ± 0.68	0.51 ± 0.08	0.60 ± 0.08	0.08 ± 0.08
	Hope (HO)	25	10.15 ± 1.54	6.29 ± 1.12	0.74 ± 0.05	0.74 ± 0.05	0
	Conwy (CO)	20	9.85 ± 1.48	6.01 ± 1.12	0.69 ± 0.06	0.72 ± 0.06	0.62 ± 0.40
	Birch Tor (BI)	13	7.00 ± 1.21	4.43 ± 0.81	0.76 ± 0.06	0.69 ± 0.05	0.08 ± 0.08
B. lucorum		**483**	**10.40 ± 0.39**	**6.30 ± 0.27**	**0.77 ± 0.01**	**0.77 ± 0.01**	**0.11 ± 0.13**
	Tongue (TO)	45	11.15 ± 1.68	6.3 ± 1.08	0.79 ± 0.04	0.77 ± 0.04	0
	Helmsdale (HE)	31	11.15 ± 1.86	6.38 ± 1.03	0.77 ± 0.04	0.78 ± 0.03	0.15 ± 0.10
	Inverness (IN)	27	10.46 ± 1.60	6.14 ± 1.10	0.71 ± 0.04	0.76 ± 0.04	0.08 ± 0.08
	Kyle of Lochalsh (KY)	20	9.23 ± 1.34	5.65 ± 1.00	0.76 ± 0.05	0.74 ± 0.04	0.08 ± 0.08
	Mergie (ME)	23	10.85 ± 1.76	6.17 ± 1.05	0.78 ± 0.03	0.77 ± 0.04	0
	Pitlochry (PI)	44	11.85 ± 2.09	7.08 ± 1.44	0.79 ± 0.04	0.77 ± 0.04	0.15 ± 0.10
	Glencoe (GL)	8	6.31 ± 0.83	4.61 ± 0.78	0.68 ± 0.05	0.72 ± 0.04	0

	Stirling (ST)	44	11.92 ± 1.84	7.11 ± 1.34	0.8 ± 0.03	0.78 ± 0.04	0.23 ± 0.12
	Rothbury (RO)	19	9.15 ± 1.35	5.96 ± 1.08	0.69 ± 0.04	0.75 ± 0.04	0.08 ± 0.08
	Moffat (MO)	27	10.15 ± 1.48	5.95 ± 0.93	0.79 ± 0.03	0.77 ± 0.03	0
	Penrith (PE)	29	10.77 ± 1.54	6.73 ± 1.20	0.79 ± 0.03	0.79 ± 0.03	0.15 ± 0.15
	Kirkbymoorside (KI)	37	11.62 ± 1.84	6.67 ± 1.15	0.78 ± 0.03	0.78 ± 0.03	0.23 ± 0.17
	Hope (HO)	17	9.38 ± 1.44	6.27 ± 1.03	0.83 ± 0.05	0.78 ± 0.03	0.23 ± 0.17
	Conwy (CO)	32	11.00 ± 1.60	6.68 ± 1.16	0.76 ± 0.04	0.79 ± 0.03	0.08 ± 0.08
	Thetford (TH)	19	10.00 ± 1.43	5.99 ± 1.02	0.77 ± 0.04	0.76 ± 0.04	0.23 ± 0.17
	Wych Cross (WY)	25	10.54 ± 1.76	6.75 ± 1.32	0.73 ± 0.04	0.77 ± 0.04	0.23 ± 0.12
	Bramshaw (BR)	36	11.23 ± 1.66	6.58 ± 1.15	0.84 ± 0.03	0.78 ± 0.04	0
B. magnus		**168**	**7.73 ± 0.46**	**5.09 ± 0.29**	**0.69 ± 0.03**	**0.66 ± 0.03**	**0.29 ± 0.09**
	Kyle of Lochalsh (KY)	37	11.62 ± 1.91	6.16 ± 1.17	0.67 ± 0.09	0.67 ± 0.09	0.85 ± 0.34
	Mergie (ME)	7	6.00 ± 0.79	4.38 ± 0.63	0.73 ± 0.09	0.66 ± 0.07	0.08 ± 0.08
	Pitlochry (PI)	16	7.85 ± 1.33	5.51 ± 1.03	0.69 ± 0.09	0.66 ± 0.09	0.23 ± 0.12
	Glencoe (GL)	46	11.46 ± 1.82	6.27 ± 1.07	0.70 ± 0.08	0.70 ± 0.08	1.08 ± 0.33
	Rothbury (RO)	6	5.54 ± 0.76	4.49 ± 0.63	0.71 ± 0.09	0.67 ± 0.07	0
	Bargrennon (BA)	5	4.92 ± 0.80	4.15 ± 0.73	0.71 ± 0.11	0.6 ± 0.09	0
	Kirkbymoorside (KI)	13	7.31 ± 1.09	5.27 ± 0.86	0.64 ± 0.09	0.68 ± 0.08	0.08 ± 0.08
	Hope (HO)	23	8.38 ± 1.35	5.12 ± 0.90	0.68 ± 0.09	0.66 ± 0.08	0.15 ± 0.15
	Birch Tor (BI)	15	6.46 ± 0.86	4.44 ± 0.59	0.69 ± 0.08	0.67 ± 0.07	0.15 ± 0.15

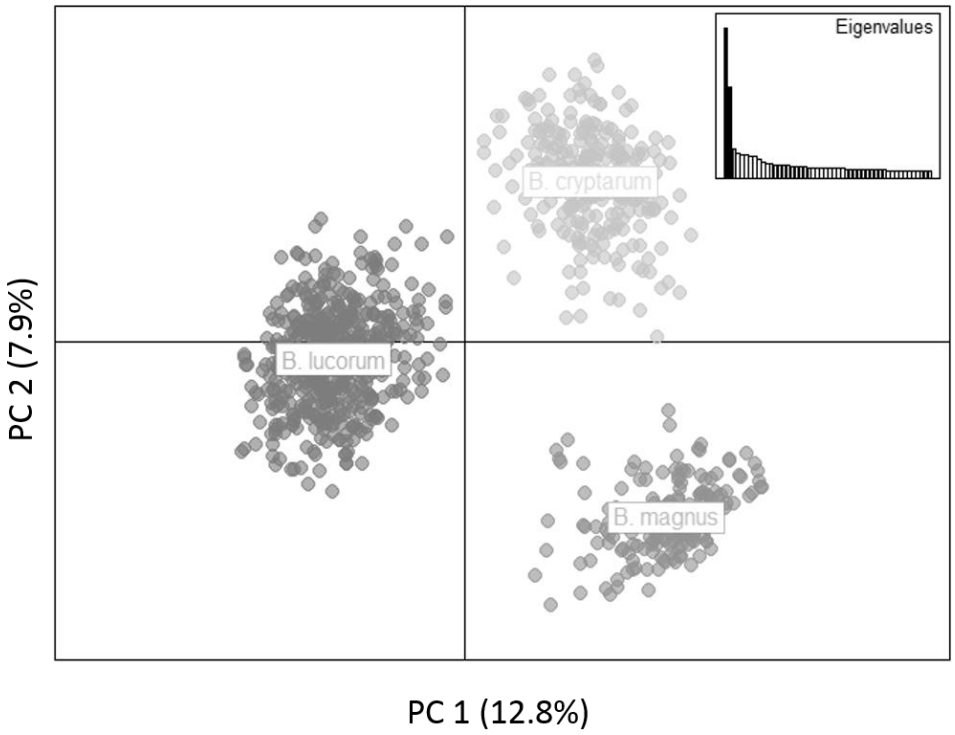


Figure 4.3. Principal component analysis (PCA) of the genetic variation between individuals of the *lucorum* complex species. The first two principal components describe 12.8% and 7.9% of the total variation respectively. Each species is represented by a different colour. The screeplot of eigenvalues is embedded showing the number of principal components retained.

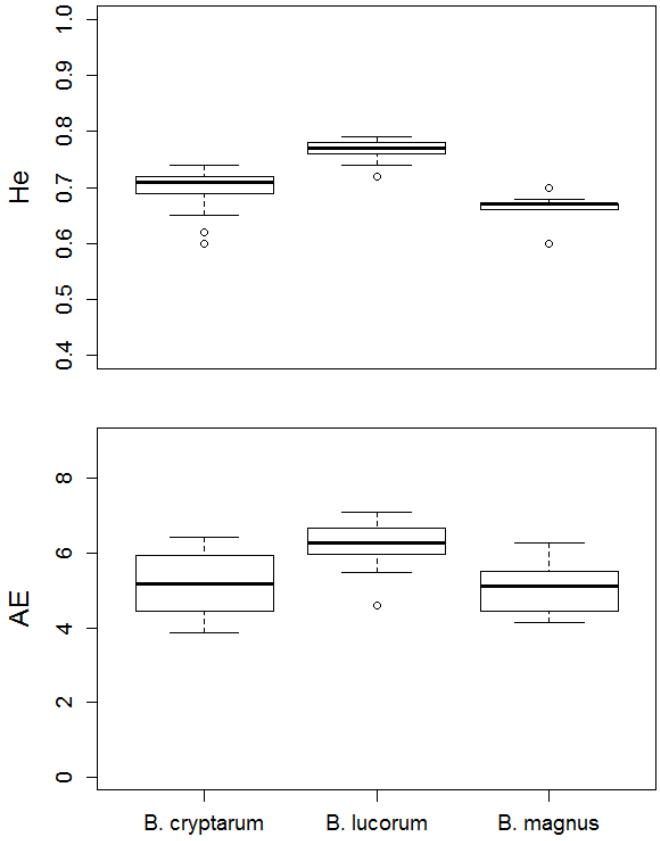


Figure 4.4. Differences in genetic diversity between *lucorum* complex species. The mean expected heterozygosity (H_e) and effective number of alleles (AE) across all loci and populations with at least five individuals varied between species. Significant differences are shown by different letters ($P < 0.01$).

Population structure

Overall, the three species show significant population structuring using all three indices ($P < 0.05$; Table 4.2). *Bombus lucorum* had the lowest value of F_{ST} and *B. cryptarum* had the highest: global F_{ST} was significantly higher in populations of *B. cryptarum* and *B. magnus* than in populations of *B. lucorum* ($\chi^2_2 = 23.9$, $P < 0.001$), there was no such pattern for the other indices. We also looked for isolation by Euclidean geographic distance (IBD; Appendix 4.11) in each species, using pairwise F_{ST} values between all populations represented by at least five colonies (Appendices 4.8-4.10). F_{ST} values among pairs of sampling locations differed significantly among the three species ($\chi^2_2 = 25.1$, $P < 0.001$): for

B. lucorum pairwise values averaged 0.005 ± 0.002 (SE), which was significantly lower than those for *B. cryptarum* (0.02 ± 0.003, W = 7322, $P < 0.001$) and *B. magnus* (0.01 ± 0.002, W = 3218, P = 0.003). *Bombus cryptarum* exhibited the greatest proportion of pairwise F_{ST} values that were significantly different from zero (35.9%), and *B. magnus* exhibited the lowest (22.2%); whereas for *B. lucorum* this figure was 24.3%. We found significant evidence for IBD for *B. cryptarum* (Fig. 4.5a. & Table 4.3), weak, non-significant evidence for IBD for *B. magnus* (Fig. 4.5c & Table 4.3) and no evidence for *B. lucorum* (Fig. 4.5b & Table 4.3). We compared the IBD slopes for *B. cryptarum* and *B. magnus* and found that the 95% confidence intervals did not overlap (Table 4.3), meaning that the slopes for these two species are significantly different: *B. cryptarum* populations exhibited the steepest slope and hence the strongest evidence for IBD.

4.5 Discussion

We studied three sympatric cryptic bumblebee species that vary along a gradient from habitat specialist to habitat generalist. Our data reveal significant interspecific differences in genetic diversity and population genetic structuring associated with interspecific variation in abundance and distribution. From the perspective of ecologically specialist species, habitats are often more heterogeneous and patchier than they are for generalists. It has been suggested that this can result in lower gene flow between populations of specialist species compared to generalists, potentially making specialists highly vulnerable to anthropogenic ecosystem fragmentation (Kelley *et al.* 2000; Brouat *et al.* 2003; Packer *et al.* 2005; Habel *et al.* 2009, 2010).

Table 4.2. Population genetics summary statistics for the three *lucorum* complex species, for all sites with at least five colonies represented. Three measures of global population structure (F_{ST}, G_{ST} & Dest) are shown with 95% confidence intervals and significance values from tests against zero (*P*). Significant values are shown in italics ($P < 0.05$).

Species	Sites	F_{ST} (95% CI)	*P*	G_{ST} (95% CI)	*P*	Dest (95% CI)	*P*
B. cryptarum	All sites	0.052 (0.044-0.063)	*0.002*	0.008 (0.001-0.017)	*0.003*	0.024 (0.004-0.043)	*0.004*
B. lucorum	All sites	0.025 (0.023-0.027)	*0.004*	0.003 (0.001-0.006)	*0.004*	0.013 (0.004-0.027)	*0.004*
B. magnus	All sites	0.048 (0.044-0.052)	*0.035*	0.007 (0.002-0.011)	*0.023*	0.018 (0.005-0.04)	*0.015*

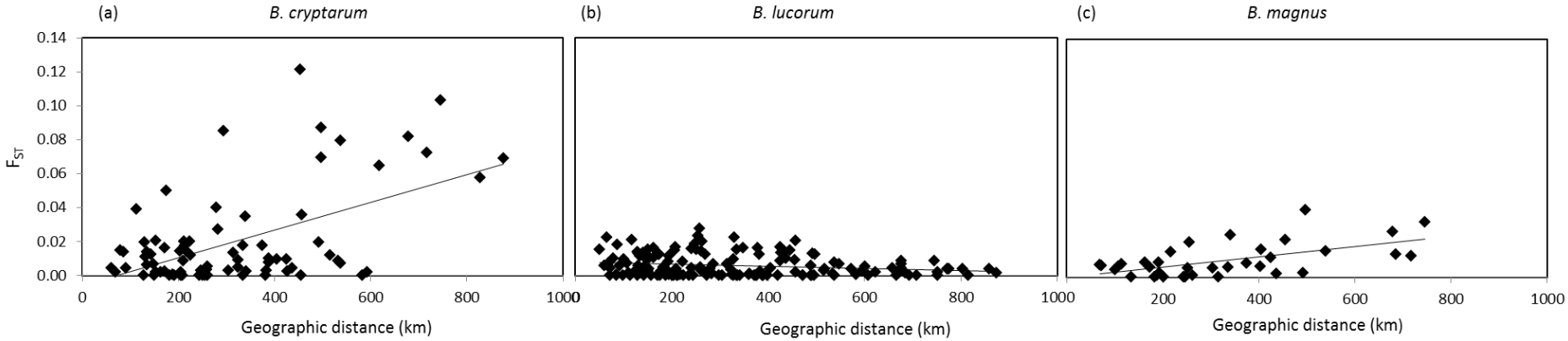


Figure 4.5. Isolation by distance (IBD) for (a) *B. cryptarum*, (b) *B. lucorum* and (c) *B. magnus*. Pairwise comparisons of genetic differentiation (F_{ST}) as a function of geographic distance for individuals from all locations with at least five individuals.

Table 4.3. Results of Mantel tests conducted to test for isolation by distance (IBD) for the three *lucorum* complex species between all sites with at least five individuals. Significant results are shown in italics ($P < 0.05$).

Species	Sites	Mantel r	*P*	IBD slope	95% CI
B. cryptarum	All sites	0.541	*0.042*	0.00015	0.00012 - 0.00018
B. magnus	All sites	0.567	0.053	0.000052	0.00004 - 0.00007
B. lucorum	All sites	-0.203	0.953	-0.000032	-0.00004 - -0.00003

Our results challenge this notion, demonstrating that the rarer heathland specialist species, *B. magnus*, exhibits less population structure across large spatial scales than the moderate generalist species, *B. cryptarum*.

Generalist species often tend to have high genetic diversities (Brouat *et al.* 2004; Packer *et al.* 2005; Louy *et al.* 2007; Li *et al.* 2014); we found this to be true for *B. lucorum*, which is a very widespread generalist species in Great Britain (Scriven *et al.* 2015; Chapter 2). All populations of *B. lucorum* had high expected heterozygosity values, comparable to levels found in other widespread and common bumblebees, such as *B. terrestris* in the UK (Moreira *et al.* 2015) and *B. bifarius* in North America (Lozier *et al.* 2011). However, we found significantly lower allelic richness and expected heterozygosity in *B. cryptarum* compared with *B. lucorum*. *Bombus cryptarum* is also a generalist species; in Great Britain, it feeds on a wide variety of plant species and is found in many different habitat types, but it has a more restricted distribution and a slightly narrower diet than *B. lucorum* (Waters *et al.* 2010a; Stanley *et al.* 2013b; Scriven *et al.* 2015; Chapter 2).

Specialist species could be expected to suffer from low genetic diversity, as they may comprise small sub-populations inhabiting discontinuous patchy habitats (Habel *et al.* 2009). *Bombus magnus* relies almost exclusively on heathland habitat and is the least abundant *lucorum* complex species in Great Britain (Scriven *et al.* 2015; Chapter 2). Accordingly, we found that *B. magnus* exhibited the lowest levels of genetic variation. Molecular studies of

Bombus species have shown that rare and declining species often tend to have reduced genetic variation (Ellis *et al.* 2006b; Charman *et al.* 2010; Darvill *et al.* 2010; Cameron *et al.* 2011; Lozier *et al.* 2011). *Bombus magnus* had higher genetic diversity than some bumblebee species that have become very rare in the UK, such as *B. distinguendus* (H_E = 0.39; Charman *et al.* 2010) and *B. muscuorum* (H_E = 0.43-0.51; Darvill *et al.* 2006, 2010), but lower than some other declining species, including *B. ruderatus* (H_E = 0.75; Dreier *et al.* 2014).

Habitat specialist species have been suggested to exhibit higher population differentiation than generalists due to reduced gene flow, resulting from patchier distribution and poorer dispersal ability (Colas *et al.* 1997; Kelley *et al.* 2000; Brouat *et al.* 2003; Packer *et al.* 2005; Louy *et al.* 2007; Groot *et al.* 2011). However, our results do not support this theory because although the specialist species, *B. magnus*, exhibited higher population genetic differentiation than *B. lucorum* (the extreme generalist), there was no significant difference in population structure between *B. magnus* and the moderate generalist *B. cryptarum*. In fact, *B. cryptarum* showed the highest level of differentiation for all three measures of population structure used, and *B. cryptarum* populations had a greater range of pairwise differentiation (F_{ST}) values than *B. magnus*. Furthermore, isolation by distance was stronger between populations of *B. cryptarum* than *B. magnus*, which could indicate that *B. magnus* may have greater dispersal ability than *B. cryptarum*. These findings indicate that specialist species may be capable of maintaining effective genetic exchange across large spatial scales and that reliance on fragmented habitat does not necessarily lead to increased population structuring.

Our conclusion could be challenged because the absence of population structure is not always indicative of contemporary gene flow. There is a time lag after population fragmentation before the population reaches a new equilibrium between migration and drift. Therefore, in cases of recent anthropogenic habitat loss, low population structure may

actually reflect gene flow that predates recent environmental disturbance (Whitlock & McCauley 1999). This might potentially explain the low population structure observed in *B. magnus* because the heathland habitat to which it is tied, previously covered several million hectares in Western Europe and has more recently suffered severe losses (Thompson *et al.* 1995; Rose *et al.* 2000; Fagúndez 2013). Nevertheless, despite this anthropogenically driven contraction of heathland, this habitat has existed in a fragmented patchwork in Western Europe throughout much of the Holocene (reviewed in Fagundez 2013); therefore it seems likely that *B. magnus* has had to contend with dispersal through a highly heterogeneous ecological network for much of its post-glacial European history.

Bumblebee species exhibit differences in both foraging and dispersal distances (Knight *et al.* 2005; Carvell *et al.* 2012; Jha & Kremen 2013b; Wood *et al.* 2015; Redhead *et al.* 2016). Some species, including both common and declining species, are relatively sedentary (Darvill *et al.* 2010; Goulson *et al.* 2011), whilst others show more elevated dispersal abilities: *B. pascuorum* showed no genetic structuring between populations spanning the whole of the UK (Ellis *et al.* 2006b), and in America several species, including *B. vosnesenskii,* showed very low population differentiation over large spatial scales (>1500 km; Lozier *et al.* 2011). Like *B. magnus, B. jonellus* and *B. monticola* are also associated with heathland to varying extents: *B. jonellus* is a heathland species in the north of the UK (Goulson *et al.* 2006; Darvill *et al.* 2010) and *B. monticola* is described as associated with upland heaths and moors (Edwards & Jenner 2005; Benton 2006). In a study on islands in the Hebrides (Scotland) Darvill *et al.* (2010) found that *B. jonellus* had a higher propensity to disperse than *B. muscorum*, a more generalist species, and speculated that this was due to the association of *B. jonellus* with a fragmented habitat. Similarly, *B. monticola* has recently colonised Ireland (Fitzpatrick *et al.* 2007a), an unusual event, as colonisations are rare among bumblebees, which suggests that *B. monticola* can also disperse over long distances.

Therefore, as a heathland specialist, *B. magnus* may have always existed in a spatially patchy metapopulation with colonisation of new patches requiring dispersal over areas of unsuitable habitat. This specialisation could have selected for the evolution of relatively high dispersal ability in *B. magnus*. Evidence from other organisms supports this theory: for example, another heathland specialist, the solitary bee *Andrena fuscipes*, showed little geographic structure and population differentiation (Exeler *et al.* 2010). Studies of other organisms, such as riverine fish (Ginson *et al.* 2015), salamanders (Feist *et al.* 2014), fig wasps (Zavodna *et al.* 2005) and bark beetles (Sallé *et al.* 2007) also suggest that specialist species can evolve dispersal strategies that overcome habitat fragmentation and compensate for the scarcity of suitable habitat in the landscape.

If broad generalist species maintain gene flow by virtue of their high abundance, tolerance of a broad range of environments and thus ubiquitous distributions, whilst specialist species can be well adapted for dispersal in patchy habitat landscapes, it may be species showing intermediate characteristics along the specialist-generalist continuum that suffer the greatest threats from habitat fragmentation. Studies on butterflies show that moderate generalist species are more endangered than would be predicted based on their degree of specialisation (Habel & Schmitt 2012) and perform less well than either extreme generalists or true specialists in response to environmental changes (Dapporto & Dennis 2013). Similarly, our study of morphologically near-identical bumblebee species showed that despite being more common, the distance between populations had a greater impact on population differentiation for the moderate generalist than for the habitat-restricted specialist, suggesting that *B. cryptarum* could be more sensitive to loss and fragmentation of habitat than anticipated. Moderate generalists may require high habitat connectivity to maintain gene flow between populations and prevent inbreeding, making them the most sensitive to environmental change (Habel & Schmitt 2012).

Previous molecular work on the species status of the *lucorum* complex species has focussed on divergence in mitochondrial DNA sequences (Bertsch *et al.* 2005; Murray *et al.* 2008; Williams *et al.* 2012b; Carolan *et al.* 2012). However, the use of mitochondrial DNA barcodes for species delimitation and identification has been strongly criticised (Will & Rubinoff 2004; DeSalle *et al.* 2005; Taylor & Harris 2012). Our present study, the first investigation of these species using highly variable nuclear markers, confirms that the discrete divergence between the three species in mtDNA is mirrored by nuclear DNA differentiation. Furthermore, the clustering results we obtained suggest that *B. cryptarum* and *B. magnus* are more closely related to each other than they are to *B. lucorum*. Whilst we have not explicitly tested for inter-specific hybridisation, our data provide no compelling evidence for frequent hybridisation within the *lucorum* complex.

Here we characterise the population genetics of the *lucorum* complex species for the first time and provide the first indication of differences in their population structure. This study reveals that sympatric cryptic species with near-identical morphology exhibit dramatic differences in population structure and genetic variation. Unidentified cryptic species are likely to be present within other keystone or endangered taxa. Our data demonstrate the importance of identifying ecological and evolutionary patterns in the population structure of cryptic species. We detected low levels of population structuring in a specialist species. This suggests that, contrary to some theory, habitat specialist species may have evolved to be effective dispersers. This could enable habitat specialists to maintain population connectivity, potentially making them well adapted to persist in, and to recolonise, habitat patches in fragmented landscapes.

4.6 Acknowledgements

We thank Andreia Penado, James Morrison and Carolyn Goldie for help collecting samples and Mairi Knight for providing further samples. We are also grateful to Lucy Woodall for assisting with DNA extractions, as well as Steph O'Connor for advice on fieldwork and analysis. The project was supported by a University of Stirling PhD studentship to JJS. MA and PRW were supported by the University of Stirling Impact Fellow program.

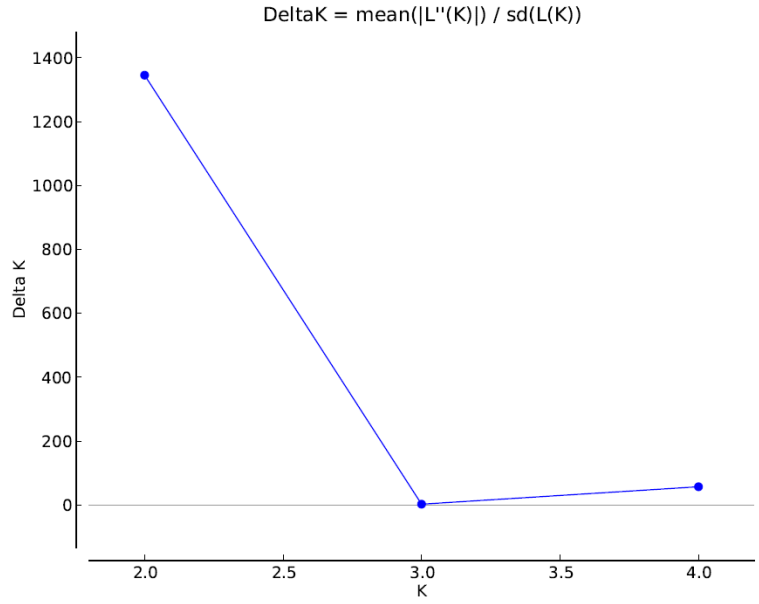
4.7 Appendix

Appendix 4.1. Details of all sampling locations for which reliable genotypes were obtained for more than one colony. The location abbreviations are used on the map in Figure 1.

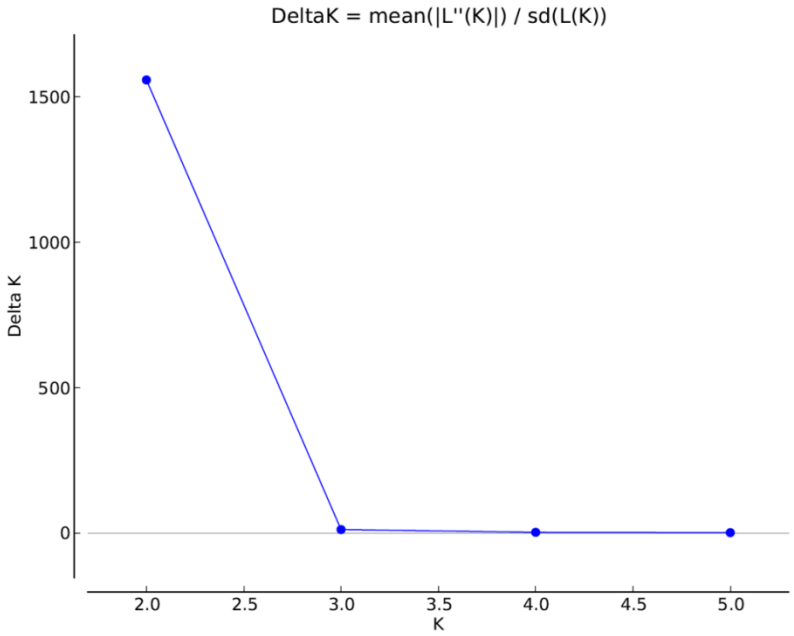
Location	Abbreviation	No. species	No. genotyped diploids	Sampling year	Latitude	Longitude
Tongue	TO	3	77	2010	58.49	-4.43
Helmsdale	HE	2	62	2010	58.05	-4.46
Inverness	IN	2	39	2010	57.49	-5.46
Kyle of Lochalsh	KY	3	76	2011	57.26	-3.27
Nethy Bridge	NE	2	2	2011	57.23	-3.68
Mergie	ME	3	98	2011	57	-2.32
Pitlochry	PI	3	65	2010	56.77	-3.91
Glencoe	GL	3	68	2011	56.67	-3.83
Stirling	ST	3	83	2010	56.17	-4.43
Rothbury	RO	3	50	2011	55.32	-1.74
Bargrennon	BA	3	34	2011	55.06	-5.09
Moffat	MO	2	84	2010	55.06	-1.99
Penrith	PE	2	48	2010	54.69	-3.93
Kirkbymoorside	KI	3	72	2011	54.28	-1.72
Hope	HO	3	70	2011	53.37	-2.8
Conwy	CO	3	66	2011	53.26	-0.91
Thetford	TH	2	24	2011	52.41	-4.52
Wych Cross	WY	1	26	2011	51.07	0.82
Bramshaw	BR	1	38	2011	50.92	-3.86
Birch Tor	BI	3	33	2011	50.61	-3.87

Appendix 4.2. Multiplex groups for microsatellite analysis

Multiplex group	Locus	Source
1	B11	Estoup *et al.* 1995 & 1996
	B118	Estoup *et al.* 1995 & 1996
	B121	Estoup *et al.* 1995 & 1996
	B10	Estoup *et al.* 1995 & 1996
	B124	Estoup *et al.* 1995 & 1996
2	BT26	Funk *et al.* 2006
	BT09	Funk *et al.* 2006
	BL11	Funk *et al.* 2006
	BT18	Funk *et al.* 2006
3	BL03	Funk *et al.* 2006
	BL06	Funk *et al.* 2006
	BT10	Funk *et al.* 2006
	BT24	Funk *et al.* 2006



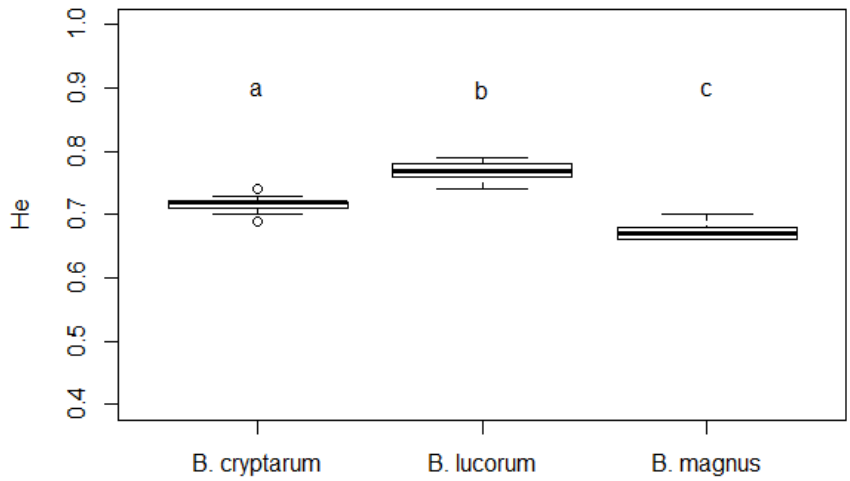
Appendix 4.3. The number of clusters (K) = 2 best fit the data obtained from samples of *B. cryptarum, B. lucorum* and *B. magnus* individuals, as shown by the plot of ΔK, an ad hoc statistic used by Evanno et al (2003) to determine the number of K groups.



Appendix 4.4. Plot of ΔK, an ad hoc statistic used by Evanno et al (2003) to determine the number of K groups that best fit the data, obtained from samples of *B. cryptarum* individuals, shows that K = 2.

Appendix 4.5. Population genetic parameters of workers of the three *lucorum* complex species at sites across Great Britain with at least ten individuals, estimated from the analysis of 13 microsatellite loci. N, number of genotyped diploid individuals, where each individual represents a unique colony; Na, mean number of different alleles; AE, mean effective number of alleles; H_O, mean observed heterozygosity; H_E, mean expected heterozygosity; R_P, mean private allelic richness.

Sampling location	N	Na ± SE	AE ± SE	H_O ± SE	H_E ± SE	Rp ± SE
B. cryptarum	**202**	**9.3 ± 0.51**	**5.6 ± 0.34**	**0.72 ± 0.02**	**0.72 ± 0.02**	**0.25 ± 0.06**
Tongue (TO)	19	9.54 ± 1.52	5.72 ± 1.00	0.7 ± 0.06	0.72 ± 0.05	0.23 ± 0.12
Helmsdale (HE)	16	8.15 ± 1.21	5.18 ± 0.85	0.71 ± 0.06	0.71 ± 0.06	0.23 ± 0.12
Mergie (ME)	62	13.38 ± 2.48	6.42 ± 1.36	0.69 ± 0.06	0.72 ± 0.06	0.85 ± 0.25
Glencoe (GL)	10	7.15 ± 0.86	4.53 ± 0.70	0.74 ± 0.06	0.70 ± 0.05	0.08 ± 0.08
Stirling (ST)	16	8.85 ± 1.34	5.89 ± 1.07	0.74 ± 0.05	0.73 ± 0.05	0
Rothbury (RO)	21	9.92 ± 1.57	5.94 ± 1.18	0.71 ± 0.06	0.72 ± 0.06	0.15 ± 0.10
Hope (HO)	25	10.15 ± 1.54	6.29 ± 1.12	0.74 ± 0.05	0.74 ± 0.05	0
Conwy (CO)	20	9.85 ± 1.48	6.01 ± 1.12	0.69 ± 0.06	0.72 ± 0.06	0.62 ± 0.40
Birch Tor (BI)	13	7.00 ± 1.21	4.43 ± 0.81	0.76 ± 0.06	0.69 ± 0.05	0.08 ± 0.08
B. lucorum	**489**	**10.49 ± 0.39**	**6.35 ± 0.26**	**0.76 ± 0.01**	**0.77 ± 0.01**	**0.11 ± 0.03**
Tongue (TO)	45	11.15 ± 1.68	6.3 ± 1.08	0.79 ± 0.04	0.77 ± 0.04	0
Helmsdale (HE)	31	11.15 ± 1.86	6.38 ± 1.03	0.77 ± 0.04	0.78 ± 0.03	0.15 ± 0.10
Inverness (IN)	27	10.46 ± 1.60	6.14 ± 1.10	0.71 ± 0.04	0.76 ± 0.04	0.08 ± 0.08
Kyle of Lochalsh (KY)	20	9.23 ± 1.34	5.65 ± 1.00	0.76 ± 0.05	0.74 ± 0.04	0.08 ± 0.08
Mergie (ME)	23	10.85 ± 1.76	6.17 ± 1.05	0.78 ± 0.03	0.77 ± 0.04	0
Pitlochry (PI)	44	11.85 ± 2.09	7.08 ± 1.44	0.79 ± 0.04	0.77 ± 0.04	0.15 ± 0.10
Stirling (ST)	44	11.92 ± 1.84	7.11 ± 1.34	0.8 ± 0.03	0.78 ± 0.04	0.23 ± 0.12
Rothbury (RO)	19	9.15 ± 1.35	5.96 ± 1.08	0.69 ± 0.04	0.75 ± 0.04	0.08 ± 0.08
Bargrennon (BA)	14	7.85 ± 1.06	5.48 ± 0.79	0.58 ± 0.05	0.76 ± 0.04	0
Moffat (MO)	27	10.15 ± 1.48	5.95 ± 0.93	0.79 ± 0.03	0.77 ± 0.03	0
Penrith (PE)	29	10.77 ± 1.54	6.73 ± 1.20	0.79 ± 0.03	0.79 ± 0.03	0.15 ± 0.15
Kirkbymoorside (KI)	37	11.62 ± 1.84	6.67 ± 1.15	0.78 ± 0.03	0.78 ± 0.03	0.23 ± 0.17
Hope (HO)	17	9.38 ± 1.44	6.27 ± 1.03	0.83 ± 0.05	0.78 ± 0.03	0.23 ± 0.17
Conwy (CO)	32	11.00 ± 1.60	6.68 ± 1.16	0.76 ± 0.04	0.79 ± 0.03	0.08 ± 0.08
Thetford (TH)	19	10.00 ± 1.43	5.99 ± 1.02	0.77 ± 0.04	0.76 ± 0.04	0.23 ± 0.17
Wych Cross (WY)	25	10.54 ± 1.76	6.75 ± 1.32	0.73 ± 0.04	0.77 ± 0.04	0.23 ± 0.12
Bramshaw (BR)	36	11.23 ± 1.66	6.58 ± 1.15	0.84 ± 0.03	0.78 ± 0.04	0
B. magnus	**150**	**8.84 ± 0.61**	**5.47 ± 0.39**	**0.68 ± 0.03**	**0.67 ± 0.03**	**0.42 ± 0.13**
Kyle of Lochalsh (KY)	37	11.62 ± 1.91	6.16 ± 1.17	0.67 ± 0.09	0.67 ± 0.09	0.85 ± 0.34
Pitlochry (PI)	16	7.85 ± 1.33	5.51 ± 1.03	0.69 ± 0.09	0.66 ± 0.09	0.23 ± 0.12
Glencoe (GL)	46	11.46 ± 1.82	6.27 ± 1.07	0.70 ± 0.08	0.70 ± 0.08	1.08 ± 0.33
Kirkbymoorside (KI)	13	7.31 ± 1.09	5.27 ± 0.86	0.64 ± 0.09	0.68 ± 0.08	0.08 ± 0.08
Hope (HO)	23	8.38 ± 1.35	5.12 ± 0.90	0.68 ± 0.09	0.66 ± 0.08	0.15 ± 0.15
Birch Tor (BI)	15	6.46 ± 0.86	4.44 ± 0.59	0.69 ± 0.08	0.67 ± 0.07	0.15 ± 0.15



Appendix 4.6. Differences in genetic diversity between *lucorum* complex species. The mean expected heterozygosity (H_e) across all loci and populations with more than ten individuals varied between species. Significant differences are shown by different letters ($P < 0.01$).

Appendix 4.7. Population genetics summary statistics for the three *lucorum* complex species, including all sites with more at least ten colonies represented. Three measures of global population structure (F_{ST}, G_{ST} & Dest) are shown with 95% confidence intervals and significance values from tests against zero (*P*). Significant values are shown in italics ($P < 0.05$).

Species	**F_{ST} (95% CI)**	***P***	**G_{ST} (95% CI)**	***P***	**Dest (95% CI)**	***P***
B. cryptarum	0.035 (0.029-0.042)	*0.001*	0.004 (-0.001-0.009)	*0.034*	0.012 (-0.003-0.027)	*0.031*
B. lucorum	0.023 (0.021-0.025)	*0.001*	0.004 (0.002-0.006)	*0.001*	0.014 (0.006-0.024)	*0.001*
B. magnus	0.03 (0.026-0.034)	*0.001*	0.008 (0.004-0.011)	*0.001*	0.021 (0.008-0.051)	*0.001*

Appendix 4.8. Population genetic structuring in *B. cryptarum*. Pairwise F_{ST} values between all population pairs with at least five individuals. Significant differentiation shown in bold ($P < 0.05$).

	ME	**BI**	**BA**	**GL**	**HE**	**KY**	**MO**	**RO**	**CO**	**KI**	**HO**	**ST**	**TO**
Mergie (ME)	0.000												
Birch Tor (BI)	0.072	0.000											
Bargrennon (BA)	0.000	**0.069**	0.000										
Glencoe (GL)	0.003	**0.082**	0.000	0.000									
Helmsdale (HE)	**0.007**	**0.057**	0.000	**0.016**	0.000								
Kyle of Lochalsh (KY)	0.000	**0.103**	0.000	0.002	0.011	0.000							
Moffat (MO)	**0.020**	**0.087**	0.015	0.020	0.018	**0.040**	0.000						
Rothbury (RO)	0.001	**0.079**	0.002	0.000	0.005	0.003	0.014	0.000					
Conwy (CO)	0.002	**0.085**	0.000	0.010	0.008	0.000	0.014	0.004	0.000				
Kirkbymoorside (KI)	**0.013**	**0.121**	0.003	0.018	**0.036**	0.004	**0.050**	0.014	0.012	0.000			
Hope (HO)	**0.010**	**0.035**	0.000	0.009	0.007	**0.019**	0.018	**0.014**	**0.012**	**0.039**	0.000		
Stirling (ST)	**0.006**	**0.065**	0.000	0.004	0.008	**0.021**	**0.020**	0.002	**0.009**	**0.027**	0.003	0.000	
Tongue (TO)	0.000	**0.069**	0.000	0.002	0.004	0.000	0.008	0.003	0.000	0.012	0.002	0.005	0.000

Appendix 4.9. Population genetic structuring in *B. lucorum*. Pairwise F_{ST} values between all population pairs with at least five individuals. Significant differentiation shown in bold ($P < 0.05$).

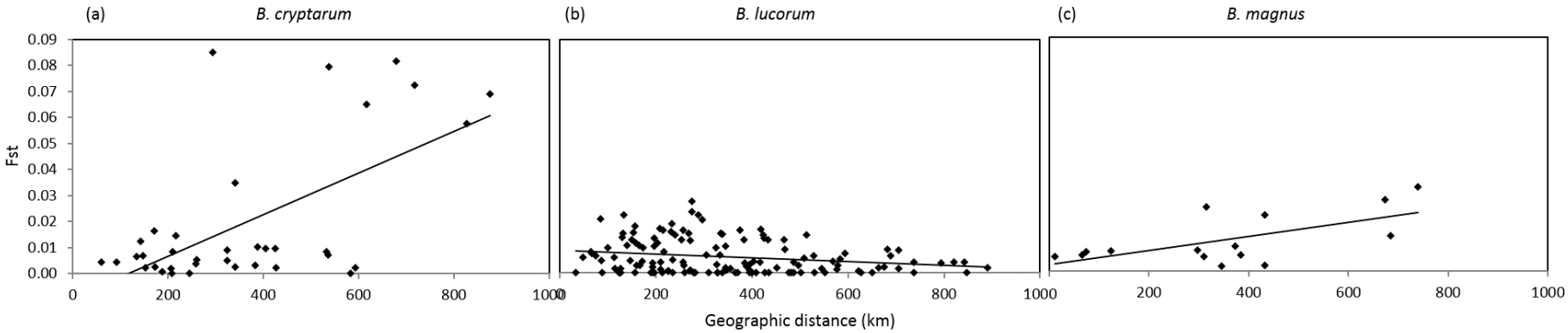
Site	ME	WY	TH	GL	HE	IN	KY	MO	RO	BR	CO	KI	HO	PE	PI	ST	TO
Mergie (ME)	0.000																
Wych Cross (WY)	0.007	0.000															
Thetford (TH)	0.007	0.000	0.000														
Glencoe (GL)	0.013	0.000	0.000	0.000													
Helmsdale (HE)	**0.010**	0.000	0.000	0.000	0.000												
Inverness (IN)	**0.013**	0.004	0.004	0.000	0.005	0.000											
Kyle of Lochalsh (KY)	**0.008**	0.004	0.002	0.007	0.006	**0.022**	0.000										
Moffat (MO)	**0.008**	0.000	0.000	0.000	0.000	0.002	0.004	0.000									
Rothbury (RO)	0.000	0.000	0.000	0.000	0.001	0.007	0.000	0.000	0.000								
Bramshaw (BR)	**0.009**	0.003	0.004	0.004	0.004	0.000	**0.009**	0.000	0.006	0.000							
Conwy (CO)	**0.013**	0.001	0.000	0.000	0.001	0.000	**0.009**	0.000	0.005	0.000	0.000						
Kirkbymoorside (KI)	**0.007**	0.001	0.000	0.000	0.002	0.000	**0.010**	0.000	0.002	0.000	0.000	0.000					
Hope (HO)	0.004	0.002	0.000	0.009	0.000	0.000	**0.013**	0.000	0.001	0.000	0.000	0.000	0.000				
Penrith (PE)	**0.028**	**0.015**	**0.016**	**0.020**	**0.016**	**0.009**	**0.022**	**0.015**	**0.018**	**0.017**	**0.013**	**0.013**	**0.010**	0.000			
Pitlochry (PI)	**0.010**	0.001	0.004	0.000	0.005	0.001	**0.007**	0.001	0.001	0.000	0.000	0.000	0.004	0.016	0.000		
Stirling (ST)	**0.011**	0.002	0.004	0.006	0.004	0.003	**0.010**	0.000	0.004	0.005	0.003	0.001	0.000	0.003	**0.006**	0.000	
Tongue (TO)	**0.017**	0.002	0.002	0.002	0.006	0.001	**0.015**	0.000	0.002	0.004	0.001	0.003	0.003	**0.014**	0.002	0.003	0.000

Appendix 4.10. Population genetic structuring in *B. magnus*. Pairwise F_{ST} values between all population pairs with at least five individuals. Significant differentiation shown in bold ($P < 0.05$).

	ME	**BI**	**BA**	**GL**	**KY**	**RO**	**KI**	**HO**	**PI**
Mergie (ME)	0.000								
Birch Tor (BI)	0.012	0.000							
Bargrennon (BA)	0.021	**0.040**	0.000						
Glencoe (GL)	0.006	**0.026**	0.000	0.000					
Kyle of Lochalsh (KY)	0.009	**0.032**	0.005	**0.007**	0.000				
Rothbury (RO)	0.001	0.015	0.009	0.000	0.005	0.000			
Kirkbymoorside (KI)	0.000	**0.022**	0.000	0.008	0.002	0.000	0.000		
Hope (HO)	0.016	**0.025**	0.001	**0.011**	0.002	0.015	0.008	0.000	
Pitlochry (PI)	0.004	**0.014**	0.002	0.007	0.006	0.000	0.006	0.006	0.000

Appendix 4.11. Pairwise distances (km) between all the populations used to determine population differentiation.

	ME	WY	BI	TH	BA	GL	HE	IN	KY	MO	RO	BR	CO	KI	HO	PE	PI	ST	TO
Mergie (ME)	0																		
Wych Cross (WY)	690	0																	
Birch Tor (BI)	717	333	0																
Thetford (TH)	529	396	205	0															
Bargrennon (BA)	275	593	501	297	0														
Glencoe (GL)	99	693	674	476	195	0													
Helmsdale (HE)	174	847	828	627	335	158	0												
Inverness (IN)	197	821	772	568	271	134	86	0											
Kyle of Lochalsh (KY)	64	737	740	545	269	73	113	134	0										
Moffat (MO)	216	481	511	338	197	213	365	345	257	0									
Rothbury (RO)	190	502	542	371	215	199	347	333	236	32	0								
Bramshaw (BR)	683	328	34	172	468	639	794	738	705	477	509	0							
Conwy (CO)	425	270	357	260	338	423	578	551	469	213	235	329	0						
Kirkbymoorside (KI)	305	395	433	278	233	298	453	427	345	89	116	400	125	0					
Hope (HO)	404	355	316	157	240	373	531	488	433	195	227	282	126	123	0				
Penrith (PE)	275	513	454	256	85	220	375	325	288	131	156	419	254	150	164	0			
Pitlochry (PI)	100	705	685	486	204	12	146	123	66	225	211	650	435	310	385	231	0		
Stirling (ST)	159	663	619	418	130	67	210	160	140	197	193	584	395	271	328	167	75	0	
Tongue (TO)	208	890	877	676	383	206	49	127	153	409	389	843	622	497	578	424	194	259	0



Appendix 4.12. Isolation by distance (IBD) for (a) *B. cryptarum*, (b) *B. lucorum* and (c) *B. magnus*. Pairwise comparisons of genetic differentiation (F_{ST}) as a function of geographic distance for individuals from all locations with at least ten individuals.

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Chapter 5

Bergmann's Body Size Rule Operates in Facultatively Endothermic Insects: Evidence from a Complex of Cryptic Bumblebee Species

A version of this chapter has been published in PlosOne as:

Scriven, J.J., Whitehorn, P.R., Goulson, D. and Tinsley, M.C. (2016) Bergmann's Body Size Rule Operates in Facultatively Endothermic Insects: Evidence from a Complex of Cryptic Bumblebee Species. PLoS ONE 11(10): e0163307. doi:10.1371/journal.pone.0163307

P. Whitehorn, D. Goulson and M. Tinsley all supervised this project and commented on draft versions of this manuscript.

5.1 Abstract

According to Bergmann's rule we expect species with larger body size to inhabit locations with a cooler climate, where they may be well adapted to conserve heat and resist starvation. This rule is generally applied to endotherms. In contrast, body size in ectothermic invertebrates has been suggested to follow the reverse ecogeographic trend: these converse Bergmann's patterns may be driven by the ecological constraints of shorter season length and lower food availability in cooler high latitude locations. Such patterns are particularly common in large insects due to their longer development times. As large and facultatively endothermic insects, bumblebees could thus be expected to follow either trend. In this investigation, we studied body size of three bumblebee species over a large spatial area and explored whether interspecific trends in body size correspond to differences in their distribution consistent with either Bergmann's or a converse Bergmann's rule. We examined the body size of queens, males and workers of the *Bombus lucorum*-complex of cryptic bumblebee species from across the whole of Great Britain. We found interspecific differences in body size corresponding to Bergmann's rule: queens and males of the more northerly distributed, cool-adapted, species were largest. In contrast, the mean body size of the worker caste did not vary between the three species. These differences in body size may have evolved under selection pressures for thermoregulation or starvation resistance. We suggest that this case study in facultatively endothermic insects may help clarify the selection pressures governing Bergmann rule trends more generally.

5.2 Introduction

The study of large-scale spatial variation in organismal traits has long been of interest to biologists, especially those studying ecology and evolution. Animal body size represents one of the most important quantitative traits as it strongly affects both physiology and fitness (Blanckenhorn & Demont 2004). Several ecogeographic rules describing correlations between morphological variation and ecological features have been formulated (Mayr 1956). Perhaps the best known is Bergmann's rule, which predicts that endothermic vertebrate species inhabiting cooler climates will be larger than related species from warmer climates (Bergmann 1847). Bergmann originally used this rule to describe interspecific trends but it was later redefined to explain intraspecific variation (Bergmann 1847; Mayr 1956, 1963; James 1970a). This rule is generally applied to endothermic organisms and has been shown to hold for many species of bird and mammal (Blackburn *et al.* 1999; Ashton *et al.* 2000; Meiri & Dayan 2003). The mechanism first proposed to explain these patterns was that in endotherms heat generation capacity increases with body volume, whereas heat loss increases with surface area; larger organisms, with relatively lower surface area, are therefore favoured in cooler environments (Bergmann 1847; Mayr 1963).

Bergmann's rule has also been demonstrated to apply to some groups of ectotherms, but not consistently, and where it does occur the mechanisms behind the trend may be different (Ashton & Feldman 2003; Blanckenhorn & Demont 2004; Tesche & Hodges 2015). Ectotherms rely on heat from their environment to thermoregulate: large bodied organisms will absorb heat more slowly than smaller organisms, which could be a disadvantage in cooler climates. On the other hand, small animals may overheat more easily in hot environments (reviewed in (Aragon & Fitze 2014)). Mousseau (1997) suggested that ectotherms follow the converse Bergmann's rule, whereby body size decreases at higher latitudes. This trend was first considered for intraspecific comparisons of body size and

reported by Park (1949) in a carabid beetle; it has since been found in many other arthropod species (Mousseau 1997; Blanckenhorn & Demont 2004; Makarieva *et al.* 2005). Such patterns appear to be mediated by season length rather than temperature: at high latitudes, seasons are shorter, reducing the time available for foraging, growth and development, and thus limiting the body size that can be attained (Mousseau 1997; Blanckenhorn & Demont 2004). More recently, both Blanckenhorn & Demont (2004) and Shelomi (Shelomi 2012) found that converse Bergmann clines are more commonly observed in larger bodied arthropods, such as Coleoptera and Orthoptera, as these species tend to have longer development times.

Bumblebees are large insects that usually exhibit an annual lifecycle with one generation per year. As large-bodied insects, this hypothesis predicts that bumblebees should exhibit converse Bergmann rule trends. However, bumblebees are also facultatively endothermic, generating considerable quantities of metabolic heat, both during active flight and when stationary (Heinrich 1974, 1975, 1979). In order to fly, bumblebees need to warm their flight muscles above the ambient temperature of the temperate regions where most species are found. This may be achieved by a combination of flight muscle contractions (shivering) while they are "uncoupled" from the wings (Heinrich 1974, 1979) and substrate cycling in the flight muscles (Newsholme *et al.* 1972; Clark *et al.* 1973). There is a limit to how much heat a bumblebee can produce and thus a minimum temperature at which they can fly (Heinrich 1975; Goulson 2010); larger individuals can produce more heat and also lose it more slowly due to their proportionally smaller surface area (Heinrich 1979). As such, the thermal explanations for Bergmann's rule that are normally applied to endothermic vertebrates may operate in bumblebees, predicting that bumblebees should exhibit a positive association between body size and latitude. Here, we test these opposing hypotheses to better

elucidate not only body size evolution in bumblebees, but also the mechanistic generality of Bergmann trends.

The widespread and economically exploited bumblebee subgenus, *Bombus sensu strictu* (Williams *et al.* 2012b), includes a complex of cryptic species, about which relatively little is known. The *lucorum* complex comprises *B. (B.) lucorum* (Linnaeus), *B. (B.) cryptarum* (Fabricius) and *B. (B.) magnus* (Vogt). All three are morphologically indistinguishable in much of their range, which makes them extremely difficult to study in the field (Waters *et al.* 2010a; Carolan *et al.* 2012). In the UK and Ireland, the *lucorum* complex species exhibit broadly overlapping distributions (Murray *et al.* 2008; Waters *et al.* 2010a; Williams *et al.* 2012b; Scriven *et al.* 2015; Chapter 2), with all three species found co-occurring at many locations (Murray *et al.* 2008; Waters *et al.* 2010a; Scriven *et al.* 2015; Chapter 2). However, recent work has found considerable differences in their ecology and distribution. *Bombus lucorum* is a very widespread, generalist species; in one study it was found at all locations surveyed across Great Britain (Appendix 5.1; Scriven *et al.* 2015; Chapter 2). In contrast, *B. cryptarum* and *B. magnus* exhibit a more restricted UK distribution than *B. lucorum* (Murray *et al.* 2008; Scriven *et al.* 2015; Chapter 2); they are absent from much of southern and eastern England, and are more commonly found at sites with lower summer temperatures (Appendix 5.1; Scriven *et al.* 2015; Chapter 2).

A previous investigation of Bergmann's rule in bumblebees found that although foraging workers of species inhabiting temperate regions were smaller than workers of species from cold climates, the largest species were found in the tropics (Peat *et al.* 2005). Their study focussed solely on workers, and included species from multiple genera, which also influenced body size variation. In this study, we use these three cryptic species, which are in most other ways ecologically and morphologically similar, as a more powerful test of Bergmann's rule.

Carolan *et al.* (2012) measured the thorax breadth of molecularly identified queens of the *lucorum* complex collected from four different European locations. While there was considerable size overlap among the species, they found that there were some significant interspecific differences in mean thorax breadth. However, these differences were inconsistent between the countries of collection: queens from Ireland showed significant variation in body size, whereas Danish queens did not (Carolan *et al.* 2012). In this study, we perform a large scale investigation into the body size variation of all three castes of the *lucorum* complex species across a broad geographic area to determine whether these reportedly highly similar species differ in size. Specifically, we aim to determine (i) whether these three species differ in body size (ii) if body size variation is consistent among castes, (iii) if geographic location or environmental temperature influences body size and (iv) whether trends in body size correspond to differences in their distribution consistent with either Bergmann's or converse Bergmann's rule.

5.3 Materials and Methods

The specimens included in this study have been used previously to assess the distribution and ecological differences of the *lucorum* complex species (see Chapter 2; Scriven *et al.* 2015). Sampling protocols were described in Chapter 2 (Scriven *et al.* 2015), but only those individuals collected in 2011 were included here. In brief, workers, queens and males were sampled at 15 sites across Great Britain from June to September of 2011 (Fig. 5.1, Appendix 5.2). The mean number of individuals collected per site was 89.4 ± 12.9. Whole bees were stored in absolute ethanol at ambient temperatures and then identified to species level using a diagnostic mitochondrial DNA RFLP assay (Murray *et al.* 2008; Scriven *et al.* 2015; Chapter 2). The thorax width between the tegulae (a standard measure of body size: (Carolan *et al.* 2012)) was measured using electronic callipers.

Figure 5.1. **Map of collection sites for *lucorum* complex individuals.**

Our statistical analysis investigated the factors influencing body size for all individuals combined and for each caste separately, fitting linear mixed effects models with normal errors in R (version 3.0.2: (R Core Team 2014)) using lmer from the lme4 package (ver. 1.1-8; (Bates *et al.* 2015)). Individual bee was the unit of replication, with site as a random effect. Firstly, we tested whether body size (thorax width) differed between the three species in a model including all three castes: the model contained the terms 'species' and 'caste' as well as their interaction. We then investigated whether thorax width differed among the three species for each caste separately and determined whether three site-level covariates, latitude (degrees), elevation (m) and mean daily temperature (°C) (UK Meteorological Office

2014) from March to August (the approximate flight period for the three species), influenced body size. For this analysis we mean-centred these covariates and tested for non-linear effects by fitting second order polynomial functions. All models were checked for constancy of variance and normality of errors. We found two queens that were sampled at each extreme of the temperature range to be highly influential in the models, so we removed these two individuals from further analyses. The data set for queens was unsuitable for effectively investigating the effect of latitude, elevation and temperature, due to smaller sample sizes and an uneven distribution across sites; therefore we did not include these variables in models of queen size variation.

We investigated whether the pattern of interspecific body size differences varied among sites by testing for a site by species random effect interaction. The significance of fixed effects and their interactions was tested using likelihood ratio tests to compare models with and without the term of interest. For random effect terms we assessed model fit using AICc values. Pairwise differences between factor means were investigated using Tukey's post hoc tests.

5.4 Results

Thorax width was recorded for 1,095 bees belonging to the *lucorum* complex species, which comprised 575 *B. lucorum*, 330 *B. cryptarum* and 190 *B. magnus* individuals (Appendix 5.2). These three cryptic species showed caste-specific differences in their mean body size (Fig 5.2): a significant interaction between species and caste (Table 5.1) demonstrated that the interspecific differences in thorax width were not consistent across the three castes. Whilst the three species exhibited significantly different thorax widths for both queens and males (Table 5.2; Fig. 5.2a & b), for workers there was no significant interspecific variation (Table 5.2; Fig. 5.2c). Posthoc tests revealed that both *B. cryptarum*

and *B. magnus* queens were significantly larger than *B. lucorum* queens (Fig. 5.2a): the mean thorax breadths were 3.3% larger in *B. cryptarum* (t = 3.2, df = 80.8, *P* = 0.005) and 9.6% larger in *B. magnus* (t = 3.2, df = 21.4, *P* = 0.01). The mean size of *B. magnus* males was larger than either of the other species (6.1% bigger than *B. lucorum* and 3.3% bigger than *B. cryptarum*); however pairwise post hoc tests for *B. magnus* compared to *B. lucorum* and *B. cryptarum* were not significant (t = 2.3, df = 12.6, *P* = 0.09 and t= 1.2, df = 13.6, *P* = 0.48 respectively; Fig 5.2b). Nevertheless, *B. cryptarum* males were significantly larger than *B. lucorum* males (t = 3, df = 216, *P* = 0.008; Fig 5.2b), which represented a 2.7% difference in mean thorax width. There were no significant differences between the body size of *B. cryptarum* and *B. magnus* for any of the castes.

Table 5.1. Caste-specific interspecific differences in thorax width across the lucorum complex species. The size differences (thorax width in mm) between the three *lucorum* complex species. Summary of the results of linear mixed effects models. *Bombus cryptarum* was the reference (intercept) species, parameter estimates for other species are given as contrasts relative to this. Significant results are shown in italics.

Fixed effects	**Estimate**	**SE**	χ^2	***P***
Intercept (*B. cryptarum* males)	5.4	0.06		
Caste (Queens)	2.02	0.08	1284.2	*<0.001*
Caste (Workers)	-0.44	0.05		
Species (*B. lucorum)*	-0.15	0.06	4.9	0.085
Species (*B. magnus)*	0.12	0.15		
Caste (Queens) : **Species** (*B. lucorum)*	-0.04	0.10		
Caste (Workers) : **Species (***B. lucorum)*	0.15	0.07	13.4	*0.009*
Caste (Queens) : **Species (***B. magnus)*	-0.05	0.22		
Caste (Workers) : **Species (***B. magnus)*	-0.18	0.16		
Random effect variance				
Site	0.01			
Residual	0.14			

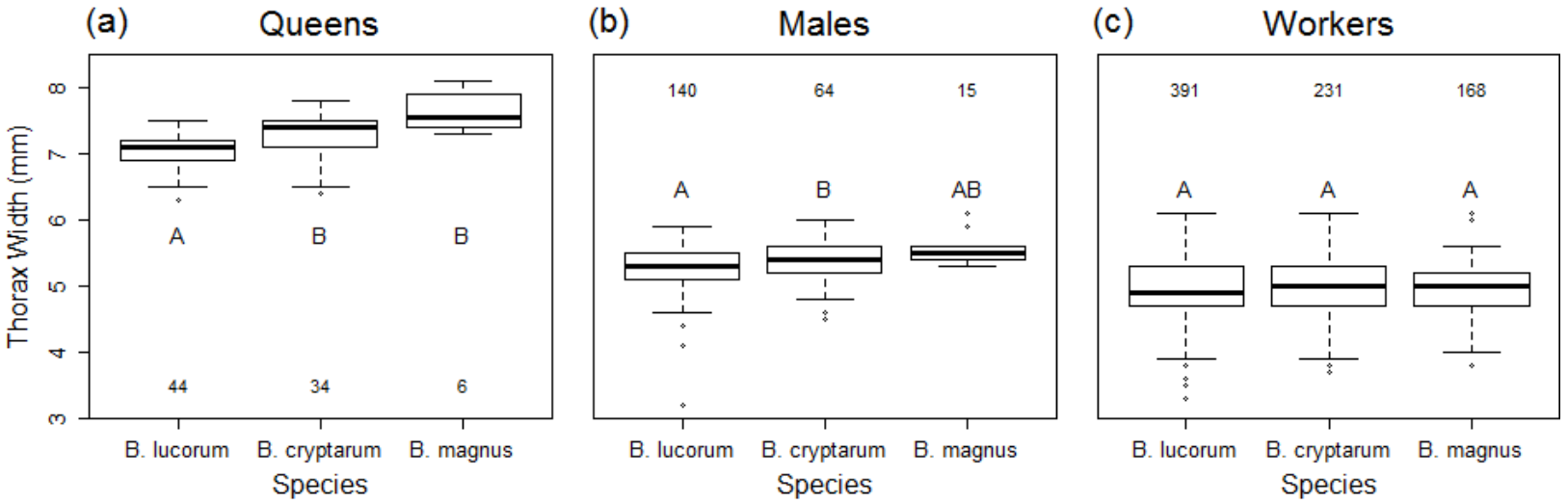


Figure 5.2. Differences in body size of the three bumblebee species.

The thorax widths of (a) queens, (b) males and (c) workers of *B. lucorum*, *B. magnus* and *B. cryptarum*. Box and whisker plots compare medians. Numbers give sample sizes. Different letters denote categories for which the means are significantly different ($P < 0.01$). The plots are based on raw data.

Table 5.2. Differential interspecific variation in thorax width among the three bumblebee castes. The size differences (thorax width in mm) between the three *lucorum* complex species for queens, males and workers. Summary of the results of linear mixed effects models. *Bombus lucorum* was the reference (intercept) species, parameter estimates for other species are given as contrasts relative to this. Significant results (testing for variation between all three species) are shown in italics.

	Queens				**Males**				**Workers**			
Fixed effects	**Estimate**	**SE**	χ^2	***P***	**Estimate**	**SE**	χ^2	***P***	**Estimate**	**SE**	χ^2	***P***
Intercept *(B. lucorum)*	6.97	0.11			5.26	0.04			4.97	0.04		
Species: *B. cryptarum*	0.23	0.07	20.5	*<0.001*	0.14	0.05	13.81	*0.001*	0	0.04	2.08	0.35
Species: *B. magnus*	0.67	0.17			0.32	0.13			-0.06	0.05		
Random effect variance												
Site	0.02				0.01				0.02			
Residual	0.09				0.09				0.16			

Our analysis provided no support for an interaction between site and species, meaning that the interspecific variation in body size was consistent across sites: including a species by site random effect interaction did not improve the model (model with all three castes: AICc = 1034.3 with interaction, and AICc = 1029.5 without). We did not detect a significant effect of mean summer temperature, latitude or altitude on body size; however, the non-significant trend that existed for workers suggested that they became larger at higher latitudes (Appendix 5.3). There was also no evidence of interactions between species and mean summer temperature, latitude or altitude; meaning that there was no variation across the three species in how these covariates were associated with body size. Lastly, there was no evidence for non-linear relationships for any continuous predictors.

5.5 Discussion

In this study we find caste-specific body size differences between the three member species of the *lucorum* complex of cryptic bumblebees, which have previously been described as near-morphologically identical (Williams 2000; Waters *et al.* 2010a; Carolan *et al.* 2012). Of the three species, *B. magnus* and *B. cryptarum*, which predominantly inhabit cooler and more northerly locations in the UK, had queens and males that were significantly larger than those of *B. lucorum*.

Bergmann's rule was originally posited to explain geographic variation in body size of endothermic vertebrates (Bergmann 1847). There has been considerable debate about how it should be applied to insects and other invertebrates, some authors suggesting that ectothermic insects with large body size should exhibit converse Bergmann clines (Mousseau 1997; Blanckenhorn & Demont 2004). Our study questioned which pattern bumblebees should adhere to, as they are both large and, unusually for insects, facultatively endothermic.

In this study of multiple populations of three cryptic bumblebee species, we find evidence for interspecific variation in body size that corresponds to Bergmann's rule.

In Great Britain, *B. cryptarum* and *B. magnus* occur more commonly where temperatures are lower, and are more abundant at northerly latitudes, a pattern that is not evident for *B. lucorum* (Appendix 5.1; Scriven *et al.* 2015; Chapter 2). They are also more active than *B. lucorum* when conditions are cooler and cloudier (Scriven *et al.* 2016; Chapter 3). Similarly, in Austria mean annual air temperature was lower for sampling sites where *B. cryptarum* was found, than for sites with *B. lucorum*; additionally *B. cryptarum* was relatively more common at higher altitudes (Bossert *et al.* 2016). This growing body of evidence suggests that *B. lucorum* is adapted for activity in warmer conditions than *B. magnus* or *B. cryptarum*. Our present results, demonstrating larger mean body size of *B. cryptarum* and *B. magnus* reproductives (queens and males) compared to *B. lucorum*, are consistent with the theory that there is divergent thermal specialisation between the species of the *lucorum* complex (Scriven *et al.* 2016; Chapter 3).

The foremost hypotheses to explain species distribution patterns in agreement with Bergmann's rule are the heat conservation hypothesis and the starvation resistance hypothesis (Blackburn *et al.* 1999). Both could explain why only the reproductive castes display Bergmann body size differences in the *lucorum* complex species. Larger bumblebees generate more heat (Heinrich 1975, 1979) and large body size creates a lower surface area to volume ratio that reduces heat loss. In the UK, queen and male bumblebees of these species are on the wing early (spring) and late (autumn) in the flight season, when conditions are likely to be coldest (Alford 1975; Goulson 2010; Scriven *et al.* 2016; Chapter 3). Thus, it is these castes that would benefit most from enhanced heat conservation in species with northerly distributions. Regarding starvation resistance, the greater likelihood of bad weather at times when these castes are active may often prevent queens and males from foraging.

Moreover, mated queens spend the winter in diapause, surviving only on the fat reserves that fill their abdominal cavity (Alford 1975; Goulson 2010). Small queens are less likely to survive this diapause period (Beekman *et al.* 1998), which is longer in the north and west of the UK (Perry 2006), meaning larger queens are better equipped to resist starvation. Selection may thus be strongest on the queens and males, which gain the highest fitness benefits from large body size in the more northerly distributed species, *B. magnus* and *B. cryptarum.* Furthermore, any differences among body size in workers may be masked by the high variation in worker body size for all three species. This is a common feature of bumblebees, where workers can vary up to ten-fold both within species and even with a single colony (Alford 1975; Goulson *et al.* 2002).

Despite the propensity for large insects to display converse Bergmann trends (Blanckenhorn & Demont 2004), our data suggest that bumblebees do not. One hypothesis to explain the existence of converse Bergmann trends is that body size is limited in cooler habitats by short growing seasons (Mousseau 1997; Blanckenhorn & Demont 2004). We suggest that the combination of facultative endothermy and colonial eusociality in bumblebees, which means offspring are reared in a warmed nest environment by numerous individuals (Heinrich 1979; Goulson 2010), may ameliorate the short growing season constraints on body size that result in converse Bergmann trends in other large invertebrates.

This study detects interspecific body size differences in accordance with Bergmann's rule but does not find strong evidence for similar intraspecific trends. Bergmann's ecogeographic rule has been modified to describe intraspecific variation in body size (Mayr 1956, 1963; Blackburn *et al.* 1999) but it was originally formulated to explain size differences between taxa (Bergmann 1847; James 1970b; Blackburn *et al.* 1999; Ashton *et al.* 2000). Since then, both intraspecific (James 1970b; Mousseau 1997; Ashton & Feldman 2003; Blanckenhorn & Demont 2004; Peat *et al.* 2005) and interspecific (Cruz *et al.* 2005;

Olalla-Tárraga *et al.* 2006; Olalla-Tárraga & Rodríguez 2007; Aragon & Fitze 2014) Bergmann's gradients and their converse have been observed. Although not significant in our study, model parameter estimates suggested a trend that workers of all three species tend to be larger at higher latitudes (corresponding to an approximate 5% size increase across the length of the UK); this is consistent with Bergmann's rule and would be worthy of future investigation.

All three of these bumblebee species have large global distributions (Williams *et al.* 2012b), so although this study included a broad geographic area, it still only represents a very small fraction of their total range, particularly for *B. cryptarum* and *B. lucorum* (see Williams *et al.* 2012b). Expanding the study to encompass a larger area might therefore reveal stronger intraspecific body size trends. Currently the best data on the worldwide distribution of these three species has been provided by Williams *et al.* (2012) but samples were limited, particularly for *B. magnus*. Based on the findings here, it might be expected that the distributions of *B. cryptarum* and *B. magnus* extend further northwards or to higher elevations than that of *B. lucorum*, but additional work would be required to confirm this.

The extent to which overlooked interspecific morphological variation exists within cryptic species complexes has received considerable interest (Wiens & Penkrot 2002; Funk *et al.* 2008; Gabaldón *et al.* 2013; García-Dávila *et al.* 2013). Here we show that, despite significant body size distribution-overlap among the bumblebee species of the *lucorum* complex, these cryptic species have diverged significantly in the mean body size of their reproductive castes. Queens and males of *B. cryptarum* and *B. magnus* were larger than those of *B. lucorum*, whereas there were no interspecific body size differences in workers. This strongly suggests that divergent caste-specific selection pressures have acted on body size in these species.

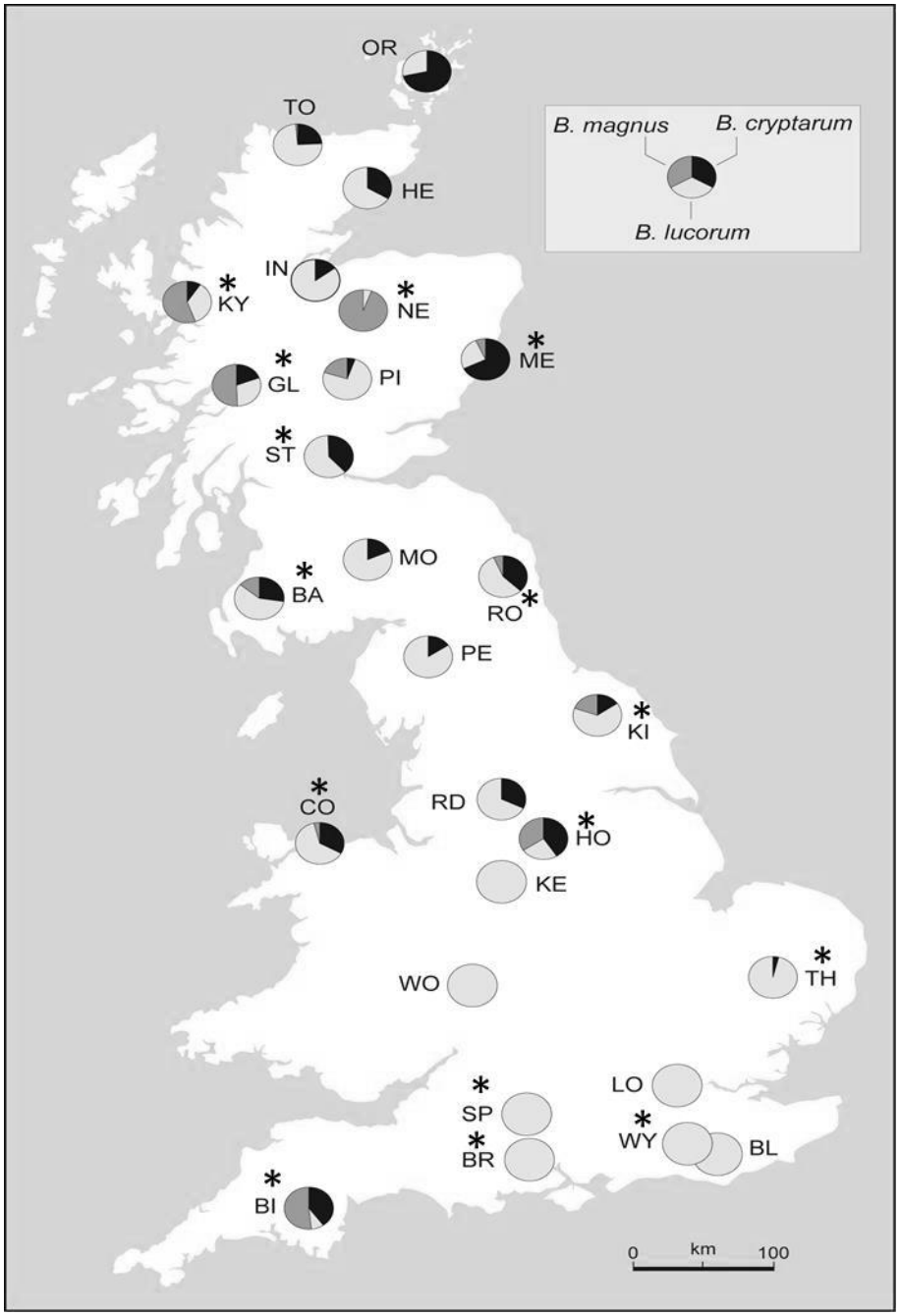
5.6 Conclusion

Bergmann's rule is a classic example of adaptive geographic variation (Ridley 1996; Futuyma 1998), nevertheless, the extent to which Bergmann's rule applies is still debated, particularly in the case of ectotherms (Blackburn *et al.* 1999; Ashton *et al.* 2000; Ashton & Feldman 2003; Meiri & Dayan 2003; Blanckenhorn & Demont 2004; Shelomi 2012). In this study we find evidence for interspecific body size variation between three closely related cryptic bumblebee species consistent with Bergmann's rule. We propose that these interspecific size differences may have been driven by selection pressures on thermoregulation and starvation resistance. Furthermore, bumblebees are an exception amongst invertebrates because they are facultatively endothermic; this case study may therefore help clarify the selection pressures that climate exerts on body size, providing indirect confirmation of the underlying explanation for the existence of Bergmann trends in other ectotherms.

5.7 Acknowledgments

We thank Mairi Knight for providing samples from Dartmoor, Steph O'Connor for advice on field work and particularly Andreia Penado for her assistance with the fieldwork.

5.8 Appendix



Appendix 5.1. The distribution of the three *lucorum* complex species across Great Britain. Sites marked with a * were sampled in 2011, those without were sampled in 2010. Taken from Chapter 2 (Scriven *et al.* 2015).

Appendix 5.2. Sample sizes from each of the sites across Great Britain following species determination. Summary of the data collected for each sampling site along with the numbers of each of the three *lucorum* complex species caught at each site. Temperature was measured as the mean daily temperature from March-August, which is the approximate flight period of these species.

Site	Habitat	Latitude	Longitude	Altitude (m)	Temp. (°C)	*B. cryptarum*	*B. lucorum*	*B. magnus*	Total
Mergie (ME)	Heathland	57.00	-2.34	165	9.3	52	6	5	63
	Non-heathland	56.99	-2.29	130	9.6	22	22	2	46
Nethy Bridge (NE)	Heathland	57.23	-3.68	260	9.9	0	1	16	17
Kyle of Lochalsh (KY)	Heathland	57.23	-5.40	15	9.0	1	7	42	50
	Non-heathland	57.28	-5.52	10	11.1	6	20	0	26
Glencoe (GL)	Heathland	56.66	-5.05	85	10.4	2	2	51	55
	Non-heathland	56.68	-5.12	15	9.9	18	29	1	48
Stirling (ST)	Heathland	56.19	-3.89	318	9.6	87	47	0	134
	Non-heathland	56.14	-3.92	50	10.3	15	87	0	102
Rothbury (RO)	Heathland	55.34	-2.12	150	9.4	25	15	6	46
	Non-heathland	55.29	-1.85	105	10.6	11	39	0	50
Bargrennan (BA)	Heathland	55.11	-4.49	270	9.3	4	1	7	12
	Non-heathland	55.01	-4.54	40	11.0	10	28	0	38
Kirkbymoorside (KI)	Heathland	54.33	-0.94	215	10.6	10	28	15	53
	Non-heathland	54.22	-0.88	30	11.7	2	23	0	25
Hope (HO)	Heathland	53.39	-1.69	340	10.6	21	3	25	49
	Non-heathland	53.35	-1.75	170	11.6	9	15	0	24
Conwy (CO)	Heathland	53.28	-3.88	190	12.6	11	26	2	39
	Non-heathland	54.22	-0.88	25	12.0	7	8	0	15
Thetford (TH)	Heathland	52.42	0.71	45	12.4	3	36	0	39
	Non-heathland	52.40	0.92	40	12.6	0	34	0	34
Wych Cross (WY)	Heathland	51.07	0.05	140	12.0	0	34	0	34
Bramshaw (BR)	Heathland	50.89	-1.69	100	12.6	0	49	0	49
	Non-heathland	50.95	-1.78	50	12.7	0	12	0	12
Birch Tor (BI)	Heathland	50.61	-3.87	421	10.5	14	3	18	35

Appendix 5.3. The size differences (thorax width in mm) between the three *lucorum* complex species and three site-level covariates, latitude (°), altitude (m) and mean daily temperature (°C), for queens, males and workers. Summary of the results of the full linear mixed effects models before model simplification. *Bombus lucorum* was the reference (intercept) species, parameter estimates are given as contrasts relative to this. Significant results are shown in italics. The data set for queens was unsuitable for effectively investigating the effect of latitude, altitude and temperature (see text); therefore we did not include these variables in models of queen size variation.

	Queens				**Males**				**Workers**			
Fixed effects	**Estimate**	**SE**	χ^2	***P***	**Estimate**	**SE**	χ^2	***P***	**Estimate**	**SE**	χ^2	***P***
Intercept *(B. lucorum)*	6.97	0.09			5.27	0.05			4.97	0.05		
Species (*B. cryptarum*)	0.27	0.08	20.50	*<0.001*	0.13	0.05	9.07	*0.01*	0.00	0.04	1.26	0.53
Species (*B. magnus*)	0.65	0.16			0.30	0.16			-0.06	0.05		
Latitude	NA	NA	NA	NA	0.001	0.04	0.05	0.83	0.03	0.02	2.54	0.11
Elevation	NA	NA	NA	NA	0.00	0.00	0.05	0.82	0.00	0.00	0.36	0.54
Mean temperature	NA	NA	NA	NA	-0.03	0.07	0.03	0.56	0.04	0.04	1.32	0.25
Random effect variance												
Site	0.02				0.02				0.02			
Residual	0.09				0.09				0.16			

Chapter 6

Non-destructive DNA sampling from bumblebee faeces

A version of this chapter has been published as:

Scriven, J. J., Woodall, L. C. and Goulson, D. (2013) Nondestructive DNA sampling from bumblebee faeces. *Molecular Ecology Resources*, 13: 225–229.

D. Goulson supervised the project, L. Woodall provided advice and both commented on draft versions of this manuscript. The published version is presented here.

6.1 Abstract

Genetic studies provide valuable data to inform conservation strategies for species with small or declining populations. In these circumstances obtaining DNA samples without harming the study organisms is highly desirable. Excrements are increasingly being used as a source of DNA in such studies, but such approaches have rarely been applied to arthropods. Bumblebees are ecologically and economically important as pollinators; however, some species have recently suffered severe declines and range contractions across much of Western Europe and North America. We investigated whether bumblebee faeces could be used for the extraction of DNA suitable for genotyping using microsatellite markers. We found that DNA could be extracted using a Chelex method from faecal samples collected either in microcapillary tubes or on filter paper, directly from captured individuals. Our results show that genotypes scored from faecal samples are identical to those from tissue samples. This study describes a reliable, consistent and efficient non-invasive method of obtaining DNA from bumblebees for use in population genetic studies. This approach should prove particularly useful in breeding and conservation programs for bumblebees and may be broadly applicable across insect taxa.

6.2 Introduction

Molecular genetic techniques are now commonly used to address questions in conservation, population and behavioural studies. For insects, these techniques have mostly been based on destructive methods that require the insect to be sacrificed. In population studies, genetic analysis can require sampling large numbers of individuals, which may reduce subsequent population size or alter the population structure (Starks & Peters 2002). This is particularly undesirable when studying small or declining populations, yet often these are the ones of most interest (Hamm *et al.* 2010). In social insect species with large colonies, workers may be sampled with little impact on colonies, but for species such as bumblebees with small colony sizes the removal of workers is likely to reduce colony performance (Schmid-Hempel *et al.* 1993). In addition destructive methods are highly unsuitable for genotyping queens that are destined to found colonies (Chaline *et al.* 2004).

Bumblebees (*Bombus*: Hymenoptera, Apidae) are ecologically and economically important as pollinators (Velthuis & Doorn 2006; Goulson 2010). Some species have recently suffered severe declines and range contractions across much of Western Europe and North America (Goulson *et al.* 2008a; Cameron *et al.* 2011). In the UK, seven out of the 27 species are listed on the Biodiversity Action Plan (BAP), a higher proportion than any other invertebrate group (Goulson 2010). Being social insects, bumblebees can have very small effective population sizes and suffer from population fragmentation and isolation (e.g. Estoup *et al.* 1996; Ellis *et al.* 2006; Goulson *et al.* 2011), which makes the conservation genetics of this group of particular interest and concern. Molecular tools have also proved to be useful in studying intractable aspects of bumblebee ecology, such as quantifying nest density, nest survival, and dispersal distances (Darvill *et al.* 2004; Knight *et al.* 2009; Goulson *et al.* 2010; Lepais *et al.* 2010; Woodard *et al.* 2015). Non-destructive sampling would therefore be valuable in studies of bumblebees, especially of rare species and of queens involved in

captive breeding or re-introduction programs. Any such sampling method should not interfere with the queen's ability to mate (Chaline *et al.* 2004), forage or found a colony.

A number of techniques have been used to non-lethally sample insect DNA such as extracting haemolymph from the defensive secretion of the forked fungus beetle, *Bolitotherus cornutus* (Donald *et al.* 2012), tibia removal in damselflies (Fincke & Hadrys 2001) and eusocial wasps (Starks & Peters 2002), wing clipping in butterflies (Hamm *et al.* 2010) and honeybees (Chaline *et al.* 2004) and tarsal clipping in bumblebees (Holehouse *et al.* 2003). Holehouse *et al.* (2003) do not recommend wing clipping as a method of non-lethally sampling DNA in bumblebees as reducing wing area most probably has an effect on flight ability and overall performance. On the other hand tarsal clipping was recommended as no significant effects on workers were detected but they concede that their analyses had relatively low power and a more extensive study could reveal significant effects of tarsal sampling. It seems likely that tarsal clipping may have more impact on queens. Bumblebee queens raise the first brood of workers alone, making this early stage in the life cycle, when she must incubate the brood but also forage regularly to provide a sufficient supply of pollen and replenish her nectar reserves, one of the most precarious (Goulson 2010). Moreover, there are situations when sampling of queen DNA is needed, such as when attempting to quantify queen dispersal (Lepais *et al.* 2010), or during reintroduction programmes.

Faeces have been shown to have the potential to provide a suitable source of DNA for genotyping individuals in mammals (Taberlet *et al.* 1997; Goossens *et al.* 2000; Frantz *et al.* 2003), birds (Idaghdour *et al.* 2003; Regnaut *et al.* 2006) and reptiles (Jones *et al.* 2008) but such non-invasive approaches have rarely been applied to studies of invertebrates. Monroe *et al.* (2010) found faecal pellets and shed exuviae from dragonfly larvae did not provide high enough quality DNA for microsatellite analyses but the frass of a phytophagous weevil, *Ceutorhynchus assimilis* (Fumanal *et al.* 2005), scarab beetles (Lefort *et al.* 2012) and

butterfly caterpillars (Feinstein 2004) have been successfully used to differentiate between morphocryptic entities and identify larvae to species. However, these studies used mitochondrial DNA and did not study genetic differences between individuals. The purpose of this study was therefore to determine whether bumblebee faeces could be used for the extraction of DNA suitable for genotyping individuals with microsatellite markers for use in population genetic studies.

6.3 Materials and methods

Sampling

The common Palearctic bumblebee species *Bombus terrestris* queens and workers collected in and around Stirling were captured and maintained in ventilated, clear plastic containers with access to sugar water. These containers had been cleaned with bleach, to ensure they could not be contaminated with DNA from other individuals, and were checked for faeces several times a day. A single faecal sample, usually all that is required, can be obtained rapidly, usually within 30 minutes of capturing an individual. Retaining individuals in this study allowed us to collect multiple samples per individual and thus assess the repeatability of our results.

Several sample storage, DNA extraction and amplification methods were used to determine which were the most suitable. Two methods of faecal collection were tested (i) using microcapillary tubes and (ii) using filter paper. The drops of liquid that form bumblebee faeces were drawn up into sterilised capillary tubes by capillary action, or gentle sucking if necessary, and then sealed with electrical tape at either end. These were used in an extraction protocol either fresh or stored immediately at -18°C. Otherwise, drops were absorbed onto small strips of Whatman Grade 3 filter paper, approximately 2-2.5cm x 0.5-

1cm. Each strip was placed into an Eppendorf tube ensuring no contamination. They were then either used in an extraction protocol fresh or allocated to one of three storage methods: (1) immediate storage at -18°C, (2) in 0.5 or 1ml of absolute ethanol at room temperature or (3) dry (dried overnight) at room temperature. In order to determine whether a single filter paper sample could be used for several extractions, some were cut in half or quarters before extraction was carried out.

DNA extraction and amplification

Two methods of DNA extraction were tested (i) using a HotShot protocol (Truett *et al.* 2000) and (ii) a Chelex® 100 protocol (Walsh *et al.* 1991). For the extractions from capillary tube samples, the faeces were gently blown from the microcapillary tubes into an eppendorf tube. Extractions from filter paper samples were carried out directly on the strips of filter paper. When testing the HotShot extraction protocol, different amounts of the buffers were tested according to the nature of the sample: 100 µl or 200 µl of both the alkaline lysis reagent and the Tris HCl buffer for the filter paper samples and 35 µl or 75 µl of each buffer for the microcapillary tube samples. All samples were incubated in the alkaline lysis reagent at 95°C for 30 min before the addition of Tris HCl buffer. In the Chelex extractions of capillary tube samples 200 µl of 5% Chelex solution, 7 µl Dithiothreitol and 2µl proteinase K were used per sample. These volumes were doubled for the filter paper samples. All samples were incubated at 56 °C for 70 min and then centrifuged at 14,000 rpm for 3 min. One hundred µl of supernatant was placed into new tubes and incubated for a further 10 min at 95 °C. DNA from tarsal tips of the queens and workers that produced the faecal samples was used to verify that the genotypes obtained from the faecal samples were correct. This was extracted using the Chelex method under the same conditions as for the microcapillary tube samples.

To investigate the effectiveness of the different collection, storage and extraction methods we initially amplified a single microsatellite locus (B118; Estoup *et al.* 1995, 1996) for all sampled individuals under the same conditions. PCR was performed in a reaction volume of 10µl containing 1 or 2 µl of template DNA, 0.2 µM of the primer, 1x QIAGEN Multiplex Master Mix and 0.5x Q-solution. All reactions were initially heated to 95°C for 15 minutes to activate the HotStarTaq DNA polymerase, before 35 cycles of 94°C for 30 s, 49°C for 90s and 72°C for 90 s followed by a final extension period of 10 min at 72°C. Amplification success was determined by electrophoresis on 2.5% agarose gels.

Tarsal tip and faecal DNA from 23 individuals that successfully amplified with B118 was then genotyped at 4 microsatellite loci: B118, B124, B11 and B10 (Estoup *et al.* 1995, 1996). Multiplex PCRs were performed using QIAGEN Multiplex PCR Kits. Each 10µl reaction volume contained 1x QIAGEN Multiplex Master Mix, 0.5x Q-solution, 0.2µM of primers for the loci B118, B124, B11 and 0.4µM of primers for B10 (all with the forward primer fluorescently labelled), and 2µl of template DNA. The thermocycler conditions were the same as for amplification of the single locus B118. All PCR reactions were performed using both negative (water) and positive controls (DNA extracted from worker wing muscle using HotShot technique). PCR products were analysed on a 3730 automated capillary DNA sequencer (Applied Biosystems) and scored with reference to an internal size-standard (GeneScan500 ROX; Applied Biosystems Inc.) using GeneMarker software version 1.97 (SoftGenetics). Amplification and analysis was carried out twice for each faecal sample to check for consistency.

6.4 Results

The Chelex 100 extraction method allowed amplification of the B118 locus from fresh samples collected on filter paper and using capillary tubes (12/13 fresh samples), whereas the amplification of DNA extracted using the HotShot method yielded very poor results regardless of the volume of buffers used (2/12). Using 2 µl of template DNA appeared to yield more PCR product than just 1 µl. Given that both sample collection methods gave positive results when amplifying a single microsatellite locus, it was decided to use the simpler method, filter paper, as the collection method for the subsequent samples.

After storage on filter paper at -18 °C, preliminary testing showed amplification of the microsatellite locus B118 to be successful (10/10) as was microsatellite amplification when a half or a quarter of a filter paper sample was used for the extraction. Dry storage of the samples at room temperature was not successful; none of the eight samples that were tested amplified.

Following microsatellite analysis at four loci, samples collected on filter paper or in capillary tubes and extracted immediately gave 100% and 80% successful amplification at all loci respectively (Table 6.1) after a single amplification. Storing filter paper samples at -18 °C was revealed to be the most effective storage method (Table 6.1). Only 45% of samples stored in 1 ml of 100% ethanol for two weeks could be genotyped at all four loci after two repeats, compared to 100% of samples frozen for two weeks. None of the samples stored in 0.5 ml of ethanol could be correctly genotyped. Four of five samples stored frozen for two months amplified successfully at all four loci with two repeats. Using fragments of each filter paper sample did not reduce the genotyping success with 100% accuracy at all loci after a single amplification.

As several faecal samples from each individual, as well as tarsal tips, were genotyped to test the different methods, we were able to verify the reliability of genotypes obtained from the faeces samples and show that the quantities of DNA obtained from the fresh and frozen samples did not cause allelic dropout during the amplifications as can sometimes occur when using very small amounts of DNA (Taberlet & Luikart 1999). All of the positive controls amplified successfully and the negative controls were always 'blank'. Sufficient DNA was extracted using the Chelex protocol from both filter paper and capillary tube samples to perform at least 50 PCR amplifications.

Table 6.1. Success rate of amplification of all four microsatellite loci for each preservation technique tested after each repeat. The cumulative total is the sum of the success rate for both repeat amplifications combined.

	Number	**Genotyping success (%)**		
Sample Treatment	**of samples**	**Repeat 1**	**Repeat 2**	**Cumulative Total**
Fresh filter paper samples	7	100	100	100
Filter paper stored frozen for 2 weeks	17	76	76	100
Filter paper stored frozen for 2 months	5	60	80	80
Filter paper stored in 1 ml ethanol for 2 weeks	11	45	45	45
Filter paper stored in 0.5 ml ethanol for 2 weeks	3	0	0	0
Half or quarter filter paper fragments stored frozen for 2 weeks	8	100	100	100
Fresh capillary tube samples	5	80	80	80
Tarsal samples	9	100	100	100

6.5 Discussion

These results show that it is possible to extract DNA from bumblebee faeces using standard and simple techniques and that the quality of the DNA is high enough to allow PCR amplification of microsatellites permitting reliable genotyping of individuals. We found that

DNA could be extracted from faecal samples collected in either microcapillary tubes or on filter paper, but the latter was much easier. The microcapillary tubes were more difficult to fill and to seal and very easy to break unintentionally, which consequently means that they would require careful storage and be more problematic to transport than samples on filter paper. The best results were achieved with DNA obtained from samples freshly collected on filter paper strips and extracted using the Chelex extraction method. Samples collected on filter paper strips can be stored frozen and still yield accurate results but the success rate may decrease with the length of storage time, testing with a larger sample size would verify this. The filter paper strips can also be divided into fragments (halved or quartered) before extraction without any negative impact on amplification success.

We obtained these positive results using very simple and inexpensive extraction methods. Further testing using more advanced extraction approaches, such as column-based techniques, could improve the method, potentially permitting consistent DNA extraction from ethanol-stored samples or the amplification of other molecular markers with alternative applications.

In this study, individual bumblebees were captured and faecal collection was carried out in the laboratory. This is, however, not a requirement; individuals may be captured and held in small containers in the field until they defecate, whereupon the faecal samples can be collected using the preferred method. If microcapillary tubes are kept sealed or filter paper samples prevented from drying out in sealed tubes, they can be kept for several hours in this way before freezing. However, this method would probably not be suitable for sampling in remote situations where access to a freezer was not available.

This study describes a reliable, consistent and efficient non-invasive method of obtaining DNA from bumblebees. Although excrements are increasingly being used as a

source of DNA in molecular and ecological studies (Beja-Pereira *et al.* 2009), such approaches have rarely been applied to arthropods. These results demonstrate that this procedure is effective both in terms of amplification success and scoring reliability. This method is ideal when no impact on survival or behaviour is required making it a particularly useful approach in breeding and conservation programs. Despite Monroe *et al.* (2010) failing to obtain DNA of sufficiently high quality for genotyping from non-invasive samples from the dragonfly, *Somatochlora hineana*, we have shown that it is possible for bumblebees and therefore it seems likely that the approach may also be applicable to other insect species.

6.6 Acknowledgements

The authors thank Steph O'Connor for help and advice with field and labwork. The work was funded by the University of Stirling.

Chapter 7

General discussion

Bumblebees are large and charismatic insects that have been very well-studied (e.g. Goulson 2010; Alford 1975); yet large gaps still remain in our knowledge and understanding of these familiar and valuable species. The *lucorum* complex represents an example of one such gap: before this PhD thesis research very little was known about this group of cryptic species. In fact, one of these three species, *B. cryptarum*, was only discovered to inhabit the UK in 2005 (Bertsch *et al.* 2005). This is perhaps even more surprising considering that *B. lucorum*, the name under which this complex has previously been grouped (Williams 1994, 2000; Benton 2006), is one of the most abundant and widespread bumblebee taxa in the UK.

Some bumblebee species have been declining throughout their worldwide distribution, both in abundance and range (reviewed in Goulson 2010). Meanwhile, other species seem to be faring comparatively well (Goulson *et al.* 2005, 2008a). The reasons why some species remain common while others have suffered severe declines remains the subject of debate (e.g. Goulson *et al.* 2005; Williams 2005; Fitzpatrick *et al.* 2007) but any assessment of the vulnerability or risks faced by a particular species requires an understanding of their ecology. Prior to the research in this thesis our knowledge of the *lucorum* complex species was very limited and in some cases inconsistent (Williams 2000; Bertsch *et al.* 2005; Murray *et al.* 2008; Waters *et al.* 2010a; Williams *et al.* 2012b; Stanley *et al.* 2013a). The results of the research presented here represent the first detailed investigation of the ecology and population genetics of *B. lucorum*, *B. magnus* and *B. cryptarum*, over a relatively large geographic area and a range of biological scales.

7.1 The ecology of the *lucorum* complex species and the implications for bumblebee conservation

This work combines information from species distribution mapping, single site study of environmental activity correlates, molecular techniques and measures of morphological

variation; it demonstrates that fundamental differences exist in the distribution, ecology and population structure among the *lucorum* complex species in Great Britain. Among the three *lucorum* complex species, *B. magnus* is the least abundant, has the most limited distribution and appears to occupy the most distinct niche. It has a narrow, highly specialised diet, feeding predominantly on species of heather (Ericacae), which results in it being restricted for the most part to heathland habitat. Furthermore, the phenology of *B. magnus* queens, workers and males appears to be delayed relative to the other two species. *Bombus lucorum* and *B. cryptarum* both have broader diets and are not constrained to a single habitat type. However, the distribution of *B. cryptarum* in Great Britain is more restricted than that of *B. lucorum*, with both *B. cryptarum* and *B. magnus* being relatively more common in areas with lower summer temperatures. Additionally, there are differences among the species in the relative activity of queens and workers according to the weather: *B. lucorum* is relatively more active than the other two when it is either warmer or sunnier. There are also interspecific differences in the mean body size of the reproductive castes: among queens, the mean body size of *B. lucorum* is smaller than that of the other two species; similarly, the males of *B. lucorum* are smaller than those of *B. cryptarum* and *B. magnus* (although not significantly so for *B. magnus*). *Bombus lucorum* exhibits the highest genetic diversity and lowest level of population genetic structure. Genetic diversity is lowest in *B. magnus* but population structure in *B. cryptarum* is more strongly affected by distance than it is in *B. magnus*.

Bombus cryptarum and *B. lucorum* appear to exploit a wide range of habitat types (Chapter 2; Murray *et al.* 2008; Waters *et al.* 2010a; Bossert *et al.* 2016), which contrasts with *B. magnus*. Chapters 2 and 3 confirm that *B. magnus* feeds predominantly on *Calluna vulgaris* and *Erica spp.*, meaning that it is very strongly associated with heathland habitat and is thus both a habitat and dietary specialist. Such a strong dietary specialisation would appear disadvantageous for a social species that needs to maintain colonies with high energy

demands beyond the flowering season of any one (or two) plant species (Williams 2005). However, because *C. vulgaris* and *Erica spp*. flower successively and for a relatively long period of time, they may provide a reliable, relatively long term, foraging resource.

In bumblebees, it has been suggested that a species' diet breadth may correlate with abundance, with rarer bumblebee species often utilising fewer flower species, implying that specialised species may be more vulnerable to population declines (Goulson *et al.* 2005, 2008b). Whilst the importance of this hypothesis has been debated (Williams 2005; Fitzpatrick *et al.* 2007a; Williams *et al.* 2007), across this cryptic species complex the species with the narrowest diet is also the rarest. Indeed, few bumblebee species in Great Britain exhibit such strong specialisation as *B. magnus* (Goulson *et al.* 2006). *Bombus jonellus* and *B. monticola* are sometimes referred to as heathland specialists (Goulson *et al.* 2005), but in the south of England, *B. jonellus* is also found in gardens and calcareous grassland, and *B. monticola* exploits a variety of food plants other than heather, particularly *Vaccinium spp.*, but also *Salix spp*. and *Lotus corniculatus* (Edwards & Jenner 2005; Macdonald & Nisbet 2006). *Bombus monticola* is a rare and declining species, particularly in England and Wales, whereas *B. jonellus* occurs widely and does not appear to have declined (Goulson *et al.* 2005; Benton 2006; Macdonald & Nisbet 2006). Some other species appear to be specialists but only as a result of severe declines: the very rare *B. distinguendus* is now mostly found on machair and nearby dunes in the far north and west of Scotland, but its past distribution demonstrates that it is not a machair specialist (Williams 2005; Goulson *et al.* 2006). As discussed in Chapter 2, specialisation by *B. magnus* on heathland could be ecologically problematic because this habitat has suffered major losses in the UK (Thompson *et al.* 1995; Price 2003). The population genetics results in Chapter 4 indicate that although *B. magnus* has lower genetic diversity than *B. cryptarum* (which we might expect if it was declining or had small effective population sizes), it has higher levels of genetic variation than some other

rare species in the UK, such as *B. distinguendus* or *B. muscuorum* (Ellis *et al.* 2006b; Charman *et al.* 2010; Darvill *et al.* 2010). This suggests that *B. magnus* is not currently showing evidence of suffering serious effects of habitat loss or fragmentation, which may be because, as explained in Chapter 4, specialists on patchy habitat types could be well-adapted to overcome habitat fragmentation (Zavodna *et al.* 2005; Sallé *et al.* 2007; Exeler *et al.* 2010; Feist *et al.* 2014). The results here indicate that although *B. magnus* has a restricted distribution, it is locally common at some sites; further long term studies of abundance would be helpful to determine whether this species is suffering from declines too recent to be detectable. Indeed, Thompson *et al.* (1995) found that 40% of the bird species using upland heather moorland were declining, therefore this merits further research.

Heathland is a semi-natural habitat that persists without management in some areas but which is usually maintained in Great Britain by rotational burning and grazing. Upland heath is a sub-montane habitat characterised by common or ling heather, *Calluna vulgaris*, found mostly in the British Isles, and along parts of the western seaboard of the northwest European mainland. *Calluna vulgaris* occurs much more widely than this, but the massive extent of rotationally burned heather is unique to the UK and Ireland (Thompson *et al.* 1995). Little is known about the range of *B. magnus* worldwide: some of the few reliable data comes from the study by Williams *et al.* (2012) and a large proportion of their *B. magnus* samples originated from the UK. It would therefore be interesting to determine whether this managed habitat represents a particularly important resource, and thus a potential stronghold, for this species in Europe, or whether it utilises different habitat types in other parts of its range.

In contrast to the data for *B. magnus*, the evidence presented in Chapters 2 and 3 indicates that *B. lucorum* and *B. cryptarum* are both relatively generalist pollinators. In their field guide for Great Britain and Ireland, Edwards and Jenner (2005) describe *B. lucorum* as "a common and widespread species found in many habitats but more frequent towards the

north". However, it is unclear whether this refers to *B. lucorum* only or the *lucorum* complex as a whole. Indeed, many studies have not distinguished between the three *lucorum* complex species (e.g. Goulson & Darvill 2004; Goulson *et al.* 2005, 2006) and those that have, have often relied on morphological identification (e.g. Peat *et al.* 2005; Macdonald & Nisbet 2006; Iserbyt & Rasmont 2012), which may not be accurate (Carolan *et al.* 2012). Using molecular techniques for species identification, *B. lucorum* has been found to be the most abundant of the three species in Ireland (Murray *et al.* 2008; Stanley *et al.* 2013b) and in Austria (Bossert *et al.* 2016). Similarly, the results presented in Chapter 2 find that on average across Great Britain, *B. lucorum* is the most common and widespread species: it was found at every sampled site. However, in the study in Chapter 3 that focussed on a single site in Scotland, *B. cryptarum* was the most abundant, as it also was in the Western Isles of Scotland (Waters *et al.* 2010a). Similar results were found for the diet breadth of these two species: across all British sites, *B. lucorum* had the broadest diet, whereas in Glencoe (Scotland; Chapter 3) and the Western Isles of Scotland (Waters *et al.* 2010a), *B. cryptarum* foraged on the widest range of plant species. Thus, at higher latitudes, it appears that the abundance and diet breadth of *B. lucorum* may become reduced relative to *B. cryptarum* when compared to lower latitudes. Thus, it is possible that the increase in abundance of *B. lucorum* in the north of the UK, reported by Edwards and Jenner (2005), may be a result of the increased presence of *B. cryptarum* and *B. magnus* alongside *B. lucorum* at these latitudes.

Chapters 2, 3 and 5 all present independent data sets supporting divergent thermal specialisation among these three species. Chapter 2 shows that *B. cryptarum* and *B. magnus* are relatively more abundant at sites with lower summer temperatures, a pattern not observed for *B. lucorum*. Chapter 3 demonstrates that *B. magnus* queens are more active in cloudy conditions than *B. lucorum,* and *B. cryptarum* workers are more active in cooler and cloudier conditions than *B. lucorum* workers. Furthermore, *B. cryptarum* and *B. magnus* queens are on

average larger than *B. lucorum* queens, as are *B. cryptarum* males (and *B. magnus* males, although not significantly), which represents a potential adaptation to cooler conditions (see Chapter 5; Bergmann 1847; Mayr 1963; Heinrich 1979). A recent study in Austria by Bossert *et al.* (2016), which found that *B. cryptarum* inhabited cooler localities than *B. lucorum*, supports these findings. As a result, we might expect the distribution of *B. cryptarum* and *B. magnus* to extend further north or to include higher altitudes than that of *B. lucorum*. Further strategic sampling would be required to determine whether this hypothesis is supported, as the current distribution information available is insufficient. As discussed in Chapter 3, rough estimates based on COI divergence and diversity, reported by Carolan *et al.* (2012) and Murray *et al.* (2008), indicate that the *lucorum* complex species may have diverged relatively recently: approximately <100,000 years ago, which falls within the last glacial period. Climatic oscillations may isolate populations in refugia, which could result in allopatric differentiation during glacial periods, followed by recolonisations during post-glacial periods (Hines 2008). Such a situation could plausibly have led to the differences in thermal specialisation observed here among the *lucorum* complex species.

All three species appear to be fairly widespread in the Palearctic, but *B. cryptarum*, which is found across Europe, Central Asia, north China, the Kuril Islands and north-western North America, has the broadest distribution of any species within the *Bombus s. str.* subgenus (Williams *et al.* 2012b). Indeed, *B. cryptarum* may still be experiencing ongoing differentiation: it includes more geographically structured lineages, than other species in the subgenus (Williams *et al.* 2012b). These comprise two principal subgroups, each exhibiting a single diagnostic genetic change, which are widespread and may represent subspecies. One of these is more northern and found in Scandinavia, northern Russia, Mongolia, to western North America, whereas the other is more southern, including individuals from Ireland, Britain, through central Europe to central Asia and both occur in Scotland (Williams *et al.*

2012b). Almost certainly both of these lineages will have been sampled during this study, meaning that the conclusions drawn for *B. cryptarum* represent average characteristics for these two lineages. Future studies could therefore investigate the extent of ecological differentiation between these two lineages within *B. cryptarum.* Expanding the genetic study in this thesis to use multiple genetic markers and encompass samples from across the worldwide ranges of these species would provide insights into their ecology and phylogeography that are so far missing, particularly in the case of *B. cryptarum*.

Chapter 3 represents the first reliable study of phenology in all three species. *Bombus cryptarum* has been considered an early spring species whose phenology precedes that of the other two species (see Bertsch *et al.* 2005). The data here did not confirm this, but do indicate that the phenology of queens, workers and males of *B. magnus* was delayed relative to *B. cryptarum* and *B. lucorum*; the production of *B. magnus* workers therefore coincided with the flowering of *C. vulgaris* and *Erica spp*. Very few *B. magnus* males were found in the course of this study, which means little could be concluded about their phenology. However, in Chapter 3 the first *B. magnus* male was caught much later than the first male of the other two species, and in the samples obtained for Chapter 2, males of *B. magnus* were only found in late September, whereas males of *B. cryptarum* and *B. lucorum* were encountered as early as mid-June. The timing of peak heather flowering appears to vary across Great Britain (pers. obs.), so it would be very interesting to determine whether *B. magnus* phenology also varies in parallel.

Bumblebee species vary considerably in the annual timing of peak worker abundance and queen emergence from hibernation: in the UK, queens of some species emerge as early as February, whilst others do not appear until the end of May (Alford 1975; Goulson 2010). These differences in phenology have been suggested to act as a mechanism to reduce inter-specific competition for resources, as a species that emerges earlier may gain a competitive

advantage. For example, in North America the bumblebee species, *B. flavifrons*, emerges several weeks ahead of *B. rufocinctus*; when *B. rufocinctus* workers appear, *B. flavifrons* workers are already numerous and have learnt where the most rewarding flowers are and how to handle them, thus outcompeting the naïve *B. rufocinctus* workers on their preferred flower species (Bowers 1985). In the UK, there are several common short-tonged bumblebee species, one of which is *B. pratorum*; unlike its competitors, this species has a very short colony duration with worker abundance peaking much earlier than most other species: in May or early June, with reproductives produced from April (Alford 1975).

Chapters 2 and 3 demonstrate that, in addition to having a delayed phenology, *B. magnus* is the rarest of the *lucorum* complex species. In the UK a correlation has been reported between bumblebee species rarity and emergence time (Goulson *et al.* 2005; Fitzpatrick *et al.* 2007a); late emerging species may have fewer nest sites remaining available to them, often have smaller colonies and may be less able to cope with environmental changes (Williams & Osborne 2009; Goulson 2010). *Bombus magnus* may avoid the problems of interspecific competition between workers by specialising on heather, which is a super-abundant resource on heathland; but the queens may suffer from competition for nesting sites. The delayed phenology of *B. magnus* could also represent a high risk strategy that suffers in years when the season finishes early, or weather/climatic conditions prevent workers foraging, or limits the amount of flowering heather available (pers. obs.), which could explain some of the variation in *B. magnus* abundance found between years.

As discussed, heathland, dominated as it can be by *C. vulgaris* and *Erica spp.*, would not appear an ideal habitat for bumblebees. It should therefore be considered that *B. magnus* could be restricted to, rather than specialised on, heathland. This could have occurred as a result of one or multiple factors, including the loss or degradation of other more suitable habitat. Since museum specimens can now be used as a source of DNA (Strange *et al.* 2009;

Lozier & Cameron 2009; Maebe *et al.* 2013) and could therefore be accurately identified to species, this hypothesis could be investigated using historical specimens from collections and museums to determine whether *B. magnus* was found in a broader range of habitats in the past.

Another factor that could cause a species to be restricted to a less favourable habitat is inter-specific competition with other pollinators. In particular, queens of *B. magnus*, emerging late, could be excluded from more suitable habitat, due to limited nesting sites, and naive workers may be outcompeted on the most rewarding forage plants. Whether these ecological differences are a result of inter-specific competition rather than preference is more difficult to solve, but it would be very interesting to determine whether the patterns found in this work remain in the absence of the other *lucorum* complex species in particular. Indeed, the patterns revealed in this thesis could potentially be driven by competition between these three species: *B. lucorum* might be able to outcompete the other two species in the optimum conditions found in the south and east of England, but to the north and west when conditions are less favourable for *B. lucorum*, *B. cryptarum* is able to persist alongside it. In these regions, *B. magnus* may persist on heathland, a habitat exploited less by the other two species, where it relies heavily on *C. vulgaris* and *Erica spp.* for forage.

7.2 Scope for further research

By focussing on a single site in Chapter 3, we were able to eliminate some of the potential sources of bias in the conclusions drawn from previous *lucorum* complex studies (e.g. Waters *et al.* 2011; Murray *et al.* 2007; Chapter 2), which have surveyed sites that are geographically broadly spaced. Using multiple sites for diet comparisons therefore introduces the possibility that the species in question does not have the option to feed on certain plants at

some sites because those plants are not present. The intensive study at a single site presented in Chapter 3, eliminates this problem, as all species have access to the same forage plants, providing a fairer test of forage preferences. This approach therefore complements previous studies, including that in Chapter 2, which were based on a single sampling time-point in the season for each site (which itself imposes some limitations on the conclusions that can be drawn). Nevertheless, repeating this study over multiple seasons would provide further interesting information, particularly concerning the fluctuation in the relative abundances of these species between years. Such further work, would also permit the investigation of the effect of a number of climatic variables on the abundance and reproductive success of the three species, which appears to vary considerably (see Chapters 2 & 3).

Observing where and when bumblebees in this species complex were foraging has revealed a number of important aspects of their ecology, including differences in their phenology, diet breadths, distribution patterns and response to weather and climatic variables. Combining this with a population genetics study has led to further important insights into the biology of these species. Population genetic studies are valuable tools for revealing many biological characteristics of species that are otherwise difficult to study. Indeed, these tools have provided insights into many aspects of bumblebee ecology, behaviour and evolution (reviewed by Woodard *et al.* 2015). Chapter 4 represents the first population genetic study of the *lucorum* complex and thus provides the first indications of genetic diversity and population structure for these species. However, there is much scope for further research. For example, in social *Bombus* species, counting the number of foraging workers is not necessarily the best representation of population health: colony abundance is more closely related to the size of the breeding population (Crozier 1979). Calculating this from observation alone is very challenging because bumblebee nests are very difficult to locate, even by highly trained dogs (Waters *et al.* 2010b; O'Connor *et al.* 2012). However, genetic

tools now exist to aid estimates of colony abundance (Goulson *et al.* 2010; Lepais *et al.* 2010; Jones & Wang 2010; Wood *et al.* 2015). It would therefore be very useful to determine the nest density and effective population size (N_e) of the three species and investigate whether this differs between the *lucorum* complex species or between habitat types.

Chapter 4 revealed differences in genetic structure among the three species that may relate to their degree of habitat specialisation and propensity for dispersal. Other work has shown that for some species, such as *B. muscuorum, B. hortorum, B. bifarius* and *B. vosnesenskii*, natural barriers, including bodies of water (Goulson *et al.* 2011; Lozier *et al.* 2011, 2013; Jha & Kremen 2013b; Jha 2015), elevation gradients (Lozier *et al.* 2011, 2013) and human-modified landscapes (Jha & Kremen 2013b; Jha 2015) appear to limit dispersal. Combining the genetic data available here with landscape information for landscape genetic analysis would thus help explain observed genetic patterns within the *lucorum* complex, be informative about environmental suitability for each species and reveal pathways of dispersal between populations. This information could be particularly important for *B. magnus* because, although this species is not currently in obvious decline, future anthropogenic modification of suitable habitat pathways could challenge dispersal and lead to population isolation (Wilson *et al.* 2005; Jha & Kremen 2013a).

The foraging range of bumblebees determines the area of habitat that an individual or a colony can exploit and is therefore a fundamental aspect of their ecology, as well as an important consideration for crop pollination (Goulson 2010). There is considerable variation in foraging ranges among bee species: a review of the foraging ranges of 62 bee species by Greenleaf *et al.* (2007) found a positive relationship between foraging distance and body size. Among bumblebees, molecular tools have revealed foraging distances that vary from 25m to more than 10km (Chapman *et al.* 2003b; Darvill *et al.* 2004; Knight *et al.* 2005; Charman *et al.* 2010; Rao & Strange 2012; Jha & Kremen 2013a; Geib *et al.* 2015). This thesis did not

study foraging distances, but no differences were found in the mean body size of workers, which suggests that, according to Greenleaf *et al.* (2007), they may have similar foraging ranges. However, exploiting heather, which is an extremely abundant resource where it is available, may mean that *B. magnus* workers do not need to travel so far to find suitable forage. Determining the foraging distances for the *lucorum* complex species would therefore contribute considerably to our understanding of how they use the landscape and forage patches within it.

Reproductive isolation between the *lucorum* complex species has also not been studied in detail. Cross breeding experiments, including those by Bučánková *et al.*

(2011) where no interspecific matings were observed, indicate that these species are reproductively isolated, but the sample sizes were small and they are not conclusive (see Bossert 2015). In bumblebees, male cephalic labial gland secretions are used for scent marking and their composition is species specific and stable across large geographic areas (Bertsch & Schweer 2012) making them useful for species recognition. The *lucorum* complex species each has a distinct labial gland secretion profile; this observation has been used as evidence for their species status and may also represent a cohesion mechanism, but not necessarily an isolation mechanism of the species (Bertsch 1997; Bertsch *et al.* 2005; Bertsch & Schweer 2012). There is also evidence suggesting that there may be some differences in colouration between males of the three species that may contribute to reproductive isolation, particularly regarding the colouration of the face: *B. cryptarum* males often appear to exhibit a much reduced extent of yellow facial hairs compared to *B. lucorum* (Scriven *et al.* Unpublished data; Rasmont *et al.* 1986). However, whether this is consistent or forms a basis for female choice remains to be confirmed. Here, the data in Chapter 4 provide no evidence for substantial levels of hybridisation or admixture between the species but the next step would be to test for it explicitly.

Studies using microsatellite loci have evidently contributed enormously to our understanding of bumblebee ecology and evolution (Estoup *et al.* 1995; Darvill *et al.* 2004; Ellis *et al.* 2006b; Charman *et al.* 2010; Goulson *et al.* 2010; Lozier *et al.* 2011; Woodard *et al.* 2015), but basing conclusions about genetic diversity on microsatellite data alone may be subject to limitations (Payseur *et al.* 2002; Haasl & Payseur 2011). Discrepancies between data sets examining a small number of microsatellite loci compared to genome-wide nucleotide diversities can occur. For example, when using microsatellite data, Lozier *et al.* (2011) found that the levels of genetic variation between stable and declining *Bombus* species in North America differed, whereas a later study based on restriction site-associated DNA sequencing data showed little difference in genetic diversities between these species (Lozier 2014). The use of these large genomic datasets can be more informative than data from a limited number of loci (Hoffman *et al.* 2014) and have many potential applications for conservation genetics; although further research into these approaches may be required (Allendorf *et al.* 2010).

The destructive nature of molecular studies of insects is problematic, particularly alongside the need for large sample sizes (Starks & Peters 2002; Donald *et al.* 2012). Despite using a non-lethal sampling method for the study in Chapter 3, this thesis still required the destruction of more than a thousand *lucorum* complex individuals. The population genetic analysis revealed that the lethal sampling method applied did not result in the destruction of large numbers of individuals from the same colony and was therefore unlikely to have had a large impact on colony success or survival. However, had any of these species been particularly rare, sampling may have had a much larger impact, as more individuals per colony would be likely to have been lost (Schmid-Hempel *et al.* 1993; Chaline *et al.* 2004). Similarly, this work avoided destroying large numbers of overwintered queens as this would have also been likely to impact local populations (Chaline *et al.* 2004). However, if similar

work was to be carried out on rare species, destroying any individuals would be undesirable. As a result, many of the population genetic studies of bumblebees have focussed on common species (e.g. Widmer & Schmid-Hempel 1999; Herrmann *et al.* 2007; Kraus *et al.* 2009; Lepais *et al.* 2010; Goulson *et al.* 2011; Rao & Strange 2012; Jha & Kremen 2013a; b; Lozier *et al.* 2013; O'Connor *et al.* 2013; Wood *et al.* 2015; Moreira *et al.* 2015; Jha 2015), and only a few have involved rare or declining species (Darvill *et al.* 2006, 2010; Ellis *et al.* 2006b; Charman *et al.* 2010; Whitehorn *et al.* 2011). The method developed in Chapter 6, which is an effective means of obtaining DNA from bumblebees without harming the individuals, therefore provides a valuable alternative to destructive sampling for studying those rarer species, or species that are being reintroduced, such as *B. subterraneus* (Gammans 2011; Lye *et al.* 2011; Brown *et al.* 2016).

7.3 The value of cryptic species complexes as model systems

Advances in molecular techniques such as PCR and DNA sequencing have led to the, often accidental, discovery of many genetically divergent but morphologically cryptic lineages. The rate of cryptic species discovery has been increasing exponentially, with such lineages distributed evenly among major metazoan taxa and biogeographical regions (Pfenninger & Schwenk 2007). Therefore "cryptic species" are commonly referred to in biology and are the focus of a considerable amount of research: a simple search for "cryptic species" in Web of Science (July 2016) finds over 38,600 publications. Yet, how special are they, and why are they so interesting? This thesis has found differences in every characteristic that has been investigated, although they are sometimes subtle. This could lead to the conclusion that cryptic species are nothing special: they are simply separate species which are hard to identify, but which still exhibit differences in many characteristics. It may also be assumed that the individuals of these species have little difficulty in distinguishing

each other, thus, they are only "cryptic" because humans have found them difficult to categorise based on a single feature: their morphology. As a consequence, perhaps cryptic species are not especially important on their own, but critically, as described throughout this thesis, they do raise many interesting and important questions for ecology and evolution (Bickford *et al.* 2007), especially when they are found in sympatry. As illustrated in Chapters 4 & 5, cryptic species are valuable as a model for a comparative approach to study ecological questions in closely comparable species. However, a limitation to this approach, and therefore these Chapters, is that conclusions are drawn from a single comparison. Ideally, such studies would involve a larger cryptic species complex to assess the generality of the conclusions that the comparative approach has produced; however, this is rarely feasible (e.g. Maingon *et al.* 2003; Racey *et al.* 2007; García-Dávila *et al.* 2013; Westram *et al.* 2013; Vodă *et al.* 2015)

Strong ecological similarity between two species should lead to strong interspecific competition, resulting in either competitive exclusion of one species or ecological differentiation (Gause 1932; Holt *et al.* 1994; Violle *et al.* 2011; Cothran *et al.* 2013). Co-occurring cryptic species therefore offer the opportunity to investigate the degree of ecological differentiation needed to facilitate coexistence. This thesis investigates the level of differentiation between three cryptic bumblebee species with overlapping distributions. In Europe and North America bumblebee communities consist of numerous species with apparently very similar niches, which we would thus expect to be shaped by strong competition (Goulson *et al.* 2008b; Goulson 2010). Such a detailed study of sympatric cryptic bumblebee species therefore provides an insight into the subtle mechanisms involved in the structure of bumblebee communities and the coexistence of species. The work here demonstrates how superficially identical species can vary in a wide range of characteristics, not initially apparent, and provide unique pollination services. Moreover, they represented an

ideal model for examining the association between habitat specialism and levels of population genetic structure, as well as patterns of body size differences in relation to Bergmann's rule, whilst eliminating some of the other confounding factors that would arise from comparing many other sets of species.

7.4 Conclusion

This thesis provides the first detailed investigation of the ecology and population genetics of three important pollinator species at a range of different biological scales. These start from the level of individuals on single flowers, through populations, to the community level, whilst including various time and spatial scales. Furthermore, it uses this cryptic species complex as a model to explore more general ecological questions. Finally, this work demonstrates that overlooking cryptic diversity, or the inability to correctly identify species, could have strong implications, not only for species conservation management, but also our understanding of ecosystem functioning and population dynamics.

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