Genetic diversity and structure in European bumblebee (*Bombus* spp.) populations.

Submitted by Sophie Jane Hedges, to the University of Exeter as a thesis for the degree of Masters by Research in Biological Sciences in December 2018.

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

In recent years there has been a global trend in declines of pollinator species primarily due to anthropogenic stressors, including habitat fragmentation and land-use change resulting from agricultural intensification. The subsequent loss of foraging plants via this process creates habitat ‘islands’, causing genetic isolation similar to that found on geographic islands. Isolated populations are at a much greater risk of inbreeding and reduced genetic diversity, which in turn increases their susceptibility to disease. To reduce the fragmentation of wildlife populations, the EU introduced Agri-environmental schemes (AES) which provide mitigation methods including offers of financial aid to farmers planting wild flower margins. This thesis compared two wild bumblebee species between island and mainland sites in the UK and France to assess the impact of geographic isolation on populations. This thesis also sampled four bumblebee species in sites with different levels of AES in the UK, to assess the efficacy of the schemes in terms of promoting genetic diversity and analyse relationships between heterozygosity and disease.

By using genetic techniques to estimate the diversity, structuring and population size of each species, comparisons between different environment types were made. Molecular analysis found significant structuring in *Bombus pascuorum* ($\theta = 0.122$) populations across the UK and French populations. Within *B. pascuorum* populations, there was found to be a higher prevalence of the gut trypanosome *Crithidia bombi* in populations with reduced heterozygosity. Molecular analysis of the agri-environmental sites found a positive relationship between floral diversity and the heterozygosity of the population, and a large proportion of genetically similar sister pairs were sampled within these sites.

The overall conclusions from the research presented in this thesis are that to sustain wild pollinator numbers, further development of agri-environmental schemes is required with a focus on increasing floral diversity. Even with low intensive sampling effort sister pairs are likely, which can impact the results of epidemiological studies of haplodiploid species. Furthermore, the genetic analysis presented here suggests a strong link between population isolation and disease prevalence, thus isolated populations are at greater risk of extinction unless intervention occurs.

List of Contents

List of Tables:

population size on the prevalence of *Apicystis bombi* and *C. bombi* within each species.

Table 3.1: Each location and the date of sampling across the four time points in 2016/2017.

Table 3.2: The number of *B. hortorum* individuals genotyped per site and sampling time point including the caste type. The heterozygosity (F_{ST}) and allelic richness per location including standard error; the number of sister pairs per site with the number of unique colonies (the number of times when only one worker represented a nest) in brackets. The effective population size and 97.5% confidence intervals after 1,000 bootstraps and max pop size equal to 1000, calculated using the ECM with those populations best described using TIRM marked with *, the floral density per sample site and the prevalence of *A. bombi* to the nearest percent. The average for ELS and HLS for each of the columns is highlighted in bold below. ELS3 and HLS5 were removed from analysis due to poor sampling efforts.

Table 3.3: The number of *B. lapidarius* individuals genotyped per site and sampling time point including the caste type. The heterozygosity (F_{ST}) and allelic richness per location including standard error; the number of sister pairs per site with the number of unique colonies (the number of times when only one worker represented a nest) in brackets. The effective population size and 97.5% confidence intervals after 1,000 bootstraps and max pop size equal to 1000, calculated using the ECM with those populations best described using TIRM marked with *, the floral density per sample site and the prevalence of *A. bombi* to the nearest percent. The average for ELS and HLS for each of the columns is highlighted in bold below.

Table 3.4: The number of *B. pascuorum* individuals genotyped per site and sampling time point including the caste type. The heterozygosity (F_{ST}) and allelic richness per location including standard error; the number of sister pairs per site with the number of unique colonies (the number of times when only one worker represented a nest) in brackets. The effective population size and 97.5% confidence intervals after 1,000 bootstraps and max pop size equal to 1000, calculated using the ECM with those populations best described using TIRM marked with *, the floral density per sample site and the prevalence of *A. bombi* to the nearest percent. The average for ELS and HLS for each of the columns is highlighted in bold below.

Table 3.5: The number of *B. terrestris* individuals genotyped per site and sampling time point including the caste type. The heterozygosity (F_{ST}) and allelic richness per location including standard error; the number of sister pairs per site with the number of unique colonies (the number of times when only one worker represented a nest) in brackets. The effective population size and 97.5% confidence intervals after 1,000 bootstraps and max pop size equal to 1000, calculated using the ECM with those populations best described using TIRM marked with *, the floral density per sample site and the prevalence of *A. bombi* to the nearest percent. The average for ELS and HLS for each of the columns is highlighted in bold below.

Table 3.6: A table to show the prevalence of the least common species within the cryptic species complexes across time points. The total number of *B. lucorum,* and *B. terrestris* caught per time point, with the prevalence of *B.*

lucorum. And the total number of *B. ruderatus* and *B. hortorum* caught per time point with the prevalence of *B. ruderatus*.

Table 3.7: A table showing the results of AMOVA performed in Arlequin for *B. hortorum, B. lapidarius, B. pascuorum* and *B. terrestris*. The groups are as follows: *B. hortorum* **(k=3)**: ELS1+ELS2+HLS4, HLS3+ELS5+HLS5, HLS1+HLS2+ELS4; *B. hortorum* **(k=7):** ELS1+ELS2, HLS3, ELS5+HLS5, HLS1, HLS2, ELS4, HLS4 *B. hortorum* **UPGMA**: ELS1+HLS4, REST AS INDIVIDUAL GROUPS. *B. lapidarius* **(k=2):** ELS1+ELS2+ELS4+HLS4, HS1+HLS2+HLS3+HLS5+ELS3+ELS5; *B. lapidarius* **UPGMA**: ELS1+ELS4, ELS2+ELS3, HLS3+HLS2, HLS1, ELS5+HLS4, HLS5. *B. pascuorum* **(K=3):** ELS2, ELS1+ELS4+ELS5+HLS1+HLS3+HLS4, ELS3+HLS2,+HLS5; *B. pascuorum* **(k=6):** ELS1, ELS2, HLS1+HLS3, ELS4+ELS5+HLS5, HLS2+HLS5, ELS3; *B. pascuorum* **UPGMA**: ELS1+HLS3+HLS1+ELS5+ELS4+HLS4, ELS2+HLS5+HLS2+ELS3. *B. terrestris* **(k=2):** HLS2+HLS5, ELS1+ ELS2+ HLS3+ ELS5+HLS1+ELS4+HLS4+ELS3; *B. terrestris* **UPGMA**: ELS1+HLS3, ELS5+HLS4+HLS1+ELS3+ELS4, ELS2, HLS2+HLS5. Results with greatest group percentage per variation per species are highlighted in bold.

Table 3.8: The results of a binomial generalised linear model (GLM) for each *Bombus* sp. (*B. hortorum, B. lapidarius, B. pascuorum and B. terrestris*), looking at the variables heterozygosity, scheme and floral density on the prevalence of *Apicystis bombi* within each population.

List of Figures:

Figure 2.6: A UPGMA (a) and NJ tree (b) showing the relationship between genetic distances of the 10 sample sites for *B. pascuorum* populations. Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L) and Ushant (U). Branch numbers represent bootstrapping percentage.

Figure 2.7: Inference of the optimal value of K using the ΔK method of Evanno (*et al.,* 2005) for *B. terrestris* populations. Figure shows support for 2 clusters (K).

Figure 2.8: Population structure analysis bar graphs from STRUCTURE for populations of *B. terrestris* with support for K=2 and K=3. Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L), Quiberon (Q) and The Isle of Scillies (S). Each individual genotype is represented by a coloured vertical bar representing the posterior assignment probability of being assigned to the clusters. The clusters are shown as different colours within the graphs.

Figure 2.9: A UPGMA (a) and NJ tree (b) showing the relationship between genetic distances of 10 sample sites for *B. terrestris* populations. Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L), Quiberon (Q) and The Isle of Scillies (S). Branch numbers represent bootstrapping percentage.

Figure 2.10: Principal Component Analysis performed in R for a) *B. pascuorum* and b) *B. terrestris* populations with each colour representing each sampling site; Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (I), Jersey (J), Liverpool (L), Quiberon (Q), The Isle of Scillies (S) and Ushant (U). Each point represents an individual sampled, and the distance between each point represents how dissimilar the samples are to each other.

Figure 2.12: The relationship between *Crithidia bombi* count per individual sample and heterozygosity of the host population in a) *Bombus terrestris* ($p<0.05$) and b) *Bombus pascuorum* ($p<0.05$). Each point represents an individual within the population.

Figure 3.1: The relationship between log bumblebee abundance and log flower abundance per m^2 on arable field margins, with each point representing a sampling point. The type of landscape is shown in the figure key. Reproduced from Pywell *et al.,* 2006.

Figure 3.2: An illustration of the four *Bombus* spp. studied; a) *B. hortorum,* b) *B. lapidarius,* c) *B. pascuorum,* and d) *B. terrestris*. (Illustration: Alice Rosen 2018)

Figure 3.3: A map of the UK showing the location of the 10 field sites sampled during 2016/2017. Entry-level farms are represented by grey squares and higher-level farms by a black circle.

Figure 3.4: The relationship between the logged geographical distance (km) and genetic distance (F_{ST}) for a) *B. hortorum* ($p<0.05$), b) *B. lapidarius* ($p=0.17$), c) *B. pascuorum* ($p=0.71$), and d) *B. terrestris* ($p=0.65$) populations. Each point represents the a pairwise comparison from genetic and distance matrices.

Figure 3.5: Neighbor-Joining trees (left) and UPGMA graphs (right) for (a) *B. hortorum,* (b) *B. lapidarius,*(c) *B. pascuorum* and (d) *B. terrestris.* Populations are as follows: 1- ELS1, 2-ELS2, 3-HLS3, 4-ELS5, 5-HLS1, 6-HLS2, 7-ELS4, 8-HLS5, 9-HLS4 and 10- ELS3.The branch numbers represent bootstrapping over loci.

Figure 3.6: Inference of the optimal value of K using the ΔK method of Evanno (*et al.,* 2005) from STRUCTUREHARVESTER. Support for a) K=3/K=7 *B. hortorum*, b) K=2 *B. lapidarius,* c) K=3, K=6 *B. pascuorum,* and d) K=2 *B. terrestris*.

Figure 3.7: Population structure analysis bar graphs from STRUCTURE for a) *B. hortorum,* b) *B. lapidarius*, c) *B. pascuorum* and d) *B. terrestris*. Each individual genotype is represented by a coloured vertical bar representing the posterior assignment probability of being assigned to the clusters. Each colour represents a different cluster within each of the graphs.

Figure 3.8: The relationship between the average floral density of each field site and the observed heterozygosity (F_{ST}) of a) *B. hortorum* ($p<0.05$), b) *B. lapidarius,* c) *B. pascuorum* ($p<0.05$) and d) *B. terrestris* ($p=0.215$) populations. Each point represents a sampling location with higher-level sites in blue and entry-level sites in orange.

Figure 3.9: The level of stewardship (HLS and ELS) and the effective population of a) *B. hortorum,* b) *B. lapidarius,* c) *B. pascuorum* and d) *B. terrestris* with the means and 95% confidence intervals. Each point represents an individual sampling location.

Figure 3.10: The prevalence of *A. bombi* given the type of agri-environment site (ELS vs HLS) for populations of a) *B. hortorum,* b) *B. lapidarius,* c) *B. pascuorum* and d) *B. terrestris*. Each point represents a sampling location and the mean, and 95% confidence intervals are given per scheme.

List of Supplementary Material

Figure S1: (a) A gel image showing the bands for PCR product from the methods of *B. terrestris* and *B. lucorum* differentiation. (b) A gel image showing the bands for digested PCR product from the methods of *B. hortorum* and *B. ruderatus* differentiation.

Table S1: A matrix of pairwise heterozygosity (F_{ST}) values for *B. pascuorum* between each sampling location using 6 loci with significant values ($p<0.05$) in bold after Bonferroni corrections. Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L) and Ushant (U).

Table S2: A matrix of pairwise heterozygosity (F_{ST}) values for *B. terrestris* between each sampling location using 8 loci with significant values ($p<0.05$) in bold after Bonferroni corrections. Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L) and Ushant (U).

Table S3: A matrix of pairwise geographical distances between sites (km). Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L) and Scillies (S).

Table S4: A matrix of pairwise heterozygosity (F_{ST}) values for *B. hortorum* between each sampling location with significant values ($p<0.05$) in bold after Bonferroni corrections.

Table S5: A matrix of pairwise heterozygosity (F_{ST}) values for *B. lapidarius* between each sampling location with significant values ($p<0.05$) in bold after Bonferroni corrections.

Table S6: A matrix of pairwise heterozygosity (F_{ST}) values for *B. pascuorum* between each sampling location with significant values ($p<0.05$) in bold after Bonferroni corrections.

Table S7: A matrix of pairwise heterozygosity (F_{ST}) values for *B. terrestris* between each sampling location with significant values ($p<0.05$) in bold after Bonferroni corrections.

Table S8: A matrix of pairwise geographical distances between sites (km).

Table S9: The GPS location of each sampling site within Chapter 1.

Chapter 1: General Introduction

1.1 The importance of wild pollinator species

There are circa 250 bumblebee species living in the world today, distributed primarily in temperate regions, with 25 species located in the UK. These insect species provide a valuable ecosystem service, which was estimated to be worth approximately $215 billion globally in 2005 (Gallai *et al.*, 2009), as pollinators both in agriculture and the natural environment. Insect pollinators account for the pollination of approximately 75% of crops and 94% of wild flower species (Klein *et al.*, 2007) due to their generalist foraging nature. However, over the past 50 years there has been a global trend for a decline of pollinator numbers and range contractions in both wild and managed populations in the Northern Hemisphere where they are primarily found (Goulson *et al.*, 2008; Williams and Osborne, 2009; Bommarco *et al.*, 2011; Cameron *et al.*, 2011; Garibaldi *et al.*, 2013; Woodcock *et al.*, 2016). Since the start of the 20th century, two bumblebee species have become extinct and eight are now listed as endangered in the UK (Ollerton *et al.*, 2014). Declines cause deserved ecological and economical concern with regards to the pollination service bumblebees provide. This reduction in numbers has been linked to a decline in wild flowers and crop provisions for humans (Biesmeijer *et al.*, 2006; Potts *et al.*, 2010).

Long term studies of these population declines have been observed, but very little is known about how pollinator populations have changed more recently and how the dynamics are influenced temporally due to interacting anthropogenic and environmental factors. These declines in bumblebee numbers are concentrated in central England (Fig. 1.1) with a comparison of pre-1960 data to post 1960s showing declines throughout England, Scotland and Wales. Analysis of European wild bee species, comprising of bumblebees and solitary bees, found 1,101 out of a total of 1,942 species were listed as Data Deficient

under IUCN criteria (Nieto *et al.,* 2014). This means there is insufficient information to make an informed assessment of the species risk of extinction.

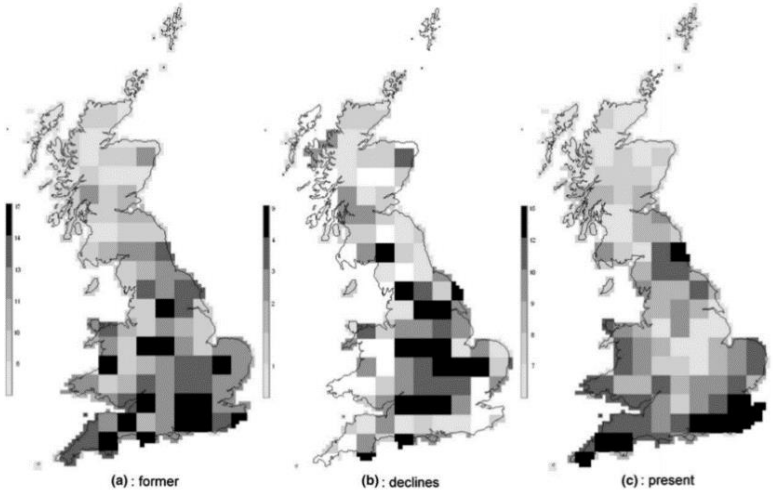

Figure 1.1: Maps of species richness for British bumblebees from the Bumblebee Distribution Maps Scheme data measured at the scale of 50 x 50 km grid cells for (a) former richness (pre 1960 and 1960 onwards records); (b) declines in richness (pre 1960s records); (c) present richness (1960 onwards records). Equal frequency grey-scale classes are used to maximise differentiation among regions. Reproduced from Williams 2005.

1.2 Force of natural geographical barriers

Islands provide unique ecological environments which have been used in many studies worldwide due to characteristics that make them distinct from mainland habitats. At a community level, these island sites are more likely to have decreased levels of fauna and flora diversity compared to the mainland (Mac Arthur & Wilson 1967), which in turn increases the level of intra-specific interactions at the species level. Biogeographical theory predicts that island populations are less genetically diverse than the mainland due to decreased migration rates, inbreeding (due to bottlenecks and small population sizes), founder effects and genetic drift (Wright, 1931; Mayr, 1978; Frankham, 1997). These populations are likely to diverge from their mainland relatives, with some examples suggesting sub-species are likely to develop dependent on the degree of isolation (Nilson and Andren, 1981; Beheregaray *et al.*, 2003; Hille *et al.*, 2003). Parallel to the limited gene pool, increasing climate change and a necessity for populations to adapt, these secluded populations are at greater risk of extinction (Frankham 1998). These high risks of extinctions and close interactions between species means a removal of a species at one trophic level

would greatly impact others, as well as the ecosystem services the species provide (Wood *et al.*, 2017a; Wood *et al.*, 2017b).

These insular populations are found not only geographically via oceanic islands, but also within fragmented habitats such as arable landscapes and housing developments with decreased floral heterogeneity. Most wild pollinators including the bumblebee are arthropods and can fly, allowing for dispersal across water. *Bombus* spp. have also successfully colonized islands such as New Zealand and Tasmania due to human importation (Hingston and McQuillan, 1999). These populations show decreased levels of genetic diversity but also the ability to withstand high levels of inbreeding (Schmid-Hempel *et al.*, 2007), as a clear genetic structure between Tasmanian, New Zealand and populations of origin in Lincolnshire was observed. By comparing the fitness of *Bombus* spp. populations of island sites with similar mainland sites, the impact of genetic structuring and heterozygosity can be studied. Oceanic islands provide important model systems into the effects of isolation over long periods of time. Anthropogenic stressors can create inland 'islands' such as within fragmented habitats, potentially causing genetic isolation as seen within these island populations.

1.3 A changing world

Since the post-war era, the UK and the rest of the world has undergone considerable economic, ecological and social transformation. Well documented advances in technology and social activism have not always benefitted natural landscapes and the biodiversity within them. Climate change as a consequence of human population growth and industrialisation is set to accelerate unless immediate intervention occurs (Sirois-Delisle and Kerr, 2018) and as a result, extinctions are likely as it is predicted that some species will be unable to adapt adequately (Sinervo *et al.*, 2010).

Increased use of pesticides, habitat fragmentation, and intensification of agriculture have all contributed to pollinator declines within the UK (Goulson *et al.*, 2015). Agricultural intensification is just one example of the pressures exerted upon the ecosystem by continual modernisation of farming methods, and consists of increased field sizes, and crop-enhancing chemicals such as pesticides and machinery. Alongside the fragmentation of arable landscape, this

increase in chemical usage aids the decline in insect species which provide valuable ecosystem services as pollinators and even as biological pest control.

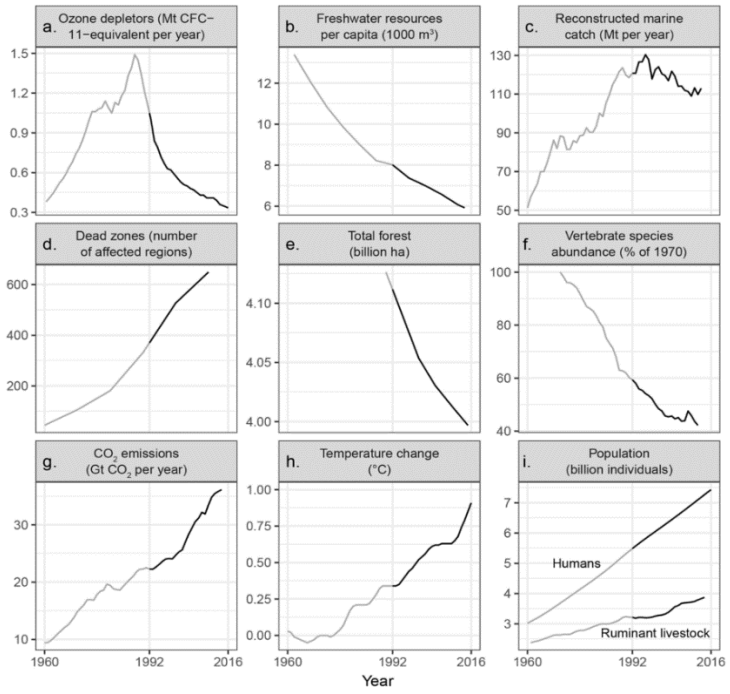

Figure 1.2: Trends over time for environmental issues. The years before and after the 1992 scientists' warning are shown as grey and black lines, respectively. Percentage change, since 1992, for the variables in each panel are as follows: (a) –68.1%; (b) –26.1%; (c) –6.4%; (d) +75.3%; (e) –2.8%; (f) –28.9%; (g) +62.1%; (h) +167.6%; and (i) humans: +35.5%, ruminant livestock: +20.5%. Figure adapted from Ripple (*et al.,* 2017).

Global environmental trends not only affect wild pollinator species, but a whole array of biota. In 1992 there were reports on the current state of human impacts on the environment to push for a more sustainable future (Ripple *et al.*, 2017). Here there was an emphasis on the limits of the biosphere before irreversible harm is made and how close to this threshold the world is. The research highlighted many areas of global change from increasing human population size to the production of carbon dioxide (fig 1.2).

1.4 Reversing impacts of human intensification of agriculture

Agricultural output increased more extensively between 1945 and 1965 than any other period in history (Brassley, 2000). This intensification of agriculture, driven by a desire for self-sufficiency, also resulted in increasing homogeneity of the UK landscape and a parallel increase in farming activities such as fertiliser use and spraying of crop pesticides. In the UK alone there has been a significant rise from 37% in 1962 to 77% by 1982 in nitrogen fertiliser use within permanent grassland (Wells and Sheail 1988). Trends in increasing agricultural

intensification are not localised to the UK (Fig. 1.3), as these factors are global issues.

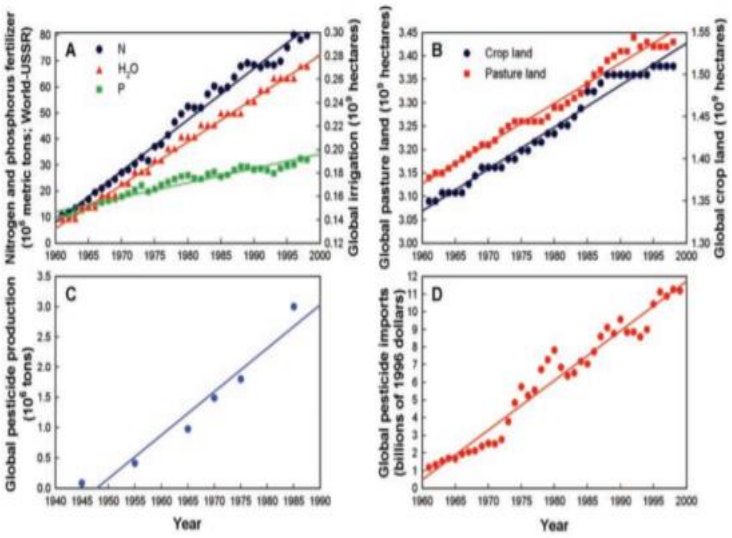

Figure 1.3: **(A)** Trends in annual rates of application of nitrogenous fertilizer (N) expressed as mass of N, and of phosphate fertilizer (P) expressed as mass of P_2O_5, for all nations of the world except the former USSR (FAO 2001), and trends in global total area of irrigated crop land (H_2O) (FAO 2001). **(B)** Trends in global total area of land in pasture or crops (FAO 2001). **(C)** Trend in global pesticide production rates, measured as millions of metric tons per year (WHO 1990). **(D)** Trend in expenditures on pesticide imports (FAO 2001) summed across all nations of the world, transformed to constant 1996 U.S. dollars. All trends are as dependent on global population and GDP as on time. Reproduced from Tilman (*et al.,* 2001).

As a mitigation tool against these trends in loss of biodiversity, the EU created agri-environmental schemes (AES) under the Common Agricultural Policy (CAP) introduced in 1992. Over the years there have been many changes to the CAP, primarily the allocation of the EU budget, with input declining from 73% in 1984 to 39% in 2003 (Stoate *et al.*, 2009). These schemes subsidise farmers to support a greater standard of living and encourage an increase in food production to meet demand of Europeans, whilst maintaining sustainable management of the landscape. One method the policy adopts is encouraging the planting of wild flower margins and less intensive farming methods whilst trying to maintain a high level of productivity. The aims behind this initiative are to protect biodiversity by reducing pesticide use and mitigating the effect of habitat fragmentation by preventing loss of wild populations (Kleijn and Sutherland, 2003).

These wild flower strips are used to encourage pollinator species and result in smaller areas of dense heterogenous foraging plants which bee species are more likely to visit (Carvell *et al.*, 2004; Wood *et al.*, 2015). The foraging behaviours of insects are shaped by the man-made landscape and have the potential to bias results of genetic diversity and estimates of population size. By sampling wild flower strips the resultant increased observation in genetic diversity could be due to increased density in the specific sampling area as opposed to the entire agricultural landscape. Investigations which suggest improvements by these schemes do not necessarily explore the population-level effects as it could be a case of manipulating forager density in the homogeneous landscape (Holland *et al.*, 2015). Pollinator richness declines with increasing distance from these foraging habitats (Garibaldi *et al.*, 2011) therefore the manipulation of the landscape through these AES need to be strategic.

1.5 Important role of disease

Stressors negatively impacting pollinator numbers such as reduced floral diversity and pesticide use may also lead to an increase in susceptibility to parasites and pathogens (Goulson *et al.*, 2015). The infection with diseases such as the trypanosome *Crithidia bombi* is likely to be contributing to the global decline of bumblebee numbers (Schmid-Hempel and Tognazzo, 2010; Koch and Schmid-Hempel, 2011). Investigations into the direct effect of reduced genetic diversity on the prevalence of disease in bumblebee populations has suggested that there is an increase in the gut parasite *C. bombi* in populations with lower genetic diversity (Whitehorn *et al.*, 2011). The study found the prevalence of *C. bombi* to be higher in populations with reduced genetic diversity; however, there was no correlation with the immune response (levels of phenoloxidase and encapsulation response) and population heterozygosity. The levels of the enzyme phenoloxidase did decline, however, with an increase in parasite abundance. Therefore, this study suggests isolated, more homozygous populations are likely to have increased parasite prevalence.

The varroa mite (*Varroa destructor*), is another emerging world-wide parasitic vector of disease and has detrimental effects on both wild and managed bee populations (Yang and Cox-Foster, 2007). Pathogens have proved to be a

significant threat to the apicultural industry in recent years, with dramatic declines observed in populations of *Apis mellifera* (European honeybee) (Smith *et al.*, 2013). Individuals and brood parasitized by the virus-vectoring *Varroa* mite are almost 100% likely to be co-infected with Deformed Wing Virus (DWV) (Genersch, 2005) and Acute Bee Paralysis Virus (AVPV) (Ball, 1985).

Spill-over of the emerging infectious disease from honeybees into bumblebee populations has been observed (Furst *et al.*, 2014), as well as from pathogens present in commercial bumblebee colonies (Graystock *et al.*, 2013) via floral resource sharing (Durrer and Schmidhempel, 1994; Graystock *et al.*, 2015). There has been an increase in the demand for managed bee populations as pollinators for crops around the world, and this has led to mass transportation of pollinator species worldwide. Parallel transportation of associated diseases such as the *Varroa* mite has also been accelerated by this global trade of commercial bees (Goulson and Hughes, 2015). It is thought that Emerging Infectious Diseases (EIDs) such as DWV have a broader host range than originally thought; of the 24 viruses isolated from honeybees many can also infect bumblebee, solitary bee, wasp, ant and hoverfly species (de Miranda *et al.*, 2013; Bailes *et al.*, 2018; McMahon *et al.*, 2018). The disease transmission route will likely influence the spread between pollinator species; for example directly transmitted diseases, such as those transmitted sexually and vertically will likely occur within and not between host species, whereas indirect transmission, such as food, faecal, and vector-borne, may increase transmissions between host species (Woolhouse *et al.*, 2005). Inter-species transmission of diseases is more likely to occur with an increase in pollinator density and sharing of floral resources such as plants within agri-environmental schemes.

Parasites and diseases are more likely to be transmitted between genetically similar hosts. An increase in heterozygosity (a measure of genetic diversity) leads to a greater chance of a resistant host being present in the population (Anderson and May, 1986) leading to a decrease in the overall disease risk within the species (Keesing *et al.*, 2006). By reducing individual and population level genetic heterozygosity, inbreeding can increase host susceptibility to infectious parasites (Ellison *et al.*, 2011). The underlying idea behind this increase in disease prevalence and reduced genetic diversity is also known as

the ‘monoculture effect’ and has been well documented in agricultural studies (Smithson and Lenne, 1996; Mundt, 2002; Reiss and Drinkwater, 2018). It has been suggested that host genetic diversity could buffer populations against epidemics in nature, but it is not clear how much is required to prevent the spread of disease (King and Lively, 2012). Current patterns of genetic diversity and disease spread have been analysed and recorded by King and Lively (2012), with research primarily focusing on endangered mammal species. The black-footed ferret for example, is believed to have been removed from its natural habitat because of low genetic diversity aiding the spread of a virulent canine distemper epizootic (Thorne and Williams, 1988).

The genetic diversity of a population greatly influences its fitness with regards to infection with parasites, with reduced heterozygosity greatly increasing a populations’ prevalence of disease. Isolated and inbred populations are therefore at a much greater risk, not only due to small population sizes but also their susceptibility to infectious pathogens. Genetic structuring within species of bumblebee could theoretically prove valuable as related hosts provide easy transmission pathways for diseases.

1.6 Aims and objectives for this thesis

To better aid conservation of declining bumblebee species, a greater understanding of their population trends in terms of dispersal and population size is required both nationally and globally. To secure pollination services and improve current and future conservation efforts with regards to invertebrate pollinator species, a clearer understanding of the relationship between genetic and environmental factors affecting populations is key. By appreciating the impact of low genetic diversity, small effective population sizes and prevalence of disease on populations, methods of buffering anthropological changes in habitat fragmentation can be improved upon. There have been previous studies demonstrating the impacts of agri-environmental schemes (Wood *et al*., 2015; Carvell *et al.*, 2017) and island biogeography (Schmid-Hempel *et al.*, 2007) on populations of wild bumblebees; however, in this thesis I link genetic diversity with measures of effective population size and pathogen prevalence and load in large scale field conditions.

This thesis aims to combine described methods of population genetics with epidemiology to analyse wild populations of bumblebees in England and France. By using these techniques, this thesis will be the first to compare current mitigation tools aimed at improving wild pollinator conservation efforts with measures of genetic diversity and disease. I will also show relationships between heterozygosity and disease prevalence in natural populations of *B. pascuorum* and *B. terrestris* in island sites. This thesis aims to contribute towards increasing the efficacy of conservation efforts of declining wild pollinator species and provide a pathway for future research studies.

I sampled naturally occurring isolated populations on islands and compared genetic structuring and impacts of heterozygosity on disease prevalence with those of mainland populations. I also sampled within agri-environmental sites and assessed how methods implemented to buffer against fragmented landscapes are impacting the genetic diversity and population structure of bumblebee species. These two investigations into bumblebee populations aim to increase understanding of the role of factors influencing heterozygosity of populations, and how this affects susceptibility to disease.

Chapter 2: Genetic structure within and between insular and mainland populations of *Bombus pascuorum* and *Bombus terrestris*

2.1 Abstract:

Island populations are at a greater risk of extinction resulting from reduced genetic diversity, due to their isolation from mainland populations, via founder effects and genetic drift. Isolated communities tend to have reduced species diversity which results in a highly complex and connected biotic structure; therefore, extinctions within one trophic level would negatively impact species within another. Insects, such as the bumblebee, provide valuable ecosystem services as wild flower and crop pollinators. A potential loss in genetic diversity within island *Bombus* spp*.* populations leads to a reduction in fitness, causing a greater risk of extinction by factors such as environmental change, pesticide use and emerging infectious diseases. Here I investigate the relationship between island and mainland sites in terms of their effective population size, genetic diversity and structuring as well as the effect of pathogen prevalence on two common bumblebee species; *Bombus terrestris* and *Bombus pascuorum*. I found genetic structuring ($\theta_{pascuorum}$=0.122, $\theta_{terrestris}$=0.069) as well as significant isolation by distance within *B. terrestris* populations. Within *B. pascuorum* populations, I found a reduction in heterozygosity lead to an increase in the prevalence of two common bee diseases; *Crithidia bombi* and *Apicystis bombi*. My research highlights the importance of understanding genetic structuring of bumblebees from within isolated populations and explores potential fitness consequences derived from inbreeding and reduced genetic diversity.

2.2 Introduction:

Islands provide a unique model system for conservation studies due to their geographic isolation and increased levels of endemism and extinctions (Jensen *et al.*, 2013). These insular ecosystems occur not only due to ocean barriers but also on mountain tops, in lakes and in ponds. Isolated islands tend to have reduced species diversity resulting in a highly complex and connected biotic community (Simberloff, 1974). Therefore, any changes in the community structure at a single trophic level would have a greater cascading impact on other species populations than within a less isolated area such as on the mainland (May, 1975; Sahasrabudhe and Motter, 2011).

Substantial evidence shows loss of *Bombus* spp. populations globally, primarily in the northern hemisphere where *Bombus* spp. are most common (Biesmeijer et al., 2006; Goulson et al., 2008; Williams and Osborne, 2009; Bommarco et al., 2011; Cameron et al., 2011; Garibaldi et al., 2013; Ollerton et al., 2014; Woodcock et al., 2016). This loss of pollinator species has a knock-on effect at other trophic levels (Brosi *et al.*, 2007). Both island and mainland populations of bumblebees and other hymenopterans are of great importance because of their role as crop and wild flower pollinators (Corbet *et al.*, 1991), ecosystem engineers (Jones *et al.*, 1994), and natural pest-predators (Van Mele and Cuc, 2000). Studying how isolation on island sites impacts the decline of wild bumblebee populations can help current understandings of reductions globally.

Genetic diversity is of great importance when studying species conservation. It has long been predicted that island populations exhibit less genetic variation than mainland populations, and endangered species experience a further reduction in genetic variation (Frankham, 1996). Increased genetic diversity can help populations buffer against a whole host of biological stressors, such as gradually increasing global temperature and environmental isolation, by facilitating genetic adaptations to these changes. Evidence supporting these hypotheses has been shown in a range of species from bighorn sheep (Fitzsimmons *et al.*, 1995) to reef-building coral, *Pocillopora damicornis* (Thomas *et al.*, 2017). Inbreeding and bottlenecks have been discovered in numerous bumblebee species, most prevalent in island or isolated populations (Darvill *et al.*, 2006; Ellis *et al.*, 2006; Schmid-Hempel et al., 2007). These

populations are characterised by low genetic diversity, smaller population sizes and weak structuring because of these bottlenecks, as well as founder effects and genetic drift (Wright, 1931; Mayr, 1978; Frankham, 1997).

A meta-analysis by Britten (Britten, 1996) supported a strong relationship between the heterozygosity of a population and fitness within a variety of plant and animal species. Within haplodiploid organisms such as the bumblebee, the effects of inbreeding and low genetic diversity are greater than in similar diploid species (Cook and Crozier, 1995). Within these haplodiploid species, sex is determined by zygosity at a specific sex determining locus; individuals heterozygous at the locus become female and those that are hemizygous develop into males. Populations with high levels of inbreeding increase the likelihood of a queen mating with a male who shares a sex determining allele, resulting in diploid, non-viable (sterile) males (Whitehorn *et al.*, 2009). Inbred populations with diploid males had a much slower growth rate and produced significantly fewer offspring and consequently demonstrated an increased likelihood of declines and extinction (Frankham, 2005; Zayed and Packer, 2005; Goulson *et al.*, 2008)

Over the years, many factors have been suggested as main causes behind island extinctions, including habitat removal, species introductions, predation, the arrival of human populations and the spread of disease (Frankham, 1998). However, some island populations may become extinct due to natural environmental and genetic factors (Shaffer, 1981). The overall size of an individual island, as well as its distance from the mainland and time elapsed since isolation, will influence the rate of species extinction, with a species' flight and dispersal capabilities affecting its own extinction rate. The likelihood of a species being able to colonise an oceanic island depends on several factors such as the distance from the mainland, the dispersal ability of the species and the opportunity to fill a niche within the new ecosystem.

The effective population size can be of great importance in conservation research and in understanding the evolutionary history of a certain species. Graur (Graur, 1985) was the first to collate data from a range of taxa to show a positive correlation between effective population size and the heterozygosity within different species. Within my study of haplo-diploid hymenopteran species,

the effective population size (N_e) is the number of colonies (or nests) in each area and not the number of individuals within them. This is due to the complex eusocial behaviour of bumblebee species; these insects live in colonies of up to 400 individuals depending on the species, with a single fertile queen representing the reproductive unit of the whole nest (Goulson, 2003).

Insular genetic isolation and resultant inbreeding depression within *Bombus* spp. lead to decreased heterozygosity and this in turn can increase an individual's and population's susceptibility to disease (Woodard *et al.*, 2015) as well as cause fitness declines, slower growth rates and lower reproductive rates (Whitehorn *et al.*, 2009). In order to optimise conservation efforts for a given species, genetic diversity as well as the distribution of disease within and among populations should therefore be considered.

My thesis studies populations of two common bumblebee species: *B. terrestris* and *B. pascuorum*. Both species are polylectic eusocial bumblebees with a wide distribution across England and France. Recently, rearing techniques of *B. terrestris* have greatly improved, producing colonies for commercial crop pollination services in the agricultural sector (e.g. to provide pollination services for crops such as tomatoes and blueberries (Vanbergen *et al.*, 2013). This has resulted in Europe-wide transportation of *B. terrestris* colonies, which affects the genetic diversity of wild populations and can also introduce diseases without careful screening processes (Otterstatter and Thomson, 2008; Graystock et al., 2013).

B. pascuorum are widespread throughout the UK and France except for on the Scilly Isles, Outer Scottish Isles and Shetland (Williams and Osborne, 2009). Recently, the species has been established on the Orkneys (Plowright and Plowright, 1997). *B. terrestris* populations have a similar range distribution, however the species is found on the Scilly Isles (Williams and Osborne, 2009) and they have been spreading in Northern Scotland (Macdonald 2001). The high prevalence of both species within the UK and France makes them the perfect study species to assess their genetic diversity and structuring within this thesis.

As well as an increase in habitat fragmentation, increases in pesticide use and other such anthropogenic stressors, disease and viruses of bee species have

become a more important area of study over the past few decades. *Varroa destructor* is an ectoparasitic mite that acts as a vector for many bee viruses such as Deformed Wing Virus (DWV) (Furst *et al.*, 2014) and has spread globally (Oldroyd, 1999) via a novel transmission route in *Apis mellifera*. This in turn results in an observed increase in the prevalence, viral load (Martin *et al.*, 2012) and virulence (Genersch *et al.*, 2010; Ryabov *et al.*, 2014) of viruses such as DWV. In places where *Varroa* is present, there has been shown to be an increase in the prevalence of pathogens in both *Apis* and *Bombus* species, suggesting there is also the ability for multi-host pathogen spill over (Manley 2017). Such pathogens include the trypanosome *Crithidia bombi* and the neogregarine *Apicystis bombi*, both of which are multi-host parasites and are likely to be transmitted via faecal-oral routes whilst workers forage on flowers. *C. bombi* parasitized workers are less likely to forage for pollen, and visit fewer flowers when foraging (Shykoff and Schmidhempel, 1991; Otterstatter et al., 2005). *A. bombi* infected workers have an increased mortality, reduced fat body content and increased sensitivity to sucrose (Graystock *et al.*, 2015), with very few bees being highly infected due to the high pathogenicity of the neogregarine. Other emerging infectious diseases (EIDs) in both managed and wild populations of bees can negatively impact pollinator services and therefore the subsequent food security this provides. Arbetman *et al.,* (Arbetman *et al.*, 2017) found that species which have no association with the three most common bee pathogens (*Crithidia bombi, Nosema* spp.*, Locustacarus buchneri)* were more prone to decline, suggesting these species have a reduced tolerance to infection. Whitehorn *et al.,* (Whitehorn *et al.*, 2011) went one step further looking into the declining bee species *B. muscuorum* in island populations, to suggest that the prevalence of *C. bombi* was higher in populations with lower genetic diversity.

Populations of *B. terrestris* have been the subject of many genetic studies globally for numerous years, as they are widespread and abundant. Studies have shown genetic variation across island/mainland populations (Widmer *et al.*, 1998); and within mainland populations (Kraus *et al.*, 2009). Subdivision within *B. terrestris* has also been observed (Rasmont *et al.*, 2008). Very few genetic studies of *B. pascuorum* populations have been conducted; however, studies that have, show a similar pattern with geographic isolation and high

genetic diversity and structuring as found in *B. terrestris* (Pirounakis *et al.*, 1998; Widmer and Schmid-Hempel, 1999; Herrmann *et al.*, 2007).

In this present study, I calculated the genetic diversity and structuring of *B. terrestris* and *B. pascuorum* populations within and between 12 island and mainland sites in the UK and France, using microsatellite markers. I used estimates of genetic diversity (F_{ST}), genetic structure (θ) and effective population size (N_e) to study the impact of geographical isolation on wild pollinator populations. I also used these measures to analyse the relationship between the heterozygosity of populations and the prevalence and abundance of the pathogens *C. bombi* and *A. bombi*.

2.3 Materials and Methods:

2.3.1 Sample species

B. terrestris and *B. pascuorum* were sampled across 12 field sites in the UK and France, resulting in a total of 283 *B. pascuorum* workers (see table 2.1) and 645 *B. terrestris* workers (see table 2.2). To allow for whole body RNA sequencing for pathogen detection, whole individuals were sacrificed. Haploid males were removed from analysis, as this would increase the levels of homozygous results. *B. terrestris* individuals were differentiated from their cryptic sister species *B. lucorum* following methods from Ellis *et al.,* (2006).

2.3.2 Sample sites

Samples were taken from 12 geographically matched island and mainland sites in England and France with the islands ranging from 40-130km from the mainland (Fig. 2.1; Table S3, Table S9). Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L), Quiberon (Q), Scillies (S) and Ushant (U). The individuals were caught whilst foraging and all within the same day, providing the temperature was greater than 15^{o}C and there was less than 80% cloud cover. The bees were collected from a 1kmx1km plot within each site, where they were caught in individual tubes, then first stored in a cool bag to be later sacrificed and kept frozen at -80^{o}C. Data collection took place in June and July 2015.

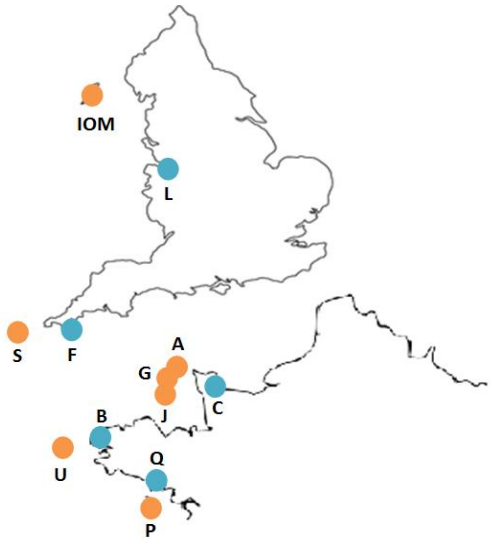

Figure 2.1: A map of the studied area with the approximate location of each sampling location in the UK and France with islands marked in orange, and mainland sites marked in blue. Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (IOM), Jersey (J), Liverpool (L), Quiberon (Q), Scillies (S) and Ushant (U).

Table 2.1: *B. pascuorum* samples per site (island or mainland location), population genetic metrics (heterozygosity and allelic richness per location including standard error) and prevalence of the parasites *Crithidia bombi* and *Apicystis bombi*. Varroa status indicates whether the honeybee population was infested with the honeybee parasite *Varroa destructor* (see Manley et al. 2019). 'Sister pairs' indicates the number of sister pairs per site with the number of unique colonies (the number of times when only one worker represented a nest) in brackets. The effective population size was calculated using the ECM (Event Capture Model) with those populations best described using TIRM (Two Innate Rate Model) marked with *; the 97.5% confidence intervals (CI) was calculated by bootstrapping (1,000 bootstraps), and max pop size equal to 1000. The average values for island and mainland populations for each measure is highlighted in bold below. Quiberon and Scillies were removed from analysis due to insufficient sample sizes.

Site	Island/ Mainland	Varroa status	Queens	Workers	Males	Workers Genotyped	Heterozygosity	Allelic Richness	Sister pairs (unique colonies)	Effective population size (CI)	*C. bombi* prevalence (%)	*A. bombi* prevalence (%)
Alderney	Island	Negative	0	30	0	30	0.62 (± 0.07)	4.95 (± 0.52)	4 (26)	208 (77, 1000)	29	57
Belle Ile	Island	Positive	0	19	0	18	0.64 (± 0.04)	7.73 (± 0.49)	2 (17)	165 (51, 1000)	53	35
Brest	Mainland	Positive	0	19	0	18	0.66 (± 0.08)	6.73 (± 0.73)	2 (16)	147 (45, 1000)	43	43
Cherbourg	Mainland	Positive	0	26	4	24	0.65 (± 0.07)	5.67 (± 0.69)	9 (15)	58* (34, 141)	10	30
Falmouth	Mainland	Positive	0	33	0	27	0.74 (± 0.06)	8.40 (± 0.52)	2 (26)	369 (117, 1000)	69	38
Guernsey	Island	Positive	0	31	0	25	0.50 (± 0.08)	4.95 (± 0.45)	6 (19)	92 (41, 292)	50	36
Isle of Man	Island	Negative	0	27	1	25	0.29 (± 0.08)	2.67 (± 0.31)	11 (13)	38 (22, 84)	37	58
Jersey	Island	Positive	0	33	1	23	0.73 (± 0.05)	6.76 (± 0.51)	6 (17)	77 (34, 245)	35	85
Liverpool	Mainland	Positive	0	30	0	25	0.67 (± 0.08)	6.36 (± 0.46)	10 (15)	52 (29, 142)	20	65
Ushant	Island	Negative	0	28	0	17	0.75 (± 0.04)	6.24 (± 0.59)	2 (15)	130 (40, 1000)	0	13
Quiberon	Mainland	Positive	0	1	0	0	n/a	n/a	n/a	n/a	n/a	n/a
Scillies	Island	Negative	0	0	0	0	n/a	n/a	n/a	n/a	n/a	n/a
Average Island	**n/a**	**n/a**	**n/a**	**23.08**	**0.50**	**n/a**	**0.50**	**n/a**	**n/a**	**118.33**	**34**	**47**
Average Mainland	**n/a**	**n/a**	**n/a**	**22.51**	**0.54**	**n/a**	**0.68**	**n/a**	**n/a**	**189.33**	**36**	**44**

Table 2.2: *B. terrestris* samples per site (island or mainland location), population genetic metrics (heterozygosity and allelic richness per location including standard error) and prevalence of the parasites *Crithidia bombi* and *Apicystis bombi*. Varroa status indicates whether the honeybee population was infested with the honeybee parasite *Varroa destructor* (see Manley et al. 2019). 'Sister pairs' indicates the number of sister pairs per site with the number of unique colonies (the number of times when only one worker represented a nest) in brackets. The effective population size was calculated using the ECM (Event Capture Model) with those populations best described using TIRM (Two Innate Rate Model) marked with *; the 97.5% confidence intervals (CI) was calculated by bootstrapping (1,000 bootstraps), and max pop size equal to 1000. The average values for island and mainland populations for each measure is highlighted in bold below. Ushant was removed from analysis due to poor sampling efforts.

Site	Island/ Mainland	Varroa status	Queens	Workers	Males	Workers Genotyped	Heterozygosity	Allelic Richness	Sister pairs (unique colonies)	Effective population size (CI)	*C. bombi* prevalence (%)	*A. bombi* prevalence (%)
Alderney	Island	Negative	0	56	1	33	0.75 (± 0.05)	7.13 ± (0.93)	0 (33)	**33**	45	45
Belle Ile	Island	Positive	0	54	5	46	0.72 (± 0.05)	7.49 (± 1.26)	19 (27)	78 (53, 132)	37	34
Brest	Mainland	Positive	0	57	4	54	0.79 (± 0.05)	9.34 (± 1.40)	20 (34)	137* (102, 322)	78	83
Cherbourg	Mainland	Positive	0	43	18	40	0.79 (± 0.05)	10.14 (± 1.74)	19 (21)	72* (52, 142)	69	42
Falmouth	Mainland	Positive	0	52	6	46	0.78 (± 0.05)	9.54 (± 1.37)	4 (42)	502 (192, 1000)	49	51
Guernsey	Island	Positive	0	43	3	36	0.77 (± 0.05)	8.37 (± 1.14)	0 (36)	**36**	64	67
Isle of Man	Island	Negative	0	53	1	51	0.64 (± 0.06)	6.13 (± 0.81)	16 (35)	162* (109, 325)	46	39
Jersey	Island	Positive	0	51	8	47	0.78 (± 0.05)	8.64 (± 1.23)	6 (41)	345 (139, 1000)	73	55
Liverpool	Mainland	Positive	0	44	15	43	0.79 (± 0.04)	8.92 (± 1.48)	9 (34)	166 (86, 437)	63	63
Ushant	Island	Negative	0	13	0	0	n/a	n/a	n/a	n/a	n/a	n/a
Quiberon	Mainland	Positive	0	56	3	44	0.77 (± 0.04)	9.21 (± 1.36)	20 (24)	71 (48, 120)	64	55
Scillies	Island	Negative	0	45	14	45	0.68 (± 0.05)	7.15 (± 0.87)	11 (35)	189 (118, 525)	64	46
Average Island	**n/a**	**n/a**	**n/a**	**47.25**	**6.50**	**n/a**	**0.73**	**n/a**	**n/a**	**136.20**	**55**	**48**
Average Mainland	**n/a**	**n/a**	**n/a**	**46.52**	**6.96**	**n/a**	**0.78**	**n/a**	**n/a**	**181.30**	**65**	**59**

2.3.3 DNA extraction

DNA was extracted from a tarsal sample removed from the individuals, using the Chelex extraction method (Walsh *et al.*, 2013). The tarsal samples were placed in liquid nitrogen where they were crushed in Eppendorf® tubes to a fine powder using sterile pestles, reducing contamination by autoclaving between uses. 200µl of 5% Chelex solution (Bio-Rad) and 2µl of Proteinase K was added to the crushed tissue and the mixture was then thoroughly vortexed. The crushed samples were placed in a 56ºC water bath for an hour and then 96ºC for 15 minutes to deactivate the enzyme. After this time, the samples were placed in a centrifuge at maximum speed for 1 minute, and 150µl of the supernatant was pipetted into 96-well plates and stored at -20ºC.

2.3.4 Microsatellite Genotyping

The extracted DNA was amplified at 9 polymorphic microsatellite loci (Estoup *et al.*, 1995; Estoup *et al.*, 1996), multiplexed in three reactions per sample (following methods in*,* (Dreier *et al.*, 2014)(Table 2.3)). Because of poor amplification results across sites, two loci (B121 and B118) were removed from the *B. pascuorum* data set.

All PCRs were 10µL in volume and contained 1µL Betaine (Q-solution), 3mM $MgCl_2$, 20µM dNTPs, 2.6µL 5x GoTaq® Flexi buffer, 1.2µL of primer (0.2µL of forward and reverse of each primer), 2u *Taq* polymerase and made up with Milliq Water and 1µL of template DNA. PCRs were performed in a thermocycler with an initial denaturing step at 95ºC for 15 minutes followed by 25 cycles of 30s at 94ºC, 90s at optimum annealing temperature (see table 2.3) and 60s at 72ºC, with a final extension step of 45 minutes at 60ºC. The PCR product was diluted 1:10 then visualised on an ABI 3730 capillary DNA sequencer using GeneScan Liz 500 size standard. Fragments were then scored using Geneious version 9.1.5 (Kearse *et al.*, 2012).

Table 2.3**:** The combination of primers used during genotyping PCR methods with their respective dyes in brackets and the optimum annealing temperature used. Within primer mix 1, B100(VIC) was replaced with B121(VIC) and in primer mix 3 B119(FAM) was replaced with B131(FAM) in *B. pascuorum* individuals – highlighted in bold.

Primer Mix	**Optimum Annealing Temperature (T_m)**
B100(VIC)/***B121*(VIC)** –*B118*(NED)-*B132*(FAM)	58ºC
B10(VIC)-*B11*(NED)(Garibaldi et al.)-*B96*(FAM)	52ºC
B119(FAM)/***B131*(FAM)**-*B124*(NED)-*B126*(PET)	56ºC

2.3.5 Gut DNA extraction

After removal from the -80ºC freezer, the gut was extracted from the abdomen using forceps, the contents mixed with 200µL insect Ringer solution and then homogenised with a pestle. Between use for each sample, the forceps and pestles were soaked in 20% bleach and autoclaved to prevent contamination. The DNA was then extracted from the solution using a Chelex method like host species DNA extraction. 35µL of the gut extract was added to 1000µL of 10% Chelex® 100 resin (Bio-Rad) solution with 2µL of Proteinase K and vortexed thoroughly before incubation. The tubes were incubated at 56ºC for one hour during which time the samples were vortexed twice and then incubated for a further 15 minutes at 95ºC. Once the incubation periods were over, the solution was centrifuged at maximum speed and the supernatant was removed and stored at -20ºC for further analysis.

2.3.6 Pathogen presence PCR

The presence of *Crithidia bombi* and *Apicystis bombi* were determined via PCR using published protocols with the following primers respectively; CB_ITS1-F/R

(Schmid-Hempel and Tognazzo, 2010), and NeoF/R (Meeus *et al.*, 2010). For the detection of *C. bombi,* a 20µL reaction mix contained 2µL DNA, 1x reaction buffer, 1.0µL dNTPS of 2.5mM each, 0.4µL of each primer of 10µM and 0.5 U of *Tag* polymerase. The PCR amplification was as follows: a denaturation step of 5min at 95^{o}C was followed by 40 cycles of 30s at 95^{o}C, annealing for 30s at 55^{o}C, and 1 min extension at 72^{o}C, the last cycle was followed by 10min at 72^{o}C. The fragmented DNA was then checked on a 1.5% agarose gel. For detection of *A. bombi,* a 20µL reaction mix contained 1.5 mmol 1^{-1} $MgCl_2$, 0.4 mmol 1^{-1} dNTPs, 0.5µmol 1^{-1} primers, 1.25 U *Taq* polymerase and 1µl of DNA. The PCR amplification was set as follows: initial denaturation steps of 2mins at 94^{o}C, followed by 35 cycles of 30s at 94^{o}C, 30s annealing at 56^{o}C, 45s at 72^{o}C, and a final 3mins at 72^{o}C to complete polymerisation. The fragmented DNA was viewed on a 1.5% agarose gel.

2.3.7 Phase-contrast microscopy

The gut samples stored at -20^{o}C were diluted 1:10, 9µL of this dilution was pipetted onto an Immune Systems FastRead 102 haematocytometer and examined at 40x magnification using a Motic BA300 light microscope. Pathogens were than counted by eye in the homogenate using the counting chamber within all positive *B. terrestris* and *B. pascuorum* individuals based on PCR presence results.

2.3.8 Statistical Analyses

MICRO-CHECKER (Van Oosterhout *et al.*, 2004) was used to identify microsatellite genotyping errors by analysing each locus for homozygote excess, which serves as an indicator for the presence of null alleles.

COLONY 2.0.6.4 (Wang, 2004; Jones and Wang, 2010) was used to identify sister individuals within the sample sites. If individuals were identified to be from the same colony with a posterior probability of >0.8, one was randomly assigned to represent the colony and the others were discarded from further population genetic analysis. This method follows previous literature studying *Bombus sp.* genetic analysis (e.g. (Carvell *et al.*, 2012; Dreier et al., 2014; Carvell et al., 2017).

From these sister-pair reconstructions, the effective population size (N_e) can be calculated. We used the DNA mark-recapture method to estimate population size based on the number of times an individual is recaptured, implemented in the software R (R Core Team 2014) using the package Capwire (Miller *et al.*, 2005). Within my study species', 'recapturing' an individual is represented as the assignment of a sister pair. Both methods implemented in the Capwire package, the Event Capture Model (ECM) and the Two Innate Rate Model (TIRM) were used to estimate the effective population size. The ECM assumes that all individuals within an area have the same probability of being caught. The TIRM, however, allows for heterogeneity in the probability of capture between individuals. To determine which model best fitted the data, Likelihood Ratio tests (LR) were conducted by simulating both ECM and TIRM and fitting both to the data. The significance of the LR was then used to determine which model best described the data. It was assumed that all workers within a colony were offspring of one singly-mated queen (Estoup et al., 1995; Schmid-Hempel and Schmid-Hempel, 2000) and a genotyping error rate of 0.05% was taken for both species following previous methods by Dreier *et al.,* (2014). With finite sampling, we expect that not all colonies at a site were sampled, i.e. that some colonies are represented by zero workers in the sample. For *B. terrestris* at sites Alderney and Guernsey, this 'zero' category could not be estimated, as all individuals caught from this site were from a unique colony, i.e. represented by only one worker. Therefore, only the minimum number of colonies can be stated for these two populations.

GENEPOP 4.1.2 (Rousset, 2008) was used to verify Hardy-Weinberg equilibrium (HWE) across populations and loci, and to detect linkage disequilibrium (LD). The genetic structure was assessed using population-specific *F*-statistics (Wright, 1965) via the hierarchical Bayesian *F*-model (Foll and Gaggiotti, 2006), in FSTAT version 2.9.3.2 (Goudet, 2001). Global statistics across all populations and pairwise θ values per sample pairs as well as the inbreeding coefficient (F_{IS}) per population were estimated. θ describes the levels of heterozygosity within the population and is therefore a measure of population differentiation due to genetic structuring. The means and standard errors reported were obtained by jack-knifing over samples and loci, with significance levels of p-values adjusted using Bonferroni corrections. Allelic

richness and expected heterozygosity were also calculated in FSTAT, using a resampling procedure to avoid bias of different sample sizes. A Pearson correlation test was conducted to analyse the relationship between genetic diversity and allelic richness.

Tests for Isolation by Distance (IBD) were calculated by firstly measuring the shortest direct distance between sites using Google Maps (Google 2018) and tests for correlation of F_{ST} and geographic distance were conducted using the R package 'vegan' (Oksanen *et al.,* 2013). The statistical significance of the correlation coefficients was estimated using 1,000 permutations. Data were analysed in RStudio, version 1.0.136 (Racine, 2012).

Structure 2.3.4 (Pritchard *et al.*, 2000) was used to conduct Bayesian analysis to investigate population structure by using a model-based clustering method. This software determines the optimal number of genetic clusters (K) using Markov Chain Monte Carlo (MCMC) methods to estimate P(XK) (the probability that the defined number of populations equals the estimate of genetic clusters). The number of clusters was set from 1 to 11 for *B. terrestris*, and 1 to 10 for *B. pascuorum* with an initial burn in of 20,000 followed by 80,000 MCMC iterations. 20 iterations of the MCMC runs were completed using the admixture model and correlated allele frequencies (default parameter settings). Results were then uploaded into StructureHarvester 0.6.94 (Earl and Vonholdt, 2012) where the Evanno method was implemented to calculate the optimum value of genetic clusters K (Evanno *et al.*, 2005; Earl and Vonholdt, 2012; Francis, 2017).

Phylogenetic trees of populations for *B. terrestris* and *B. pascuorum* were constructed using the UPGMA (Unweighted Pair-Group Method with Arithmetic Mean; (Sneath, 1987) based on F_{ST} values. Additionally, Neighbor-Joining trees were calculated using Nei's chord distance (Nei *et al.,* 1983). The trees were built using Poptree2 (Takezaki *et al.*, 2010) and were constructed after 1000 bootstraps.

In order to define genetic structuring of populations, Principal Component Analyses (PCA) of the heterozygosity of the populations were conducted per population in R using the package "prcomp()". PCA allows for the representation of raw genetic data in a low-dimensional Euclidean space using a matrix of dissimilarity (in this case, F_{ST}), visually representing the data within

two axes. PCA allows for multiple factors (in this case alleles) to be presented on two principle coordinate axes, via explaining the variance with Eigen Vectors. To test for population differentiation, AMOVA (Analysis of Molecular Variance) was conducted in ARLEQUIN ver 3.5.2.2 (Schneider *et al.,* 2000) to detect population differentiation using the molecular markers. This statistical method studies the patterns and degree of relatedness within populations, within individuals within specified groups and between specified groups by multidimensional scaling and the clustering dendrogram.

2.4 Results:

2.4.1 Null alleles and Hardy-Weinberg

A total of 232 *B. pascuorum* were successfully genotyped for 7 markers, with loci B121 and B118 removed from analysis due to poor amplification. Null alleles were only present in locus B10 (>10% in 9 out of 10 of the sites sampled), B10 was therefore removed from further analysis. No significant deviations from HWE or linkage disequilibrium were observed ($p>0.05$, Benjamini-Hochberg correction for multiple testing). Subsequent analysis was performed using the remaining 6 loci for *B. pascuorum* (B132, B96, B11, B131, B124 and B126).

In total, 486 *B. terrestris* were genotyped. Null alleles were present in locus B126 (>10%) for more than half of *B. terrestris* populations, therefore the locus was removed from further analysis. Global tests by population showed no significant departure from HWE or linkage disequilibrium across all loci ($p>0.05$; Benjamini-Hochberg correction for multiple testing). Subsequent analysis was carried out using the final 8 remaining loci for *B. terrestris* (B132, B100, B118, B96, B10, B11, B120 and B124).

2.4.2 Colony Reconstruction

A total of 232 *B. pascuorum* individuals were sampled and grouped into 203 colonies (an average of 1.14 workers per colony) between 10 of the field sites. 486 *B. terrestris* individuals were sampled which were grouped into 411 colonies (an average of 1.21 workers per colony) across 11 field sites in the UK and France. The number of sister pairs can be seen in tables 2.1 and 2.2.

2.4.3 Effective Population Size

Due to no assignment of sib-ships from COLONY, the effective population size of *B. terrestris* could not be estimated for the populations in Alderney and Guernsey. This left a total of 9 sites with estimated effective population size *for B. terrestris* and 10 sites for *B. pascuorum*. Based on a log likelihood comparison in the CAPWIRE program, ECM was the best representing model for 5/9 *B. terrestris* sites and 10/10 *B. pascuorum* sites (tables 2.1 and 2.2).

There was no significant difference between the number of colonies estimated for island and mainland sites (*B. pascuorum*: t=-0.49 df = 8 p=0.64, *B. terrestris*: t=-0.54 df=9 p=0.60) (tables 2.1 and 2.2; fig. 2.2). A Pearson's correlation test revealed no significant relationship between the effective population size and the heterozygosity of each site (*B. pascuorum:* r = 0.43, p= 0.21, *B. terrestris:* r = 0.15, p= 0.69).

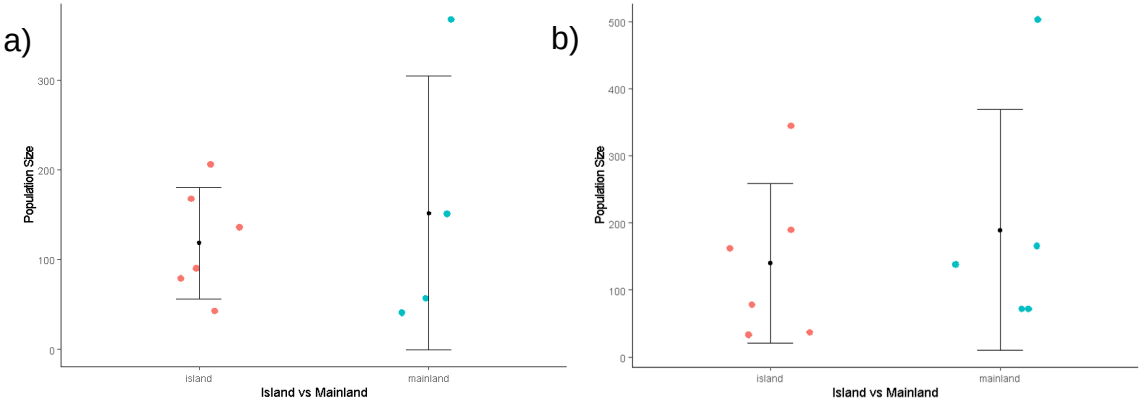

Figure 2.2: The effective population size within island (orange) and mainland (blue) sites estimated for a) *B. pascuorum* and b) *B. terrestris*, estimated using CAPWIRE. Figure shows error bars of 95% confidence intervals and the mean.

2.4.4 Population Structure

Significant global genetic structuring was found within *B. pascuorum* (θ = 0.122 (± 0.022), p<0.05), and all pairwise θ values between sites were significant except Liverpool and Falmouth (p = 0.378). Global genetic structuring was not significant in *B. terrestris* (θ = 0.069 (± 0.0009), p=0.53). For both species, genetic diversity is significantly correlated with allelic richness (Pearson's

correlation, *B. pascuorum* n=206, r=0.865, p<0.001; *B. terrestris* n=410, r=0.357, p<0.001).

2.4.5 Genetic isolation by distance (IBD)

There was no evidence for a pattern of IBD among *B. pascuorum* population pairs (Mantel test, r=0.26, p=0.15; fig. 2.3a), whereas *B. terrestris* analysis showed a significant relationship between pairwise F_{ST} values (θ) and geographic distance (km) (Mantel test: r=0.36, p=0.04; fig. 2.3b).

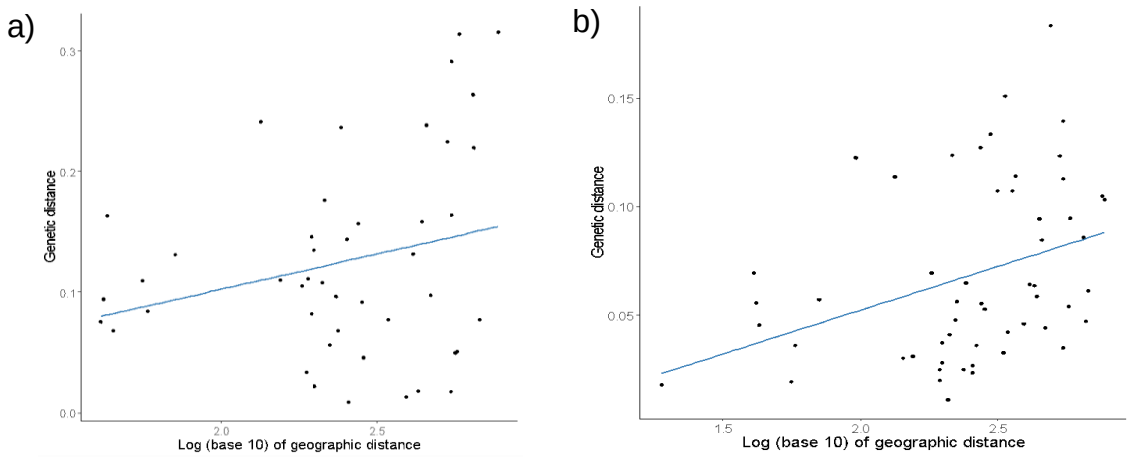

Figure 2.3: The (logged) geographical distance and the genetic distance (F_{ST}) between each of the sampled field sites in a) *B. pascuorum* (p=0.15) and b) *B. terrestris* (p=0.04).

2.4.6 Genetic structure analysis

Results from STRUCTURE revealed support for two clusters (K=2) given by the high ΔK value from downstream STRUCTURE HARVESTER analysis (fig. 2.4) for *B. pascuorum,* with weaker support for K=3. All populations of *B. pascuorum* show high levels of cluster 1 (red), with fewer individuals from A, G and M included within the second cluster (green) (fig. 2.5). A third cluster (blue) is underrepresented in the island (A, G, M, J and U) populations than compared to the mainland sites (fig. 2.5).

The ΔK statistic was greatest at K=2, with significantly weaker explanatory power when including additional clusters across *B. terrestris* populations (fig. 2.7). Support for genetic clustering of individuals from the Isles of Scilly can be

observed by the lack of contribution in the second cluster (green, fig. 2.8). The addition of a third cluster (K=3), shows further genetic structuring of the Isles of Scilly in the second cluster, and the Isle of Man populations dominating the third cluster (blue, fig. 2.8).

Such weak support for genetic structuring between populations of both species is further evident from constructed UPGMA and NJ trees, both with weak bootstrapping values (fig. 2.6, fig. 2.9).

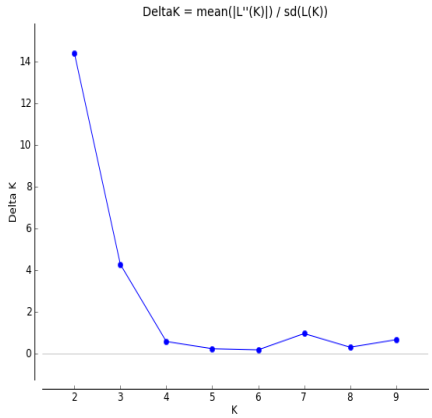

Figure 2.4: Inference of the optimal value of K using the ΔK method of Evanno (*et al.,* 2005) for *B. pascuorum* populations. Figure shows support for 2 clusters (K) and weaker support for K=3.

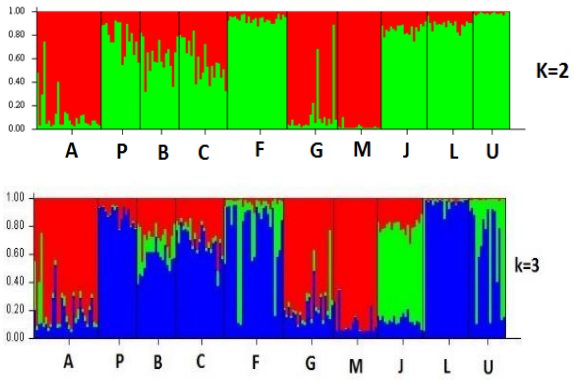

Figure 2.5: Population structure analysis bar graphs from STRUCTURE for populations of *B. pascuorum* with support for K=2 and K=3. Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L) and Ushant (U). Each individual genotype is represented by a coloured vertical bar representing the posterior assignment probability of being assigned to the clusters. The clusters are shown as different colours

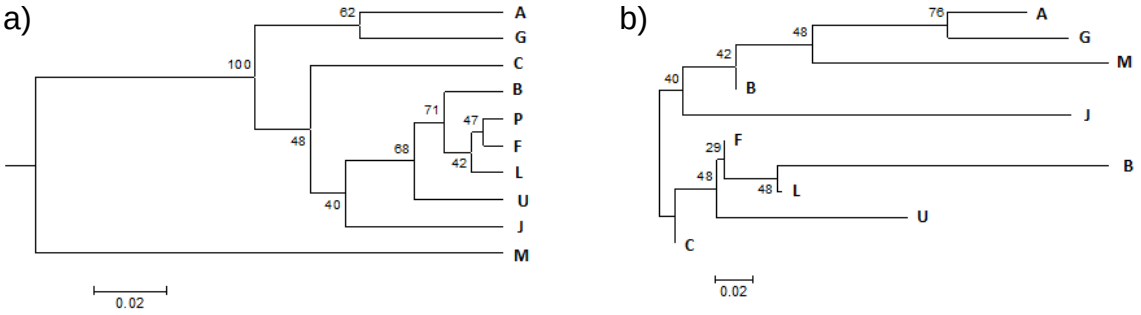

Figure 2.6: A UPGMA (a) and NJ tree (b) showing the relationship between genetic distances of the 10 sample sites for *B. pascuorum* populations. Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L) and Ushant (U). Branch numbers represent bootstrapping percentage.

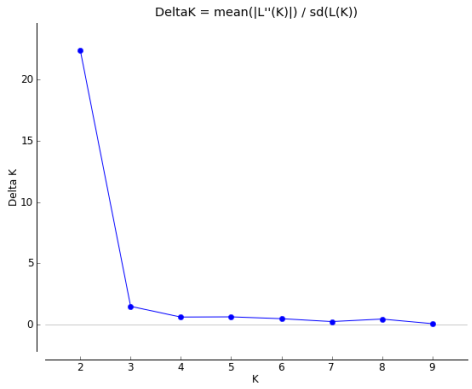

Figure 2.7: Inference of the optimal value of K using the ΔK method of Evanno (*et al.,* 2005) for *B. terrestris* populations. Figure shows support for 2 clusters (K).

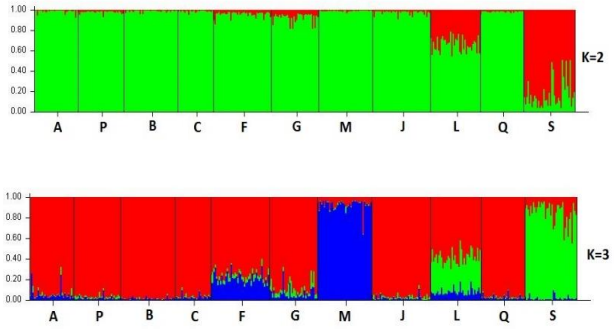

Figure 2.8: Population structure analysis bar graphs from STRUCTURE for populations of *B. terrestris* with support for K=2 and K=3. Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L), Quiberon (Q) and The Isle of Scillies (S). Each individual genotype is represented by a coloured vertical bar representing the posterior assignment probability of being assigned to the clusters. The clusters are shown as different colours within the graphs.

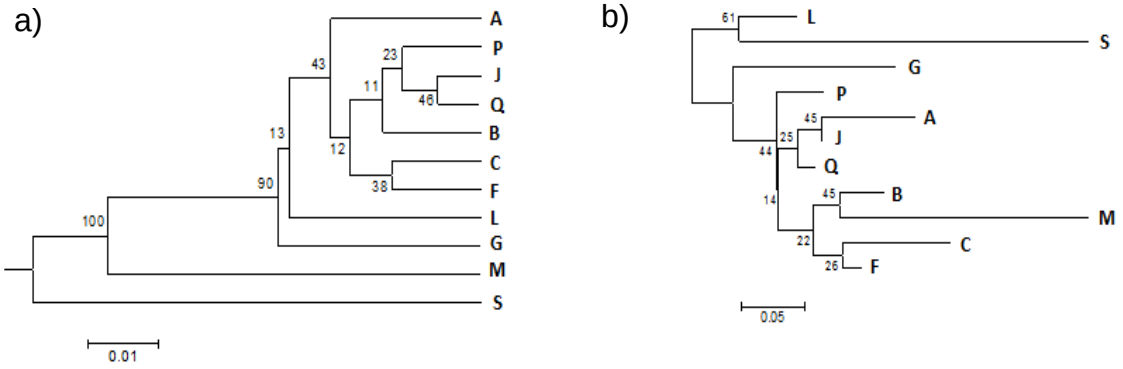

Figure 2.9: A UPGMA (a) and NJ tree (b) showing the relationship between genetic distances of 10 sample sites for *B. terrestris* populations. Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L), Quiberon (Q) and The Isle of Scillies (S). Branch numbers represent bootstrapping percentage.

2.4.7 Principal Component Analysis

Results from PCA show no distinct clustering in multivariate space on the PC1 and PC2 axes for both *B. pascuorum* and *B. terrestris* (fig. 2.10), with very high levels of admixture between sample sites.

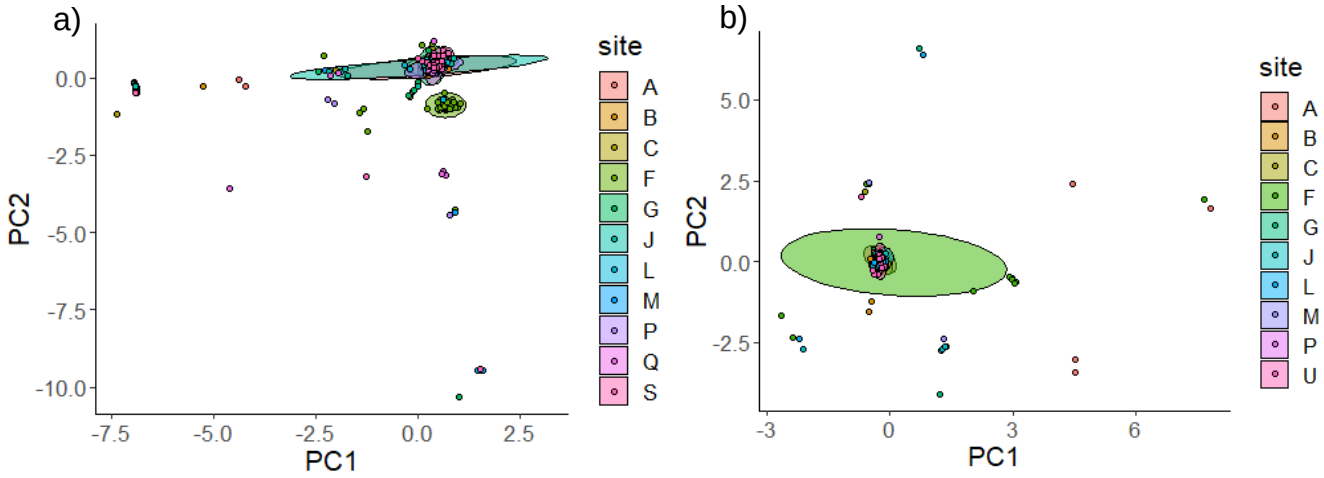

Figure 2.10: Principal Component Analysis performed in R for a) *B. pascuorum* and b) *B. terrestris* populations with each colour representing each sampling site; Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (I), Jersey (J), Liverpool (L), Quiberon (Q), The Isle of Scillies (S) and Ushant (U). Each point represents an individual sampled, and the distance between each point represents how dissimilar the samples are to each other.

2.4.8 Analysis of Molecular Variance

Results from the AMOVA show the landscapes producing the greatest significant among-group percentage variation for *B. pascuorum* and *B. terrestris* were using the groupings of the STRUCTURE analysis (Table 2.4). Within *B. pascuorum*, 10.4% (n=6, $p<0.001$) of genetic variation is explained by grouping together the Channel Islands (A, G+J), taking the other islands as their own groups (M, U, P) and grouping the remaining mainland populations together. Within *B. terrestris* populations grouping the four UK sites (S, M, F, L) individually and then grouping the remaining sites, accounted for 5.21% (n=5, $p<0.05$) of genetic variation between groups.

Neither *B. pascuorum* nor *B. terrestris* showed genetic variation between island and mainland sites ($p>0.05$); however, *B. terrestris* results show a small variation between UK and French populations (n=2, 1.04%, $p<0.05$).

Table 2.4: Analysis of Molecular Variance (AMOVA) of microsatellite data from (a) *B. pascuorum* and (b) *B. terrestris* populations within the UK and France grouped by different criteria. Values shown are percentage variation within populations of the species, among populations of the species and between the designated groups within the species. Results in bold represent the grouping with the greatest among-group variation percentage.

Landscape	Total Number of Groups	Within-population % variation, F_{IS} (*P-value*)	Among-population % variation, F_{SC} (*P-value*)	Among-group % variation, F_{CT} (*P-value*)
Island and Mainland	2	82.8, 0.0559 (0.00119)	11.9, 0.11939 (0.000)	0.37, 0.00374 (0.295)
UK and France	2	83.1, 0.0559 (0.00119)	12.3, 0.12240 (0.000)	-0.20, -0.00292 (0.510)
UK Island, UK Mainland, France Island and France Mainland	4	82.1, 0.0559 (0.00119)	9.7, 0.10009 (0.000)	3.38, 0.0338 (0.0580)
Varroa and Non-Varroa	2	81.2, 0.0559 (0.00040)	10.5, 0.108 (0.000)	3.58, 0.0358 (0.0640)
STRUCTURE (M+U+P+A,G,J+Rest)	**6**	**81.5, 0.0559 (0.00158)**	**3.25, 0.0363 (0.000)**	**10.4, 0.1042 (0.00079)**
Landscape	Total Number of Groups	Within-population % variation, F_{ST} (*P-value*)	Among-population % variation, F_{SC} (*P-value*)	Among-group % variation, F_{CT} (*P-value*)
Island and Mainland	2	84.8, 0.0913 (0.000)	7.22, 0.0718 (0.000)	-0.57, -0.00567 (0.942)
UK and France	2	84.2, 0.0913 (0.000)	6.31, 0.0638 (0.000)	1.04, 0.0104 (0.0324)
UK, Channel Islands and France Mainland	4	84.5, 0.0913 (0.000)	6.44, 0.0648 (0.000)	0.61, 0.00608 (0.113)
Varroa and Non-Varroa	2	84.2, 0.0913 (0.000)	6.46, 0.0652 (0.000)	0.91, 0.00907 (0.133)
STRUCTURE (S+M+F+L+Rest)	**5**	**83.1, 0.0913 (0.000)**	**3.35, 0.0353 (0.000)**	**5.21, 0.0521 (0.0397)**

2.4.9 Relationships with disease

Two species of parasite were detected and analysed in this current study: the gut trypanosome *Crithidia bombi* and the neogregarine *Apicystis bombi*.

C. bombi was detected at a significantly greater prevalence in *B. terrestris* populations than *B. pascuorum* populations (Two sample T test: $t_{14.87}$=-3.22, p=0.006; tables 2.1 and 2.2). There was no difference in *A. bombi* prevalence between *B. terrestris* and *B. pascuorum* populations ($t_{15.80}$=-0.87, p=0.40; tables 2.1 and 2.2).

2.4.10 Individual Level Results

There was no significant effect of heterozygosity on the individual count of *C. bombi* or *A. bombi* within populations of *B. pascuorum* or *B. terrestris* (table 2.5).

Table 2.5: The results of a generalised linear mixed effect model (GLMM) for *B. pascuorum* (left) and *B. terrestris* (right) looking at the heterozygosity, *A. bombi/C. bombi* prevalence, island/mainland with site as a random effect on the abundance of *A. bombi* and *C. bombi* within each species.

***C. bombi* count**					***C. bombi* count**				
	Co-efficient estimate	Standard Error	z-value	p-value		Co-efficient estimate	Standard Error	z-value	p-value
Heterozygosity	-2.1	3.54	-0.6	0.55	Heterozygosity	-0.63	1.88	-0.34	0.74
Island	0.3	0.94	9.26	0.75	Island	0.51	0.19	2.75	<0.05
A. bombi count	0.005	0.0005	0.32	<0.001	*A. bombi* count	0.0004	0.0002	1.77	0.08
***A. bombi* count**					***A. bombi* count**				
Heterozygosity	1.1	3.08	0.4	0.72	Heterozygosity	4.86	4.18	1.16	0.25
Island	-0.85	0.82	-1.04	0.3	Island	-0.24	0.43	-0.55	0.58
C. bombi count	0.05	0.002	27.21	<0.001	*C. bombi* count	0.004	0.0007	4.91	<0.001

2.4.11 Population Level Disease Results

Results from the glm showed that heterozygosity had a significant negative effect on *C. bombi* prevalence within *B. pascuorum* populations (z=-2.87, p=0.004), but positive effect on *B. terrestris* populations (z=2.38, p=0.017). *C. bombi* prevalence was strongly correlated with *A. bombi* prevalence within both species (*B. pascuorum*: z=3.69, p=<0.001; *B. terrestris*: z=2.75, p=0.006). The presence of *Varroa* and the effective population size increased *C. bombi*

prevalence in *B. pascuorum* populations only. The type of site (island/mainland) had no effect on the prevalence of *C. bombi* (table 2.6).

The heterozygosity of the population only effected the prevalence of *A. bombi* within *B. terrestris* populations (z=2.02, p=0.043), and not within *B. pascuorum*. Similar to *C. bombi* results, the prevalence of *C. bombi* significantly affected the prevalence of *A. bombi* within both species (*B. pascuorum*: z=3.57, p=<0.001; *B. terrestris*: z=2.74, p=0.006). The *Varroa* status, type of site and effective population size did not affect the prevalence of *A. bombi* for either of the two species sampled.

Table 2.6: The results of a binomial generalised linear model (GLM) for *B. pascuorum* (left) and *B. terrestris* (right) looking at the heterozygosity, *A. bombi/C. bombi* prevalence, *Varroa* status, island/mainland and effective population size on the prevalence of *Apicystis bombi* and *C. bombi* within each species.

C. bombi prevalence					*C. bombi* prevalence				
	Co-efficient estimate	Standard Error	z-value	p-value		Co-efficient estimate	Standard Error	z-value	p-value
Heterozygosity	-5.08	1.77	-2.87	**0.004**	Heterozygosity	9.48E+00	3.98E+00	2.38	**0.017**
A. bombi prevalence	1.23	0.332	3.69	**<0.001**	*A. bombi* prevalence	5.70E-01	2.07E-01	2.75	**0.006**
Varroa status	1.25	0.453	2.77	**0.006**	*Varroa* status	-3.67E-01	3.91E-01	-0.937	0.349
Island	0.159	0.378	0.421	0.672	Island	-4.21E-01	2.68E-01	-1.57	0.117
Effective Population Size	0.005	0.00178	2.55	**0.011**	Effective Population Size	8.46E-05	7.44E-04	0.114	0.909
A. bombi prevalence					*A. bombi* prevalence				
	Co-efficient estimate	Standard Error.	z-value	p-value		Co-efficient estimate	Standard Error	z-value	p-value
Heterozygosity	-0.238	1.52	-0.157	0.876	Heterozygosity	7.89	3.9	2.02	**0.043**
C. bombi prevalence	1.17	0.327	3.57	**<0.001**	*C. bombi* prevalence	0.569	0.207	2.74	**0.006**
Varroa status	0.000621	0.361	0.002	0.999	*Varroa* status	-0.137	0.388	-0.35	0.723
Island	0.621	0.361	1.72	0.085	Island	-0.124	0.256	-0.48	0.63
Effective Population Size	-0.00303	0.00169	-1.8	0.072	Effective Population Size	-0.000286	0.000734	0.39	0.697

2.5 Discussion

Understanding the genetic diversity and population structure of bumblebees greatly aids their conservation. Here, I investigated population structuring, genetic diversity and the association with disease for two common bumblebee species (*B. terrestris* and *B. pascuorum)* across island and mainland sites in England and France. This thesis aimed to test if there was population

structuring between island and mainland sites, whether the isolated island populations studied had reduced genetic diversity and population size, and how the heterozygosity of a population affects the association with disease. The results suggest that *B. pascuorum* but not *B. terrestris* show levels of genetic structuring on a larger scale, that island sites did not have reduced effective population sizes, and that in *B. pascuorum*, a reduction in genetic diversity increases the prevalence of both *C. bombi* and *A. bombi*.

2.5.1 Geographical barriers and genetic structuring

Natural geographic barriers have led to significant genetic differentiation between island and mainland sites (Lozier and Cameron, 2009; Cameron et al., 2011; Goulson *et al.*, 2011a; Lozier *et al.*, 2013; Jha, 2015). The greatest geographical barrier within this study is the English Channel, which for *B. terrestris* populations, but not B. *pascuorum* populations, showed significant among-group variation. Around Europe, *Bombus* species have been observed offshore several kilometres away from land, and it has been shown that *B. terrestris* colonised islands up to 30km from mainland New Zealand (Macfarlane & Gurr 1995). The minimum distance between mainland UK and France is approximately 35km, so it would not be unfeasible for individuals to cross this barrier. However, this distance doesn't consider small oceanic islands such as Alderney, Jersey and Guernsey which could allow for further gene flow between the two countries. The high levels of admixture seen from this study would be determined by the dispersal capabilities of the newly emerging queens and males of each colony; i.e. the reproductive individuals produced at the end of a colony's life cycle. However, a more detailed study highlighting the exact dispersal abilities of queens within these island populations would prove valuable.

Other factors which could influence the levels of genetic structuring observed within this study include the size of the island and the islands' distance from the mainland. Populations with greater migration of reproductives would observe an increase in the effective population size as well as avoiding reduced genetic diversity by genetic drift (Estoup et al., 1996; Widmer et al., 1998; Shao *et al.*, 2004; Darvill et al., 2006; Schmid-Hempel et al., 2007; Darvill *et al.*, 2010; Goulson *et al.*, 2011b; Lozier *et al.*, 2011; Lye *et al.*, 2011; Darvill *et al.*, 2012;

Lecocq *et al.*, 2013; Moreira *et al.*, 2015). It should also be recognised that the lack of genetic structuring observed within this study could be a result of limited genotypic markers used. Previous studies investigating genome-wide heterozygosity tend to use 10-30 loci (Chapman *et al.*, 2009; Maebe *et al.*, 2019), however this thesis followed methods comprehensively used in the literature and well suited to bumblebee genetic analysis (Dreier *et al.,* 2014; Wood *et al.,* 2015). Clearly, further studies into these populations would ideally sample all 9 loci (if not more) from all 12 field sites for more representative results.

2.5.2 The effective population size

The estimated number of colonies per site was relatively high with a range of 71-345 for *B. terrestris* and 52-369 for *B. pascuorum*, and despite there being no significant difference island sites had on average a smaller population size. These figures are likely to incur type II errors due to extrapolation and model assumptions as well as missed sister pairs, i.e. due to both amplification and human scoring error some sisters will go undetected which would then result in an overestimation of the number of nests in a population. Current methods surrounding estimates of effective population size need refining, but these values still provide great insight into the estimation of colony numbers based on caught workers.

There is much discrepancy as to what constitutes a minimum viable population size (Frankham, 1996), but it is believed that anything less than 50 would be at a much greater risk of inbreeding and consequently extinction (Frankham, 1998). Given this, and assuming the estimates are accurate, some of the bumblebee populations sampled could be at risk of inbreeding resulting in reduced fitness and increased susceptibility to extinction. These small populations were primarily observed within island sites which is predicted by the theory of island biogeography, which links small population size with increased levels of inbreeding.

Despite the ratio of *B. pascuorum* to *B. terrestris* colonies (Island: 1:1.12; Mainland: 1:1.2) being much smaller than previously studied (Darvill *et al.,* 2004; 1:2.68), the overall effective population sizes for both species were much greater. This would suggest that islands within the Scotland study were more

isolated, and populations studied in this thesis had almost similar population sizes between the two species.

Numerous previous studies within a range of species including plants and insects have provided evidence for a positive relationship between population size and genetic diversity (Frankham, 1996). For example, a study looking at the effects of over exploitation due to overfishing of the New Zealand Snapper, suggested that the subsequent isolated populations have a much lower effective population size than originally believed, with these populations also having lower genetic diversity (Hauser *et al.*, 2002). The results of this study show no significant relationship between population size and heterozygosity of the population, although low intensive sampling effort could account for this. A reduced genetic diversity could affect the adaptive genetic variability of these extreme isolated populations.

Previous studies looking to estimate effective population size within an area have assumed the number of 1 worker, 2 workers, 3 workers… etc sampled per colony follow a Poisson distribution (Chapman *et al.*, 2003; Darvill *et al.*, 2004; Knight *et al.*, 2005; Ellis et al., 2006; Knight *et al.*, 2009). These studies acknowledge that assuming all nests are equally likely to be sampled is inaccurate due to differences in size and distance from the sampling site. Following recent methods, using CAPWIRE allows two models to be simulated with the data, the ECM (equal probability of capture) and TIRM (probability of capture varies) with comparisons to detect the best model to fit to the data.

2.5.3 Relationship with disease

This study is the first large scale island/mainland study to highlight the relationship between the genetic diversity of wild bumblebee populations and the prevalence and abundance of key *Bombus* spp. parasites. This thesis set out to test; i) if the individual level heterozygosity affects the abundance of disease, and ii) if the population level heterozygosity affects the prevalence of disease. Contrary to previous studies, this thesis suggests that increasing levels of genetic diversity does not increase the pathogen load of an individual (van Baalen and Beekman, 2006; Whitehorn et al., 2011). However, increasing the heterozygosity decreases the prevalence of both *C. bombi* and *A. bombi* in *B.*

pascuorum populations (Sherman *et al.* 1988; Schmid-Hempel 1998; Whitehorn *et al.* 2010).

It is hypothesised that due to a reduced level of heterozygosity, homogenous populations have a reduced fitness and an increased susceptibility to infections. This trend has been observed in many mammalian species (Frankham, 1998) as well as in insects such as polyandrous leaf cutting ants (Hughes and Boomsma, 2004) but has been little studied in invertebrates and especially not in wild populations. The pathogens studied in this thesis which are observed at greater prevalence have been shown to reduce the fitness of populations as well as increasing mortality rates (Meeus *et al.*, 2010). As island populations have the potential to become more genetically isolated and fragmented from the mainland, this negative impact on population health could be a major driver in species declines (De Castro and Bolker, 2005).

2.5.4 Conservation and further study

Understanding the role of population connectivity in conservation strategies is vital (Lecocq *et al.*, 2016). It is suggested that in species with low population connectivity, the focus should be on preserving local populations to maintain species throughout its range. In those species with high population connectivity, the management is aimed at preserving gene flow throughout the species.

Impacts of disease are often overlooked in pollinator conservation studies with anthropological factors such as pesticide use and landscape changes appearing more frequently in the media for example. More evidence to suggest that inbreeding and geographical isolation is a hindrance to bumblebee health by loss of genetic diversity would improve the current understanding of isolated populations. Mitigation methods such as potential reintroductions to small island populations will increase heterozygosity and subsequently fitness (Woodard *et al.*, 2015). These unique island sites provide the foundations for isolation studies at a much greater scale.

2.6 Conclusions

The population genetic structure of *B. terrestris* and *B. pascuorum* in the UK and France is not as distinct as originally thought, with the English Channel restricting gene flow in *B. terrestris* populations only. The study also revealed

differences in the genetic structure between the two species, suggesting behavioural and ecological differences could also influence the genetic diversity of a *Bombus* spp*.* These results highlight the importance of careful identification of cryptic bumblebee species mentioned within this chapter. This chapter also revealed how low genetic diversity and greater homogeneity within *B. pascuorum* is associated with higher prevalence of disease. The findings from this chapter highlight how multiple factors can have varying effects upon genetic diversity within a bumblebee species, especially within isolated populations, and how emerging infectious diseases associated with these fitness costs are likely to increase with homogenous populations. As a result, small, isolated populations of bumblebees may be more at risk of extinction due to reduced genetic diversity and the associated impacts of disease.

Chapter 3: Population Genetics and Agri-environmental Schemes

3.1 Abstract:

In recent years intensification of the agricultural landscape has caused an increase in associated habitat fragmentation, pesticide use and a reduction in flower-rich habitats. Across the EU, agri-environmental schemes have been implemented to buffer against the rapidly changing landscape with methods such as wild flower strips. Here I investigated the impact of these wild flower margins on the population size and genetic diversity of four *Bombus* spp. within farm sites in the UK using molecular microsatellite markers, as well as the effect on disease prevalence and abundance in these localised populations. There was very little genetic structuring across the four bumblebee species; *B. hortorum* (θ=0.005), *B. lapidarius* (θ=0.009), *B. pascuorum* (θ=0.025), and *B. terrestris* (θ=0.005). The study revealed there was no significant impact of the level of stewardship on the effective population size or genetic diversity within all wild bee species sampled. However, there were positive relationships observed between floral density and genetic diversity in all but *B. pascuorum* populations. This research is contradictory to previous studies suggesting that targeted agri-environment schemes have a positive effect on population size and diversity. However, despite no relationship with the level of stewardship from this thesis, these results show increasing floral diversity has a positive impact on wild pollinator species.

3.2 Introduction:

In 2018, it was estimated that 38% of the world's total land surface (or approximately half of its habitable area) was occupied by agricultural land (Benton *et al.*, 2018). Farmland accounted for around 70% of land area in the UK (Defra, 2016), and this trend in intensification of farmland and changing of land use is predicted to rise with increasing human population size. An increase in arable landscape has resulted in a reduction in the floral diversity and consequently in the resources available for insect pollinators. The subsequent reduced heterogeneity of the landscape has resulted in a decline in wild pollinator numbers (Biesmeijer et al., 2006; Ollerton et al., 2014; Scheper *et al.*, 2014). These declines have been observed primarily in the Northern hemisphere (Goulson et al., 2008; Williams and Osborne, 2009; Bommarco et al., 2011; Cameron et al., 2011; Garibaldi et al., 2013; Woodcock et al., 2016) and can be attributed to agricultural intensification as well as associated habitat fragmentation, pathogens and effects of pesticides (Potts *et al.*, 2010). Parallel documented collapse of many honeybee colonies in both the UK and America (Potts *et al.*, 2010) signifies the value of these wild pollinators and the importance of this continued ecosystem service.

Many methods have been proposed as mitigation tools to restore and maintain biodiversity in these landscapes in order to sustain and boost ecosystem productivity. Agri-Environmental Schemes (AES) have been introduced in 26 out of the 44 European countries (Kleijn and Sutherland, 2003) to buffer habitat fragmentation under the EU agricultural policy. All EU member states are required to develop these agri-environment programmes under the Common Agricultural Policy. The current budget for the 7-year period from 2014-2020 is £22.3bn in direct payments and £2.3bn in rural development funds (GOV.UK, 2013). Agri-Environmental Schemes (AES) provide financial rewards to farmers of EU member states to implement Agri-Environment Measures (AEM) currently based on a two-tier system; Entry-Level Stewardship (ELS, Defra 2005a), consisting of more generalised requirements and standards, and Higher-Level Stewardship (HLS, Defra 2005b), with more specific environmental requirements and therefore a more limited participation (Quillerou and Fraser, 2010). As of 2014 around 72% of agricultural land in England was under ELS management and around 21% under HLS (JNCC 2014), with methods of

mitigation varying between sites. Both ELS and HLS can adopt WFS, however, the HLS is more targeted than ELS, creating a more competitive scheme and one for which not all land is eligible. This scheme holds the basics of ELS but also provides grants for additional conservative work for example restoration of farm buildings. Additionally, ELS agreements last for five years in contrast to the 10 year contracts held by HLS farms (Emery and Franks, 2012). These schemes aim to target pollinator conservation via different techniques, one example being the provision of Wild Flower Strips (WFS); however, HLS sites require a more active management with visits from Natural England. WFS are sown within field margins to attract insect pollinators and, by including a mix of nectar and pollen-rich annual and perennial flowering species, these WFS aim to provide valuable foraging resources between early and late summer. These implemented schemes aim not only to increase numbers of pollinating insect species such as bumblebees, solitary bees, butterflies, and hoverflies, but also to promote biological pest control by increasing plant biodiversity and supporting farmland bird populations in the form of seeds and invertebrate food resources (Haaland *et al.*, 2011).

The efficacy of field margins as a targeted method to increase pollinator abundance and diversity has been previously studied (Carvell et al., 2004; Carvell *et al.*, 2011; Dreier et al., 2014; Wood et al., 2015; Carvell et al., 2017; Marja *et al.*, 2018). Previous studies into WFS have demonstrated a positive effect of attracting wild honeybees and bumblebees (Carvell *et al.*, 2011) as well as enhancing and establishing pollinator nests (Wood *et al.*, 2015; Carvell *et al.*, 2017). These studies show that sown WFS can provide floral resources all year round to improve bumblebee reproduction in intensively farmed areas by increasing floral density (fig 3.1).

One study by Wood *et al.,* (2015) found that the difference between HLS farms and ELS was significant with regards to the abundance of bumblebees observed within the transect walks, as well as increased nesting densities for *B. hortorum* and *B. lapidarius*. This suggests that the targeted agri-environment schemes which increase the availability of suitable foraging sites significantly increase the size of wild bumblebee populations. Population level studies of eusocial bumblebees focus on the number of colonies as opposed to the

number of individuals in each area as the effective population size of species, due to their complex social structure.

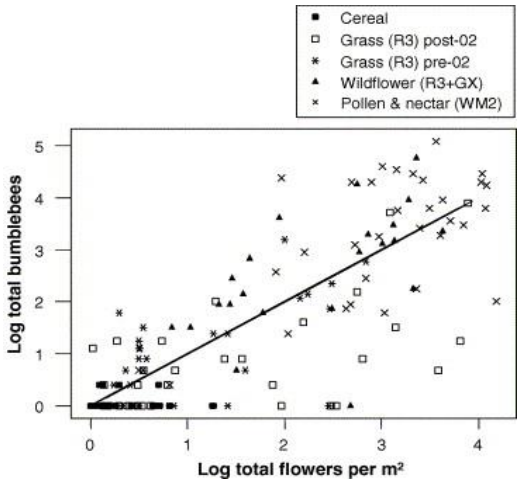

Figure 3.1**:** The relationship between log bumblebee abundance and log flower abundance per m^2 on arable field margins, with each point representing a sampling point. The type of landscape is shown in the figure key. Reproduced from Pywell *et al.,* 2006.

Bumblebees have a complex social structure as haplodiploid hymenopterans, with the queen considered as the only reproductive within a colony. Therefore, despite an abundance of (sterile) workers, effective population size may be small and haplo-diploidy may reduce genetic variation (Chapman and Bourke, 2001). In hymenopteran species, the sex is determined by zygosity at the sex determining locus. Individuals homozygous at the sex locus develop into non-viable sterile males which incurs a great cost upon the colony (Cook and Crozier, 1995). Inclusive fitness and altruism within the colony is greater due to this complex social structure. Females are developed from fertilised eggs and males from unfertilised eggs, therefore sisters within a colony are more related to each other than they are to their own offspring (0.75). Genetically related individuals are similar in their susceptibility to pathogens (Bremermann, 1980; Hamilton, 1980; Tooby, 1982; Hamilton et al., 1990), and within these eusocial insects transmission of disease between nest mates is high (Shykoff and Schmidhempel, 1991). The role of population genetics in epidemiological studies is often overlooked and is vital especially within study species like bumblebees. In this study where field margins have been shown to increase bee abundance and visitations (Doublet *et al.,* in press), the transmission routes of emerging infectious diseases are important. These agri-environment schemes have the potential to alter transmission between individuals by increasing the density within which pollinator species can forage, i.e.

bumblebees are visiting flowers which are frequented by others, therefore increasing the chance of pathogen transmission. In order to assess this mode of transmission fully, sisters much be identified as it is difficult to determine if pathogens are passed between these individuals whilst foraging or within the nest.

Genetic diversity is vital to allow *Bombus* spp. populations the ability to adapt to short-term environmental fluctuations and reduce the impact of pathogen infections. However, due to fragmentation, it is inferred that gene flow and admixture between populations could be limited, creating isolated populations at a greater risk of disease.

I genotyped the four most common bumblebee species (*Bombus hortorum, B. lapidarius, B. pascuorum* and *B. terrestris* (fig 3.2)) across 10 field sites, to estimate the genetic diversity within and between populations and predict effective population sizes. These species were chosen due to their widespread ecology, therefore allowing for comparison across a range of sites. These species are also well documented in terms of their genetic diversity in England and across Europe. I also used sequenced RNA of the individuals to assess disease prevalence (the proportion of individuals carrying the disease) within populations as well as pathogen load (the amount of each pathogen observed) of individuals.

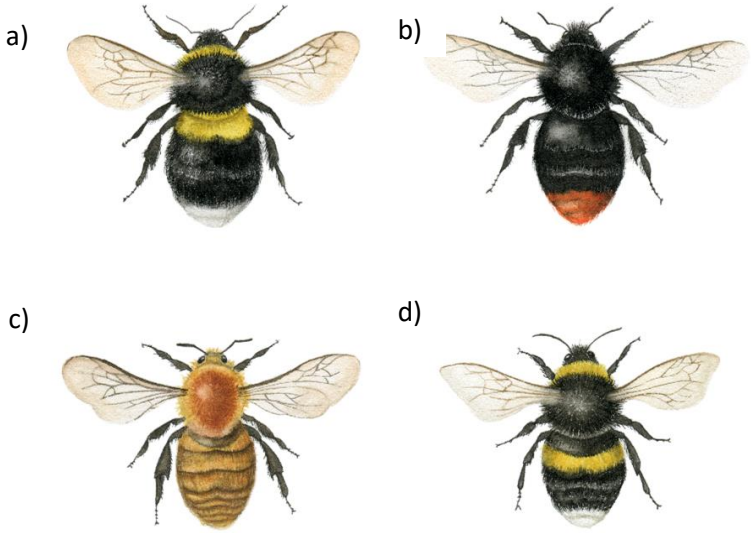

Figure 3.2: An illustration of the four *Bombus* spp. studied; a) *B. hortorum,* b) *B. lapidarius,* c) *B. pascuorum,* and d) *B. terrestris*. (Illustration: Alice Rosen 2018)

This is the first study of its kind to combine methods of genetic diversity to explain disease prevalence and load within agri-environment schemes in England. This thesis applies previously described methods of microsatellite analysis to a unique sampling set to analyse populations within agri-environment schemes.

3.3 Materials and Methods

3.3.1 Study area

Five paired field sites across the south of England were selected and sampled from March to August 2016 and again during the spring of 2017 (fig. 3.3; table S8; table S10). Farms were renamed with respect to their scheme type (ELS and HLS) to provide anonymity and to ensure independence of data. Farms were at least 10km apart to compensate for the maximum foraging range of honeybee workers (which were also sampled for further studies) (Schneider and McNally, 1993; Steffan-Dewenter and Kuhn, 2003). At this distance, bumblebees are also unlikely to travel between sites to forage (Knight et al., 2005; Osborne *et al.*, 2008). The primary crops within and surrounding the field sites were oilseed rape, wheat/barley and either arable or dairy farmlands.

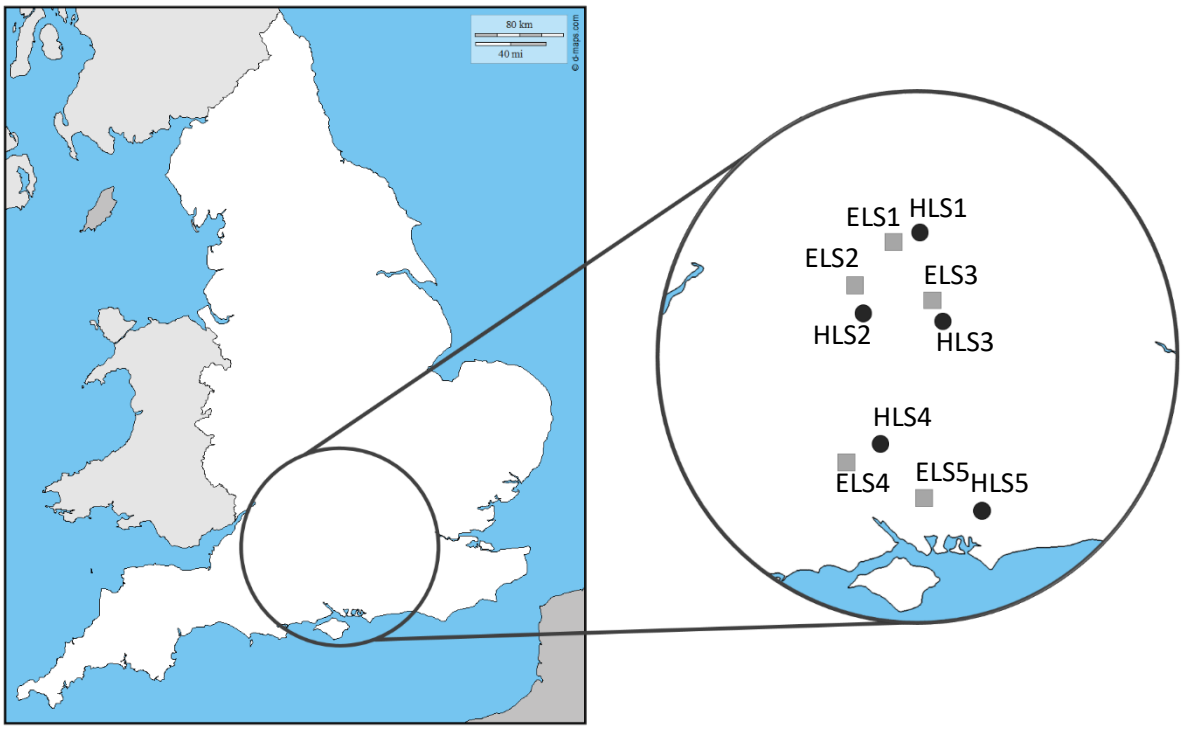

Figure 3.3: A map of the UK showing the location of the 10 field sites sampled during 2016/2017. Entry-level farms are represented by grey squares and higher-level farms by a black circle.

3.3.2 Sample collection

Three standardised 100m transects were set out for each farm and each site was sampled over four time points (TP1 – Early Spring 2016; TP2 – Late Spring 2016; TP3 – Summer 2016 and TP4 Spring 2017; table 3.1). Insect collection was conducted in favourable conditions, including wind speeds at a maximum of 5 on the Beaufort scale, and minimum shade temperature of 15^{o}C in the summer and 9^{o}C in the spring.

Table 3.1: Each location and the date of sampling across the four time points in 2016/2017.

	Date of Sampling			
Site	**TP1**	**TP2**	**TP3**	**TP4**
ELS1	07/05/2016	28/06/2016	09/08/2016	08/04/2017
ELS2	06/05/2016	26/06/2016	08/08/2016	07/04/2017
ELS3	20/04/2016	24/06/2016	06/08/2016	03/04/2017
ELS4	17/04/2016	21/06/2016	04/08/2016	05/04/2017
ELS5	13/04/2016	19/06/2016	31/07/2016	26/03/2017
HLS1	08/05/2016	30/06/2016	10/08/2016	09/04/2017
HLS2	04/05/2016	27/06/2016	07/08/2016	06/04/2017
HLS3	03/05/2016	26/06/2016	05/08/2016	02/04/2017
HLS4	19/04/2016	23/06/2016	03/08/2016	30/03/2017
HLS5	12/04/2016	18/06/2016	30/07/2016	25/03/2017

The five most abundant pollinator species were collected from each location (species used in future studies if they are not represented here, e.g. *Apis mellifera*) and to allow for RNA sequencing for pathogen detection, whole individuals were sacrificed. The samples were stored in -80^{o}C freezers at which temperature they remained until further investigative steps were required. In the case of a *Bombus* sp. being collected for sampling, the caste type of each individual bee was recorded (table 3.2, 3.3, 3.4 and 3.5). Queens were primarily sampled in the early spring, workers in the summer, and males later in the year.

Bombus terrestris was sampled at all 10 samples sites as were *B. pascuorum and B. lapidarius;* however, *B. hortorum* was collected from 5 HLS and only 4 ELS sites (tables 3.2, 3.3, 3.4 and 3.5).

Within each field site, the average floral density was calculated using results of observed floral density per transect. A quadrat was thrown randomly along each

of the three transects, within which the number of open flowers was counted. An average was then calculated between the three transects.

3.3.3 Genetic sampling

DNA was extracted from individual tarsal samples, crushed in liquid nitrogen, via the Chelex extraction method (Walsh *et al.*, 2013) using 5% Chelex and 2 µl of Proteinase K. Extracted DNA was stored at -20^{o} C before downstream analyses.

3.3.4 PCR based differentiation and identification

B. terrestris and *B. lucorum* individuals were differentiated using the primer pair BBMI_IGSF1 and BBMI_IGSR1 (*pers comms* Regula Schmid-Hempel). The PCR reaction mix contained 4µL of 5x GoTaq® Flexi buffer, 2µL of $MgCl_2$, 2µL of dNTPs, 0.5µL of the forward and reverse primers, 0.2µL of GoTaq® Flexi DNA polymerase and 2µL of template DNA. The total reaction volume was 20µL made up with Milliq H_2O. PCRs were performed in a thermocycler with an initial denaturing step of 95^{o}C for 1 minute, followed by 38 cycles of 15s at 95^{o}C, 15s at 55^{o}C, 45s at 72^{o}C, with a final extension step of 7 minutes at 72^{o}C. The amplified products were then visualised with UV transilluminator on a 2% gel stained with RedSafe, running at 120V with 1kB ladder. The resultant fragmented DNA bands are expected at 180 bp for *B. terrestris* and at 210 bp for *B. lucorum* (Supplementary Material).

B. hortorum and *B. ruderatus* individuals also required differentiation by molecular analysis to prevent incorrect species assignment leading to inaccurate results during the investigation. DNA was once again sampled and extracted as above, and the individuals were then amplified at the mitochondrial region cytochrome *b* by polymerase chain reaction following methods from Ellis *et al.,* (2006). The PCRs contained: 0.8U of *Taq*, 30pmol MgCl2, 1x PCR buffer (containing an extra 45pmol MgCl2), 6pmol dNTPs and 6.5 pmol primer (forward: TTCAGCAATTCCATATATTGGAC; and reverse: ATTACACCTCCTCATTTATTAGG). The PCRs were conducted in an Applied Biosystems® Veriti Thermal Cycler under the following conditions: 4 min at 94^{o}C; 35 cycles of 30 s at 94^{o}C, 30 s at 48^{o}C and 1 min at 72^{o}C, followed by a final extension step of 10 min at 72^{o}C. After this, the PCR product was then

digested using the restriction enzyme Tsp45I (New England Biolabs®). The individual reactions contained 15µL PCR product, 1U enzyme, 1x buffer N.E number 1 with reactions made up to 20µL using H_2O. The DNA was digested at 65ºC for 4 hours. Upon completion of the digestion, the products were run via electrophoresis on a 3% agarose gel for 180 min at 60V. Fragments were compared for a 100bp ladder (Promega®). B. *ruderatus* are identified by their singular band of undigested PCR product at 426 bp, and *B. hortorum* were identified by the same undigested band at 426 bp as well as two digested bands at 306 bp and 125 bp (Supplementary Material). The band at 306 bp is most prominent, with the other two bands being faint or absent (methods from Ellis *et al.,* 2006).

In order to identify the presence of *Apicystis bombi* per individual the following primer pair was developed: Forward: TGATCCATAATAATTTTGTGAAT and reverse: AGTGCTATGTTTGTTTTTAACGA (Anderson per comms). The reaction mix contained 5µL of cDNA, 4µL DNTPs, 2µL $MgCl_2$, 4µL PCR buffer, 0.2µL enzyme and made up to a total reaction volume of 20µL with H_2O. The PCR was then run in a thermocycler under the following conditions: 2 min at 94ºC; 35 cycles of 30ºs at 94ºC, 30s at 60.7ºC and 1 min at 72ºC, followed by an extension step of 3 min at 72ºC. The product was run on a 1.5% agarose gel at 110v for 35 minutes with a band present at 392bp if *Apicystis bombi* is present.

3.3.5 Fragment analysis

The extracted DNA was amplified at 9 polymorphic microsatellite loci (Estoup et al., 1995; Estoup et al., 1996). The same nine markers were used for all species (*B100, B118, B132, B10, B11, B96, B119, B124, B126*), but *B100* was replaced with *B121* for *B. pascuorum* and *B119* replaced with *B121* in *B. pascuorum* and *B. lapidarius*. Three multiplex PCRs were run with the following loci:

B100(VIC)/*B121*(VIC) –*B118*(NED)-*B132*(FAM), *B10*(VIC)-*B11*(NED)-*B96*(FAM) and *B119*(FAM)/*B131*(FAM)-*B124*(NED)-*B126*(PET) following methods from Wood *et al.,* (2015).

PCRs were 10µL in volume and contained 1µL Betaine (Q-solution), 3mM $MgCl_2$, 20µM dNTPs, 2U *Taq* polymerase, 1.2µL of primer (0.2µL of forward and reverse of each primer) made up with 1.8µL Milliq Water and 1µL of

template DNA. PCRs were performed in a thermocycler with an initial denaturing step at 95ºC for 15 minutes followed by 25 cycles of 30s at 94ºC, 90s at 54ºC and 60s at 72ºC, with a final extension step of 45 minutes at 60ºC. The PCR product was diluted 1:10 then visualised on an ABI 3730 capillary DNA sequencer using GeneScan Liz 500 size standard. Fragments were then scored using Geneious version 9.1.5 (Kearse *et al.*, 2012).

3.3.6 Identification of unique colonies and assigning sib-ship

Individuals with more than 3 poorly amplified loci were removed. MICRO CHECKER (Van Oosterhout *et al.*, 2004) was used to identify microsatellite genotyping errors by analysing each locus for homozygote excess, which serves as an indicator for the presence of null alleles.

The software COLONY version 2.0 (Jones and Wang, 2010) allows full reconstruction of family relationships between individuals for each species within each sample site and time point. COLONY uses a full-likelihood approach to sibship assignment by estimating posterior probabilities and is the most reliable method of estimating sibship in bumblebees (Lepais *et al.*, 2010). Following methods from Carvell *et al.,* (2017), the threshold for the estimated posterior probability of these sibships was 0.8, any estimates below this value were ignored. Based on previous studies (Estoup et al., 1995; Bourke, 1997; Schmid-Hempel and Schmid-Hempel, 2000), it was assumed that all workers within a colony were the offspring of a single monogamous queen and the genotyping error rates were set at 0.05% for all four species. Once all sisters were identified, a random number generator was used to select a single individual from within each sib-ship assignment and all other sisters were removed from further analysis. This was so a single nest mate represented each colony throughout the analysis.

3.3.7 Estimating effective population size

In sites where all the individuals caught were from unique colonies (i.e. only one bee caught per colony with no sibship assignment), an effective population size could not be predicted. In these instances, the total number of bees caught was used as an estimate of minimum colony size.

The DNA mark-recapture method estimates population size based on the

Table 3.2: The number of *B. hortorum* individuals genotyped per site and sampling time point including the caste type. The heterozygosity (F_{ST}) and allelic richness per location including standard error; the number of sister pairs per site with the number of unique colonies (the number of times when only one worker represented a nest) in brackets. The effective population size and 97.5% confidence intervals after 1,000 bootstraps and max pop size equal to 1000, calculated using the ECM with those populations best described using TIRM marked with *, the floral density per sample site and the prevalence of *A. bombi* to the nearest percent. The average for ELS and HLS for each of the columns is highlighted in bold below. ELS3 and HLS5 were removed from analysis due to poor sampling efforts.

	Number of Individuals Genotyped									
Site	**TP1 (Queens)**	**TP2 (Workers)**	**TP3 (Workers)**	**TP4 (Queens)**	**Heterozygosity (F_{ST})**	**Allelic Richness**	**Sister pairs (unique colonies)**	**Effective population size (CI)**	**Floral Density (per m^2)**	***A. bombi* prevalence (%)**
ELS1	2	n/a	13	8	0.84 (± 0.04)	6.16 (± 0.55)	12 (11)	75 (22, 1000)	33.97	n/a
ELS2	3	27	n/a	17	0.84 (± 0.02)	6.29 (± 0.43)	22 (19)	87* (48, 356)	15.38	41
ELS3	6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
ELS4	2	n/a	13	5	0.86 (± 0.02)	6.60 (± 0.53)	13 (12)	86 (26, 1000)	25.90	36
ELS5	n/a	1	24	n/a	0.83 (± 0.02)	6.04 (± 0.48)	18 (13)	34 (21, 67)	24.48	42
HLS1	n/a	30	11	15	0.86 (± 0.02)	6.42 (± 0.50)	31 (24)	79 (48, 172)	40.25	20
HLS2	8	20	6	15	0.85 (± 0.03)	6.45 (± 0.63)	18 (17)	88 (31, 1000)	7.80	38
HLS3	31	28	n/a	34	0.86 (± 0.02)	6.67 (± 0.56)	26 (24)	180 (66, 1000)	27.26	40
HLS4	n/a	10	1	5	0.88 (± 0.02)	6.60 (± 0.51)	10 (9)	51 (15, 1000)	62.20	30
HLS5	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Average ELS	**3.3**	**14.0**	**16.7**	**10.0**	**0.84**	**6.27**	**16.25**	**64.7**	**24.9**	**39.8**
AverageHLS	**19.5**	**22.0**	**6.0**	**17.3**	**0.86**	**6.54**	**21.25**	**99.5**	**34.4**	**32.1**

Table 3.3: The number of *B. lapidarius* individuals genotyped per site and sampling time point including the caste type. The heterozygosity (F_{ST}) and allelic richness per location including standard error; the number of sister pairs per site with the number of unique colonies (the number of times when only one worker represented a nest) in brackets. The effective population size and 97.5% confidence intervals after 1,000 bootstraps and max pop size equal to 1000, calculated using the ECM with those populations best described using TIRM marked with *, the floral density per sample site and the prevalence of *A. bombi* to the nearest percent. The average for ELS and HLS for each of the columns is highlighted in bold below.

ELS1	7	27	13	12	0.74 (± 0.04)	5.09 (± 0.72)	34 (29)	116 (64, 247)	33.97	46
ELS2	29	27	24	26	0.77 (± 0.04)	5.36 (± 0.73)	37 (32)	136 (75, 437)	15.38	42
ELS3	17	19	16	1	0.73 (± 0.05)	4.65 (± 0.54)	28 (23)	73 (42, 187)	14.41	50
ELS4	21	28	18	3	0.74 (± 0.04)	5.00 (± 0.63)	42 (39)	243 (114, 1000)	25.90	33
ELS5	n/a	8	26	n/a	0.75 (± 0.05)	4.85 (± 0.65)	33 (32)	550 (176, 1000)	24.48	40
HLS1	24	56	15	4	0.76 (± 0.04)	5.07 (± 0.59)	34 (27)	86 (49, 166)	40.25	38
HLS2	12	28	24	12	0.73 (± 0.05)	4.78 (± 0.62)	46 (42)	268* (163, 1000)	7.80	37
HLS3	29	33	29	1	0.72 (± 0.05)	4.72 (± 0.60)	53 (46)	189 (114, 357)	27.26	45
HLS4	26	32	18	3	0.78 (± 0.04)	5.13 (± 0.58)	39 (32)	131* (94, 311)	62.20	46
HLS5	4	12	21	n/a	0.77 (± 0.04)	4.79 (± 0.60)	31 (29)	253 (94, 1000)	44.11	48
Average ELS	**18.5**	**21.8**	**19.4**	**10.5**	**0.75**	**4.99**	**34.8**	**223.6**	**22.8**	**42.2**
AverageHLS	**19.0**	**32.2**	**21.4**	**5.0**	**0.75**	**4.90**	**40.6**	**185.4**	**36.3**	**42.8**

Table 3.4: The number of *B. pascuorum* individuals genotyped per site and sampling time point including the caste type. The heterozygosity (F_{ST}) and allelic richness per location including standard error; the number of sister pairs per site with the number of unique colonies (the number of times when only one worker represented a nest) in brackets. The effective population size and 97.5% confidence intervals after 1,000 bootstraps and max pop size equal to 1000, calculated using the ECM with those populations best described using TIRM marked with *, the floral density per sample site and the prevalence of *A. bombi* to the nearest percent. The average for ELS and HLS for each of the columns is highlighted in bold below.

	Number of Individuals Genotyped									
Site	**TP1 (Queens)**	**TP2 (Workers)**	**TP3 (Workers)**	**TP4 (Queens)**	**Heterozygosity (F_{ST})**	**Allelic Richness**	**Sister pairs (unique colonies)**	**Effective population size (CI)**	**Floral Density (per m^2)**	***A. bombi* prevalence (%)**
ELS1	3	29	32	n/a	0.58 (± 0.10)	6.01 (± 1.05)	41 (28)	74 (53, 102)	33.97	44
ELS2	2	31	29	9	0.67 (± 0.08)	6.99 (± 1.02)	42 (31)	83 (54, 130)	15.38	41
ELS3	2	26	26	1	0.64 (± 0.06)	5.57 (± 0.80)	39 (33)	133*	14.41	31
ELS4	3	30	n/a	12	0.60 (± 0.10)	5.76 (± 0.81)	25 (20)	77 (38, 208)	25.90	29
ELS5	n/a	4	29	n/a	0.57 (± 0.12)	6.19 (± 0.94)	29 (24)	87 (47, 187)	24.48	41
HLS1	1	29	13	24	0.58 (± 0.11)	6.66 (± 1.00)	34 (27)	93 (57, 201)	40.25	39
HLS2	19	31	28	31	0.64 (± 0.07)	6.45 (± 0.96)	49 (40)	151 (94, 265)	7.80	38
HLS3	12	31	29	12	0.62 (± 0.10)	7.09 (± 1.09)	46 (34)	118 (77, 217)	27.26	32
HLS4	1	31	30	21	0.57 (± 0.11)	6.14 (± 0.96)	43 (34)	127* (99, 229)	62.20	49
HLS5	n/a	30	30	n/a	0.65 (± 0.08)	7.08 (± 1.01)	45 (36)	144* (108, 305)	44.11	30
Average ELS	**2.5**	**24.0**	**29.0**	**7.3**	**0.612**	**6.10**	**35.2**	**80.3**	**22.8**	**37.2**
AverageHLS	**8.3**	**30.4**	**26.0**	**22.0**	**0.612**	**6.68**	**43.4**	**120.7**	**36.3**	**37.6**

Table 3.5: The number of *B. terrestris* individuals genotyped per site and sampling time point including the caste type. The heterozygosity (F_{ST}) and allelic richness per location including standard error; the number of sister pairs per site with the number of unique colonies (the number of times when only one worker represented a nest) in brackets. The effective population size and 97.5% confidence intervals after 1,000 bootstraps and max pop size equal to 1000, calculated using the ECM with those populations best described using TIRM marked with *, the floral density per sample site and the prevalence of *A. bombi* to the nearest percent. The average for ELS and HLS for each of the columns is highlighted in bold below.

	Number of Individuals Genotyped									
Site	**TP1 (Queens)**	**TP2 (Workers)**	**TP3 (Workers)**	**TP4 (Queens)**	**Heterozygosity (F_{ST})**	**Allelic Richness**	**Sister pairs (unique colonies)**	**Effective population size (CI)**	**Floral Density (per m^2)**	***A. bombi* prevalence (%)**
ELS1	9	17	9	11	0.58 (± 0.10)	6.01 (± 1.05)	23 (20)	100 (45, 1000)	33.97	47
ELS2	12	23	4	12	0.67 (± 0.08)	6.99 (± 1.02)	27 (26)	369 (117, 1000)	15.38	44
ELS3	17	18	n/a	25	0.64 (± 0.06)	5.57 (± 0.80)	16 (14)	71 (32, 1000)	14.41	67
ELS4	31	22	11	4	0.60 (± 0.10)	5.76 (± 0.81)	28 (25)	132* (72, 534)	25.90	44
ELS5	26	10	14	27	0.57 (± 0.12)	6.19 (± 0.94)	22 (20)	130 (47, 1000)	24.48	59
HLS1	19	14	3	10	0.58 (± 0.11)	6.66 (± 1.00)	15 (14)	115 (35, 1000)	40.25	60
HLS2	8	13	23	9	0.64 (± 0.07)	6.45 (± 0.96)	35 (31)	172 (79, 728)	7.80	39
HLS3	18	21	22	5	0.62 (± 0.10)	7.09 (± 1.09)	33 (25)	81 (52, 158)	27.26	33
HLS4	28	26	16	22	0.57 (± 0.11)	6.14 (± 0.96)	37 (33)	158 (81, 417)	62.20	42
HLS5	16	n/a	25	15	0.65 (± 0.08)	7.08 (± 1.01)	22 (19)	92 (41, 1000)	44.11	61
Average ELS	**19.0**	**18.0**	**9.5**	**15.8**	**0.61**	**6.10**	**23.2**	**167.5**	**22.8**	**52.3**
AverageHLS	**17.8**	**18.5**	**17.8**	**12.2**	**0.61**	**6.68**	**28.4**	**123.6**	**36.3**	**47.0**

number of times an individual is recaptured, implemented in the software R (R Core Team 2014) using the package Capwire (Miller *et al.*, 2005). Here, the effective population size is represented by the number of colonies as opposed to the number of individuals within the sample sites, so that each sister represents a recapture event within the model. Both methods implemented in the Capwire package, the Event Capture Model (ECM) and the Two Innate Rate Model (TIRM), were used to estimate the effective population size. The ECM assumes that all individuals within an area have the same probability of being caught. The TIRM, however, allows for heterogeneity in the probability of capture between individuals. To determine which model best fit the data, Likelihood Ratio tests (LR) were conducted by simulating both ECM and TIRM and fitting both to the data. The significance of the LR was then used to determine which model best described the data.

3.3.8 Identification of population structure

GENEPOP 4.1.2 (Rousset, 2008) was used to test for deviations from Hardy-Weinberg Equilibrium (HWE) across populations and loci, as well as to detect Linkage Disequilibrium (LD) using a Markov-chain approximation to exact tests and likelihood-ratio tests, respectively. The results from HWE and LD were corrected for multiple testing using the Benjamini-Hochberg procedure, with both tests significant ($p<0.05$).

Estimates of global F_{ST} and population heterozygosity were calculated using FSTAT version 2.9.3.2 (Goudet 2001) using the hierarchical Bayesian *F*-model (Foll and Gaggiotti, 2006). The genetic diversity per site was calculated both by grouping all time points together, as well as per individual time point. For the individual time point analysis, only samples of at least 5 individuals per time point/site were included to allow for accurate measurement of genetic diversity. Standard errors were calculated by jack-knifing across loci. FSTAT was also used to calculate the allelic richness per sampling site.

PopTree (Takezaki *et al.*, 2010) was used to create Neighbor-Joining trees and UPGMA (Unweighted Pair Group Methods of Arithmetic Mean) diagrams per species, bootstrapping with 1000 replications.

AMOVA (Analysis of Molecular Variance) were conducted in ARLEQUIN (Schneider *et al.,* 2000); a method of estimating population differentiation from the molecular data obtained. This statistical method studies the patterns and degree of relatedness between populations and groups by multidimensional scaling and the clustering dendrogram; allowing for a greater understanding of genetic structuring.

3.3.9 Data Analysis

All statistical analyses were conducted in R (R Core Team 2014). Isolation by Distance was analysed via a Mantel test using the package 'vegan', allowing the comparison of pairwise genetic distance (F_{ST}) with geographical distance (km) between sites. The differences between ELS and HLS sites in terms of floral density, population size and heterozygosity were compared with Welch's two-sample t-tests. Generalised linear models were constructed with the variables of pathogen prevalence, heterozygosity, floral density and scheme set as factors and site as a random factor. Relationships between genetic heterozygosity and ecological traits (floral density and population size) were analysed using Pearson's correlation. All linear graphs were constructed using the package 'ggplot2'.

3.4 Results

3.4.1 Species differentiation

B. terrestris was found to be more prevalent that *B. lucorum,* and *B. hortorum* was more prevalent than *B. ruderatus* after correct identification using molecular techniques (table 3.6). Both species were more prevalent in TP2 sampling (18.46% and 10.20% respectively).

3.4.1 Removal of males, sisters and poor amplification

After the removal of males and females with poor amplification of loci (i.e. greater than 1/3 of the loci failed to amplify) the remaining females were assessed in COLONY for maximum-likelihood of sibship assignment. As bumblebees are haplodiploid organisms, male diploids of the species are removed as they will skew the data appearing as homozygotes giving a false over estimation of homozygosity. A total of 46 *B. hortorum* (n=289), 71 *B.*

lapidarius (n=597), 108 *B. pascuorum* (n=503) and 129 *B. terrestris* (n=430) were removed prior to further analysis (males, poor amplification and sisters).

Table 3.6: A table to show the prevalence of the least common species within the cryptic species complexes across time points. The total number of *B. lucorum* and *B. terrestris* caught per time point, with the prevalence of *B. lucorum.* And the total number of *B. ruderatus* and *B. hortorum* caught per time point with the prevalence of *B. ruderatus*.

Time Point	*B. lucorum*	*B. terrestris*	Prevalence (%)	*B. ruderatus*	*B. hortorum*	Prevalence (%)
1	17	194	8.06	0	31	0
2	48	212	18.46	15	132	10.20
3	22	290	7.05	11	141	7.24
4	21	151	12.21	7	94	6.93
Total	108	847	11.31	33	398	7.66

3.4.2 Identification of unique colonies and effective population size

A total of 48 *B. hortorum* sister pairs (loci=6), 103 *B. lapidarius* sister pairs (loci=9), 203 *B. pascuorum* sister pairs (loci = 8) and 66 *B. terrestris* sister pairs (loci = 6) were identified within the sample field sites (tables 3.2, 3.3, 3.4 and 3.5). These figures were used to reconstruct estimates of the effective population size as the samples caught are predominantly workers during the time of increased colony productivity. There were no 'non-circular' nests in which there are two sisters related to a third sister but not to each other identified within all sister pairs. Global analysis across all field sites and TPs 1,2&3 per species discovered sister pairs between sites, 19 *B. hortorum (*n=234*),* 86 *B. lapidarius (n=615),* 50 *B. pascuorum (n*=503*)* and 107 *B. terrestris (n*=614*).*

Even Capture Model (ECM) best represented the data obtained in *B. hortorum* (7/8 sites), *B. lapidarius* (8/10 sites), *B. pascuorum* (10/10 sites), *B. terrestris*

(9/10 sites) using the Likelihood Ratio Test (LRT) compared with Two Innate Rates Model (TIRM).

3.4.3 Null alleles, Hardy-Weinberg and linkage disequilibrium

Null alleles were present in loci B100 and B118 for more than half of *B. hortorum* sites and were removed from further analysis. The presence of null alleles in the other three *Bombus* spp*.* occurred in less than half of the sites so remained in subsequent analysis. Significant deviations from HWE were present in loci B124 and B126 in *B. lapidarius* sites and B96, B11 and B126 in *B. terrestris* sites; these loci were removed from further analysis. After Benjamini-Hochberg procedure was conducted on the significant values of linkage disequilibrium (LD) tests, there was no LD detected between loci across all *Bombus* spp*.* The total number of loci used during the investigation per species were as follows; *B. hortorum* -6 loci, *B. lapidarius* -9 loci, *B. pascuorum* -8 loci, and *B. terrestris* -9 loci.

3.4.4 Population structure

The overall F_{ST} values for each of the four species are relatively low and significant: *B. hortorum* θ=0.005 (p=0.016), *B. lapidarius* θ=0.009 (p=0.014), *B. pascuorum* θ= 0.025 (p=0.006), and *B. terrestris* θ= 0.005 (p=0.011). These values were as expected over such a short geographical distance. Parallel to this low global structuring within the species, there was also no isolation by distance observed for any of the species (fig. 3.4). Results from a Mantel test show the lack of significant interaction of geographical range on the genetic distance of all but *B. hortorum* (*B. hortorum:* r=0.60, p<0.05; *B. lapidarius:* r=0.14, p=0.17; *B. pascuorum:* r=-0.09. p=0.71; *B. terrestris:* r=-0.06, p=0.65).

Neighbor-Joining trees and UPGMA diagrams were created per species (fig 3.5), also showing very little genetic structuring within each species.

The STRUCTURE analysis revealed weak support for structural clustering within the four species of bumblebee (fig. 3.6, fig 3.7). 2 clusters were estimated for *B. lapidarius* and *B. terrestris*, whereas support for K=3 with weaker K=7 for *B. hortorum* and K=3 and K= 6 for *B. pascuorum* were estimated. The bar plots for each species allow assignment of each cluster to each sample site, separated by the individuals themselves.

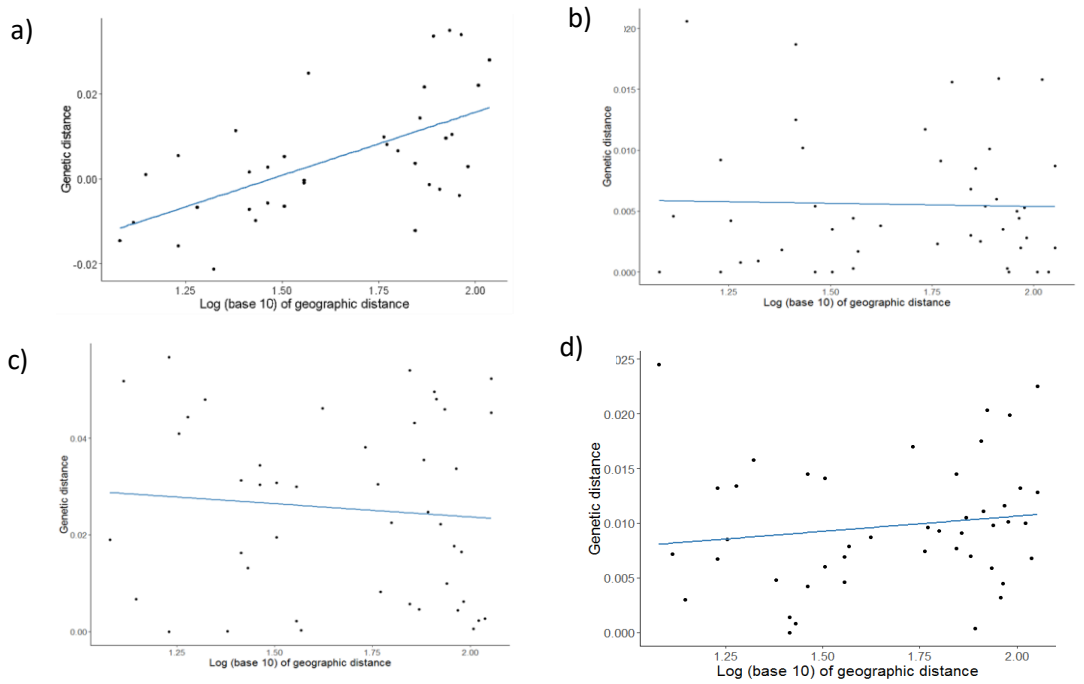

Figure 3.4: The relationship between the logged geographical distance (km) and genetic distance (F_{ST}) for a) *B. hortorum* (p<0.05), b) *B. lapidarius* (p=0.17), c) *B. pascuorum* (p=0.71), and d) *B. terrestris* (p=0.65) populations. Each point represents a pairwise comparison from genetic and distance matrices.

Given the results observed from the NJ, UPGMA trees and STRUCTURE analysis, Analysis of Molecular Variance was calculated in Arlequin (table 3.7). Results from the UPGMA provided the greatest variation between groups of *B. hortorum* populations. The structuring included ELS1 and HLS4 in one group whilst the other sites occupied their own individual grouping. This number of clusters (K=8) was weakly supported for by the results from the STRUCTURE analysis. The UPGMA also provided the greatest variation between groups of *B. lapidarius* and *B. pascuorum* with 6 and 2 groups respectively. These groupings were not estimated by the STRUCTURE analysis, and the variation was still very low for *B. lapidarius* (0.99%). Grouping ELS2, HLS2, HLS5 and ELS3 and then the rest in another group for *B. pascuorum* gave a variation of 3.08%. Finally, the grouping giving the greatest variation of 0.63% in *B. terrestris* used results from STRUCTURE analysis using K=2, by separating HLS2 and HLS5 from the other sites, this structuring is also observed in the NJ and UPGMA models. From looking at geographical data and information describing these sites the reasoning behind this grouping is not clear, and it is likely these 'groups' are spurious. Due to such low population structure within the species,

groupings of these locations are unlikely to exist as high levels of admixture between sites occurs.

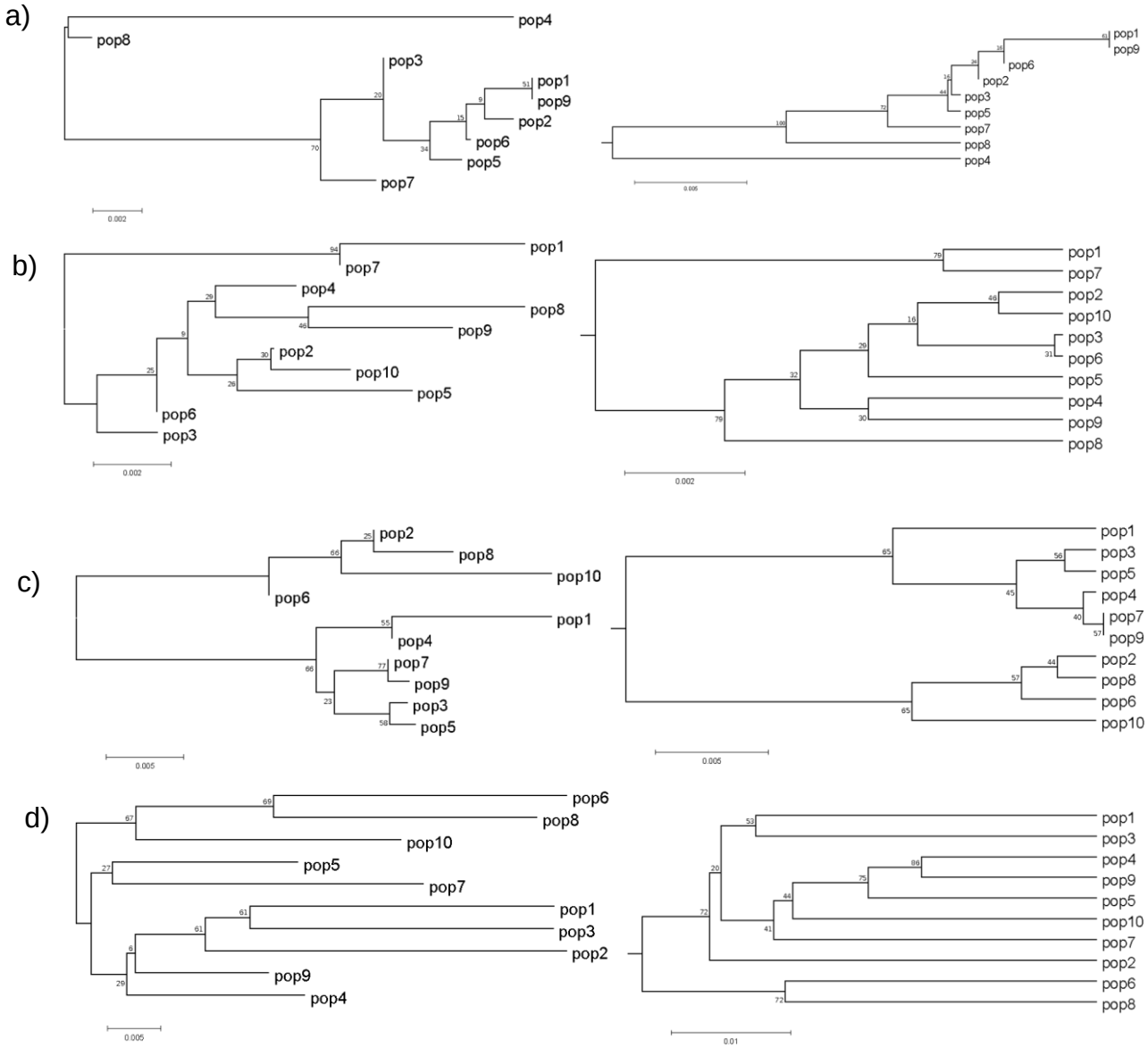

Figure 3.5: Neighbor-Joining trees (left) and UPGMA graphs (right) for (a) *B. hortorum,* (b) *B. lapidarius,* (c) *B. pascuorum* and (d) *B. terrestris.* Populations are as follows: 1- ELS1, 2-ELS2, 3-HLS3, 4-ELS5, 5-HLS1, 6-HLS2, 7-ELS4, 8-HLS5, 9-HLS4 and 10- ELS3.The branch numbers represent bootstrapping over loci.

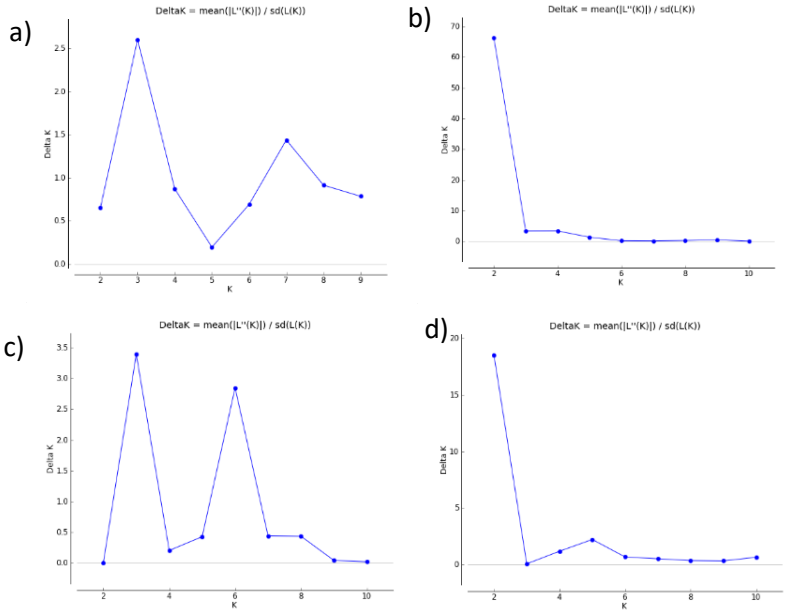

Figure 3.6: Inference of the optimal value of K using the ΔK method of Evanno (*et al.,* 2005) from STRUCTUREHARVESTER. Support for a) K=3/K=7 *B. hortorum*, b) K=2 *B. lapidarius,* c) K=3, K=6 *B. pascuorum,* and d) K=2 *B. terrestris*.

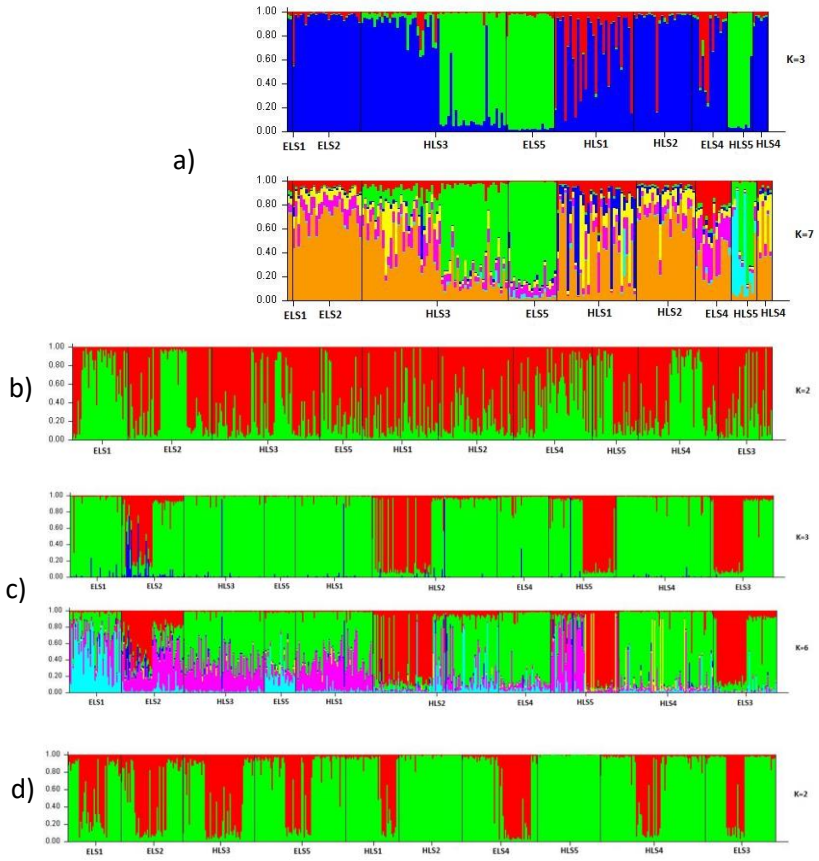

Figure 3.7: Population structure analysis bar graphs from STRUCTURE for a) *B. hortorum,* b) *B. lapidarius*, c) *B. pascuorum* and d) *B. terrestris*. Each individual genotype is represented by a coloured vertical bar representing the posterior assignment probability of being assigned to the clusters. Each colour represents a different cluster within each of the graphs.

Table 3.7: A table showing the results of AMOVA performed in Arlequin for *B. hortorum, B. lapidarius, B. pascuorum* and *B. terrestris*. The groups are as follows: *B. hortorum* **(k=3)**: ELS1+ELS2+HLS4, HLS3+ELS5+HLS5, HLS1+HLS2+ELS4; *B. hortorum* **(k=7):** ELS1+ELS2, HLS3, ELS5+HLS5, HLS1, HLS2, ELS4, HLS4 *B. hortorum* **UPGMA**: ELS1+HLS4, REST AS INDIVIDUAL GROUPS. *B. lapidarius* **(k=2):** ELS1+ELS2+ELS4+HLS4, HS1+HLS2+HLS3+HLS5+ELS3+ELS5; *B. lapidarius* **UPGMA**: ELS1+ELS4, ELS2+ELS3, HLS3+HLS2, HLS1, ELS5+HLS4, HLS5. *B. pascuorum* **(K=3):** ELS2, ELS1+ELS4+ELS5+HLS1+HLS3+HLS4, ELS3+HLS2,+HLS5; *B. pascuorum* **(k=6):** ELS1, ELS2, HLS1+HLS3, ELS4+ELS5+HLS5, HLS2+HLS5, ELS3; *B. pascuorum* **UPGMA**: ELS1+HLS3+HLS1+ELS5+ELS4+HLS4, ELS2+HLS5+HLS2+ELS3. *B. terrestris* **(k=2):** HLS2+HLS5, ELS1+ ELS2+ HLS3+ ELS5+HLS1+ELS4+HLS4+ELS3; *B. terrestris* **UPGMA**: ELS1+HLS3, ELS5+HLS4+HLS1+ELS3+ELS4, ELS2, HLS2+HLS5. Results with greatest group percentage per variation per species are highlighted in bold.

Grouping Criteria	Total Number of Groups	Within-population % variation, F_{IS} (*P-value*)	Among-population % variation, F_{SC} (*P-value*)	Among-group % variation, F_{CT} (*P-value*)
B. hortorum				
STRUCTURE (K=3)	3	84.0, 0.157 (0.000)	0.32, 0.00317 (0.111)	-0.00, -0.00005 (0.431)
STRUCTURE (K=7)	7	84.0, 0.157 (0.000)	-0.89, -0.00900 (0.954)	1.23, 0.0122 (0.142)
Scheme ELS vs HLS	2	84.0, 0.157 (0.000)	0.30, 0.00304 (0.000)	0.02, 0.160 (0.368)
UPGMA	**8**	**84.0, 0.157 (0.000)**	**-5.03, -0.0532 (0.957)**	**5.36, 0.0536 (0.0269)**
B. lapidarius				
STRUCTURE (K=2)	2	98.6, 0.0163 (0.0485)	-0.20, -0.00200 (0.982)	0.21, 0.0137 (0.222)
Scheme ELS vs HLS	2	98.6, 0.0136 (0.0517)	-0.28, -0.00281 (0.997)	0.35, 0.0143 (0.0906)
UPGMA	**6**	**98.6, 0.0136 (0.0486)**	**-0.97, -0.00979 (1.00)**	**0.99, 0.00994 (0.00653)**
B. pascuorum				
STRUCTURE (K=3)	3	86.0, 0.108 (0.000)	0.84, 0.00862 (0.00000)	2.71, 0.02713(0.00178)
STRUCTURE (K=6)	6	68.7, 0.108 (0.000)	0.48, 0.00489 (0.00356)	2.27, 0.02266 (0.00277)
Scheme ELS vs HLS	2	87.0, 0.108 (0.000)	2.76, 0.02754 (0.00000)	-0.35, -0.00353 (0.64208)
UPGMA	**2**	**58.7, 0.108 (0.000)**	**0.82, 0.00847 (0.00000)**	**3.08, 0.03080 (0.00416)**
B. terrestris				
STRUCTURE (K=2)	**2**	**85.1, 0.141 (0.000)**	**0.30, 0.00305 (0.00069)**	**0.63, 0.00626 (0.0211)**
Scheme ELS vs HLS	2	85.3, 0.141 (0.000)	0.41, 0.00410 (0.00000)	0.18, 0.00179 (0.0734)
UPGMA	4	85.4, 0.141 (0.000)	0.13, 0.00127 (0.07218)	0.55, 0.00554 (0.00129)

3.4.4 Floral density and genetic diversity

As the floral density increases, the genetic diversity increases significantly in *B. hortorum* (R^2=0.62, F(1,5)=8.101, p<0.05), *B. lapidarius* (R^2=0.42, F(1,8)=5.74 populations, p=<0.05) but not significantly in *B. terrestris* populations (R^2=0.148, F(1,8)=1.813, p=0.215) whereas a negative correlation was shown in *B. pascuorum* (R^2=0.42, F(1,8)=5.875, p<0.05). The graphs show the floral density during TP2 sample collection (fig. 3.8). HLS sites had a greater floral density (mean = 36.3) than ELS (mean = 22.8), although this was non-significant (p=0.22).

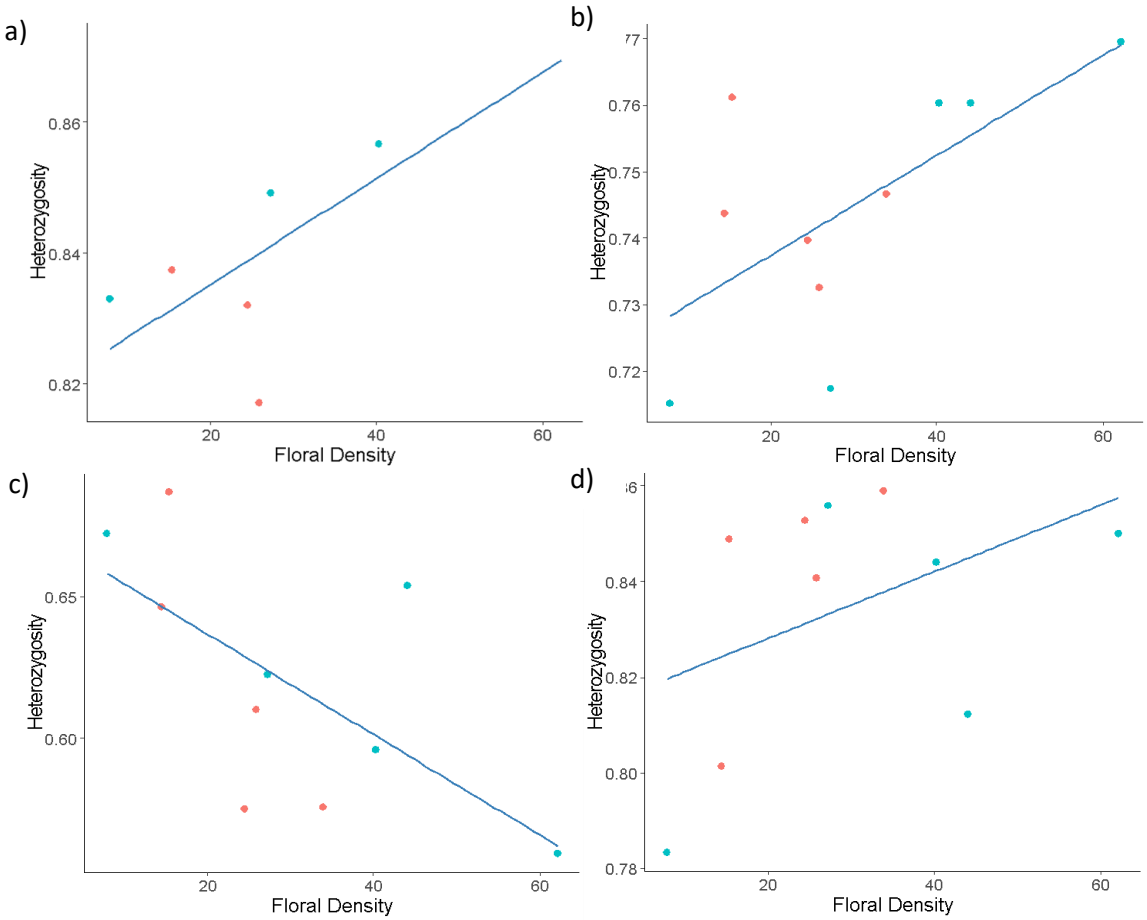

Figure 3.8: The relationship between the average floral density of each field site and the observed heterozygosity (F_{ST}) of a) *B. hortorum* (p<0.05), b) *B. lapidarius,* c) *B. pascuorum* (p<0.05) and d) *B. terrestris* (p=0.215) populations. Each point represents a sampling location with higher-level sites in blue and entry-level sites in orange.

3.4.5 Effective Population Size

The level of stewardship had a significant positive relationship with the effective population size for *B. pascuorum* but not in the other species (*B. hortorum*: t=-

0.95, df=4.15, p=0.40; *B. lapidarius*: t=0.41, df=5.27, p=0.70; *B. pascuorum*: t=-2.41, df=8.0, p=0.04; *B. terrestris*: t=0.65, df=4.89, p=0.54) (fig. 3.9).

The genetic diversity of each site showed no significant relationship with the effective population size of each species (*B. hortorum*: cor=0.001, t=0.002, df=5, p=0.998; *B. lapidarius*: cor=-0.328, t=-0.98, df=8, p=0.36; *B. pascuorum*: cor=0.386, t=1.182, df=8, p=0.27; *B. terrestris*: cor=0.127 t=0.36, df=8, p=0.73).

A Pearson's correlation also found no relationship between the floral density of a site and the effective population size of each *Bombus* sp. sampled in the area (*B. hortorum:* cor=-0.257, t=-0.65, df=6, p=0.54; *B. lapidarius:* cor=-0.178, t=-0.51, df=8, p=0.62; *B. pascuorum:* cor=0.017, t=0.05, df=8, p=0.96; *B. terrestris:* cor=-0.258, t=-0.76, df=8, p=0.47).

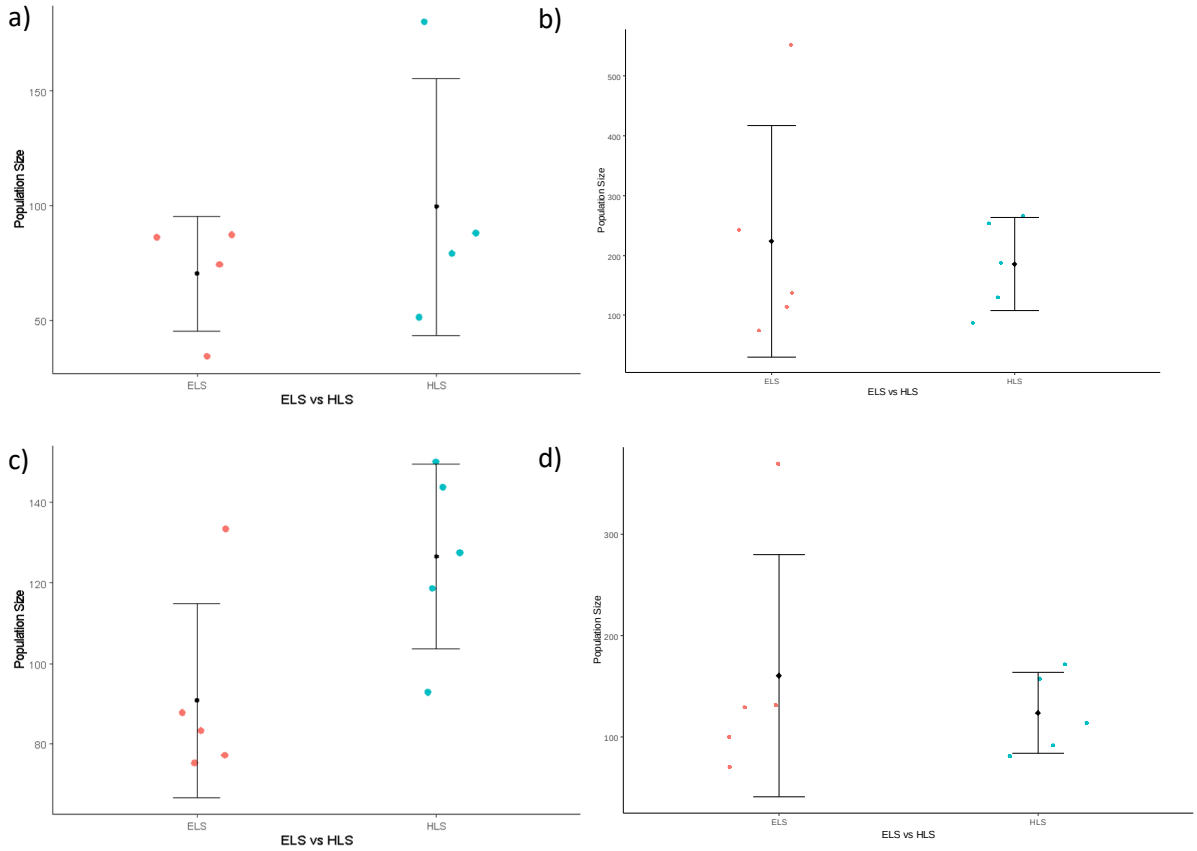

Fig 3.9: The level of stewardship (HLS and ELS) and the effective population of a) *B. hortorum,* b) *B. lapidarius,* c) *B. pascuorum* and d) *B. terrestris* with the means and 95% confidence intervals. Each point represents an individual sampling location.

3.4.6 Relationship with disease

Apicystis prevalence was estimated per site and per species then comparisons between HLS and ELS sites were conducted (fig 3.10.). There were no

differences found between the two levels of stewardship and the prevalence of *Apicystis* (*B. hortorum:* t=1.36, df=5, p=0.23; *B. lapidarius:* t=-0.14, df=8, p=0.89; *B. pascuorum:* t=0.07, df=8, p=0.95; *B. terrestris:* t=0.72, df=8, p=0.49)

Finally, I tested interactions between heterozygosity of a population, the type of scheme and floral density with the prevalence of *Apicystis bombi*, by conducting Generalised Linear Mixed Effect Models (table 3.8). There were no significant interactions between the variables (heterozygosity, scheme and floral density) on the prevalence of *A. bombi* for any of the four species studied.

Table 3.8: The results of a binomial generalised linear model (GLM) for each *Bombus* sp. (*B. hortorum, B. lapidarius, B. pascuorum and B. terrestris*), looking at the variables heterozygosity, scheme and floral density on the prevalence of *Apicystis bombi* within each population.

				B. lapidarius				
Co-efficient estimate	s.e.	Z	p		Co-efficient estimate	s.e.	Z	p
2.95	26.2	0.113	0.91	**heterozygosity**	3.55	8.13	0.437	0.662
-0.33	0.54	-0.611	0.541	***scheme***	0.194	0.254	0.785	0.432
-0.017	0.021	-0.804	0.422	***floral density***	0.0032	0.011	0.3	0.764
				B. terrestris				
Co-efficient estimate	s.e.	Z	P		Co-efficient estimate	s.e.	Z	P
-256	17920	-0.014	0.989	**heterozygosity**	-10.3	6.77	-1.517	0.128
8.51	2845	0.003	0.998	***scheme***	-0.63	0.331	1.892	0.059
-1.303	94	-0.014	0.989	***floral density***	0.018	0.011	1.693	0.091

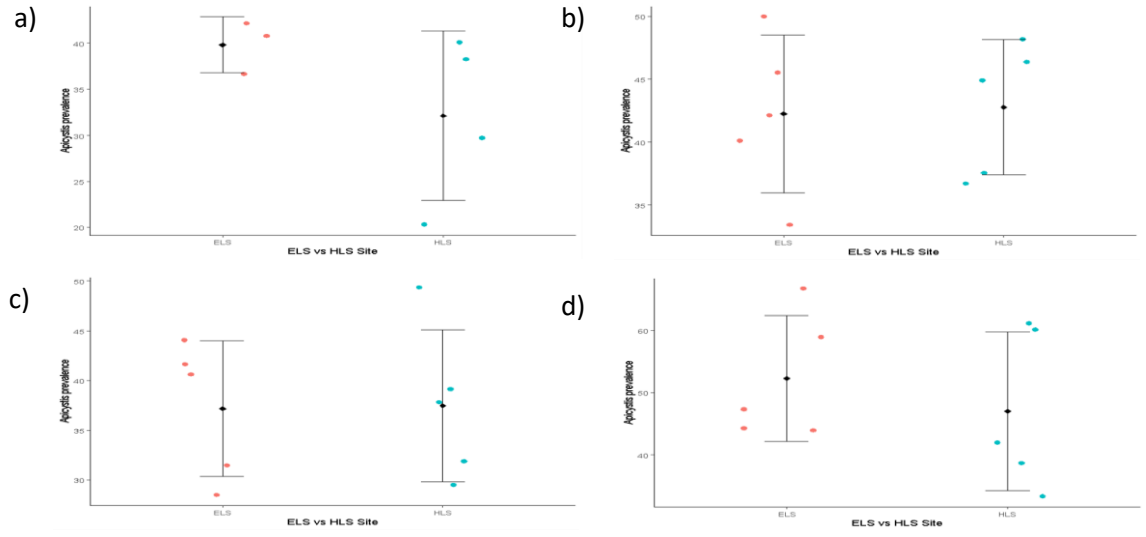

Figure 3.10: The prevalence of *A. bombi* given the type of agri-environment site (ELS vs HLS) for populations of a) *B. hortorum,* b) *B. lapidarius,* c) *B. pascuorum* and d) *B. terrestris*. Each point represents a sampling location and the mean, and 95% confidence intervals are given per scheme.

3.5 Discussion

Unlike previous studies looking into the impact agri-environments schemes have on the genetic diversity of different bumblebee species (Carvell et al., 2004; Carvell et al., 2011; Wood et al., 2015; Carvell et al., 2017), this thesis found that the type of scheme had no effect on the effective population size, heterozygosity or disease prevalence. However, the floral density significantly increased the genetic diversity of 3/4 bumblebee species sampled in this study. This suggests that further investigative work with more thorough meta-data for each site would prove more valuable than a two-tier categorical system (ELS vs. HLS) that is currently in place.

3.5.1 Cryptic bumblebee species

False identifications of cryptic bumblebee species have the potential to invalidate the results of population genetics and epidemiology, weakening conclusions drawn from conservation studies. In this thesis, I molecularly differentiated between individuals of *B. terrestris/B. lucorum* and *B. hortorum/B. ruderatus*. *B. lucorum* and *B. ruderatus* were found at a much lower prevalence (11.31% and 7.66% respectively) than their sympatric, cryptic pair. The low prevalence of *B. lucorum* in these lowland sites reflect previous work (Scriven *et al.*, 2015) in which they highlight the species preference for a cooler climate. Different species occupy different ecological niches, so the first step in accurate conservation studies involves correct identification of sample species.

3.5.2 Genetic structuring

The overall genetic structuring for the four-bumblebee species sampled was low (0.005-0.025) compared to other similar population-genetic studies (Goulson *et al.*, 2011b). The high number of sister pairs globally across sites also confirms this.

B. pascuorum was the only species to show significant IBD as well as this higher level of genetic structuring. These results suggest there is a lot of admixture between the sites, as well as ‘stepping stone’ populations which were not sampled allowing gene flow between populations. Non-specific groupings were observed from the results of the UPGMA and NJ trees as well as cluster reconstruction with STRUCTURE harvester. Geography therefore has no clear

impact on the groupings and neither does the type of scheme, suggesting high levels of inter-population breeding within the area. This thesis found that most of the variation observed in these hymenopteran species is present within populations rather than among populations. On account of the lack of genetic differentiation between geographic regions, it suggests that the distance alone is not restricting gene flow and there is no genetic divergence between populations.

3.5.3 Impacts of field margins

The impacts of field margins providing valuable resources have been shown to increase nest density, pollinator abundance and diversity as well as population size (Wood *et al.*, 2015). However, this study suggests that although the type of scheme does not affect pollinator diversity, the floral density positively increases the genetic diversity of pollinator visitors (*B. hortorum, B. lapidarius* and *B. terrestris*). Using the estimated foraging distances for all four species derived from Wood *et al.* study (*B. hortorum*- 566m*, B. lapidarius*-714m*, B. pascuorum*-363m and *B. terrestris*-799m), it can be concluded that there would be no overlap in ranges between field sites (minimum separation distance of 12km). Parallel to the lack of positive association between the level of stewardship and the impact on genetic diversity of bumblebee species, there seems to be no significant relationship between the assigned level of stewardship and the observed floral density. This would suggest that current efforts invested into implementing these schemes are not having the desired outcome. Proxies for assessing the efficacy of these schemes include the biodiversity of selected species, but these may need to be investigated further. My results suggest field margins are not positively impacting the density or species richness within the field site under the current two-tier scheme, and therefore there may be an expected decrease in genetic diversity of the study species within these 'improved' HLS sites. The lack of genetic structuring observed across the sampling area suggests that these fragmented arable landscapes are not restricting gene flow, but instead create areas with high foraging densities within more floral rich areas. Attracting individuals from many colonies to share these resources could mean an increase in the transmission of bumblebee diseases.

In addition to the environment scheme and the floral density observed within each field site, the landscape context would also play a role in the genetic diversity and effective population size of each bumblebee species (Heard *et al.,* 2007). The surrounding area would greatly impact the density of foraging pollinator species, for example the percentage of arable habitat was shown to greatly influence the density of all bumblebee species (Carvell *et al.*, 2011). Further studies looking into the sample sites and their surrounding would prove valuable.

3.5.4 The effective population size

Contrary to previous studies, there was no significant relationship between the level of agri-environment scheme and the effective population size of *Bombus* sp. other than in *B. pascuorum* populations. Exploring this further revealed that even the density of the flowers within each site had no direct impact on the number of bumblebee colonies estimated from the microsatellite data. Thus, suggesting the positive impact of HLS sites on *B. pascuorum* population size is most likely spurious, a result of another untested ecological variable or less intensive sampling methods. Further to this, the sample sizes of each species between sites was not equal (i.e. the sampling effort varied). In order to accurately predict and compare the effective population sizes, greater care should be taken to ensure equivalent sample sizes for each site and species.

3.5.5 Temporal influence

Temporal changes in external factors can have a great impact on the results of an individual study, for example a poor winter could greatly influence the success of newly emerging queens (Owen *et al.,* 2013). This study suggested there were temporal changes between the two sampling seasons, however in order to assess this fully, assessing seasonal changes over a longer time period would be of greater use.

3.5.6 The efficacy of targeted agri-environment schemes

In order to increase the efficacy of agri-environmental schemes in terms of wild pollinator management, to conserve rare species and maintain stable populations, a more accurate and intensive study of current population diversity and structure is required. To be able to track these important variables over

time and correlate with the surrounding landscape will aid towards the conservation of these declining species. This will also ensure correct changes are made to current policy to enable these schemes to continue to enhance pollinator abundance and buffer against the surrounding intensification of agricultural landscape. This increased knowledge of variables influencing population fitness allows inference into the overall stability of individual species in the area. A vital aspect of effective conservation is to accurately assess each species alone instead of pooling species together, as this could lead to inaccurate assumptions of population declines or for increases to be overlooked, resulting in a lack of intervention to prevent species loss.

An issue with assessing the efficacy of field margins is determining whether the abundance of pollinator species is increasing or if the high density of sown wildflowers is attracting them to a more confined area. Previous studies have shown that the Higher-Level Stewardship schemes increase bee population sizes significantly in *B. hortorum* and *B. lapidarius* (Wood *et al.,* 2015). Sampling efforts between the two investigations were not equivalent, with Wood *et al.,* sampling over a much larger area; similar methods across projects would make it easier to compare results. Despite having a two-tier system, the current agri-environment process can have different targeting methods within the entry- and higher-level schemes. It would suggest that despite being given the status of an HLS site, this does not always accurately represent the ecological landscape of the farms as some ELS were found to have greater floral density and diversity than similar HLS sites. These levels reflect the heterogeneity of the landscape and suggests that to some extent the measure implemented on ELS farms can be highly effective. The designated grouping of ELS and HLS may be misleading given a whole range of other factors which could influence these figures. It is therefore vital to sample the environment of these sites, as using the stewardship level alone does not accurately represent the ecology. Similarly, as the investigation was not experimental and therefore ecological conditions were not standardised both within the sample sites and the surrounding area, it would be suggested that farms which opted in to the HLS schemes may already have greater levels of semi-natural vegetation as well as a greater awareness of the positive impacts of increased biodiversity.

3.5.7 Disease prevalence and genetic diversity

The prevalence of disease within these schemes can have serious consequences on bumblebee fitness at the population level. Although the relationships between the type of agri-environmental scheme and the prevalence of *Apicystis bombi* was found to be non-significant, the general trends suggested these HLS sites had a decreased prevalence of the disease. From the results of the GLM however, it is unclear which factors are influencing these results as the heterozygosity, type of scheme and the floral density did not impact the prevalence of the pathogen. This is unlike previous similar studies which suggest an association with the genetic diversity of the population (Whitehorn *et al.*, 2011). The heterozygosity of a population increases the fitness, making it less susceptible to diseases. Sisters found within these sites are more genetically related and would bias results of epidemiological studies with transmission occurring at a greater rate within the colonies. By regressing multiple factors including genetic diversity within the sites, management schemes used to buffer effects of habitat fragmentation can adopt these methods to decrease disease prevalence.

3.5.8 Further study

This research study focused on the four most abundant *Bombus* spp. within these agri-environment schemes. To further improve upon this research into their efficacy, assessing the genetic structuring for less common species would be valuable. *Apis mellifera* were also sampled from every site and it would be beneficial to sequence the COI-COII mitochondrial region (which is representative of the honeybee lineage) to analyse the diversity of this polyandrous bee species, with a much larger foraging range and different colony structuring.

There has been a plethora of case studies and meta-analysis investigating the efficacy of agri-environmental schemes, providing evidence that these wildflower field margins greatly improve the diversity and community of wild pollinators (Carvell et al., 2011; Marja et al., 2018) as well as other fauna (Kleijn and Sutherland, 2003). The consensus is that while these schemes do positively affect the conservation of many threatened species, there is a trade-off with the cost of providing better managed and targeted methods. Even with

low intensity sampling effort such as during this study, sister pairs are still caught at a high frequency within sites and time points. The relatively high frequency of sib-ships can skew results of epidemiological studies as the individuals are more genetically related and likely to have similar susceptibility characteristics.

3.6 Conclusions

As with many conservation studies, the efficacy of the implemented schemes is subjective as the optimal genetic diversity and structure required to sustain a healthy population within these arable landscapes is difficult to estimate. It would be useful to combine studies from sites all over the globe to estimate a threshold at which a bumblebee population's genetic diversity, structure and population size would prove sustainable and not under threat of extinction. By creating a timeline of how these agri-environmental schemes impact the genetic diversity year after year, a general trend could be estimated. This will help determine if these schemes are having the desired effect on the biodiversity they hope to sustain.

Many methods of reversing human impact on the reduction of biodiversity and population numbers tend to target a specific group of animals, for example foraging plants for pollinator species. However, this research suggests that trends in one species of bumblebee are not always exhibited in another. As with most conservation efforts, analysing the effects of different interacting factors down to the species level proves valuable. What might be working for one species could be having disastrous effects on another, thus reiterating the importance of molecular analysis to distinguish between cryptic bumblebee species.

Sister pairs are frequent across these types of landscapes, even with relatively low intensity sampling effort. Future epidemiological studies should provide full sib-ship reconstruction in order to prevent bias of results from increased colonial transmission between these genetically similar sisters. Current disease data for this sample group is only complete for *Apisystis;* however, ongoing research should be completed for this data set, to allow for further comparisons of factors potentially influencing disease abundance and prevalence. Relating the density of flowers within these schemes would hopefully show if there is a correlation,

and if so whether these floral-rich areas despite increasing genetic diversity might be increasing the transmission of pathogens within populations and between species.

Chapter 4: General Discussion and Synthesis

Bumblebees provide a great model species for studying the impacts upon genetic diversity and structuring because of their great amount of publicity and media attention, as well as the ease of sampling many individuals in a short space of time. The general decline of bumblebee species in the UK and France since the post-war era can be attributed to a combination of factors such as habitat loss and fragmentation, pesticides, parasites, pathogen spill-over and climate change. Here we investigated the genetic structuring and association with disease for four common bumblebee species in England and France, studying the impact of islands and agri-environment schemes on the species.

4.1.1 Island and Mainland Populations

4.1.2 Genetic diversity

The results from this thesis show that structuring for *B. pascuorum* populations across island and mainland populations in the UK and France is high (θ =0.122 (±0.0 22), $p<0.05$). Whereas results from *B. terrestris* analysis show a lower non-significant level of genetic structuring between these populations (θ = 0.069 (±0.009), p=0.053). This would suggest greater 'stepping-stone' populations of *B. terrestris,* by which gene flow can occur more often between isolated populations. Contrary to these global F_{ST} values, *B. terrestris* populations showed a significant isolation by distance whereas this was not observed in *B. pascuorum* populations.

Interestingly, cluster analysis for both species revealed island populations being more genetically dissimilar to the mainland. *B. pascuorum* showed the greatest variation grouping all mainland sites with the Channel Islands and the Isle of Man and Ushant creating their own cluster. Grouping within *B. terrestris* samples grouped all sites together however there was clear genetic diversity with individuals from the Isle of Scilly.

The results from this thesis provide further evidence of genetic structuring of bumblebee species between island and mainland sites.

4.1.3 Effective Population Size

This thesis found that population size did not differ between island and mainland sites in either species, and neither was there an effect of heterozygosity on the populations. However, despite similar sampling efforts between sites, the confidence intervals for the estimated population sizes vary greatly. In order to improve upon the accuracy of these predictions, more samples should be taken from each site. This was not achievable for the current study as whole body sampling was conducted. In the case of tarsal sampling (as has been shown in previous studies (Wood, 2015 #142) more individuals could be genotyped, leading to more sister pairs being identified which would increase the confidence of the CAPWIRE models.

4.1.4 Prevalence of Disease

This thesis combines genetic studies with disease data, an assessment of bumblebee health which could serve as a proxy looking into the effect of inbreeding within isolate island sites. My results suggest that as the heterozygosity of a population increases (i.e. it becomes more diverse), the abundance of *Crithidia bombi* (i.e. the parasite load of each individual) also increases. Although contradictory to what would be expected, these results are similar to experimental work by Whitehorn *et al.,* (2011). As has been suggested before, individuals are more likely to be able to survive with higher pathogen loads at these higher levels of genetic diversity.

At the population level, although *C. bombi* prevalence decreased significantly in *B. pascuorum* populations with increased genetic diversity there was no effect of the type of site (island or mainland). The positive result from *B. terrestris* populations is likely spurious due to the lack of genetic structuring within the species.

Geographical barriers are present all over the world, extending from vast mountain ranges, oceanic islands and fragmented agricultural land. All these landscape features can prevent admixture between populations, inhibiting gene flow, thus creating more genetically isolated populations such as the Isle of Man and Scillies from this thesis. Inbreeding within these populations is not rare, and the associated fitness costs are high (Whitehorn et al., 2009; Woodard et al., 2015).

4.2 Agri-Environment Schemes

4.2.1 Genetic Diversity

Population structure (global F_{ST} values) for all four study species were significantly low as was expected over such short geographical distances. Parallel to this, weak clustering support with no clear patterns between sites provides further evidence for high levels of admixture between these arable landscapes. One surprisingly significant result was the positive association between floral density and heterozygosity, unexpected due to the lack of significant difference in floral density between scheme levels.

4.2.2 Effective Population Size

The type of scheme implemented by the farms only significantly affected populations of *B. pascuorum* which is likely to be explained by the highest levels of genetic structuring calculated of the species. As the type of scheme (ELS vs HLS) has very little impact on any of the other variables of this study, this significant result is likely to be spurious and due to other non-recorded factors.

4.2.3 Impact on agricultural policy

The results obtained from these studies could be of great value to EU countries partaking in the scheme under the Common Agricultural Policy. Positive results from such studies would also prove favourable in the UK after leaving the EU, to secure monetary subsidies for UK farmers to implement similar localised schemes to aid pollinator conservation from the UK government. The current OECD average for government support in agriculture is 18% and 22% within the EU; Brexit will no doubt impact the agricultural schemes sampled in this study. A primary issue with these higher-level stewardship schemes is they are usually shaped over a long period of time, sometimes even a decade, so any interruption would have a considerable impact on the efficacy of these schemes.

Current policy only allows for WFS subsidies for farmers, preventing gene flow by fragmenting the habitats between semi-rural and rural landscape. By providing similar subsidies and methods to other possible sources of biodiversity and ‘green space’ within the landscape, corridors can be created to

prevent genetic isolation and inbreeding. The current method of high-level stewardship not only leaves patches of land available but also leaves the farming areas subject to further intensification.

4.3 The Study Species

The focus of this study was on the four most common species found within the UK and France; however, of greater concern would be species listed as ‘rare’ and ‘declining’ such as *B. ruderatus, B. humilis* and *B. sylvarum* (Goulson *et al.,* 2005). Understanding how geographical barriers and agri-environment schemes could influence genetic diversity, population size and disease prevalence of threatened species is vital. Being able to target specific specialist pollinator species through intervention would greatly improve the ecosystem.

4.4 Final Remarks

With the world changing at an unprecedented rate, primarily linked with the exponential growth of the human population and associated anthropogenic activities, research into current buffering methods is of great importance. This thesis demonstrates a broad understanding of the current structuring and disease status of two bumblebee species in the UK and France, and four bumblebee species within agri-environment schemes.

From the results of this thesis, further research in this area of study should focus on how the landscape can be better managed to mitigate against habitat fragmentation. Geographic isolation of bumblebee populations does affect their genetic diversity and structuring, as well as disease prevalence. Using this information, and the positive results of floral diversity, management schemes across the UK should be better assessed to prevent species loss. A larger global study of *Bombus* spp. populations would prove valuable in understanding where the primary declines are occurring and exploring gene flow between populations. Finally, it is important to appreciate the impact of sister pairs on the results of epidemiological studies by understanding that increased genetic similarity increases disease prevalence which would only worsen with inbreeding.

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Supplementary Material

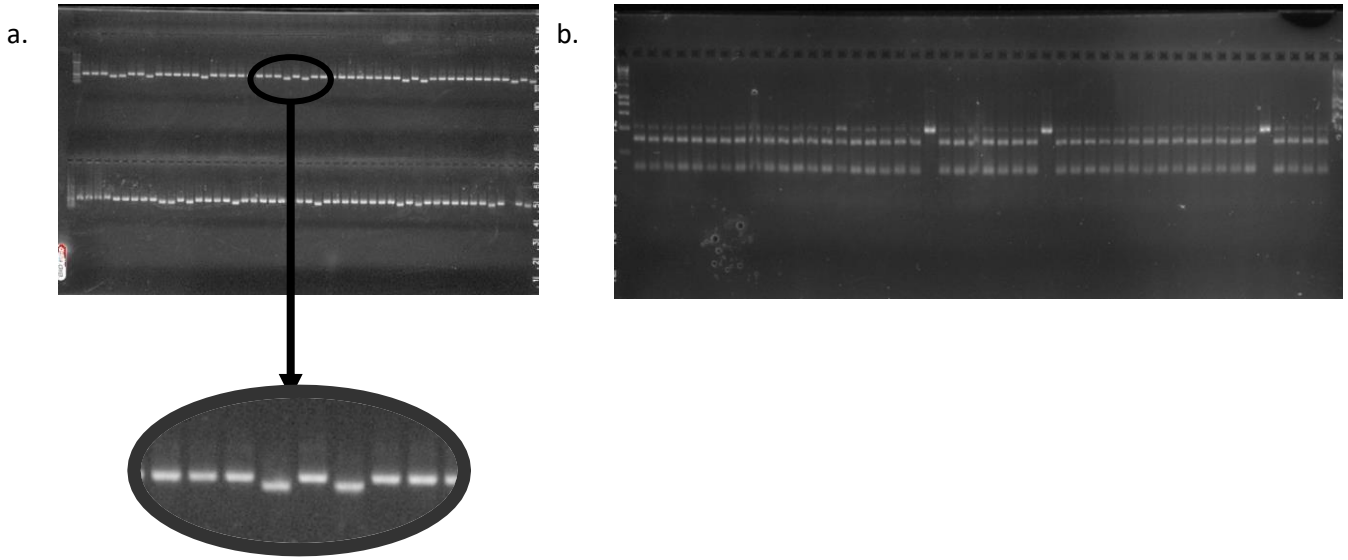

Figure S1: (a) A gel image showing the bands for PCR product from the methods of *B. terrestris* and *B. lucorum* differentiation. (b) A gel image showing the bands for digested PCR product from the methods of *B. hortorum* and *B. ruderatus* differentiation.

Table S1: A matrix of pairwise heterozygosity (F_{ST}) values for *B. pascuorum* between each sampling location using 6 loci with significant values ($p<0.05$) in bold after Bonferroni corrections. Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L) and Ushant (U).

	A	P	B	C	F	G	M	J	L	U
A	0	**0.1569**	**0.0563**	**0.0942**	**0.1078**	**0.0753**	**0.2245**	**0.1094**	**0.1317**	**0.1437**
P		0	**0.1099**	**0.0917**	**0.0771**	**0.2364**	**0.3154**	**0.146**	**0.0772**	**0.1112**
B			0	0.0174	0.0222	**0.1049**	**0.2196**	**0.0818**	**0.0508**	**0.0682**
C				0	0.0087	**0.1311**	**0.1641**	**0.084**	**0.0181**	**0.0458**
F					0	**0.135**	**0.2382**	**0.0681**	0.0132	0.0333
G						0	**0.2912**	**0.1636**	**0.1584**	**0.1759**
M							0	**0.3134**	**0.2409**	**0.2636**
J								0	**0.0971**	**0.096**
L									0	**0.0496**
U										0

Table S2: A matrix of pairwise heterozygosity (F_{ST}) values for *B. terrestris* between each sampling location using 8 loci with significant values ($p<0.05$) in bold after Bonferroni corrections. Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L) and Ushant (U).

	A	P	B	C	F	G	M	J	L	Q	S
A	0	0.0553	0.0478	0.0557	0.0412	0.0694	0.1235	0.0195	0.0642	0.0267	0.1334
P		0	0.0312	0.0529	0.0424	0.0648	0.1034	0.0251	0.0611	0.0178	0.114
B			0	0.035	0.0281	0.0696	0.086	0.02	0.0541	0.0301	0.1236
C				0	0.0233	0.0572	0.1128	0.0361	0.0637	0.036	0.151
F					0	0.0374	0.0847	0.0249	0.046	0.0327	0.1223
G						0	0.1394	0.0454	0.0587	0.0562	0.1272
M							0	0.0946	0.1138	0.1048	0.1835
J								0	0.0441	0.011	0.1073
L									0	0.0471	0.0942
Q										0	0.1072
S											0

Table S3: A matrix of pairwise geographical distances between sites (km). Alderney (A), Belle Ile (P), Brest (B), Cherbourg (C), Falmouth (F), Guernsey (G), Isle of Man (M), Jersey (J), Liverpool (L) and Scillies (S).

	A	P	B	C	F	G	M	J	L	Q	S
A	0	275	222	42	211	41	530	56	413	256	297
P		0	155	283	343	242	772	195	675	19	366
B			0	545	199	182	646	195	570	143	215
C				0	255	71	546	58	428	264	336
F					0	198	455	237	392	330	96
G						0	546	43	439	224	274
M							0	579	134	754	491
J								0	469	208	316
L									0	659	448
Q										0	357
S											0

Table S4: A matrix of pairwise heterozygosity (F_{ST}) values for *B. hortorum* between each sampling location with significant values ($p<0.05$) in bold after Bonferroni corrections.

	ELS1	ELS2	HLS3	ELS5	HLS1	HLS2	ELS4	HLS5	HLS4
ELS1	0	-0.0068	-0.0065	0.022	-0.0146	-0.0057	-0.0039	0.0096	-0.0213
ELS2		0	**0.0053**	**0.034**	0.0027	0.001	-0.0014	**0.0143**	-0.0072
HLS3			0	**0.0216**	-0.001	0.0015	0.0037	0.0081	-0.0103
ELS5				0	**0.028**	**0.0337**	**0.0248**	0.0113	**0.035**
HLS1					0	-0.0004	0.0029	0.0105	-0.0158
HLS2						0	0.0066	**0.0099**	-0.0099
ELS4							0	0.0054	-0.0025
HLS5								0	-0.0122
HLS4									0

Table S5: A matrix of pairwise heterozygosity (F_{ST}) values for *B. lapidarius* between each sampling location with significant values ($p<0.05$) in bold after Bonferroni corrections.

	ELS1	ELS2	HLS3	ELS5	HLS1	HLS2	ELS4	HLS5	HLS4	ELS3
ELS1	0	**0.0134**	**0.0141**	0.0132	**0.0245**	**0.0145**	0.0032	0.0225	**0.0203**	**0.0158**
ELS2		0	**0.006**	0.0045	0.0042	0.003	0.007	0.01	**0.0091**	0.0014
HLS3			0	0.0105	0.0069	0	**0.0077**	0.0111	**0.0096**	0.0072
ELS5				0	0.0068	0.0004	**0.0079**	0.0085	0.0048	0.0059
HLS1					0	0.0046	**0.0199**	0.0128	**0.0098**	0.0067
HLS2						0	0.0093	0.0116	0.0074	0.0008
ELS4							0	0.017	**0.0132**	0.0175
HLS5								0	0.0087	0.0101
HLS4									0	0.0145
ELS3										0

Table S6: A matrix of pairwise heterozygosity (F_{ST}) values for *B. pascuorum* between each sampling location with significant values ($p<0.05$) in bold after Bonferroni corrections.

	ELS1	ELS2	HLS3	ELS5	HLS1	HLS2	ELS4	HLS5	HLS4	ELS3
ELS1	0	**0.0443**	**0.0195**	0.0006	**0.019**	**0.0344**	**0.0177**	**0.0523**	**0.0222**	**0.048**
ELS2		0	**0.0308**	**0.0337**	**0.0304**	**0.0067**	**0.0355**	0.0023	**0.0431**	**0.0163**
HLS3			0	0.0046	0.0022	**0.0313**	0.0057	**0.0481**	**0.0082**	**0.0518**
ELS5				0	0.0027	0.0247	0.0003	**0.0409**	0.0001	**0.0459**
HLS1					0	**0.0299**	0.0062	**0.0452**	**0.0099**	**0.0567**
HLS2						0	**0.0225**	**0.0044**	**0.0305**	**0.0132**
ELS4							0	**0.0381**	0	**0.0496**
HLS5								0	**0.0461**	**0.0165**
HLS4									0	**0.054**
ELS3										0

Table S7: A matrix of pairwise heterozygosity (F_{ST}) values for *B. terrestris* between each sampling location with significant values ($p<0.05$) in bold after Bonferroni corrections.

	ELS1	ELS2	HLS3	ELS5	HLS1	HLS2	ELS4	HLS5	HLS4	ELS3
ELS1	0	0.0008	0	0	0	**0**	0.005	**0.0087**	0.0035	0.0009
ELS2		0	0.0035	0.0044	0.0054	**0.0206**	0.0054	**0.0158**	0.0085	**0.0125**
HLS3			0	0.0025	0.0044	**0.0187**	0.0068	**0.0159**	**0.0091**	**0.0046**
ELS5				0	0	**0.0101**	0.0017	0.0042	0.0018	0.0003
HLS1					0	0.0003	0.0028	0.002	0	0
HLS2						0	**0.0156**	0.002	**0.0023**	0.0102
ELS4							0	**0.0117**	**0.0092**	**0.006**
HLS5								0	0.0038	0.0053
HLS4									0	0.003
ELS3										0

Table S8: A matrix of pairwise geographical distances between sites (km).

	ELS1	ELS2	HLS3	ELS5	HLS1	HLS2	ELS4	HLS5	HLS4	ELS3
ELS1	0	16	24	92	105	13	31	37	85	117
ELS2		0	24	78	94	29	17	34	72	108
HLS3			0	77	84	24	24	13	67	94
ELS5				0	35	100	62	68	17	56
HLS1					0	108	78	72	25	21
HLS2						0	40	37	91	118
ELS4							0	26	55	92
HLS5								0	56	81
HLS4									0	45
ELS3										0

Table S9: The GPS location of each sampling site within Chapter 1.

Location	GPS Coordinates
alderney	49.715004,-2.197356
belle ile	47.347009,-3.156406
brest	48.390394,-4.486076
cherbourg	49.633731,-1.622137
falmouth	50.169173,-5.107088
guernsey	49.454168,-2.549707
iom	54.152337,-4.486123
jersey	49.180502,-2.103233
liverpool	53.408371,-2.991573
quiberon	47.720529,-2.997077
scilly	49.925002,-6.298672