Quantifying effects of interventions on genetic diversity and functional trait composition of pollinators

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Safeguard
Safeguarding European wild pollinators

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Table of contents

Preface

Pollinators form a substantial part of global biodiversity and play a crucial role in the maintenance of food security and human well-being. However, pollinator populations are facing significant declines due to anthropogenic pressures, including land-use change, climate change, and pesticide use. To mitigate loss and degradation of suitable habitats in agroecosystems, conservation and restoration measures, such as the maintenance of semi-natural habitats and the implementation of agri-environment schemes, have been fostered during the last decades through environmental and agricultural policies in Europe. Although we have a thorough understanding of the effects of such conservation interventions on pollinator diversity and abundance, their long-term impacts on genetic diversity, genetic population structure, effective population sizes, and on functional (intraspecific) trait diversity remain largely unknown. In Task 4.1 we address these knowledge gaps by implementing a paired sampling design across 66 sites in Europe, collecting focal wild bee species from agricultural landscapes with contrasting proportions of long-term interventions and by analyzing data through combined molecular and trait-based methods.

Summary

Understanding how conservation interventions impact wild pollinators is crucial for maintaining ecosystem services in agricultural landscapes. In Task 4.1, We combined population genetics and trait-based methods to quantify levels of genetic diversity and differentiation, as well as body size and variation in wild bee species with contrasting traits; large-bodied *Bombus pascuorum* and medium-/small-bodied *Halictus scabiosae* and *Lasioglossum villosulum*. Specimens were collected from 66 sites (33 pairs) across multiple European countries. Sites pairs were selected with contrasting proportions of long-term (≥10 years) agri-environment interventions. Genetic analyses, conducted using double digest Restriction Site Associated DNA sequencing (ddRADseq), indicate population structure in both *B. pascuorum* and *H. scabiosae* at the European scale, but a lack of structure within regions. Locally, there were little to no differences in genetic diversity measures among areas with low or high proportions of long-term interventions, suggesting high levels of gene flow and connectivity among sites. While our results indicate that *B. pascuorum* and *H. scabiosae* are likely well adapted to agricultural landscapes, there were two exceptions. In the United Kingdom and Switzerland, *B. pascuorum* exhibited divergent responses in body size and intraspecific trait variation, suggesting potential plastic responses to environmental factors. These diverging patterns warrant further investigation into resource allocation strategies under different landscape conditions. *L. villosulum* exhibited higher intraspecific trait variation in low-proportion landscapes, indicating a potentially adaptive response to limited or patchy floral resources. By integrating population genetics with functional trait analyses, we highlight how gene flow and phenotypic plasticity can shape pollinator responses to land use. Finally, establishing baseline genetic and trait data is essential for long-term monitoring, enabling early detection of population declines and ensuring the resilience of pollinators in human-modified landscapes.

List of abbreviations

CV	**Coefficient of Variation**
ITD	**Inter-tegular distance**
ITV	**Intraspecific trait variation**

DNA	**Deoxyribonucleic acid**
ddRADseq	**Double Digest Restriction Site Associated DNA sequencing**
AES	**Agri-environment schemes**

1. Introduction

Pollinators, both managed and wild, form a substantial part of global biodiversity and play a vital role in the maintenance of food security, ecosystem stability and human well-being. Wild pollinators, including wild bees, provide key pollination services for native plant species and commercial crops, contributing significantly to global crop yields and the persistence of diverse plant communities (Klein *et al.*, 2007; Potts *et al.*, 2010). Despite their undeniable importance, wild bees, alongside other insect pollinators, are experiencing global declines due to anthropogenic pressures. Among the primary drivers to pollinator loss are human induced land use change and intensification, climate change and pesticide-use (Klein *et al.*, 2018; Lebuhn & Vargas, 2021; Potts *et al.*, 2016). These factors often interact, potentially leading to population declines, which can subsequently disrupt plant-pollinator networks, reduce plant reproductive success, and compromise overall ecosystem function (Mathiasson & Rehan, 2020; Lebuhn & Vargas, 2021; Potts *et al.*, 2010). In response to these challenges, there has been a growing recognition of the importance of implementing conservation measures that promote the preservation and restoration of pollinator habitats. In agricultural landscapes, the creation and restoration of habitat for pollinators and pollinator-friendly management through the implementation of agri-environment schemes (AES) are, besides the maintenance of semi-natural habitats, among the most important measures to promote pollinators (Batary *et al.*, 2015; Kovács-Hostyánszki *et al.*, 2017). Semi-natural habitats, such as calcareous grasslands or wildflower strips, and extensively managed agricultural meadows promoted through AES have been shown to promote pollinator diversity by providing crucial resources, including nesting sites, floral resources and shelter for pollinators (Maurer *et al.*, 2022; Boetzl et al 2021; Langlois *et al.*, 2020; Albrecht *et al.*, 2007). While many studies have demonstrated the short-term impacts of these interventions on pollinator abundance and diversity, their long-term effects on population genetics and functional trait diversity remain largely unexplored.

Body size is a functional trait that plays an important role in bee ecology: it can affect foraging and dispersal distances (Kendall *et al.*, 2022), pollination capacity (both in terms of quantity of pollen carried, as well as the diversity of flower species visited) (Jauker *et al.*, 2016; Renauld *et al.*, 2016), brood provision, temperature regulation, and predation risk (Chole *et al.*, 2019; Gavini *et al.*, 2020). In turn, bee body size is influenced directly by diet, temperature, and brood cell size, and in eusocial bees, complex interactions between the queen, workers, and brood (Chole *et al.*, 2019). It can also be influenced by land use change (i.e., declines in habitat quality and quantity, and fragmentation), with potential consequences for pollination services in simplified landscapes (Gérard *et al.*, 2020; Grab *et al.*, 2019; Oliveira *et al.*, 2016). However, there is no one-size-fits-all response to these external pressures. Smaller body sizes may be more energetically efficient when resources are limited (Chole *et al.*, 2019; McNab, 2010), yet larger body sizes can be beneficial in fragmented landscapes, given that foraging distance increases with body size (Greenleaf *et al.*, 2007; Warzecha *et al.*, 2016). Larger bee body sizes have also been associated with population declines, possibly due to their higher requirements for pollen (Scheper *et al.*, 2014).

Intraspecific variation in bee body size can also play a role in a species' ability to adapt to adverse conditions and thus reduce their susceptibility to decline (Austin & Dunlap, 2019). Higher within-colony intraspecific trait variation (ITV) in bumblebees can expand the variety of flowering plant species the colony is able to forage from (Peat *et al.*, 2005; Vaudo *et al.*, 2015), increasing their resilience to changes in floral resource availability. This can also increase the pollen variability in their diet, which can positively affect their immune systems and thus resilience to parasites and disease (Vaudo *et al.*, 2015). Further, intraspecific variation in body size can affect how pollinators interact with the plant community: solitary bees with high ITV, and social bees with low ITV, have been found to be more central in their plant-pollinator networks, and interact with a more diverse range of flowering plants (Peralta *et al.*, 2024). As a result, changes to their population dynamics could significantly influence the plant community.

Additionally, population genetics can provide valuable insights into the response of pollinator populations and communities to ecological factors and conservation interventions. Theory predicts that fragmented populations will experience increased genetic drift due to smaller effective population sizes, as well as reduced gene flow resulting from limited inter-patch dispersal (Wright, 1943). These processes can subsequently lead to decreased genetic diversity within populations and increased genetic differentiation among them. In turn, these responses may then reduce adaptability and increase inbreeding, ultimately leading to greater extinction risks (Frankham, 2005). By examining the interactions between landscape features and microevolutionary processes such as gene flow, genetic drift or selection, we can gain a deeper understanding of pollinator diversity trends, better identify species of concern, and better understand how species react to applied measures (Lopez-Uribe *et al.*, 2017; Manel *et al.*, 2003). In agreement, previous studies have shown that stable species exhibited limited population structure when faced with land-use changes, whereas rare species displayed increased population structure across their range, along with locally reduced genetic diversity and smaller population sizes (Dreier *et al.*, 2014; Mola *et al.*, 2024). Since AES often aim to improve connectivity for pollinators, among other resources, in intensively managed landscapes, and since connectivity plays a crucial role in gene flow, investigating the local genetic processes in response to such interventions will allow us to better assess current management schemes. It will also provide crucial information to better assess population viability, compare population genetic measures with stable species, identify and set targets for declining species and to effectively develop and implement future management efforts.

Recent developments of sequencing technologies, particularly of double digest Restriction Site Associated DNA (ddRADseq) sequencing, enables an affordable approach for the genotyping of non-model organisms without the use of a reference genome (Andrews *et al.*, 2016). Genetic analyses can therefore complement traditional species abundance and diversity descriptors, as well as functional trait measures to better understand and predict pollinator responses to applied conservation strategies. In particular, integrating population genetics with functional trait analyses provides insight into both long-term population stability and the ecological performance of species in human-modified environments. Since functional traits influence fitness and subsequently population persistence (McGill *et al.*, 2006; Ostwald *et al.*, 2024), examining their variation alongside genetic structure can reveal whether phenotypic divergence is driven by selection or by environmental plasticity (Crispo, 2008).

In Task 4.1, we implemented a paired sampling design across Europe to assess the impacts of long-term agri-environment interventions on wild bee populations through genomic and functional trait analyses. Specifically, we examined how areas with high- compared to low-proportions of interventions influence wild bee population genetics, including genetic diversity, differentiation and effective population sizes, as well as their impacts on functional trait diversity. We chose *Bombus pascuorum*, *Halictus scabiosae*, and *Lasioglossum villosulum* as our study species, as these reflect a range of body sizes, with *B. pascuorum* being large-bodied, while *H. scabiosae* and *L. villosulum* being medium-/small-bodied. Assessing species with varying traits such as these allowed us to not only gather important baseline genetic information for a variety of taxa, but also to investigate whether smaller, potentially more restricted species differ in their responses to AES compared to larger, likely more widespread species. We also investigated whether these effects varied among these species, and across intervention types and countries.

2. Methods

2.1. Population sampling

In the spring and summer of 2023 or 2024 (Italy), paired populations of two bee species with differing life history traits (*Bombus pascuorum* and a second smaller species, either *Halictus scabiosae* or *Lasioglossum villosulum*), were collected from a total of 66 sites (33 pairs) with contrasting proportions of long-term (≥10 years) intervention areas (e.g., calcareous grasslands, extensively managed meadows, etc. see Table 1) separated by ≥6km within a pair and ≥10km among pairs (Figure 1). Focal habitat amounts were quantified by Geographic Information System (GIS) within 2km buffer radii, and specimens were collected within the inner 1km radius. This sampling scheme was replicated in five countries for *B. pascuorum*, in three countries for *H. scabiosae* and in one country for *L. villosulum* (Table 1). Since *L. villosulum* was collected in only one country, it was omitted from genetic analyses (but maintained in the trait analyses) as it could not contribute to cross-country genetic structure or diversity comparisons. Unfortunately, additional partners were unable to participate, either due to scheduling conflicts or to unusually low numbers in the sampling year.

Within each site, we aimed to collect 20+ workers of *B. pascuorum* and 15+ specimens of either *H. scabiosae* or L. villosulum by hand netting specimens directly into 95% ethanol. Samples were stored at -20°C until later processing. To minimize the probability of sampling related individuals, efforts were made to spread sampling across the site area and on different visits to avoid capturing two individuals of the same species at the same place and time.

It is also to be noted that our sampling design has omitted a third landscape type (the control, protected habitat area). On the one hand, selecting sufficient numbers of replicates, with all three landscape types, while maintaining the necessary within- and among- site minimum distances to ensure site independence was simply not feasible given the relatively small size of many participating countries. Secondly, the time required to sample specimens in high enough numbers while ensuring minimal relatedness for genetic analyses across additional sites would have been extremely demanding. Therefore, we preferred to focus on a well selected, paired design with sufficient numbers to allow for statistically powerful analyses, while still enabling us to address questions regarding the implementation of long-term agri-environment schemes in the landscape.

Table 1: Sampling scheme overview within each participating country and the number of specimens measured per country, species, caste (if applicable), and landscape type. Low = low-proportion of long-term interventions; high = high-proportion of long-term interventions.

Country	Intervention type	n° Sites (n° pairs)	Species	Caste	Landscape type	n° Specimens Trait analyses	n° Specimens Genetic analyses (after filtering)
Italy	Semi-natural areas. Established for 25+ years.	12 (6)	*B. pascuorum*	Worker	Low	118	68
					High	120	97
		12 (6)	*H. scabiosae*	N/A	Low	121	70
					High	119	72
Netherlands	Semi-natural, flora-rich grasslands that are part of the Nature Network (NNN) in the Netherlands. Established for 15+ years.	12 (6)	*B. pascuorum*	Worker	Low	141	110
					High	143	106
		12 (6)	*L. villosulum*	N/A	Low	111	0
					High	141	0
Germany	Calcareous grasslands. Established for 60+ years.	14 (7)	*B. pascuorum*	Worker	Low	136	106
					High	138	96
		12 (6)	*H. scabiosae*	N/A	Low	54	70
					High	78	73
United Kingdom	Calcareous grassland, protected and extensively managed primarily through conservation grazing. Variable years of establishment.	12 (6)	*B. pascuorum*	Worker	Low	136	108
					High	136	117
Switzerland	Extensively managed meadows under the Swiss agri-environment scheme for meadow extensification. Established for 14+ years.	16 (8)	*B. pascuorum*	Queen	Low	33	N/A
					High	57	N/A
				Worker	Low	137	155
					High	116	145
		16 (8)	*H. scabiosae*	N/A	Low	115	105
					High	105	91

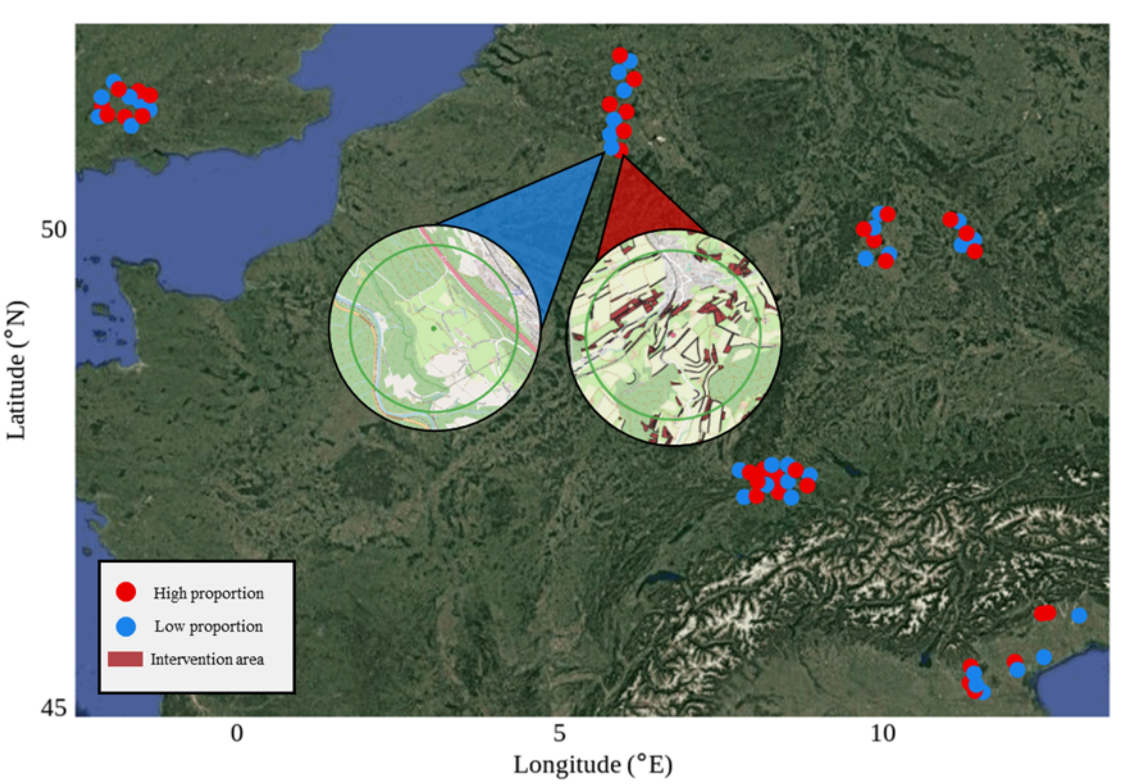

Figure 1: Location of the 66 study sites across Italy, Germany, Netherlands, Switzerland and the United Kingdom. Highlighted are examples of a site pair (high-proportion of long-term intervention area and low-proportion of long-term intervention area), showing landscape configurations within a 2km buffer radius.

2.2 Genomic library preparation and processing

DNA was extracted from the legs of 1,356 *B. pascuorum* and of 539 *H. scabiosae* using the LGC Sbeadex Plant Kit adapted for KingFisher Sample Purification System (Thermo Fisher Scientific). DNA quality was assessed visually by electrophoresis on 1% agarose gels and quantified using PicoGreen fluorescent nucleic acid stain (Invitrogen). Samples were then randomized on 96-well plates and diluted to 5ng/uL for *B. pascuorum* and to 3ng/uL for *H. scabiosae*. The Double Digest Restriction Enzyme Associated DNA sequencing (ddRADseq) libraries were prepared according to the protocol by Westergaard *et al.* (2019). Briefly, DNA was double-digested with EcoRI-HF and TaqI-v2 restriction enzymes and ligated to Illumina barcoded adapters. Barcoded samples were then pooled by species and size selected to ~550pb libraries using 0.58x AMPure beads. Libraries were then washed and purified to select for P2-biotin labeled adaptors, and 12 PCR cycles were run to enrich the libraries with Illumina indexes. Finally, libraries were quantified by Qubit, fragment-size assessed on an Agilent 2200 Tape station, and pooled libraries were sequenced on a NovaSeq6000 platform with 150bp paired-end reads (Novogene). Resulting raw sequences were demultiplexed and processed (based on R packages stacks and ddocent) by the Genetic Diversity Center ETH Zürich.

In parallel, 24 specimens of *B. pascuorum* (6 from each country, except Italy) and 24 *H. scabiosae* (all from Switzerland) were randomly selected and sequenced on an Element

AVITI platform with 300bp paired-end reads (Functional Genomics Center, Zürich, Switzerland) to generate deNovo reference catalogs for each species. Various parameters were tested during the raw sequence processing, which aimed at maximizing the re-mapping rate (as in Pedrazzini *et al.*, 2023). Optimal settings resulted in reference catalogs including ~77K RAD loci for *H. scabiosae* and ~52K for *B. pascuorum*.

Processed NovaSeq reads were then mapped to the newly created reference catalogues and quality filtered with the following criteria: minimum quality score of 30, a minor allele count of five, a minimum depth of three, a minor allele frequency of 1%, a mean depth of 10 and successfully genotyped in 50% of individuals. Additional filtering steps included the removal of loci based on allele balance, mapping quality, loci with extremely high coverage and individuals with extreme heterozygosity values. Only bi-allelic SNPs were retained and sites with more than 5% missing data were removed. Linkage disequilibrium (LD) pruning was performed using a 10 bp window. All filtering steps were conducted using bcftools v1.20 with htslib v1.20. As a final filtering step, we also detected and removed putative siblings. To do this, coefficients of relatedness between all individuals were calculated using vcftools, and for each pair with a coefficient >0.25, the individual with the lowest read depth was removed from all downstream genetic analyses.

2.3. Analyses of genetic diversity, population structure and effective population size

Basic measures of genetic diversity (He, Ho and Fis) were calculated using the R package hierfstat (Goudet, 2005). To account for biases related to sample size differences and to facilitate later comparisons, we first standardized each site population by randomly subsampling individuals without replacement (per population: *B. pascuorum* N=10 except in 2 sites with lower sample sizes in Germany, *H. scabiosae* N = 6). Measures of nucleotide diversity (π) were calculated using vcftools and averaged in R.

Overall population structure across each species was assessed using a sparse non-negative matrix factorization (sNMF) approach from the LEA package in R (Frichot *et al.* 2014). A range of values for K (1–10) were tested to determine the optimal number of ancestral populations and cross-entropy values were computed to identify the best-supported K. In addition, a discriminant analysis by principal component (DAPC) was implemented without prior population information using the find.clusters function from the adegenet R package (Jombart, 2008), which transforms the data using principal component analysis and employs sequential K-means. Bayesian information criterion was used to assess the optimal number of clusters. A DAPC with a priori population designation was also performed to visualize genetic differentiation among populations within each country. To test for isolation by distance (IBD) among populations for each species within a country, a Mantel test was performed using the VEGAN R package with 1000 permutations between pairwise Nei's genetic distances and pairwise Euclidean geographic distance matrices. For both species, unbiased pairwise Fst values using the Weir and Cockerham (1984) method were also calculated among populations within each country.

Contemporary effective population size (Ne) for each standardized population was calculated using the linkage disequilibrium method (LDNe) based on Pearson correlation approximation (Waples *et al.*, 2016) from the stataG R package (Archer *et al.*, 2016). We report the results based on 8000 randomly selected loci without replacement (for computational efficiency), employing a 0.01 minor allele frequency threshold with no missing values. 95% Confidence intervals were generated using the jack-knife option. Since LD methods often underestimate values of Ne in haplodiploid species (Waples *et al.*, 2016), we highlight that the focus of this analysis is on the relative differences in Ne associated with intervention proportions, rather than absolute Ne measures.

Finally, linear mixed effect models (LMM) were used to compare heterozygosity, the inbreeding coefficient (F_{is}), mean π and Ne between sites with high- and low-proportions of long-term intervention areas. Sampling pair was included as a random effect factor in each model. We first modeled each species per country separately, however these models either lacked significance, had singularity issues, or showed poor model fit. Given the limited sample sizes and the need for a more robust estimation, we opted to combine data from all countries per species while including country as an interaction term, allowing us to account for potential country-specific differences while maintaining statistical power and model stability. Below we report values only from the combined, cross-country models.

2.4 Trait measurements

The inter-tegular distance, as defined in Cane (1987) (ITD; Figure 2), was measured three times for each specimen using a Euromex Stereoblue microscope and 10x/21 eyepiece (Euromex, n.d.). At the start of each measuring session, the eyepiece was calibrated using a micrometer. ITD can be used as a proxy for bee body size (Cane, 1987), and is a useful indicator of pollen carrying capacity (Warzecha *et al.*, 2016), dispersal/foraging distance (Greenleaf *et al.*) and consequently, the spatial scale at which bees can provide pollination services (Ostwald *et al.*, 2024). Additionally, the coefficient of variation (CV) was calculated for each species and landscape. CV is a dimensionless measure of intraspecific trait variation that can be calculated by dividing the standard deviation of the trait by the mean value of the trait (Lande, 1977). An overview of how many specimens were measured per country, species, and landscape type can be found in Table 1.

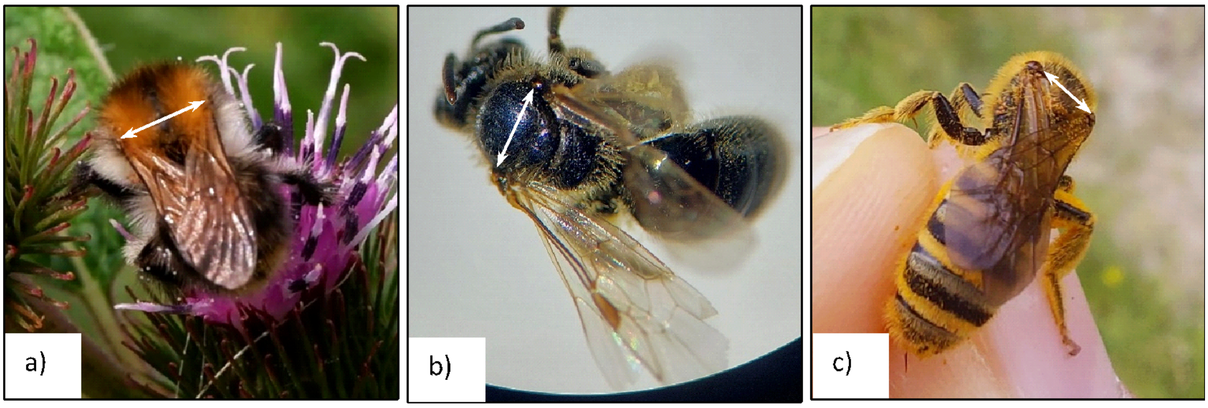

Figure 2: Inter-tegular distance, indicated with white arrows, for a) *Bombus pascuorum*, b) *Lasioglossum villosulum*, and c) *Halictus scabiosae*.

2.5 Data analyses of trait measurements

ITD and CV were modelled using linear mixed models with the proportion of long-term interventions in the landscape (landscape type) as a categorical predictor variable. Each country and species were analysed separately. As some queens were potentially accidentally collected in Switzerland an additional categorical predictor (caste) was included in both *B. pascuorum* models (ITD and CV) to differentiate between worker and queen specimens, with queens defined as having an ITD > 4.00 mm. The model outcome of the Swiss dataset with queens removed was similar (Supplementary Information: Tables S1–2). Thus, we chose to keep queens in the final analysis to maintain a balanced sample size between landscapes. No solitary bee species was collected in the United Kingdom, so this country was excluded from those analyses. No *H. scabiosae* specimens were collected for one high-proportion landscape in Germany, so the corresponding pair was dropped from all analyses, leaving a total of ten paired landscapes.

3. Results

3.1 Genomic data

Following initial mapping and quality filtering, an average of 237,582 (range: 192,377-319,380) high quality SNPs remained among *Bombus pascuorum* individuals and an average of 197,487 (range: 177,854-213,812) remained among *Halictus scabiosae* individuals. After the subsequent filtering steps, 42,805 (range: 38,779-49,775) SNP sites were identified among 1,276 *B. pascuorum* and 37,440 (range: 28,248-46,850) among 507 *H. scabiosae*, respectively. Following the removal of putative siblings, 1,108 *B. pascuorum* and 481 *H. scabiosae* individuals remained for downstream genetic analyses.

3.2 Genetic diversity of populations

Observed heterozygosity ranged from 0.119 to 0.1877 in *B. pascuorum* and from 0.2548 to 0.3189 in *H. scabiosae* across populations. For *B. pascuorum*, values of observed and expected heterozygosity were similar in Italy and the United Kingdom, whereas observed values differed and were slightly lower than expected across populations in Germany, Netherlands and Switzerland (mean difference = -0.005, p < 0.0001 for each country). For *H. scabiosae*, observed and expected heterozygosity were comparable within each country. The inbreeding coefficient was low among all countries and for both species (Table 2). For both species within, and across each country, linear mixed models revealed no significant differences in Ho, He and Fis between populations established in areas with high-proportions of long-term interventions and areas with low-proportions of long-term interventions (all p-values > 0.1 for *B. pascuorum* and p-values > 0.7 for *H. scabiosae*). Values of mean nucleotide diversity within and across countries did not differ significantly among site pairs for *B. pascuorum* (all p-values > 0.1). However, the effect of landscape type on mean π did differ significantly across countries for *H. scabiosae* (χ2 =9.71, p=0.0078). Post-hoc contrasts indicated that in Switzerland, mean π was significantly larger in low-proportion compared to high-proportion landscapes (β=−0.6938, SE = 0.191, t=−3.639, p=0.0022). Effective population sizes ranged from 60 to 1,924 for *B. pascuorum* across all sites and from 8 to 25 in *H. scabiosae*, with no significant differences among intervention proportions (all p-value >0.6 for *B. pascuorum* and p-value >0.9 for *H. scabiosae*).

Table 2: Genetic diversity estimates and effective population sizes averaged per country and landscape type for standardized populations of *B. pascuorum* (N=10) and *H. scabiosae* (N=6): Expected (He) and observed heterozygosity (ho), inbreeding coefficient (Fis), effective population size (Ne) and mean nucleotide diversity (mean π).

Country	Landscape type	He	Ho	Fis	Ne	Mean π
a) ***Bombus pascuorum***						
Italy	High	0.1802	0.1744	0.0238	680	0.1799
	Low	0.1822	0.1801	0.0081	579	0.1814
Germany	High	0.1272[a]	0.1217[a]	0.0301	1019	0.1264
	Low	0.127 [a]	0.1219[a]	0.0281	670	0.1263
Netherlands	High	0.1273[a]	0.122[a]	0.0299	850	0.1262
	Low	0.1269[a]	0.121[a]	0.0337	655	0.1261
Switzerland	High	0.1271[a]	0.1216[a]	0.031	956	0.1262

	Low	0.1271[a]	0.1216[a]	0.0306	750	0.1261
United Kingdom	High	0.1884	0.1827	0.023	561	0.1879
	Low	0.189	0.1836	0.0217	687	0.1889
b) *Halictus scabiosae*						
Italy	High	0.2639	0.2628	-0.0055	24	0.2642
	Low	0.2633	0.2629	-0.0074	24	0.2637
Germany	High	0.3058	0.3104	-0.0229	20	0.3057
	Low	0.3062	0.3078	-0.0155	20	0.3062
Switzerland	High	0.2726	0.2743	-0.0144	22	0.272[b]
	Low	0.2727	0.2744	-0.014	22	0.2732[b]

[a] Significantly different values per country

[b] Significantly different values among landscape types

3.3 Quantifying genetic structure

Across the full dataset, sNMF analyses revealed population structure in *B. pascuorum* for K=3, clustering populations from mainland Europe north of the Alps into one group (Germany, Netherlands and Switzerland), mainland Europe south of the Alps into another group (Italy), and those from the United Kingdom clustering into a third group (Figure 3a). sNMF also revealed clear population structure across the *H. scabiosae* dataset, suggesting an optimal K value of thee and clustering the populations by countries separately (Germany, Italy and Switzerland) (Figure 3b). Within country however, sNMF analyses revealed no clear population structure, suggesting single genetic units (K=1) across each countries' study area for each of the two species (data not shown). In agreement, the find.clusters function indicated three genetic clusters based on BIC for *B. pascuorum* across all countries, again clustering populations from Germany, the Netherlands and Switzerland together, with populations from Italy and the United Kingdom clustering each into separate groups (Figure 4 a-b). A separate analysis to assess finer structure across the mainland cluster, without samples from Italy and the United Kingdom, did not reveal any further structuring (BIC value supporting 1 genetic cluster, Fst values (≥0.005)) (Figure 4 c-d). Three genetic clusters were also indicated by BIC among populations of *H. scabiosae*, which also clustered by separate countries (Figure 4 e-f). Within country BIC values again indicated the presence of only one genetic cluster across entire study areas for both *B. pascuorum* and *H. scabiosae* (data not shown). For both species, DAPC analyses performed with a priori group information within countries did cluster individuals into sampling populations, however slight structural patterns were only observed for *B. pascuorum* in Switzerland and in Italy, and for *H. scabiosae* in Italy (Figure 5). These results are congruent with populations that revealed levels of isolation by distance (IBD). Within countries, Mantel tests between Nei's genetic distance and geographic distance matrices showed no IBD among populations of *B. pascuorum* in the Netherlands, the United Kingdom or in Germany, but did reveal a slight yet significant positive correlation in Switzerland (r=0.260, p= 0.044) and a positive correlation in Italy (r=0.493, p=0.001). For *H. scabiosae* a significant positive correlation was also found among populations in Italy (r=0.321, p=0.045), but not in Switzerland or in Germany. Genetic differentiation among populations within each country was low in both species (*B. pascuorum* all Fst ≤ 0.01, *H. scabiosae* all Fst ≤ 0.02).

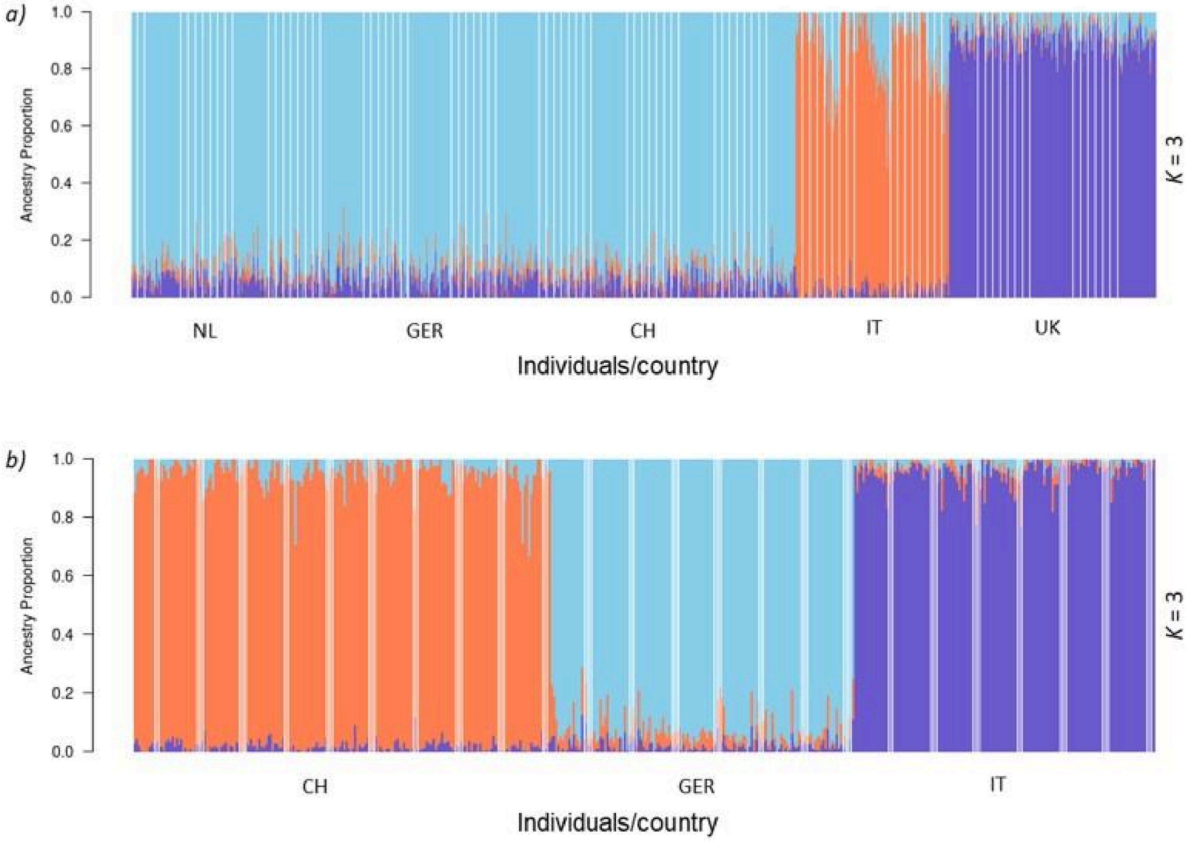

Figure 3: Bar plots of sNMF analyses across all countries for a) *Bombus pascuorum* and b) *Halictus scabiosae*. Each individual is represented by a vertical bar, which is partitioned into K colored segments showing the individual's probability of belonging to the cluster with that color.

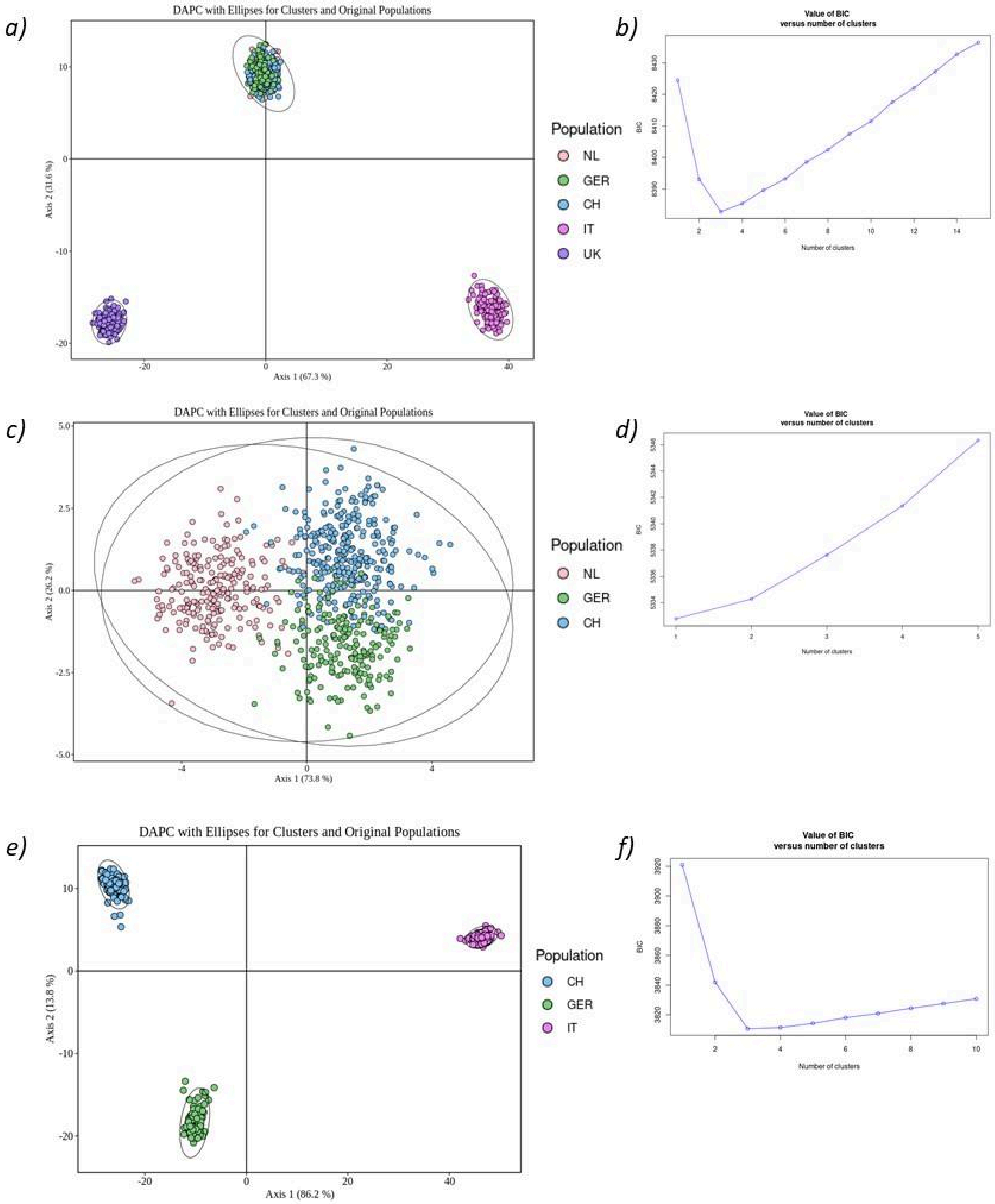

Figure 4: Discriminant Analyses of Principal Components without a priori group assignment across all countries for a) *Bombus pascuorum* and e) *Halictus scabiosae* and c) *Bombus pascuorum* across mainland countries only. Bayesian information criterion plots indicate the optimal number of clusters as three across b) *Bombus pascuorum* and d) *Halictus scabiosae* and an optimal number of one across d) *Bombus pascuorum* mainland sites. NL = Netherlands, GER = Germany, CH = Switzerland, IT = Italy, UK = United Kingdom.

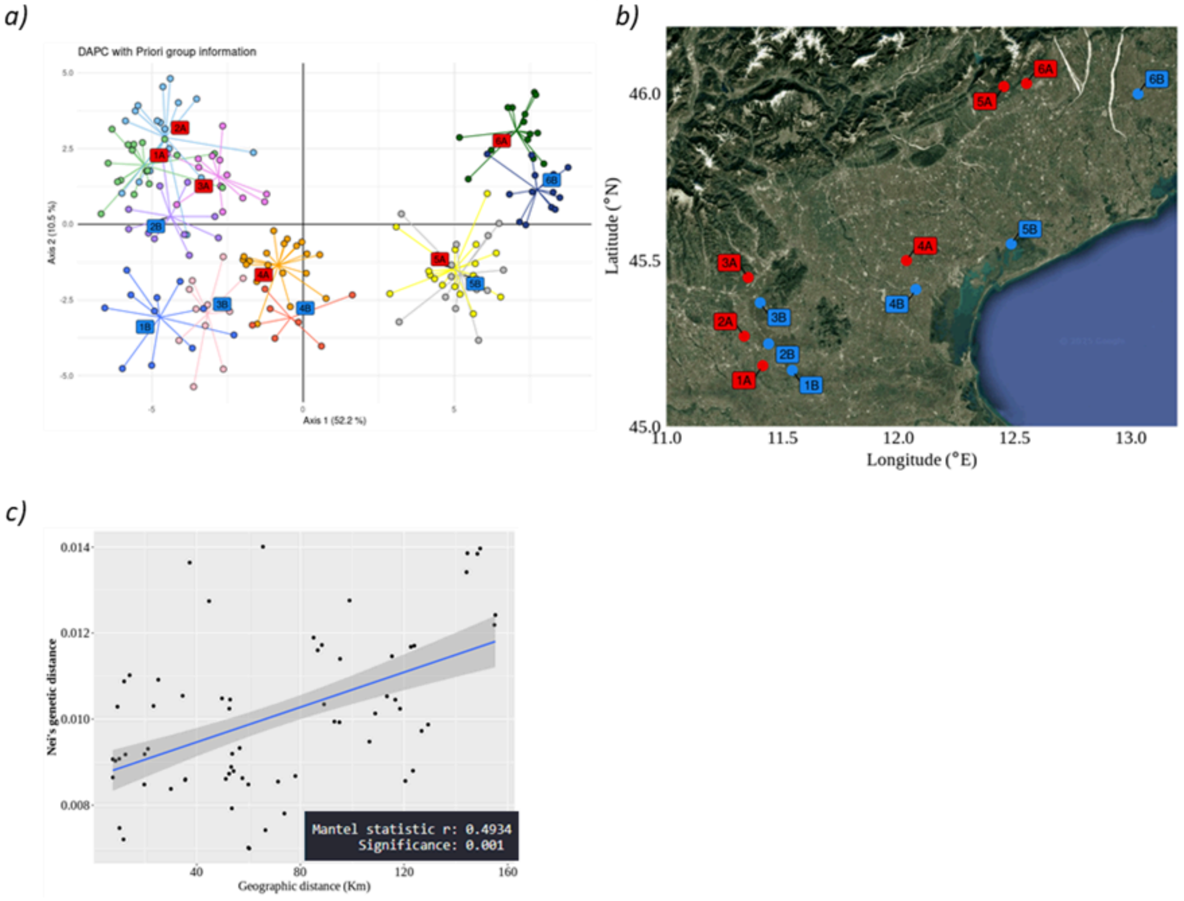

Figure 5: Example of within country population structure in *Bombus pascuorum* in Italy a) Discriminant Analyses of Principal Components with a priori group assignment showing slight structure along Axis 1, associated with geographic distance. b) Sampling sites in Italy. Red labels indicate sites with high-proportions of long-term interventions and blue labels indicate sites with low-proportions of long-term interventions. c) Pairwise genetic distance by pairwise geographic distance indicating isolation by distance with Mantel statistics.

3.4 Landscape-level habitat influence on body size

Bombus pascuorum
ITD differed significantly between landscape types in the United Kingdom ($\chi2(1) = 15.373$, $p < 0.001$) and Switzerland ($\chi2(1) = 5.22$, $p = 0.022$) (Figure 6d–e). We found no difference in *B. pascuorum* ITD between high- and low-proportion landscape types in the Netherlands ($\chi2(1) = 3.058$, $p = 0.08$), Germany ($\chi2(1) = 1.051$, $p = 0.305$) or Italy ($\chi2(1) = 0.106$, $p = 0.745$) (Figure 6a–c). Average ITD was significantly higher in high-proportion landscapes in the United Kingdom ($\beta = 0.129$, $SE = 0.032$, $z = 3.98$, $p < 0.001$), and significantly lower in Switzerland ($\beta = -0.057$, $SE = 0.025$, $z = -2.29$, $p = 0.022$).

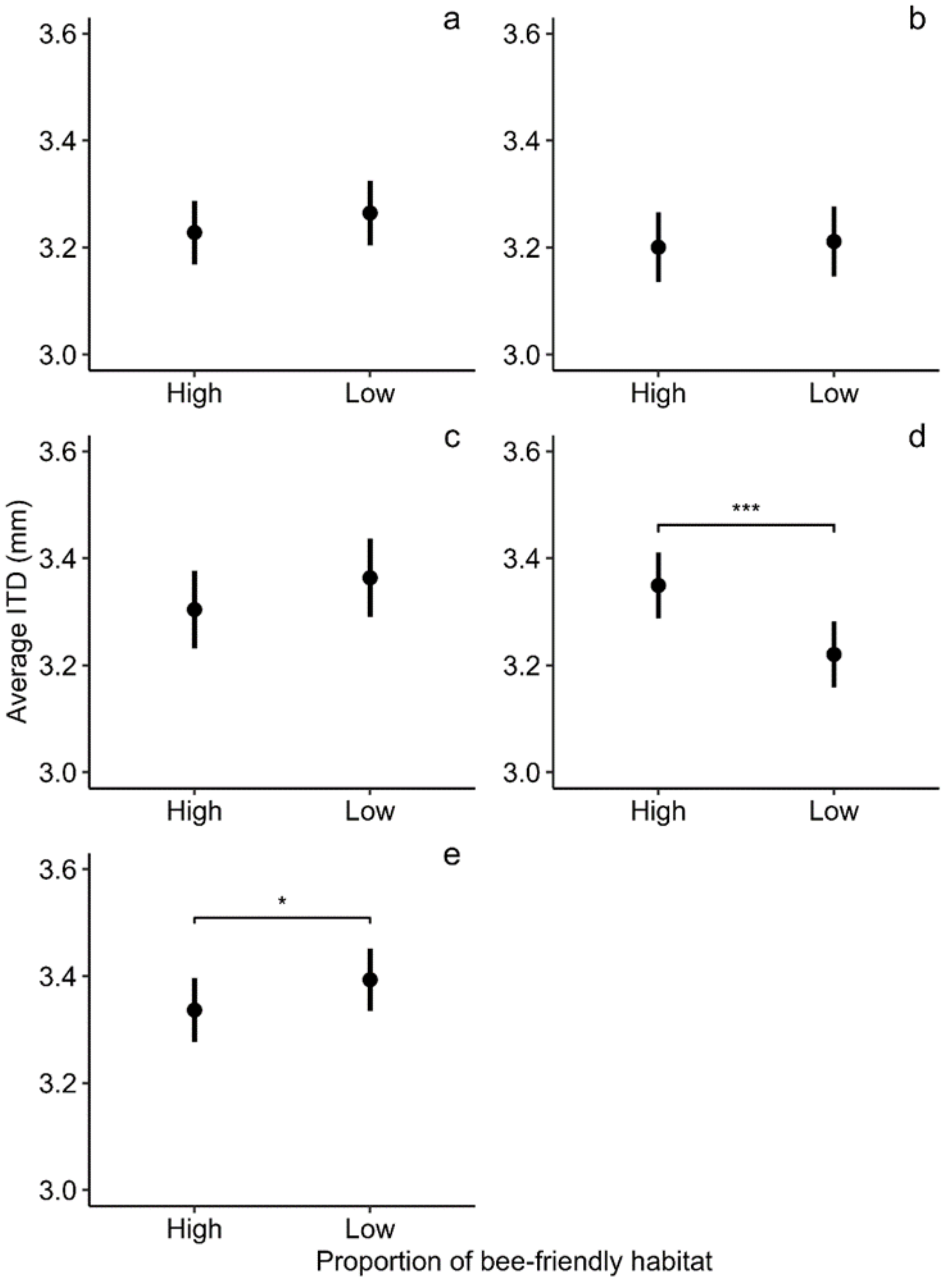

Figure 6: Estimated marginal means of inter-tegular distance (ITD, mm) of *Bombus pascuorum* by landscape type. Panels are split by country: a = Germany, b = Italy, c = the Netherlands, d = United Kingdom, and e = Switzerland. Bars and asterisks indicate significant differences between the two landscape types. < 0.05 = *, < 0.01 = **, < 0.001 = *.**

Halictus scabiosae & Lasioglossum villosulum

We found no difference in average ITD between high- and low-proportion landscapes in Germany (χ2(1) = 0.05, p = 0.824), Italy (χ2(1) = 0.958, p = 0.328), Switzerland (χ2(1) = 1.468, p = 0.226) or the Netherlands (χ2(1) = 2.624, p = 0.105) (Figure 7a–d).

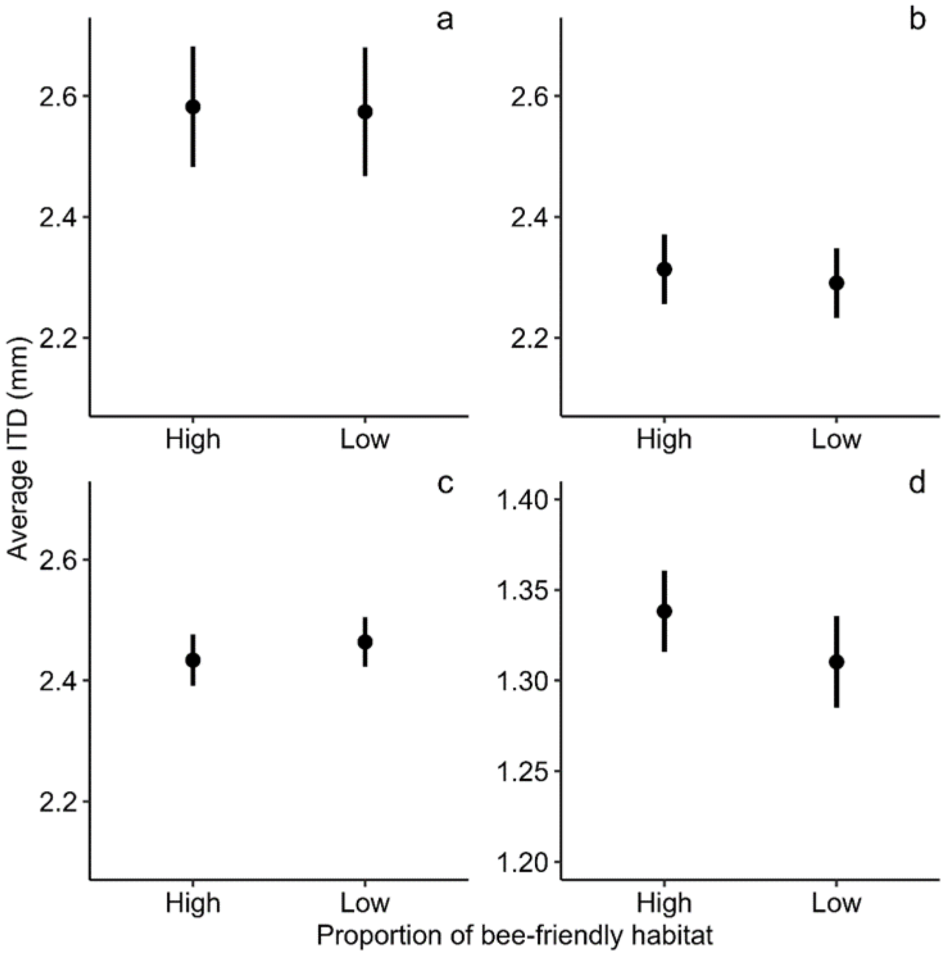

Figure 7: Estimated marginal means of inter-tegular distance (ITD, mm) of solitary bee species by landscape type. Panels are split by country: a = Germany, b = Italy, c = Switzerland, d = the Netherlands. *Halictus scabiosae* was the study species in Germany, Italy and Switzerland. *Lasioglossum villosulum* was the study species in the Netherlands.

3.5 Landscape-level habitat influence on intraspecific trait variation

Bombus pascuorum
CV differed significantly by landscape type in the United Kingdom ($\chi^2(1) = 13.796$, $p < 0.001$) (Figure 8d). In Switzerland, there was a significant interaction between caste and landscape type ($\chi^2(1) = 3.96$, $p = 0.047$) (Figure 8e). We found no difference in *B. pascuorum* ITD between high- and low-proportion landscape types in the Netherlands ($\chi^2(1) = 1.178$, $p = 0.276$), Germany ($\chi^2(1) = 2.833$, $p = 0.092$) or Italy ($\chi^2(1) = 2.941$, $p = 0.0864$) (Figure 8a–c). Average CV was significantly lower in high-proportion landscapes in the United Kingdom ($\beta = -2.115$, SE = 0.369, $z = -5.727$, $p < 0.001$). In Switzerland, CV was only significantly higher in high-proportion landscapes for the worker caste ($\beta = 2.38$, SE = 0.702, $t = 3.383$, $p = 0.013$)

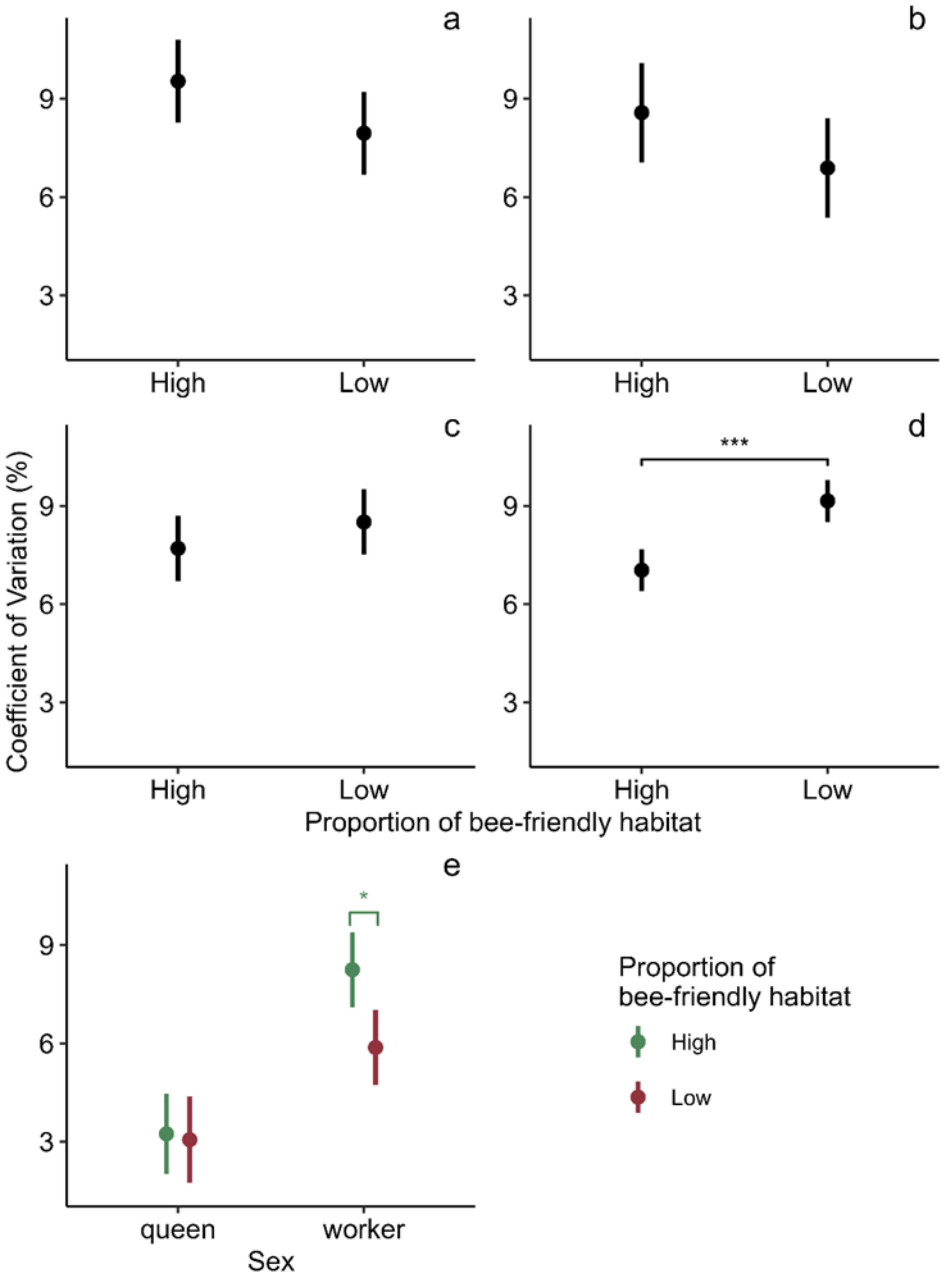

Figure 8: Estimated marginal means of the coefficient of variation (CV, %) of *Bombus pascuorum* by landscape type. Panels are split by country: a = Germany, b = Italy, c = the Netherlands, d = United Kingdom, e = Switzerland. Swiss *Bombus pascuorum* specimens (panel e) were split into two categories (queens, workers). Bars and asterisks indicate significant differences between the two landscape types. < 0.05 = *, < 0.01 = **, < 0.001 = *.**

Halictus scabiosae & Lasioglossum villosulum
CV was significantly different between high- and low-proportion landscapes in the Netherlands (χ2(1) = 4.93, p = 0.026) (Figure 8d). We found no difference in CV between landscape types in Germany (χ2(1) = 0.176, p = 0.675), Italy (χ2(1) = 0.005, p = 0.947), or Switzerland (χ2(1) = 0.648, p = 0.421) (Figure 9a–c). In the Netherlands, CV was

significantly lower in high-proportion landscapes (β = -1.316, SE = 0.408, z = -3.224, p = 0.00126).

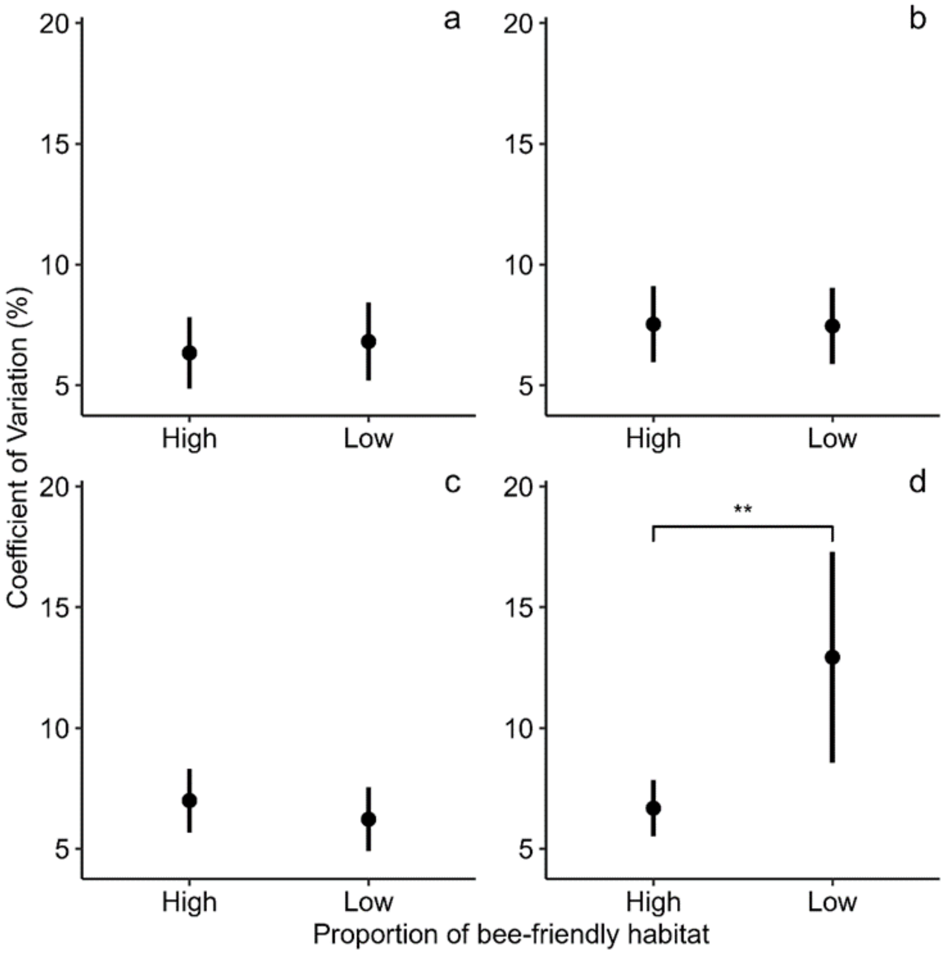

Figure 9: Estimated marginal means of the coefficient of variation (CV, %) of solitary bee species by landscape type. Panels are split by country: a = Germany, b = Italy, c = Switzerland, d = the Netherlands. *Halictus scabiosae* was the study species in Germany, Italy and Switzerland. *Lasioglossum villosulum* was the study species in the Netherlands. Bars and asterisks indicate significant differences between the two landscape types. < 0.05 = *, < 0.01 = **, < 0.001 = *.**

4. Discussion

Across the study range, our results support the presence of three genetic clusters in *Bombus pascuorum*, with a mostly panmictic cluster across mainland Europe north of the alps, and limited gene flow across both the Alps and the English Channel. In contrast, populations of *Halictus scabiosae* clustered into distinct groups at smaller geographic scales, with separate groups in Italy, Germany and Switzerland. Within each county however, populations of both species exhibited little genetic structure, clustering into single genetic units. Further, we found little to no genetic differences among areas with contrasting amounts of long-term interventions, suggesting that local land-use types do not act as barriers to gene flow for these mobile species at this scale. Additionally, we found no differences in the mean body size and ITV of *H. scabiosae* between landscapes with low- and high- proportions of long-term interventions, or of *B. pascuorum* in Italy, Germany, and the Netherlands. In Switzerland and the United Kingdom, we found diverging responses in the body size of *B. pascuorum* to the proportion of long-term interventions in the landscape, suggesting that multiple factors might influence whether populations of *B. pascuorum* invest in more, smaller-bodied workers versus fewer, larger-bodied workers. These differences in body size, despite the lack of population structure, suggest potential phenotypic plasticity as a response to local landscapes. In the Netherlands, *L. villosulum* body size was not different between landscape types, however ITV was significantly higher in low-proportion landscapes, and is potentially an adaptive response to limited or patchy floral resources.

4.1 Genetic diversity

Among the different sampling regions, observed heterozygosity (Ho) in *B. pascuorum* was comparable to expected heterozygosity (He) in Italy and the United Kingdom. However, in Germany, Netherlands and Switzerland, Ho was slightly but significantly lower than He. While this could indicate potential bottleneck or drift events, the relatively small difference (-0.005), the overall low Fis values, and the lack of population structure provide little evidence for declining genetic diversity. Further, these genetic diversity estimates are comparable to those found in other common bumblebee species assessed using ddRADseq methods, whereas restricted or declining populations typically resulted in lower estimates (Jackson *et al.*, 2018; Huml *et al.*, 2021). In *H. scabiosae*, estimates of Ho and He were similar across the study range, suggesting relatively stable genetic diversity. At the local scale, we found no differences in heterozygosity, inbreeding or effective population sizes in either species among sites with contrasting proportions of long-term interventions. This suggests that, at these scales, land-use types do not significantly hinder the dispersal of either *B. pascuorum* or *H. scabiosae*. Rather, it is likely that the study regions contain sufficient high-quality habitats to maintain connectivity and to allow for gene flow among populations. In *H. scabiosae* in Switzerland, nucleotide diversity was slightly higher in sites with low-proportions of intervention types than in those with high-proportions. This may be the result of recent range expansions of *H. scabiosae*, as recently described by Gil-Tapetado *et al.* (2024) in Switzerland and across Europe, greatly extending its choice of habitats.When considering the generally low levels of inbreeding and lack of barriers to gene flow, another possible explanation is that multiple surrounding populations are spilling over into these low intervention areas, subsequently contributing to increased genetic mixing. While these results indicate that genetic variation in *B. pascuorum* and *H. scabiosae* has not, or possibly not yet, been largely influenced by local interventions, we cannot exclude that such impacts may need more time to appear.

4.2 Genetic differentiation

Cross country sNMF and DAPC analyses indicate three distinct clusters of *B. pascuorum*, with populations across mainland Europe north of the Alps forming one group (despite many sites being separated by >500km), while populations in the United Kingdom and Italy cluster into two separate groups (Figures 3a and 4a-b). These separations may be the result of long-term isolation following historical events, such as different post glacial refugia. Within countries, we found a lack of marked differentiation among high- and low- intervention proportions, with each sampling site clustering into single genetic units. Differentiation in this species appears to be driven by natural barriers, in this case with limited gene flow across the Alps and the English Channel- consistent with findings by Widmer *et al.* (1999), who previously described two genetic clusters in continental Europe. Such panmictic structure could be explained by the relatively high dispersal abilities of *B. pascuorum* queens, which range from 3-5km (Lepais *et al.*, 2010), as well as the species' relatively high population densities (Dreier *et al.*, 2014). Slight isolation-by-distance (IBD) effects were detected in Switzerland and Italy. However, considering the generally low Fst values, there is little evidence for differentiation across these sampling regions. One possible explanation is that *B. pascuorum* has been found to forage more locally (Chapman *et al.*, 2003). While they are capable of dispersing over longer ranges, should sufficient local resources be available, they may not need to expend energy flying further. Additionally, if suitable habitats are spread across the sampling range, sequential gene flow between neighboring sites could explain the lack of differentiation across the region. *H. scabiosae* also clustered into three groups across the study region. However, unlike *B. pascuorum*, mainland populations were clustered into separate groups. This is in line with likely shorter dispersal ranges often associated with smaller body sizes, compared to the larger *B. pascuorum* (Greenleaf *et al.*, 2007). Locally, however, *H. scabiosae* was similarly characterized by single clusters and a lack of genetic differentiation, indicating gene flow across local landscapes. In Italy, the IBD effects detected for *H. scabiosae* appear to be associated with geographic distance rather than to proportion of interventions, given the low Fst values across sites. *H. scabiosae* have been shown to disperse up to 4.5km (Ulrich et al, 2009), suggesting that, if necessary, individuals could travel to other suitable habitats. However, the patterns observed here suggest that local resources in Italy are likely sufficient, reducing the need for long-range dispersals.

4.3 Functional traits

The advantages and trade-offs associated with body size can be wide-ranging in both primitively eusocial (*B. pascuorum* and *H. scabiosae*) (Packer & Richards, 2021; Rubenstein & Abbot, 2017) and solitary species *(L. villosulum*) (Plateaux-Quénu *et al.*, 1989). In bumblebees, larger workers have been found to be more efficient foragers (Spaethe & Weidenmüller, 2002) and are better at providing for brood (Cnaani & Hefetz, 1994), while smaller workers are more resilient to starvation (Couvillon & Dornhaus, 2010), and having more, smaller workers is a form of insurance against the loss of workers due to predation or disease (Chole *et al.*, 2019). Higher within-colony variation also allows a colony to exploit a wider range of floral resources (Peat *et al.*, 2005). In solitary species, larger females have been found to carry much larger pollen loads and thus can provision larger or more brood cells (Renauld *et al.*, 2016), but smaller females have lower food requirements and thus are less limited by floral resource availability.

We expected to see differences in body size and the coefficient of variation between landscape types, under the assumption that in more fragmented and lower quality habitat, there would be more pressure for reproductive females to adjust brood provisioning according to floral resource availability. What we found was largely the opposite: *B. pascuorum* body size and CV differed between habitat types in Switzerland and the United Kingdom, but not in any other country. There was no difference in body size or CV of *H. scabiosae* in any country, and only a difference in CV in *L. villosulum* in the Netherlands. *B. pascuorum* is already a relatively large-bodied bee species whose foraging range may be sufficiently large to overcome the lesser quality of low-proportion landscapes in Italy, Germany, and the Netherlands (Greenleaf *et al.*, 2007, Redhead *et al.*, 2016). It is also possible that other habitats, such as private gardens and green infrastructure, were supplementing the floral resources available to *B. pascuorum*, *H. scabiosae*, and *L. villosulum*, all of which are polylectic species. In the Netherlands, *B. pascuorum* was regularly seen and caught on flowering lavender (Lavandula sp.) inside private gardens, and *L. villosulum* was largely caught on road verges. Though the landscapes in all countries were selected so that urban area was similar between paired sites, it is possible that there were sufficient floral resources in gardens and green infrastructure to mitigate the negative effects of an otherwise resource-poor landscape, acting, in essence, like a habitat enhancement (Grab *et al.*, 2019; Phillips *et al.*, 2020; Samnegård *et al.*, 2011).

We found contrasting patterns in Switzerland and the United Kingdom. In Switzerland, the body size of *B. pascuorum* was larger in low-proportion landscapes, where it might be favourable to invest in larger-bodied workers as it increases flight distance and thus their ability to forage in a fragmented landscape (Gérard *et al.*, 2020; Greenleaf *et al.*, 2007). In the United Kingdom, we found the opposite response, where *B. pascuorum* workers were larger in high-proportion landscapes. Here, it may have been more beneficial to invest in more, smaller-bodied workers who require fewer resources to produce and maintain and thus can make better use of limited floral resources (Chole *et al.*, 2019; McNab, 2010). Bumblebee colonies also favour producing larger workers, which are more costly to produce, when resources are abundant (Kerr *et al.*, 2019; Malfi *et al.*, 2019), which may be the case in the high-proportion landscapes in the United Kingdom. Bumblebees have been shown to have diverging responses to land use change: in the Netherlands, large-bodied bees shrank over time, likely in response to a worsening of habitat conditions (Oliveira *et al.*, 2016); conversely, in Belgium, bumblebee queen body sizes increased over the last century, possibly as a result of agricultural intensification and habitat fragmentation (Gérard *et al.*, 2020). In general, either response to a decline in habitat quality (more, smaller vs. fewer, larger workers) can be adaptive in bumblebees, though the reason why one response might occur over the other is not yet well understood (Kerr *et al.*, 2021). Habitat fragmentation may favour larger workers (such as in Switzerland), as increased mobility compensates for patchy resources, whereas limited floral resources in the landscape may favour smaller workers (such as in the United Kingdom), as they require fewer resources to produce (Kerr *et al.*, 2019). In Switzerland, CV was higher in the high-proportion landscape compared to the low-proportion landscape. Higher CV in worker size could enable better resource utilization in species-rich environments (Vaudo *et al.*, 2015), which is potentially the case for high-proportion landscapes, where semi-natural habitat is present in larger amounts and is therefore less likely to be fragmented (Lindgren & Cousins, 2017). In the United Kingdom, CV was higher in the low-proportion landscape, which may be an adaptive response that allows for broader resource use, and thus survivability in a degraded environment (Peralta *et al.*, 2024).

Other studies investigating the effect of land use change on solitary bee species have found that bee body size decreased along a gradient of agricultural land (Grab *et al.*, 2019; Renauld *et al.*, 2016). However, we found minimal variation in body size and CV between landscape types in *H. scabiosae*. The exception was *L. villosulum*, sampled in the Netherlands, where CV was significantly higher in low-proportion landscapes. A higher CV in low-quality landscapes may indicate an increase in phenotypic plasticity, as greater variation in body size allows for broader resource use, potentially enhancing survival in degraded environments (Peralta *et al.*, 2024). *L. villosulum* is also, compared to *H. scabiosae*, a particularly small-bodied species (*H. scabiosae*: mean ITD = 2.41 mm, SD = 0.22; *L. villosulum*: mean ITD = 1.33 mm, SD = 0.14). They may be more sensitive to resource scarcity due to their small foraging range (Gathmann & Tscharntke, 2002; Greenleaf *et al.*, 2007) and thus require greater flexibility in their foraging strategies in low-quality environments compared to *H. scabiosae*. Further, floral resources may be more discontinuous in the low-proportion landscapes, which contain a higher proportion of non-mass-flowering crops (Hemberger *et al.*, 2023). Such inconsistent resource availability may increase body size variation in offspring, and subsequently CV (Peralta *et al.*, 2024). *H. scabiosae* may be buffered against the negative effects of habitat fragmentation, as they likely have a larger foraging distance due to their larger body size (Greenleaf *et al.*, 2007). *H. scabiosae* has also been shown to be well-adapted to urban environments, and thus may rely less on floral resources in agricultural and semi-natural habitats (Gil-Tapetado *et al.*, 2024). Further, *H. scabiosae* is a primitively eusocial species wherein queens can influence the body size of their first brood by restricting food provisions, producing worker females that help raise the second brood rather than reproducing themselves (Brand & Chapuisat, 2012). Similar to bumblebees, primitively eusocial bee species may be less affected by habitat fragmentation or degradation as the queen can rely on workers to provision the next brood (Packer & Richards, 2021).

4.4 Conclusion

Together, our results support high genetic connectivity between populations established in areas with low and high proportions of long-term interventions. This suggests that current interventions and habitat resources are sufficiently well distributed across regions to support the maintenance of *Bombus pascuorum* and *Halictus scabiosae* populations, or that both species are already well-adapted to agricultural landscapes, and thus do not respond strongly to lower proportions of long-term interventions. However, Switzerland and the United Kingdom were exceptions, where we found divergent responses in both body size and ITV in *B. pascuorum*, indicating that further research is needed to determine which factors are playing a role in the selection for either small-bodied or large-bodied workers. However, given the high gene flow and lack of within-country population structure, we believe that body size differences are likely the result of phenotypic plasticity, rather than local adaptation (Austin *et al.*, 2022).

Given the scale of the landscapes involved in this study, we could not quantify landscape-wide floral resources beyond the percent area of semi-natural habitat. While we ensured large contrasts between low- and high-proportion landscapes, we recommend directly testing resource allocation strategies in bumblebee colonies under different environmental conditions (e.g., increased habitat fragmentation, decreased habitat area, decreased flower diversity) to better understand what factors might drive a colony to invest in fewer, larger-bodied workers versus more, smaller-bodied workers. We also suggest that any future studies at the landscape-level attempt to quantify habitat fragmentation and floral resource availability (including urban habitat, such as gardens and green infrastructure) within the landscape despite the effort involved, as it could shed light on the degree to which floral resource limitation and/or habitat fragmentation are influencing bee body size and intraspecific trait variation.

Since taxonomic groups can vary greatly in their levels of genetic diversity, further studies should be conducted across a broader range of species, both common and rare, to ensure that interventions effectively support and promote diverse communities. Studies such as these are not only important first steps in generating baseline knowledge of current genetic states, they are also critical for future monitoring schemes. By comparing later genetic diversity measures, monitoring efforts will be better equipped to assess and quickly detect potential population declines. This is particularly important since it has been shown that sometimes seemingly common species can be in decline, as census sizes are often larger than effective population sizes (Huml *et al.*, 2023).

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