

**The Ecology and Conservation of the
Adonis Blue (*Polyommatus bellargus*) and
the Chalkhill Blue (*Polyommatus coridon*)
Butterflies in the UK**

Rory S. O'Connor

Linacre College

University of Oxford

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Supervisors:

Professor Rosemary S. Hails

Professor Jeremy A. Thomas

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Abstract

A combination of habitat loss, degradation and fragmentation are causing declines in butterfly species across Europe, with habitat specialists more vulnerable than generalists. The influence of climate change is serving to complicate matters, particularly at the edge of geographic ranges. Improving the quality of habitats is a key tool for the conservation of threatened species at both a local and landscape scale and this requires a thorough understanding of the ecology and habitat requirements of species. From this perspective this thesis examines and compares the ecology of two closely butterfly species, *Polyommatus bellargus* (the Adonis Blue) and *Polyommatus coridon* (the Chalkhill Blue) in southern England; calcareous grassland specialists, found in a highly fragmented ecosystem at the northern most edge of their geographic range.

Chapter 2 explores whether there has been an expansion in the larval niche of *P. bellargus* due to a warming climate in recent decades, and finds no indication of change once concurrent local habitat change has been accounted for. Chapter 3 examines the role that differences in microhabitat requirements play in differentiating the larval niche of *P. bellargus* and *P. coridon*, showing *P. coridon* has broader microhabitat requirements. Chapter 4 experimentally examines the specificity of mutualistic interactions between the larvae of each butterfly species with the ants *Myrmica sabuleti* and *Lasius niger*. Chapter 5 uses microsatellites to explore the genetic structure of both *P. bellargus* and *P. coridon*, showing (as predicted) that *P. coridon* has a less fragmented population structure than *P. bellargus*, but both species have high levels of inbreeding.

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Rory S O'Connor wrote all chapters with feedback on the drafts of each from Jeremy A. Thomas and Rosemary S. Hails. John C. Day also provided feedback on chapter 5. The concept for this DPhil was originally conceived as a component of the CEH Environmental Change Integrating Fund project - The role of pathogens in butterfly population declines, by R. S. Hails, J. A. Thomas, Helen Roy and Helen Hesketh.

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Chapter 1 – General Introduction

1.1 Insect conservation in Northern Europe

Unprecedented declines in biodiversity are occurring across the globe, with increasing numbers of species and ecosystems threatened with extinction (Gaston and Fuller 2007; Pimm and Raven 2000; Rands et al. 2010; Thomas et al. 2004). The causes of decline are multifactorial, including habitat loss, degradation and fragmentation, over exploitation, invasive species and climate change, but all stem from human activity (Millennium Ecosystem Assessment 2005). Insects, with around 950,000 described species and an estimated 5-10 million species in existence, are the most diverse group of animals on the planet and show a corresponding diversity of ecological functions and requirements: as such, they present unique challenges to conservation biology (Gaston 1991; New 2012; Samways 2007; Speight et al. 2009). Further, different regions of the world present different challenges for insect conservation, and so different approaches are needed. The tropical regions, with their large diversity of species across taxa means that a habitat or ecosystem protection approach to their conservation, focusing on species diversity, is most common (Bonebrake et al. 2010). With its temperate conditions and historical land-use, and much lower diversity of species, Europe presents different challenges and these too vary from southern to northern Europe (Merckx et al. 2013; Stewart and New 2007).

Europe's landscape has been heavily modified by centuries of human agricultural activity; today an estimated 45% of land cover in the Europe Union is devoted to agriculture (FAO 2014), and for the UK alone this figure rises to 70% (Defra 2012). Given Europe's long history of arable and pastoral agriculture, the majority of habitats of conservation concern are semi-natural, maintained for millennia in a particular successional stage by human activities (e.g. grazing and coppicing) (Bignal

and McCracken 1996; Bignal and McCracken 2000); some highly valued assemblages and ecosystems, notably heathlands, were actually created by ancient land usage (Webb 1986). However, with the advent of industrial agriculture after the second-world war in western Europe, and for the purposes of this thesis specifically in the UK, traditional practices were replaced, leading to the large scale conversion of most flatter land to intensive agriculture or the abandonment of other areas of semi-natural habitat, especially on slopes with thin soils (Henle et al. 2008). The areas of semi-natural habitat that remain are fragmented, with formerly contiguous biotopes now found in patches, often small and isolated, embedded within a more homogenous agricultural landscape (Jongman 2002).

Conserving insects in these degraded and fragmented habitats is complicated further by a warming climate (Stewart 2012). Being poikilotherms, insects are particularly sensitive to changes in temperature (Bale et al. 2002), and as the global climate has warmed the ecology of insects has already begun to change, with range shifts, advancing phenologies, and the consequential disruption to plant-insect trophic interactions (Musolin 2007; Parmesan 2006). Much evidence comes from the relatively well documented insect fauna of Britain; the phenology of some butterflies and Odonata has advanced (Hassall et al. 2007; Roy and Sparks 2000) and their geographical range, along with those of other insect groups, has expanded northwards as warmer temperature allow them to utilise habitat previously unavailable (Hickling et al. 2006; Hickling et al. 2005; Hill et al. 2002; Parmesan et al. 1999). However, the capacity of a species to track climatic change can be impeded by a lack of suitable habitat, fragmented habitats and by its own intrinsic capacity for dispersal. Northern expansions are also coupled with a decrease in southern range limits, which will leave

some species subject to range retractions but unable to track changing temperatures, increasing the threat of extinction (Hill et al. 2006; Hill et al. 2002; Thomas et al. 2006; Warren et al. 2001).

1.2 Butterflies: models for insect conservation and monitoring

Butterflies are one of the, if not the, most studied insect groups globally (Lewis and Senior 2010), and the butterfly fauna of North -Western Europe is unparalleled in the current understanding of species distributions, population dynamics and ecology (Settele et al. 2009). This is due to long traditions of professional and non-professional entomology and more recently dedicated monitoring schemes that harness the efforts of numerous skilled amateurs (citizen science) (Merckx et al. 2013; Settele et al. 2009; Stewart and New 2007; Thomas 2005; Warren 2012). Given the comparatively large amount of data available on butterflies, they have come to be representative as an indicator group for terrestrial insects, in two senses. Firstly, the well-documented changes in their distributions and populations are indicative of broader impacts of habitat and climate changes on other less conspicuous terrestrial insect taxa, and provide an early warning for species whose population dynamics are less sensitive (Fleishman et al. 2005; Thomas 2005). Secondly, as resources for ecological study and conservation implementation are often scarce, and given the comparatively detailed information available on their habitat needs, butterflies can act as flagship or umbrella species for conservation management programmes (New 1997).

There is clear evidence that most butterfly species are in decline across Europe, with losses occurring in nearly all European countries for which distribution data are

available, and trends showing that specialist species are in general more threatened than generalists (van Swaay et al. 2011; van Swaay et al. 2006). The most recent assessment of European butterflies according to current IUCN Redlist criteria classed 19% of 483 of Europe's butterfly species as under threat, but the authors emphasise that given the limitations of the data available, this is likely to be a conservative estimate (van Swaay et al. 2011). For the 62 butterfly species found in Great Britain (58 resident and four regularly breeding) the picture is similar, if not worse, with 37% of species being classed as Threatened (19 species) or Regionally Extinct (4) and a further 11 (18%) species classified as near threatened (Fox et al. 2011b). Based on changes in the occupancy of 10km grid squares across Great Britain, Thomas et al. (2004) also showed that 71% of all butterfly species had declined in range, with a median loss (based on all species) of 13%, an overall rate of decline that greatly exceeded those of breeding birds and vascular plants during the same period. The most recent assessment, utilising the UK Butterfly Monitoring Scheme abundance data, show that these declines continue; again specialist species experienced the most pronounced declines (Fox et al. 2011a).

One of the most prominent examples of a depleted and fragmented ecosystem in north-western Europe is that of unimproved calcareous grasslands, growing on shallow, well-drained soils on a limestone or chalk base. These valued assemblages and communities were largely created by, and are highly dependent on, traditional agricultural practices such as livestock grazing and haymaking. These maintain the land in plagioclimaxes or successions with a high frequency of early seral stages. Subsequently, with the advent of modern agriculture, calcareous grasslands have undergone severe declines throughout Europe in the 20th century, either being

converted for high input agriculture or being abandoned (Poschlod and WallisDeVries 2002). The UK has seen an 80% loss of calcareous grassland since the 1940s (Blackstock et al. 1999; Fuller 1987). The thin, low nutrient soils of calcareous grasslands support some of the most diverse plant and invertebrate communities in northern Europe, including a high number of rare species, and are consequently of high conservation concern (Balmer and Erhardt 2000; Dolek and Geyer 1997; Mortimer et al. 1998; Polus et al. 2006; Steffen-Dewenter and Tschardt 2002; WallisDeVries 2002). For example, van Swaay (2002) suggested that of an estimated 576 butterfly species found in Europe 261 are found on calcareous grasslands, as are 37 of the 71 species he designates as 'Threatened'. The conservation of insect communities found on the calcareous grasslands of the UK and other high latitude countries present amplified challenges to those in mainland Europe. Britain represents the northern most edge of the geographic range for many of its resident butterfly species, and their specific microhabitat requirements increasingly include thermal constraints the closer populations approach their northern range limits (Thomas 1993). For many of these species, breeding is restricted to a subset of their food plants growing both in early seral habitats and on south-facing aspects in these parts of their ranges, which provide their larvae with suitably warm microclimates. This type of thermophilous habitat was historically scarce in northern Europe, and largely disappeared under modern agriculture: its restoration and maintenance requires complicated habitat management prescriptions which not only vary in detail under different climates (and with climate warming) but which also account for habitat fragmentation (Bourn and Thomas 2002; Oliver et al. 2009; Oliver et al. 2012; Thomas et al. 2001a).

It is within this context of degradation and fragmentation that this thesis examines the ecology of two specialist species of calcareous grassland butterflies in the UK; *Polyommatus bellargus* (the Adonis Blue) and *Polyommatus coridon* (the Chalkhill Blue) (fam: Lycaenidae, subfam: Polyommatainae, genus: *Polyommatus*, subgenus: *Lysandra*). The work presented is concerned with quantifying the larval habitat requirements of these two species, to identify what constitutes a high quality habitat for both, and how they might differ in their ecology and requirements. Before introducing this system and the specific hypotheses that are tested, a short review is presented to contextualise the importance of such research into the habitat requirements of threatened species.

1.3 Butterfly conservation – populations and metapopulations

The drastic declines observed for many species of the European butterfly fauna led to a re-orientation of conservation practices for butterflies in the 1980s, with detailed ecological studies of habitat use by a species being applied to inform conservation management strategies (Bourn and Thomas 1992; Merckx et al. 2013; Morris et al. 1993; Thomas 1983; Thomas 1995; Thomas et al. 1986; Warren 1987; Warren 1993). The resultant “single-species approach” arguably saved several specialist butterfly species from extinction in the UK and enabled the Large blue (*Maculinea arion*) to be re-established there (Merckx et al. 2013). Counter-intuitively, for the 20 British species for which precise habitat requirements have been quantified, population size is not constrained by any adult resource and is seldom correlated with the abundance of a host plant on a site; instead, both relative colony size and population trends over time correlate closely with the subset of host plants found growing in a (defined) suitable condition for each species on a site (Thomas et al. 2011). Consequently,

managing habitats to increase the availability of an essential resource, namely host plants growing in optimal conditions for the larval stage, allowed the recovery of populations on nature reserves and much additional land managed, for example, under agri-environmental schemes.

However, improving the quality of local habitats only addresses part of the problem; habitat destruction has left species in a landscape composed of islands of suitable habitat located within a matrix of unsuitable land. In the 1990s, the concept of the metapopulation was established as an important tool in understanding the ecology and landscape-scale conservation of butterflies. A metapopulation is a population composed of smaller subpopulations, occupying discrete patches, maintained by the extinction – colonization dynamic between patches (Hanski 1998; Hanski et al. 1996). A metapopulation persists via the balance of extinction of local populations events and colonisation/re-colonisation of empty patches (Hanski 1994; Hanski 1999). Each patch is conceived as existing as a discrete area of suitable habitat within a network of suitable patches, embedded within a matrix of unsuitable habitat.

Central to the translation of metapopulation theory into real world scenarios is the area-isolation paradigm (Hanski 1994). This emphasises how the geometric attributes of a patch network, the size of patches and the amount of isolation from other patches, influences the metapopulation dynamics of a species. The larger patches become, the less susceptible they are to stochastic extinction events, and the more isolated from other patches a patch is the lower the probability that it will be re-colonised. The spatially explicit incidence function model (IFM) for metapopulations integrates these assumptions with regard to patch size and isolation to model patch occupancy (Hanski

1994; Hanski 1999; Hanski and Gilpin 1997). The IFM has explained a large amount of the variation in the patch occupancy dynamics and persistence for several butterfly species, but has been criticised more recently for limiting its perspective to area and isolation only, and specifically for not including patch quality (Dennis et al. 2006; Moilanen and Hanski 1998; Shreeve and Dennis 2004).

With the success of the metapopulation approach came a paradigm shift, emphasising the maintenance of landscape structure in a way thought to promote the regional persistence of a species (Hanski 1999). However, an assumption implicit in the initial metapopulation framework was that the habitat quality of different patches is uniform and constant (Hanski 1998). In reality there is heterogeneity in the condition of habitat both between and within patches and this may influence the persistence of individual populations of different species. Indeed, it is the management of habitat quality within patches that has proved so successful in conserving some species and many have argued for the inclusion of this variation in the metapopulation framework (Anthes et al. 2003; Dennis and Eales 1997; Dennis and Shreeve 2003; Thomas et al. 2001b; Thomas et al. 2011).

This debate is of real pertinence for metapopulation theory, the conservation of butterflies, and also for conservation in fragmented habitats in general. Emphasis on maintaining patch network structure over the quality or attributes of habitat within patches, or vice versa, without being properly informed could have detrimental effects on the populations we aim to conserve. Habitat loss, degradation and fragmentation are arguably the three largest drivers of the current extinction crisis (Pimm and Raven 2000). In a recent review of UK wildlife reserves, Lawton et al. (2010) suggest that

our priority should be to make sure that the quality of our existing nature reserves is high, but that maintaining area and connectivity in a network is also key; it is likely that a mixture of the two perspectives will best serve the goals of conservation.

1.3.1 Habitat quality – the third metapopulation variable?

Introducing variation in habitat quality into a patch network could in theory alter metapopulation dynamics through three major routes; changes in carrying capacities for a given patch, influencing patterns of emigration/immigration, and altering the probability of successful patch colonisation / re-colonisation.

- I. **Carrying capacity** If there can be variation in the quality of a prescribed habitat, then the quality of a habitat patch will influence population size independently of area. A higher quality patch will support a larger number of individuals per unit area and so increase the carrying capacity compared to an area of the same size but lower quality. The key assumption that patches of smaller area are more susceptible to stochastic extinction events might then not stand, as this is hinged on smaller patches supporting smaller populations. In an analysis of UK butterflies, Thomas et al. (2001b) found that the carrying capacity of optimum habitat for a species was about 100-fold greater than that of the same area of sub-optimal source-habitat which had nevertheless proved capable of supporting a population for more than 7 generations.
- II. **Emigration** Patches that maintain larger populations are also likely to produce a larger number of dispersing individuals, which in turn will increase the amount of recolonisation / supplementation of other populations occurring within a system (Schooley and Branch 2011). Higher quality habitat is therefore likely both to reduce patch extinction rates within a system and increase the

colonisation of empty neighbouring patches. Where patch quality is higher emigration rates might initially be lower, as there may be less benefit in individuals leaving a patch. If this holds a patch will retain higher population densities, which could lead to density dependent effects operating within a patch (Dennis et al. 2006), at which stage emigration may again become beneficial (Poethke and Hovestadt 2002). Indeed, experimental manipulation of habitat quality (through grazing) in a metapopulation of *Boloria eunomia*, found that not only did the rate of emigration from patches increase with reductions in habitat quality but also emigration rates from high quality patches decreased (Baguette et al. 2011). On the other hand, Poethke and Hovestadt (2002) showed that, in theory and practice, sites that supported very high densities of insects (i.e. those with the highest possible quality of habitat in high density years) generated a disproportionately large number of emigrants.

- III. **Colonisation** The condition of habitat within a patch will also influence the success rate of colonisation “attempts”. Higher quality habitat will result in higher levels of survivorship in the offspring of emigrants and in turn faster rates of population growth. As such, newly established populations will be less vulnerable to the stochastic events and the inverse density dependent effects (Allee effects) that frequently cause extinction in their early years (Bonsall et al. 2014; Thomas et al. 2011).

1.3.2 Empirical evidence for the effects of habitat quality in fragmented populations

Table 1.1 presents a summary of studies that have considered the effect of habitat quality as an explanatory variable of patch occupancy in metapopulations. The studies

were found by searching both Web of Science and Google Scholar using the terms Habitat Quality, Patch Quality, Metapopulation, Fragmentation, and Butterfly in all combinations, and by searching the citations of those studies found. Only studies that have explicitly considered the role of habitat quality in relation to patch size and isolation are considered here. Each evaluates how much of the variation in patch occupancy within a landscape is explained by patch area, patch isolation and patch habitat quality. Two have also addressed the role of each variable in determining patch extinction rates and density (Heisswolf et al. 2009; Poniowski and Fartmann 2010). For the purposes of this thesis the main emphasis will be on the consideration of butterfly and insect studies, but efforts for other groups are also reported.

Of the 20 studies in Table 1.1, 15 have concluded that habitat quality has a significant effect on patch occupancy, and for 9 of these that it is the most significant determinant of patch occupancy. In terms of taxonomic attention, butterflies are by far the most represented group with 11 studies devoted to them, 6 concluding it is the most significant determinant of occupancy. A further 5 studies have considered other invertebrate groups, with all concluding that habitat quality is important, and 3 concluding it was the most important determinant of patch occupancy. Four studies have considered vertebrates (2 mammals and 2 bird), with three of these supporting the role of habitat quality.

As shown in Table 1.1, measures of habitat quality for different studies vary in the aspects of an organism's environment considered and the scale at which their variation is addressed. Before research can demonstrate the role habitat quality plays in sustaining fragmented populations, clear definitions are needed of what constitutes

a study species' habitat and how variation in its quality should be quantified. If results are to be comparable between studies, the working definition of habitat should be the same. In practice, making generalisations from the evidence (Table 1.1) is currently difficult, as some studies may be testing different questions.

1.3.3 Defining habitat

The habitat is a ubiquitous concept for ecology and conservation biology, yet its definition within the literature has been ambiguous (Dennis and Shreeve 2003; Hall et al. 1997; Mitchell 2005; Morrison and Hall 2002). Between studies and practitioners there is much variation in the factors (both biotic and abiotic) considered to constitute an organism's habitat. A review by Hall et al. (1997) of 50 studies and their use of habitat and related terms (e.g. habitat type, habitat structure, habitat quality) concluded that 82% of the articles used habitat terminology that was vague and imprecise. For instance habitat was regularly used interchangeably with terms for vegetation classification or for ecosystem: throughout this thesis, the strict definition of the term 'habitat' is used to describe the specific requirements of a single species within an ecosystem, in this case the habitats of *P. bellargus* and of *P. coridon*.

1.3.4 Measures of habitat quality in current studies

Though it may be more appropriate to assess the effect of habitats on the fitness of individuals, the logistical difficulties of tracking survivorship or fecundity for most insects are great. The presence or density of individuals is generally the most practical indicator of quality. The most common measures of habitat quality have measured habitat attributes at the patch level, based on the assumption that the presence or

Table 1-1 The presence of circle (○) within a box indicates that the variable was significant in determining patch occupancy. Where a study indicated the relative importance of each variable, that which explained the most variation in occupancy is marked with a filled circle (●). A minus symbol (-) denotes that a variable was not significant in explaining variation in occupancy. NC denotes a variable was not considered. **References:** 1. Dennis and Eales (1997); 2. Moilanen and Hanski (1998); 3. Thomas et al. (2001b); 4. Fleishman et al. (2002); 5. Anthes et al. (2003); 6. Krauss et al. (2005); 7. Habel et al. (2007); 8. Thomas et al (2009); 9. Betzholtz et al. (2007); 10. Rabasa et al. (2007); 11. Bauerfeind et al. (2008); 12. Bonte et al. (2003); 13. Yamanaka et al. (2009); 14. Heisswolf et al. (2009); 15. Franzen and Nilsson (2010); 16. Poniatowski and Fartmann (2010); 17. Jaquierey et al. (2008); 18. Franken and Hik (2004); 19. Vogeli et al. (2011); 20. Robles and Ciudad (2012).

Species	Measures of Habitat Quality	Results		
		Habitat Quality	Area	Isolation
1. <i>Coenonympha tullia</i> (Large Heath)	23 factors - designated either positive (i.e. presence of host plants, vigorous host plant growth, wetness, overlap of larval and adult resources) or negative (i.e. burning, overgrazing, succession) in terms of <i>C. tullia</i> 's requirements. Each author also conducted a subjective assessment.	●	●	○
2. <i>Melitaea cinixia</i> (Glanville Fritillary)	Grazing severity scored 1-3. Percentage cover values for the host plant, dry host plant, low vegetation and flowering plants. Percentage of patch perimeter bordering onto – field, forest, semi-open, water.	-	○	○
3. <i>Melitaea cinixia</i> (Glanville Fritillary), <i>Polyommatus bellargus</i> (Adonis Blue), <i>Thymelicus acteon</i> (Lulworth Skipper)	The larval niche of each species was quantified in terms of the probability of a food plant growth form (successional stage, leaf size) or microhabitat measures (i.e. turf height, shelter) for a site containing eggs or larvae. Patch quality was then quantified using an index of habitat quality derived from these measures.	●	-	○
4. <i>Speyeria Nokomis apacheana</i> (Nokomis Fritillary)	Number of larval host plants, number of adult nectar sources in a patch were recorded, percentage cover for host plants, nectar sources and dead vegetation/litter along with average vegetation height, human generated disturbance (non, heavy, mild).	●	-	-
5. <i>Euphydryas aurinia</i> (Marsh Fritillary)	Microhabitat and microclimate measures of plants for oviposition preferences and larval web densities. Density of 'suitable' host plants within a patch was then used to measure patch habitat quality. Patch management regimes was	●	○	○

	also used.			
6. <i>Polyommatus coridon</i> (Chalkhill Blue)	Percentage cover of plants in flower, shrub layer and bare ground. Height of the herb layer, wind protection (estimated from the adjacent tree and shrub layer).	-	●	-
7. <i>Maculinea alcon</i> (Alcon Blue)	Percentage cover - shrubs, bare soil, plant detritus and sphagnum moss. Host plant (marsh gentians) distribution within the patch was categorised as either dispersed or clumped. Individual plants were classified as one of four successional stages (seedling, juvenile, vegetative or senile), which were used to identify a general demographic stage for each patch. Patch micro-relief (tussock-hollow) structure was also recorded.	●	○	○
8. <i>Maculinea arion</i> (Large Blue)	Presence, absence and egg population size of <i>M. arion</i> were measured annually on 21, together with sward structure, microclimate, the attributes of <i>Myrmica sabuleti</i> ant colonies, and 20 other habitat parameters affecting larvae or the young stages plus site size, area and isolation.	●	-	○
9. <i>Euphydryas aurina</i> (Marsh Fritillary)	6 microclimate variables - Vegetation height, insolation around host plants, humidity, risk of summer flooding, vegetation structure and overgrowth. 3 food plant variables - density of food plant, density of a food plant used occasionally by larvae and total nectar supply for adults.	○ (only larval food plant density)	-	●
10. <i>Iolana iolas</i> (Iolas Blue)	Fruit production per plant was measured to estimate nectar resource quantity within a patch. Slope, aspect, insolation (direct solar radiation received by a patch during summer solstice) and surface curvature were also recorded.	-	●	○
11. <i>Lycaena helle</i> (Violet Copper)	Percentage cover - host plant, preferred vegetation, nitrophilous plant (indicators of eutrophication) and cover by trees and shrubs. Also measured - Trees and shrub along patch edge, abundance of flowering plant, mean vegetation height and current land use.	○	●	●
12. <i>Pardosa monticola</i> (Dune Wolf Spider)	Mean vegetation height. Patch area and patch perimeter were considered measures of quality.	●	○	

13. <i>Copera annulata</i> (Damselfly)	A pond equated to a patch. 6 variables - predatory fish abundance, benthos fauna abundance, coverage of aquatic and riparian vegetation, the thickness of the debris layer, pond area and perimeter revetment (slope to protect against erosion).	●	○	
14. <i>Cassida canaliculata</i> (Leaf Beetle)	Occurrence of <i>C. canaliculata</i> - Patch slope, host plant size and density	○	○	●
	Density of <i>C. canaliculata</i> – Host plant density	●	○	-
15. <i>Andrena hattorfiana</i> (Solitary Bee)	Extinction and colonisation – Flowering plants of <i>Knaulia arvensis</i> , the main pollen source for <i>A. hattorfiane</i>	●	- (once population size accounted for)	-
16. <i>Metrioptera Brachyptera</i> (Flightless Bush Cricket)	Patch Occupancy for <i>M. brachyptera</i> - Mean Precipitation, Mean Temperature.	○	●	-
	Density of <i>M. brachyptera</i> – Aspect, daily sunshine, total vegetation Cover, bare ground cover, litter layer, cryptograms cover, vegetation height, horizontal vegetation density, land use type.	●	NC	NC
17. <i>Crocidura russula</i> (Greater White Toothed Shrew)	Plant productivity, altitude, northness and eastliness, winter solar radiation, number of frost days per year. No. of winter heated buildings in 100m radius and no. of buildings in a 100m radius weighted by distance.	○	-	●
18. <i>Ochotona collaris</i> – (Collared Pika)	The average survival value for a patch over all years was used as a measure of quality. The amount of vegetation within a patch and patch aspect were also recorded.	○	-	○
19. <i>Chersophilus duponti</i> (Dupont's Lark)	Occurrence, Density and Extinction – Vegetation density, Arthropod availability, bird species density and richness, predator abundance	-	● (Density)	● (Ext'tn)

20. <i>Dendrocopus medius</i> (Middle Spotted Woodpecker)	The density of large oaks (≥ 37 cm dbh, 1.3 m above ground) in a patch. These provide forage for Juveniles and Adults.	●	– (once population density accounted for)	–
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quantity of a certain variable (such as host plant coverage) may be correlated with the quality of that patch.

Several studies have scored each patch for a factor (such as grazing intensity) or derived coverage values for vegetation and substrate types (such as larval host plant, flowering plants or shrubs). Dennis and Eales (1997) defined habitat quality using questions of a binary (presence/absence) nature, based on the observation of the broad environmental requirements of *Coenonympha tullia* (See Table 1.1). Moilanen and Hanski (1998) used percentage cover values for various vegetation attributes, as well as factors that might affect the quality of patch perimeter, to modify the effective area of patches within an IFM. Other studies have also used percentage cover measurements though within regression analyses (Fleishman et al. 2002; Habel et al. 2007; Krauss et al. 2005) (see Table 1.1). Such measures are relatively easy to make, and so are useful in terms of time and resources for large field studies (for instance much can be estimated from aerial photographs). However, such measures based on the association of a species with broadly defined habitat characteristics are not necessarily made at a scale or with a precision most relevant to the species in question, and can cause misleading model results (Armstrong 2005). It would be productive if metrics of habitat quality were based on data that demonstrate causation between variation in a factor and (at least) the density of a species.

Mortelliti et al. (2010) provide a useful framework for this dilemma between the accuracy and ease of recording. Three levels at which quality can be measured are identified: 1) measuring the actual effect of a habitat on a population; 2) measurements of key (limiting) resources within a patch; 3) measurements of patch structure. With each measure, accuracy decreases at the expense of ease of measurement. Mortelliti et al. (2010) conclude that measurements of level 2 will often be the most appropriate, as this is grounded in an empirical understanding of an organism's habitat requirements, but will allow for a relatively simple procedure in grading the quality of a patch. This is basically an exercise in reconciling problems of scale. As with many ecological questions (Weins 1989), the choice of scale (that is the spatial extent and grain) at which one defines a habitat and subsequently its quality will influence the results (Hall et al. 1997; Mitchell 2005; Morrison and Hall 2002). Level 2 will produce metrics of quality that consider the environment from the perspective of the organism in question. Level 3, which can be defined as patch scale measures, will miss the finer details that actually dictate the fitness of the individuals within a patch. Level 1 becomes circular as, by definition, it can only be applied to patches that are occupied.

A minority of studies have done more to account for variation in habitat at the scale of the individual. Having previously established that larval rather than adult resources determine population size and persistence in their study species, Thomas et al. (2001b) constructed measurements of habitat quality for three butterfly species by quantifying the larval niche directly (*Melitaea cinixia* and *Thymelicus acteon*) or from female oviposition preferences (*Polyommatus bellargus*) in terms of food-plant growth form or microhabitat variables (see Table 1.1). Habitat quality for each patch

was then quantified from the densities of source (sub-optimal to optimum) growth-forms of host plants, as defined by observed preferences. Anthes et al (2003) also quantified female oviposition preferences for *Euphydryas aurina* (Marsh Fritillary butterfly) in terms of plant form and microhabitat structure (Table 1.1). Oviposition increased with plant height and leaf number but decreased with vegetation density. These methods quantify variation in available habitat in terms of its effect on the density of individuals, at a scale appropriate to the species in question.

Interestingly, all of the butterfly studies that have subsequently cited the conclusions of Thomas et al (2001) to support their investigations of habitat quality have actually used patch scale measures in their own models, such as percentage cover measures of host plants and other surfaces, sward height and slope (Krauss et al. 2005, Habel et al. 2007, Betzholtz et al. 2007, Rabasa et al. 2008, Bauerfeind et al 2008). Further to this, two studies on other invertebrate taxa have actually included patch area in their metric of habitat quality, which confounds any attempts to discern the relative importance of either (Bonte et al. 2003; Yamanaka et al. 2009). Two studies, however, of a solitary bee (*Andrena hattorfiana*) and a leaf beetle (*Casida cananiculata*) have considered factors demonstrated to affect the density of these species in their metrics of quality (Franzen and Nilsson 2010). The obvious deterrent to using more detailed measures of habitat quality is that it will increase the amount of data collection required for a study. Below, a strategy is proposed that would reduce the time required to create a usable, and comparable, measure of habitat quality.

1.3.5 A resource based definition

Recently, specifically within the context of butterfly ecology, some have advocated a “Functional definition” of habitat, based around the resource requirements of a species (Dennis et al. 2003, Shreeve et al. 2004, Dennis et al. 2006, Dennis 2010). This considers that an organisms’ habitat can be defined in terms of its resource requirements of each stage in its life cycle, or that it should be possible to map the bounds of a habitat in terms of life history requirements (Dennis and Shreeve 2003). Resources are partitioned in terms of requirements at each life stage and further divisions can be used to define particular resources into consumables (larval host plants, adult nectar sources) and utilities (physical sites for various activities). In a very real way this represents what Mitchell (2005) notes; a movement of the habitat concept closer to that of Hutchinson’s (1958) realised niche, and Dennis et al (2006) acknowledge this.

This resource based perspective is attractive as it could standardise methods used by researchers to distinguish the different habitat needs of co-occurring species, with regard to how important particular consumables and utilities are in defining the habitat requirements of a species (i.e. a particular suite of plants for nectaring may be an important factor in defining the habitat of species A, but species B is much less constrained in the plants it utilises). The prospect of describing the habitat preferences of a species at this resolution might seem unfeasible, but informed decisions based on observation of an organisms’ habits can be used to focus descriptions on resources that appear to be particularly important (i.e. if species A appears to be utilising a subset of plants for larval feeding, but unselective in the areas it chooses nectaring as adults).

To aid this focus, metrics of habitat quality could begin with identifying the life stage of the organism most susceptible to resource limitation; that is the life stage for which habitat components have the strongest impact on survival or fitness, and which drive the population dynamics of a species (i.e. the 'key factor(s)' among the many parameters). Based on this, the resources that are most limiting within this stage, and which exhibit a large degree of variation between habitats, can be chosen as the basis of a metric of habitat quality. Focusing on the life stage that has the most influence on population density will also go some way to dealing with problems in reconciling the diffuse picture of a species habitat, where the resources for one life stage might occur in a separate location to another, with that of delimiting habitat into distinct patches within the landscape (see Dennis et al. 2003, Shreeve et al. 2004, Baguette and Mennechez 2004 for further discussion).

The insights of Thomas et al (2011) with regard to the conservation of UK butterflies are particularly helpful here. In a meta-analysis of 20 species of butterfly found in the UK they demonstrate that the precise requirements of many UK butterfly species with regard to the conditions in which host plants are found, leads to the availability of larval host plants found in optimal condition being strongly correlated with population size. When the absolute host plant abundance was considered as metric of quality, no correlation was found with population size (Thomas et al 2011). A major conclusion from this study is that the most successful and cost efficient conservation management regimes are likely to be those that aim to modify to a few essential resources dictating the larval niche of a species, such as those aimed at *Polyommatus bellargus* in the UK. The same can be said for effective metrics of habitat quality, but

with the focus on simply identifying a small set of variables that account for a large amount of variation in the abundance or survivorship of a species.

1.3.6 Conclusions

From the summary of studies in Table 1.1, it can be surmised that there is a strong case for considering the role that variation in the quality of a habitat patches might play in determining its metapopulation dynamics of a species. However, due to the lack of consistency in methodology between studies, it is hard to make further conclusions, such as whether particular species' traits are associated with the effects of quality. It is essential, therefore, that future studies considering the role of habitat quality in determining the patch occupancy of a species should use a metric of quality that is a reliable indicator of how likely a patch will be to sustain a population. A few suggestions are made above that might help those considering how to quantify habitat quality, emerging from the confusion evident in previous research. These would help to standardise approaches and provide clearer metrics of quality, resulting in more reliable conclusions about individual species, more reliable comparisons between the conclusions of studies, and eventually, a better-informed approach to conservation of species in fragmented landscapes.

1.4 *Polyommatus bellargus* and *Polyommatus coridon* in the UK

Polyommatus bellargus (the Adonis Blue) and *Polyommatus coridon* (the Chalkhill Blue) are specialist butterflies of calcareous grassland, distributed throughout southern Europe, with the northern most edge of their distributions in the South of England (see Figure 1.1). The range of *P. coridon* in the UK extends further north

than *P. bellargus* and shows a wider distribution among 10km square in general, but there is an almost complete overlap in the distribution of these two species and many grassland sites exist where they co-occur.

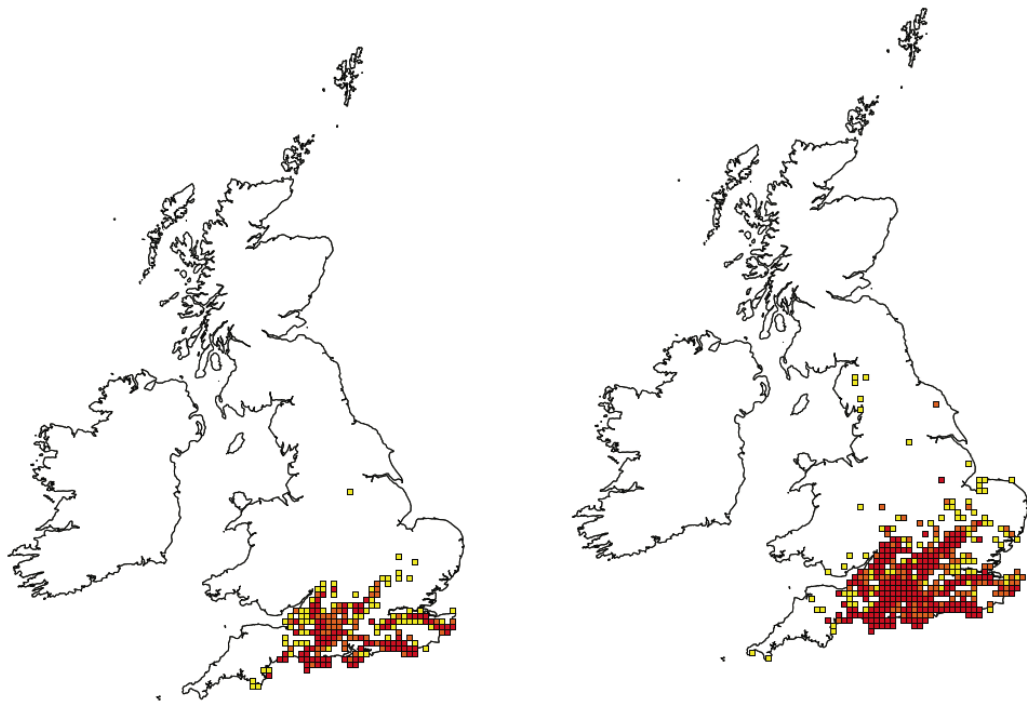


Figure 1.1 UK distribution maps for a) *Polyommatus bellargus* and b) *Polyommatus coridon* at a 10km² resolution. Yellow squares represent the pre 1970 distribution, orange squares the 1971 -1982 distribution and red squares their current distribution. Images created using the National Biodiversity Network Gateway - <https://data.nbn.org.uk>.

These species utilise the same, and sole, larval host plant in UK, *Hippocrepis comosa* (Horseshoe vetch). Also a specialist of calcareous grassland, *H. comosa* has a wider distribution than both *P. bellargus* and *P. coridon* in England, but is found in small fragmented populations further north, a factor that (Asher et al. 2001) suggest may limit the distribution of *P. coridon*. Both species also engage in mutualistic interactions with ants as larvae and pupae, which though classed as facultative (the

larvae can survive in captivity without ant attendance) are extremely common in the wild and little understood beyond species associations. Both butterflies are known to associate with the same species of ants as larvae; *Lasius niger*, *Myrmica sabuleti* and *Lasius flavus* (Thomas 1983; Thomas and Lewington 2010).

1.4.1 Decline and recovery

Most species of butterfly in the UK have undergone drastic reductions in abundance and retractions in range since the 1950s (Asher et al. 2001), and *P. bellargus* and *P. coridon* are no exception. Between the 1950s and 1980s *P. bellargus* experienced a 42% decline in range (Warren et al. 1997), and *P. coridon* a decline of 43% since 1800 with a further 25-33% loss in 1970-1982 (Asher et al. 2001). With the large scale loss and fragmentation of species-rich calcareous grasslands, post 1950 populations of each species became increasingly fragmented, and by the 1980s *P. bellargus* in particular persisted in just 70-80 small colonies (Thomas 1983). These reductions are directly linked to a loss of grasslands due to conversion for agricultural use and inappropriate habitat management (Brereton et al. 2008; Fox et al. 2006; Thomas 1983). Many formerly suitable grassland areas were converted for agricultural production, and a decline in rabbit populations due to myxomatosis, paired with a lack of appropriate grazing by livestock, allowed much of what remained to develop a taller sward structure and scrub encroachment.

Due to these declines, and being an indicator of good health for calcareous grasslands, *P. bellargus* was given 'Priority Species' status on the UK Biodiversity Action Plan (Bourn and Warren 1998). *Polyommatus coridon* was given the lesser status of 'Species of Conservation Concern', due to its wider distribution and much greater

number of colonies (Fox et al. 2006; Fox et al. 2011a). These designations have meant that the requirements of these species, particularly *P. bellargus*, have directly informed both nature reserve management plans and agri-environment schemes, which appear to have been effective. In 2006, following an increase in abundance of 63% since 1995, *P. bellargus* was downgraded in status to a ‘Species of Conservation Concern’ (Fox et al, 2006). *P. coridon* has also undergone an overall recovery in abundance of 31% since the 1970s, but in 1995 – 2004 underwent a decline of 34%. The prospects for these species have improved since the 1980s, especially for *P. bellargus*, that was declining so dramatically that an extrapolation of its 20th century losses suggested national (UK) extinction by about 2000 (Thomas et al. 2011). Conservation initiatives have undoubtedly played a large role in these recoveries, but the recovery of rabbits from myxomatosis and the effects of climatic warming may also be influential (Oliver et al. 2012), as has also been suggested for *Hesperia comma* (the Silver Spotted Skipper) (Davies et al. 2005; Davies et al. 2006; Warren et al. 2001).

P. bellargus and *P. coridon* populations have always existed as discrete colonies within the landscapes, as does *H. comosa*. However, the loss and further fragmentation of calcareous grasslands in the South of England has inevitably led to increased spatial structuring in the populations of both species, most often found on nature reserves and other Protected Areas. However, the degree to which these species are actually fragmented, that is sub-populations are isolated from the wider population, is unlikely to be the same for both species. Factors such as dispersal ability, degree of habitat specialism and the habitat surrounding sites will determine the movement of individuals between patches, and to what degree populations

function as a metapopulation. The dispersal ability of species can be crudely quantified through mark release recapture (MRR) experiments, as has been done for both *P. bellargus* and *P. coridon*, but population genetics offers a way to quantify the degree to which fragmentation affects the gene flow, and so the movement of individuals (including rare long-distance dispersal), between populations. For UK *P. bellargus* populations, Harper et al. (2003) found relatedness of populations between regions (i.e. counties) was strongly related to linear distance between them. Within regions there was also a large genetic differentiation between colonies over relatively short geographical distance, supporting previous observations of no exchange of individuals detectable by MRR between colonies in Dorset that were separated only by a few hundred metres (Thomas 1983). The analysis also suggested that a population bottleneck, caused by a drought in England in 1976, had reduced the effective population size of *P. bellargus* in England, a conclusion further supported by the analysis of old museum specimens (Harper et al. 2003; Harper et al. 2006). Evidence for *P. coridon* in both southern and northern Germany shows low genetic differentiation between populations (Schmitt and Seitz 2002; Krausse et al. 2004). This again corroborates with observations of greater dispersal ability in *P. coridon*, with individuals being found travel 2km from their natal site in the UK, and further in Germany (Asher et al. 2001).

1.5 Summary of aims

The primary concern of this thesis is to understand how *Polyommatus bellargus* and *Polyommatus coridon* differ in their ecology in the UK, and the implication of this for their conservation. These two closely related species share the same larval foodplant and inhabit the same ecosystem, and hence have historically been subject to the same

process of habitat fragmentation within the same landscapes in the South of England. The microhabitat requirements of the larval niche of *P. bellargus*, for *H. comosa* plants growing in short turf and highly sheltered spots, have already been documented and used to great effect in prescribing suitable habitat management for its conservation. Here, the consequences of climatic warming on the habitat preferences and abundance of *P. bellargus* will be explored, as have already been documented for the similarly thermophilous calcareous grassland specialist *Hesperia comma*. The larval niche of *P. coridon* has not previously been described, so this thesis also examines the putative role microhabitat plays in differentiating its larval niche from that of *P. bellargus*. Further, the specificity of mutualistic interactions with different ant species will be investigated in both species of butterfly, to test whether ants play a role in differentiating the niche of each species and to explore the trade-offs in fitness that may result from interacting with ants. Finally, again with a focus on comparing the ecology of these two species, the genetic structure of their population within the same fragmented landscape will be explored for the first time.

The following four questions will be addressed, each comprising a separate chapter aimed at better understanding the ecology and informing the conservation of these two species in Britain, and more broadly of other specialist species in Europe.

Chapter 2 - Has the narrow larval niche of *P. bellargus* expanded in response to climatic warming?

Chapter 3 - Do *P. bellargus* and *P. coridon* differ in their larval microhabitat preferences?

Chapter 4 - Does myrmecophily play a role in defining and differentiating the larval niches of *P. bellargus* and *P. coridon*?

Chapter 5 - Does population genetics indicate differences in the population structure of *P. bellargus* and *P. coridon*?

**Chapter 2 - Accounting for habitat when
considering climate: has the niche of the Adonis
blue Butterfly changed in the UK?**

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2.1 Abstract

The dramatic recovery of three species of grassland specialist butterfly threatened with extinction at their high latitude range limits in the 1980s has been attributed to two factors; increased grazing on calcareous grassland sites and warmer air temperatures. Both result in the warming of soil surface temperatures, favourable to the larvae of these species. We address the influence of both of these factors on the habitat usage of the butterfly *Polyommatus bellargus*, undergoing recovery at its northern range edge. We test the hypothesis that the larval niche of *P. bellargus* has become less constrained in the past three decades, whilst controlling for changes in habitat structure. Once habitat change has been accounted for we find no evidence for a broadening of the larval niche of *P. bellargus*. Further, we show that co-incident with the recovery of *P. bellargus* there have been drastic reductions in average turf height across UK chalk grasslands, but changes in air temperature have been highly variable. We conclude that changes to soil surface temperatures caused by reducing turf heights will have been a more consistent influence than air temperature increases and so habitat improvements through increased grazing will have been the major driver of recovery in *P. bellargus*. We consider the need to account for changes in habitat when exploring the impacts of recent climate change on local habitats in thermophilous species, and emphasise the continued importance of habitat management to support such species under variable local climates.

2.2 Introduction

Insects, like many small poikilotherms, respond strongly to changes in temperature, especially at higher latitudes within species' ranges, where populations are often restricted to small patches of habitat that possess microclimates warm enough for larval development (Dennis 1991; Thomas 1993). During recent decades in West Europe, two factors have influenced the ambient temperature of the received microclimates for many grassland species whose larval stages live at or near the soil surface. Firstly, climate warming has elevated spring-autumn air temperatures (Roy and Sparks 2000; Trenberth et al. 2007). Secondly, agricultural changes have directly or indirectly altered the structure of most semi-natural grasslands, transforming the shading and insulation properties of swards (Thomas 1983; Thomas 1991). The combined impact of both factors can be synergistic (Thomas et al. 2009) or opposing (Warren et al. 2001) but their relative contributions to changes in habitat use by a thermophilous species has not been quantified. The relative degree of influence that each factor has had on the quality of habitats will inform how conservation management strategies are adapted, or not, in the future.

Many ground-breeding butterflies and other insects are restricted to warm, early successional stages within ecosystems near the (cooler) climatic edges of their ranges (Thomas 1993; Thomas et al. 1999). The numerous population extinctions of UK butterfly species in semi-natural grasslands were largely attributable to a cooling of their habitat following the near universal abandonment of semi-natural swards by farmers during the first 80 years of the 20th century, exacerbated by the loss of rabbits through myxomatosis in the 1950s (Thomas 1991). The resulting taller swards lead to

cooler microclimates at ground level. For example increasing the mean sward height of a grassland from just 1cm to 5cm can reduce daytime soil surface temperatures in March-October by up to 5°C (Thomas 1983; Thomas 1993). Since the 1980s, many semi-natural grasslands in lowland England have gradually returned to close-cropped states following targeted conservation management, agri-environment schemes and the recovery of rabbit populations. For example, the re-introduction and recovery of the butterfly *Maculinea arion* (Large blue) was driven by carefully controlled (heavier) grazing regimes in selected protected areas, which support higher densities of *M. arion*'s main larval resource, the ant *Myrmica sabuleti* (Thomas et al. 2009). The grazing intensity needed to generate the threshold density of the ant host to support the butterfly had eased under the respective air temperatures of the 1970s and 2000s (Thomas et al. 2009; Thomas et al. 2011), but the relative influence of habitat management and changes in air temperature in improving habitat quality has not been addressed.

Global warming is an equally well-documented driver of change in insects, affecting phenology, voltinism, niche breadth, population stability and density, and geographic ranges (Altermatt 2010; Davies et al. 2006; Hill et al. 2002; Oliver et al. 2012; Parmesan 2006; Parmesan et al. 1999; Roy et al. 2001; Roy and Sparks 2000; Thomas et al. 2001a). Thermal constraints at higher latitudes can be relaxed, broadening the amount of habitat available to a species by enabling its larvae to occupy cooler aspects and slopes, and to exploit food plants in later successional stages; this in turn may facilitate range expansion and recovery in species that have undergone declines (Oliver et al. 2012; Thomas et al. 2001a). The role of climate change in the range expansion of the butterfly *Hesperia comma* in the UK has been extensively studied

(Davies et al. 2005; Thomas et al. 2001a; Wilson et al. 2009; Wilson et al. 2010). Davies et al. (2006) show that a key factor enabling this expansion was a widening of *H. comma*'s realized larval niche under warmer air temperatures. However, since the 1980s the habitat of many *H. comma* sites has been managed to optimise larval habitat (as defined by Thomas et al. (1986)), and so warmer temperatures and habitat management will have been acting on *H. comma* populations concurrently.

2.2.1 *Polyommatus bellargus* in the UK

Polyommatus bellargus is bivoltine in the UK and its sole larval host plant is *Hippocrepis comosa*, with the offspring of the second adult generation overwintering as larvae. Between 1950 and the 1980s, 70% of *P. bellargus* colonies in the UK had gone extinct and its range had retracted by 42% (Thomas 1983; Warren et al. 1997). Since receiving Priority Species status in the UK Biodiversity Action Plan in the 1990s, the number of UK colonies of *P. bellargus* has increased fourfold compared to its nadir in the 1980s, including a 30% range increase in the past decade; consequently in 2006 its national status was downgraded to a UK Biodiversity Action Plan Species of Conservation Concern (Asher et al. 2001; Bourn and Thomas 2002; Fox et al. 2006).

The recovery of *P. bellargus* coincided with the restoration of grazing to many former sites, but it has also been suggested that a warmer climate has broadened its realized niche (Asher et al. 2001; Fox et al. 2006; Roy and Thomas 2003). A seasonal switch exists between the niches occupied by successive generations of larvae, with the summer-feeding cohort exploiting *H. comosa* plants growing in 2-7cm tall swards (in 2000), and the autumn/spring-feeding larvae constrained to those growing in short

turf (<3 cm) within sheltered depressions. These microhabitat constraints ensure a warm microclimate but also impose an annual population bottleneck on overwintering generations (Roy and Thomas 2003). A broadening of the realized niche of this second, temperature-constrained generation might therefore have contributed to the UK recovery of *P. bellargus*. Here we explore the hypothesis that the larval niche of the second, more constrained generation of *P. bellargus* has become broader in its habitat preferences than in previous decades, whilst accounting for changes in habitat structure that have occurred. Further we consider how both air temperatures and habitat structure have changed within the study region, and how this might explain their respective influence on the habitat usage by *P. bellargus* in recent decades.

2.3 Materials and methods

All analysis was carried out in the statistical computing package R, version 2.15.2 (R Core Development Team, 2012).

2.3.1 Study sites

In 2009 three nature reserves in Dorset, UK, were selected to record the oviposition preferences of second-generation *P. bellargus* females (Ballard Down, Swanage (SZ0281); Fontmell Down, Shaftesbury (ST8817); Five Marys, East Chaldon (SY7884)). These are managed nature reserves of calcareous grassland on south facing slopes known to sustain stable populations of *P. bellargus*. These sites were selected because historical data on the oviposition preferences of *P. bellargus* exist for Ballard Down in 1981 (J. A. Thomas, unpublished data) and for Five Marys Down in 2000 (Roy and Thomas 2003).

2.3.2 Oviposition preferences of *P. bellargus*: 1981 and 2009

Stratified random sampling was used for both data sets; to make sure that the different habitat structures within each site were sampled proportionally. Prior to sampling each site was surveyed and divided into broad areas based on the habitat structure, classed as short turf, long turf or scrub. Sampling of host plants was then conducted at random within each stratum, with the search time being proportional to its area within the site.

In late August and early September 1981 data were collected on Ballard Down using stratified, random egg searches across the site. For each *H. comosa* plant sampled the turf height (see Stewart et al. (2001)) and degree of shelter were recorded. Shelter was defined as one of three categories: (1) open downland with even, unsheltered terrain; (2) limited shelter from tracks, scrapes and depressions; and (3) complete shelter in tracks, scrapes, hoofprints and similar depressions (after Thomas (1983)). The number of eggs found on each plant was also recorded. To account for the zero inflation in the data set a hurdle model was employed for the analysis, as recommended by Zuur et al. (2009), and was implemented using the VGAM package in R (Yee 2012). This is a two step modelling processes, whereby a glm with binomial errors is used to model the data set as presence-absence, to examine the cause of the zeros. A second model is then run which does not use the zeros in the data set, utilising a zero truncated, negative-binomial error distribution to account for overdispersion.

Data for 2009 were collected in early August to mid September, the flight period of the second generation of *P. bellargus*. For each site host plants were first sampled

from the entire population using stratified random sampling, to describe the available population of host plants. Plants that had been oviposited on were at first sampled by following ovipositing females and recording plants on which they selected to lay eggs (after Roy and Thomas, 2003). However this technique proved time consuming, and so to ensure adequate data were collected stratified egg searches were used. A quadrat was thrown to a random point within each stratum and *H. comosa* plants found within it were searched for eggs, and the search was terminated when an egg was found or all plants had been searched. These searches were repeated in all strata for 15 - 30 minutes at a time depending on the size of the strata, and this was repeated as necessary until around 50 plants with eggs on had been located. For each plant the variables of turf height (cm) and shelter category were recorded, along with the percentage cover of bare ground and host plant using a 25cm x 25cm quadrat. Due to differences in topography between sites, as a matter of caution the shelter categories 2 and 3 were amalgamated, to avoid any false distinctions between sites. The two shelter categories were recorded as (1) open downland with even, unsheltered terrain (2) shelter from tracks, scrapes and depressions (adapted from the shelter categories of Thomas (1983)). Bare ground was recorded as it increases the temperature of the surrounding soil, and is specifically recommended as favourable to *P. bellargus* in the Biodiversity Action Plan (Bourn and Warren 1998).

A total of 146 *P. bellargus* eggs were observed across the three field sites (around 50 per site), with 37 of these located by observing the oviposition events and 109 by stratified egg searches. Data were also collected for 174 random plants, split approximately equally between sites, again using stratified randomly thrown quadrats to select each plant sampled and collecting data for only one plant per quadrat. A

presence only logistic regression model was used to estimate the effect of turf height and shelter category on oviposition preferences. This utilised the expectation maximisation method of Ward et al. (2009), which accounts for the fact that although the presence of eggs recorded in this data set are true presences, the random plants represent pseudo-absences, in that eggs were present on some of these plants. The degree of contamination with eggs in the random host plant population was estimated to be 4 in 100 plants by checking each host plant sampled for eggs. The expectation-maximisation procedure utilises this estimate to adjust a logistic regression model to provide more reliable parameter and probability estimates (Ward et al. 2009). Checks for co-linearity between explanatory variables, using variance inflation factors (VIFs) revealed that no variables were correlated enough to cause concern, as all VIF values were less than two 2 (following Zuur et al (2009)).

2.3.3. The effect of year on oviposition preferences

Two analyses were carried out to assess if the oviposition preferences of *P. bellargus* had altered with regard to the turf height and shelter category; one comparing preferences on Ballard Down in 1981 with those in 2009 and another comparing preferences on Five Marys in 2000 with those in 2009.

Oviposition data for Five Marys in 2000 were collected from observations of ovipositing females. Turf heights and shelter categories were recorded for these plants. Stratified sampling of random plants for turf height and shelter category was also carried out, for a total of 700 plants from the available host plant population.

Two problems had to be overcome in analysing these dataset pairings.

1. *The 1981 data set being a true presence absence dataset:* This data was made comparable to the 2009 sampling regime by generating a random population of host plants through sampling from the entire 1981 dataset 200 times, with replacement. As the 1981 dataset represents a random sample of the original host plant population, this provided an equivalent of the random host plant datasets collected in 2009.
2. *Changes in habitat usage being confounded with changes in habitat structure:* Comparing single year models of oviposition preferences or running a logistic regression with year as a categorical variable would not distinguish as to whether this was due to changes in habitat structure or climate. To overcome this a methodology was used that would distinguish between change that resulted from habitat change and that caused by year. The proportion of plants falling into turf height – shelter category combinations was calculated to create a response variable that was standardised across years. Plants were allocated to a class based on their turf height value (1-15) and their shelter category (1 or 2). For the 1981 and 2001 shelter categories 2 and 3 were amalgamated into one category to make them comparable with the 2009 data set. For each site, within each year, the proportion of all egg bearing plants falling into each class was then derived (PE). The same process was also carried out for the random plants sampled, to give the proportion of plants for the population of a whole site that fell into the categories represented for egg bearing plants (PR).

These data were then analysed using a linear model defined below, with PE representing the proportion of egg carrying plants falling into each category, PR

representing the proportion of random plants falling into each category and Yr representing year.

$$\text{arcsine-square root(PE)} = \text{PR} + \text{Yr}$$

An arcsine square root transformation was applied to PE to normalise the distribution of the residuals. The null hypothesis is that the variance in PE is explained by PR, that is the variation in plants selected for laying is explained purely by the availability of plants within the larger population. The addition of year (Yr) to the model would show whether year explains any residual variation in oviposition preferences, so indicating if a shift in oviposition preferences has occurred over the intervening decades.

2.3.4 The effect of turf height and shelter on the temperature of the soil underneath host plants

In October 2011, while the second instar larvae were still feeding between resting at the base of the plant or in soil crevices below it, the soil temperature was measured underneath 102 *H. comosa* plants. Measurements were made on Ballard Down over two days (40 plants on 01-Oct-2011 and 62 plants on 02-Oct-2011), along with the ambient air temperature and the surrounding turf height and shelter category. All measurements were made under clear sky and low wind conditions, between 13.00 and 15.00 the first day and 13.30 and 15.30 on the second day. Soil temperature was measured at a depth of 5mm under the soil surface and the probe (EcoScan Temp 5, Eutech Instrumental, ± 0.5 accuracy) was left there until the temperature reading became stable. Ambient air temperature was measured by placing an air temperature probe (E.T.I. ltd, Precision Thermometer, ± 0.5 accuracy) approximately 10cm above the soil surface, left to stabilise for the duration of taking 10-15 soil readings. The

soil-ambient temperature difference was calculated (soil temperature – ambient temperature). Initial model fitting with a quadratic term for turf height suggested that the relationship was non-linear and so a generalised additive model was employed to fit a non linear trend in the final model, using the mgcv package in R (Wood 2011).

2.3.5 Changes in turf height structure on *P. bellargus* sites

Surveys of the sward structure of 48 calcareous grassland sites in Dorset were made in 1979 and again in 1998-99. For each site turf height was measured at 30 random points, with stratified sampling where necessary and the mean turf height was calculated. A pairwise t-test was used to examine whether turf heights for 1979 were significantly greater than in 1998-99. Both data sets were square root transformed to achieve normality prior to analysis. The turf heights of Ballard Down in 1981 and 2011, derived from all plants searched for eggs in 1981 and recordings of turf structure across the site in 2011, were tested with a Mann-Whitney U test to examine if turf height in 2011 was significantly shorter than in 1981.

2.3.6. Changes in temperature in the Dorset region

Soil surface temperature reflect those experienced by caterpillars at the host plant level and are known to be dependent on air temperatures, with higher air temperatures resulting in a warmer surface temperature (Morecroft et al. 1998). Mean temperature data for August and September, the months in which *P. bellargus* fly and oviposit, for the Dorset region was taken from the UK MET office (the National Weather Service) data from the Hurn weather station from 1970 onwards, which is approximately 30 miles away from each of the sites. A linear model was as used to assess if mean temperatures in August and September, when adults select the plants for their

autumn/spring-feeding larvae, had increased significantly over the past 40 years in the local region. This would in turn imply that soil surface temperatures would also have increased on average.

2.4 Results

2.4.1 Oviposition preferences in *P. bellargus*

1981 Zero adjusted model

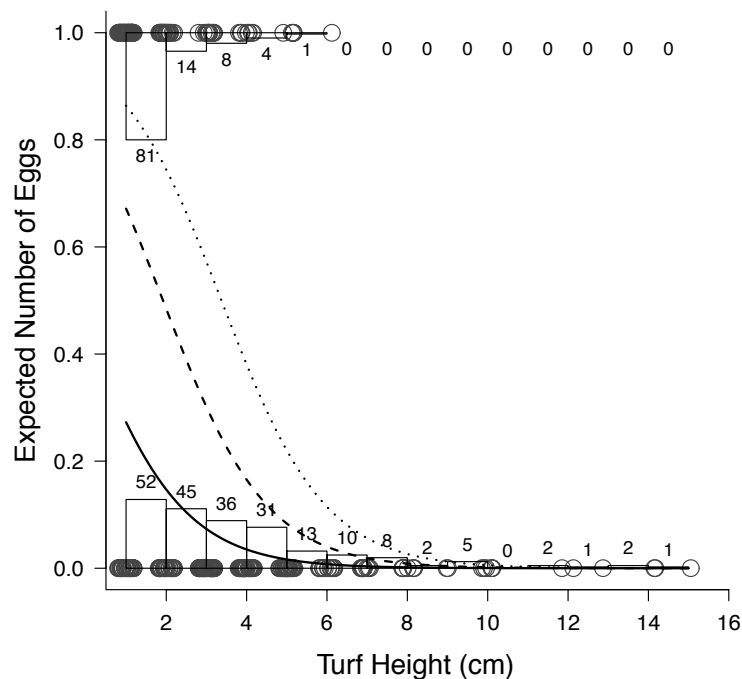
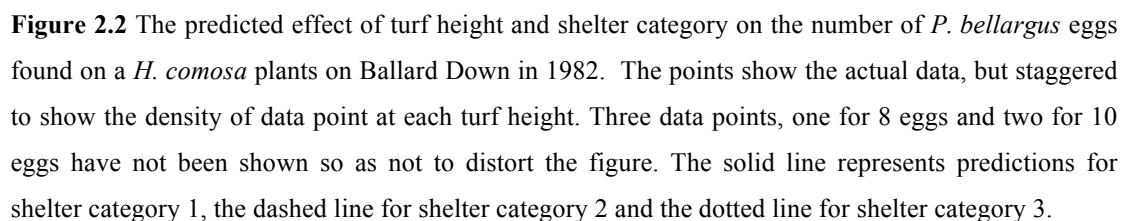


Figure 2.1 The logistic regression curve for the probability of finding *P. bellargus* eggs on a *H. comosa* plant on Ballard Down in 1981. The solid line represents predictions for shelter category 1, the dashed line for shelter category 2 and the dotted line for shelter category 3. Shelter categories are as follows: (1) open downland with even, unsheltered terrain; (2) limited shelter from tracks, scrapes and depressions; and (3) complete shelter in tracks, scrapes, hoofprints and similar depressions. The top histogram shows the number of plant with eggs present found in each turf height and the bottom histogram show the number of plants found without eggs present found in each turf height.

For the presence – absence component of the model the main effect terms for both turf height and shelter were significant predictors of oviposition, explaining around 36% of the variation in oviposition preference ($\chi^2_{(3,312)} = 147.12$, $p < 0.001$). Turf height accounted for around 62% of the deviance explained ($\chi^2_{(1,314)} = 91.295$, $p < 0.001$), with the probability of oviposition decreasing with an increase in turf height ($\beta = -0.778 \pm 0.117$). The probability of oviposition increased with an increase in shelter, with shelter category 2 having a significantly higher probability of oviposition than shelter category 1 ($Z_{(1,313)} = 3.917$, $\beta = 1.695 \pm 0.433$, $p < 0.0001$), as did shelter category 3 ($Z_{(1,313)} = 6.541$, $\beta = 2.827 \pm 0.432$, $p < 0.0001$) (Figure 2.1).

For the zero truncated component of the zero inflated model the main effects of turf height and shelter were significant predictors of the number of eggs found on a plants (LogLik = -143.82, $\chi^2_{(3,211)} = 16.975$, $p < 0.001$). An increase in turf height cause a decrease in number of eggs found on a plant ($\beta = -0.448 \pm 0.149$, $z_{(1, 105)} = -3.009$, $p < 0.01$). An increase from shelter category 1 to shelter category 2 did not have a significant effect on the number of eggs found on a plant ($\beta = 1.056 \pm 0.699$, $z_{(1, 104)} = 1.511$, $p = 0.131$), but an increase to shelter category 3 significantly increased the number of eggs laid ($\beta = 1.481 \pm 0.674$, $z_{(1, 103)} = 2.199$, $p < 0.05$). (Figure 2.2).



For 2009, the additional variables of bare ground and *H. comosa* cover were removed from the model in a stepwise fashion as they were not significant predictors of egg presence. This permitted 105 extra random plants with records only for turf height and shelter category to be added to the data set to improve the statistical power of the model, and the model re-run with just these variables. This model explained 23% of the variation in oviposition preference (pseudoR² = 0.23, $\chi^2_{(6,400)} = 127.0$, p<0.0001), with turf height again having a negative effect on the probability of a plant bearing an egg ($\beta = -1.026 \pm 0.136$, $z_{(405)} = -7.5$, p<0.001) and accounting for the majority of the deviance explained ($\chi^2_{(1,405)} = 57.4$, p<0.001) (Figure 2.3). Shelter category was not a

significant predictor of oviposition preferences for Ballard Down ($\beta = -0.4671 \pm 0.4784$, $z_{(402)} = -0.9$, $p = 0.329$) (Figure 2.3a), but the interaction term between shelter category and site showed that shelter category two increased the probability of oviposition on Fontmell ($\beta = 1.590 \pm 0.695$, $z_{(401)} = 2.2$, $p < 0.022$), and Five Marys downs ($\beta = 1.480 \pm 0.650$, $z_{(400)} = 2.3$, $p = 0.023$) (Figures 2.3b and 2.3c). Though significant the overall explanatory power of shelter was much lower than that of turf height ($\chi^2_{(2,400)} = 6.9$, $p = 0.0305$).

2.4.2 Niche expansion in *P. bellargus*

The minimum adequate model for the years 1981 and 2009 (Ballard Down) contained only PR (the proportion of plants within the overall population in each turf-shelter category selected for egg laying), as year was not a significant predictor of PE ($\beta = 0.001 \pm 0.003$, $t_{(15)} = 0.6$, $p = 0.583$). Increased representation of plants in a given turf height–shelter category in the host plant population increased the proportion of plants with eggs found in that category ($\beta = 1.999 \pm 0.589$, $t_{(16)} = 3.4$, $p = 0.004$), explaining around 38% of the variation in PE ($\text{AdjR}^2 = 0.38$, $F_{(1,16)} = 11.5$) (Figure. 2.4a).

The model for data from the years 2000 and 2009 provides a similar picture with year removed from the model as it was not a significant predictor of PE ($\beta = -0.008 \pm 0.005$, $t_{(39)} = 0.4$, $p = 0.725$). The final model explained 30% of the variation in PE ($\text{AdjR}^2 = 0.30$, $F_{(1,10)} = 5.7$, $p = 0.038$) with PR being a significant, positive predictor of PE ($\beta = 2.195 \pm 0.362$, $t_{(39)} = 7.9$, $p < 0.0001$), (Figure 2.4b).

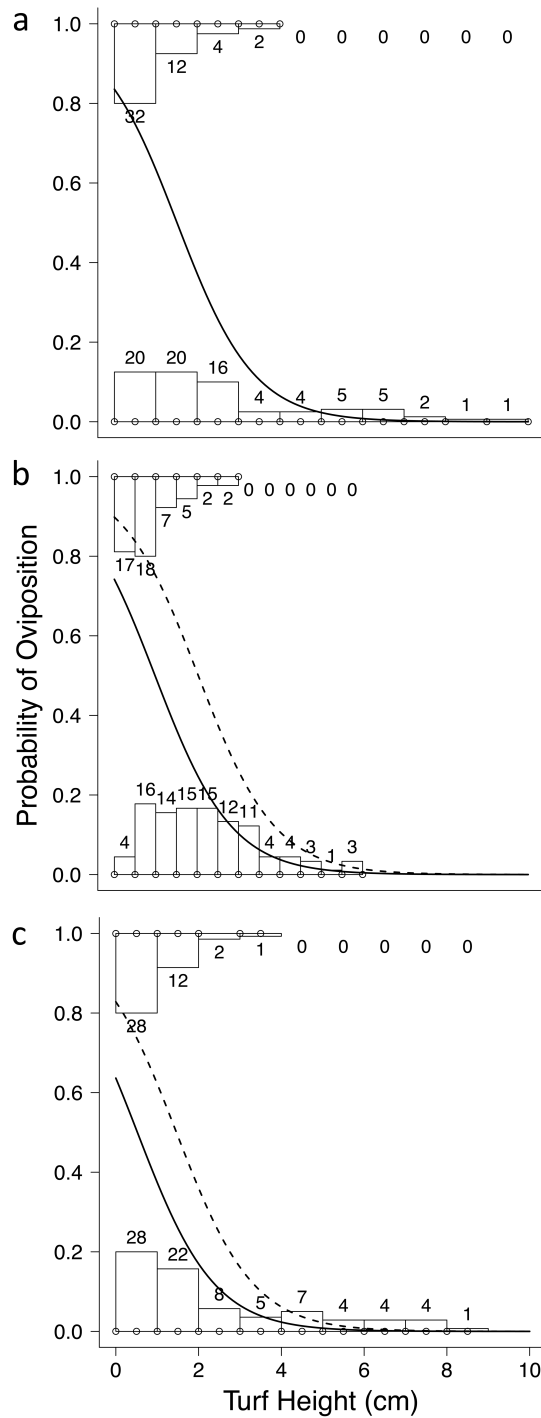


Figure 2.3 Logistic regression curves for the probability of a plant having *P. bellargus* egg upon it in relation to height of the turf surrounding it, in 2009, for a) Ballard Down b) Fontmell Down and c) Five Marys. The histograms across the top and bottom represent the turf height distribution of plants with eggs on and the turf height distribution of random plants within the host plant population respectively. The solid line represents predictions for shelter category 1 and the dashed line for shelter category 2.

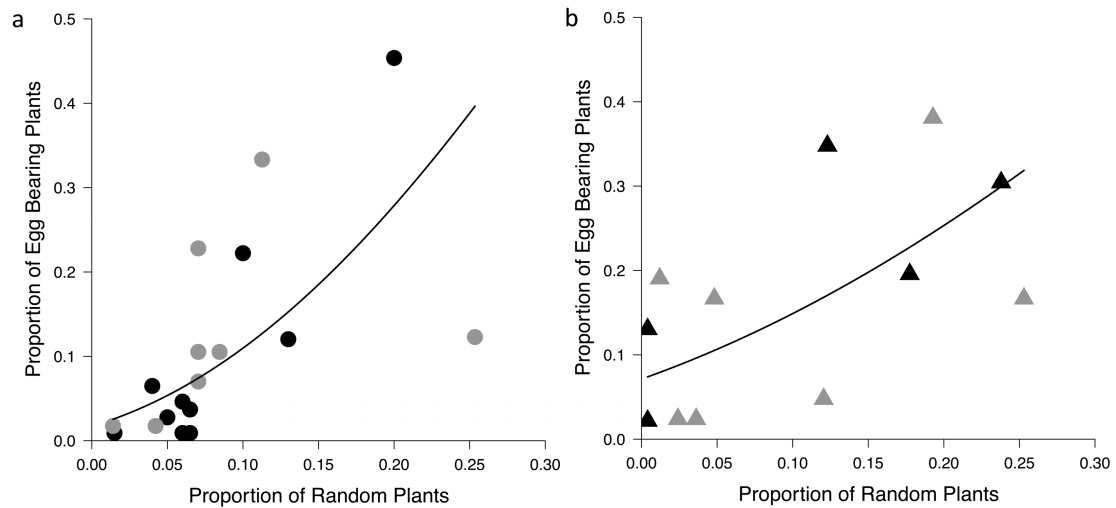


Figure 2.4 The relationship between the proportion of egg bearing plants falling into turf height-shelter category and the estimated proportion of plants in the host plant population falling into this category for a) Ballard Down from 1981 – 2009 and with black circles representing 1981 and grey circles representing 2009, and b) Five Marys Down from 2001 – 2009, with black triangles representing 2001 and grey triangle representing 2009. The predicted values have been square root-arcsine back transformed.

For both of these analyses, year was also fitted as a categorical variable, as differences between years may not follow a linear trend, but this did not alter the significance of the term in either the model.

2.4.3 The effect of turf height and shelter on the temperature of the soil underneath *H. comosa* plants

The final model included terms for turf height, shelter category and day, explaining around 45% of variation in air-soil temperature difference ($\text{AdjR}^2 = 0.45$). Turf height was a significant, negative predictor of air-soil temperature difference ($F_{(2, 99)} = 28.5$, $p < 0.001$) (Figure 5). Shelter category explained a significant amount of variation in temperature difference model ($F_{(1, 98)} = 6.4$, $p < 0.05$), with an increase in shelter increasing the difference between soil and air temperature ($\beta = 1.078 \pm 0.424$) (Figure

2.5). Recording day was also significant ($F_{(1,97)} = 6.3$, $p < 0.05$), with temperature difference increasing between days ($\beta = 1.044 \pm 0.417$). This difference between days is likely due to the fact that on the second day (October 2nd) the temperature records were begun 30 minutes later in the day, meaning the ground will have been more heated.

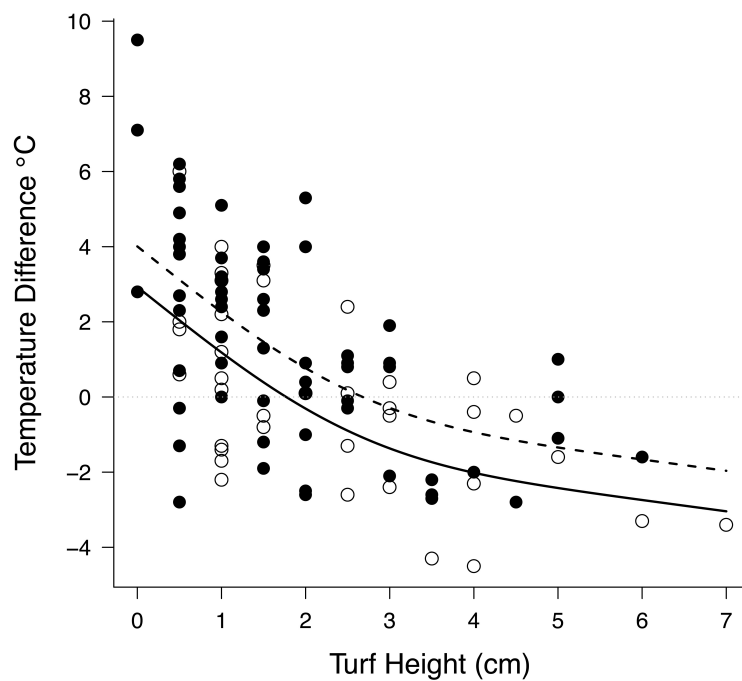


Figure 2.5 The predicted effect of turf height on soil temperature relative to air temperature under *H. comosa* plants on 1st and 2nd of October 2011 on Ballard Down, from a Generalized Additive model. Soil temperature was measured with the probe tip 5mm below the soil surface and air temperature was measured at 10cm above the soil surface. The solid line represents shelter category 1 (open downland with even, unsheltered terrain) ($n=38$) and the dashed line shelter category 2 (shelter from tracks, scrapes and depressions) ($n=64$) and points representing the original data. The points are the original data, with clear circles representing shelter category 1 and black circles representing shelter category 2. The coefficient for day was kept at the average temperature difference between days (0.521°C) to represent the intermediate scenario.

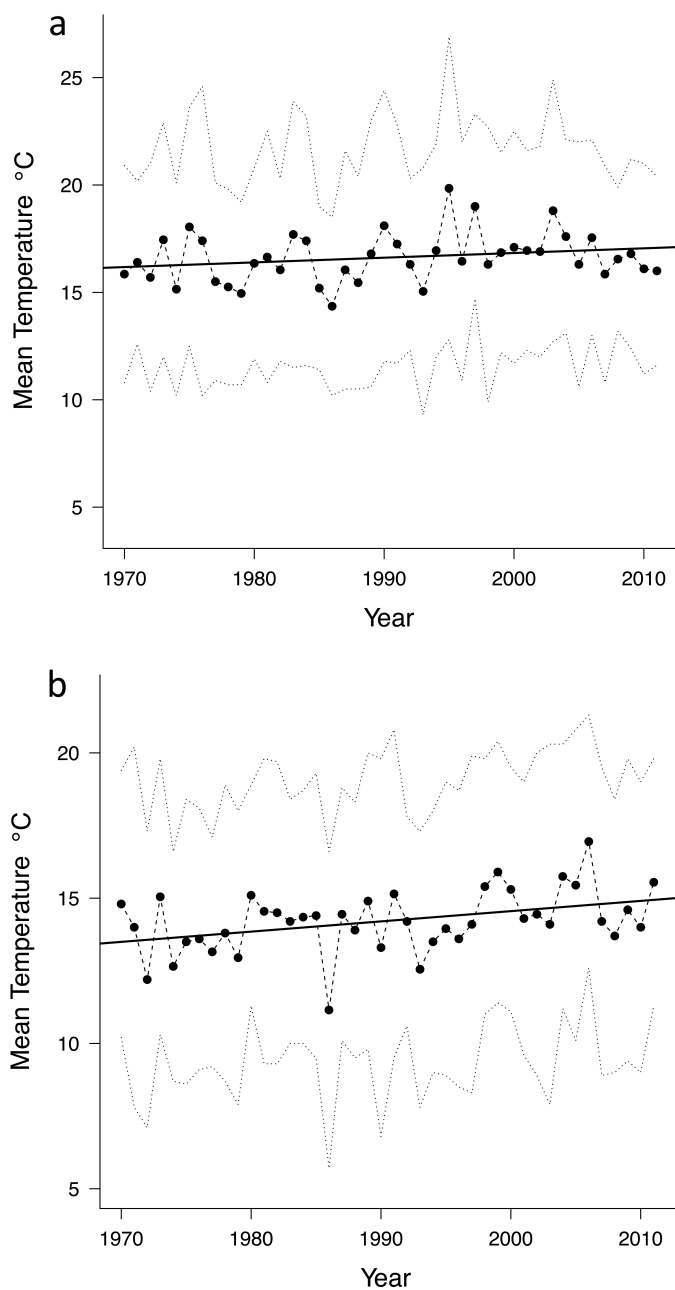


Figure 2.6 Mean temperatures in Hurn, Dorset, from 1970 – 2010 for a) August and b) September, showing the line of best fit as predicted by a linear model and the maximum and minimum temperatures as dotted lines above and below the mean values. The model for August temperatures was not significant ($F_{(1,39)} = 2.3$, $\text{Adj}R^2 = 0.031$, $p = 0.137$) and the model for September temperature showed a significant increase in temperatures ($\text{Adj}R^2 = 0.142$, $F_{(1,39)} = 7.7$, $p = 0.008$).

2.4.4 Changes in sward height of calcareous grassland sites in Dorset

The average sward height of sites in 1979 was significantly taller than in 1998-99 (1979 $\bar{x} = 20.75 \pm 1.76\text{cm}$, 1998-99 $\bar{x} = 4.92 \pm 0.33\text{cm}$, $t_{(47)} = 11.4$, $p = <0.001$). The turf height on Ballard down in 1981 was also significantly taller than in 2009 (1981 $\bar{x} = 3.42 \pm 0.14\text{cm}$, 2009 $\bar{x} = 2.197 \pm 0.11\text{cm}$, $W = 28684.5$, $p < 0.001$).

2.4.5 Changes in air temperature in the Dorset region over four decades

Year did not explain a significant amount of variation in mean August temperature over the past four decades ($\text{AdjR}^2 = 0.031$, $F_{(1,39)} = 2.3$, $p = 0.137$). For September, year explained around 14% of the variation in mean temperature ($\text{AdjR}^2 = 0.142$, $F_{(1,39)} = 7.7$, $p = 0.008$), with average temperature increasing with year ($\beta = 0.035 \pm 0.012$) (Figure 2.6).

2.5 Discussion

2.5.1 The larval niche of *P. bellargus*

It has been speculated that a warmer climate has broadened the larval niche of *Polyommatus bellargus* in the UK, and that habitat management practices for this species may need to change as a result (Asher et al. 2001; Fox et al. 2006; Roy and Thomas 2003). However, once changes in the availability of host plants in optimal microhabitats over the past three decades have been accounted for there is no evidence to suggest an expansion in the realized niche of *P. bellargus* in the UK.

Historical turf height data for 48 calcareous grassland sites in the UK clearly shows large changes in habitat structure, between 1979 and 1998-99, with turf height across sites being reduced by 15cm on average. These reductions will have resulted in warmer microclimates at the host plant level; we found that a decrease in turf height from 3cm to 1cm could increase the temperature of the soil (and larval niche) below a plant by around 2.0°C, but turf heights above 3cm will result in soil surface temperatures cooler than that of the air. Parallel air temperature increases in the region over the past 40 years (significant for September but not August) show high levels of inter-annual variation, meaning a warming effect has not been constant across the years. For instance the mean September temperature for 1981, 2000 and 2009 were 14.6°C, 15.3 and 14.6°C respectively. A lack of change in the realized niche of *P. bellargus* is consistent with the variability in the magnitude of temperature differences between years. In some years warmer temperatures certainly may have relaxed the microhabitat constraints on *P. bellargus*, but the inter-annual variability will make a consistent effect upon habitat availability, and so populations, unlikely. The changes in microclimate caused by increased grazing pressure on sites will therefore have been larger and, where management has been sustained, a more consistent influence on microclimate than those caused by rises in air temperature.

It must be acknowledged that the reduction in turf height over the decades makes the prospect of observing a shift in the niche of *P. bellargus* more remote. The number of host plants found in tall turf will have been reduced, which in turn reduces the opportunities available for females to oviposit in longer turf. *Polyommatus bellargus* may also have responded to climate change in other ways that would benefit its populations, such as earlier emergence and longer flight periods (Altermatt 2010; Roy

and Sparks 2000). Our results also show the influence of shelter category on oviposition was less than that of turf height, especially in 2009, and for Ballard Down in 2009 it was not a significant predictor of egg presence. The influence of shelter may be related to local wind speeds and topographies, and further data on soil temperatures at different sites are required to explore this further.

Overall our results suggest that periods of heavy grazing of calcareous grassland, to maintain areas of short turf, should remain a priority in the management of habitats for *P. bellargus*. This will serve to ensure the availability of suitably warm microhabitats in the face of variable temperatures, and will be particularly important in maintaining populations in cooler regions of the species' range (Lawson et al. 2012) and cooler than average years. These findings also show that when considering the impacts of climatic warming on species we must be careful to consider the actual magnitude of local temperature changes. Global temperatures have undoubtedly risen in recent decades, altering the ecology of many systems (Parmesan, 2006), but the degree of warming has been variable between regions and across time and this should be accounted for. Further, changes to a species' environment that have occurred concurrently with climate change, such as those influenced by management, should be controlled for. If we had compared the single models of the 1981 and 2009 oviposition preferences we may have concluded that larval niche of *P. bellargus* had become less constrained, as the importance of turf height and shelter explained 36% of variation in oviposition preferences in 1981, but only 22% in 2009. By controlling for alteration in the structure of microhabitats over the decades, we show that the influence of turf height on habitat preferences has been reduced not because longer turf is more suitable, but because turf heights are on average much shorter. This is

particularly important if habitat management protocols for a species are modified, as has been suggested for *P. bellargus*, because taking the wrong course of action could be detrimental.

2.5.2 Broader implications

Mair et al. (2012) found that in the past two decades there have been significant increases in the range expansion rates of specialist butterfly species, without significant changes in air temperatures. This corroborates our findings and suggests that habitat management is not only helping populations locally, but facilitating larger scale processes such as colonisation. Others have suggested that the importance of local habitat quality in generating connectivity in landscapes has been under emphasised recently, both as a tool for conservation and with regard to facilitating range expansion in itself (Hodgson et al. 2009; Lawson et al. 2012). Indeed, metapopulation models of the expansion of *H. comma* suggest that an increase in the quality of habitat patches (i.e. nature reserves) has facilitated its population increase and range expansion (Wilson et al. 2009). From this perspective, more work considering the role that habitat change within nature reserves has played in species undergoing range expansion would be valuable.

We have demonstrated that changes to the sward structure of grassland sites has made habitats more favourable to *Polyommatus bellargus*, but the relationship between management activities and population change has yet to be quantified in the way that it has for *Maculinea arion* (Thomas et al 2009). Future work to examine how populations of *P. bellargus* have responded to the management regimes on nature reserves across its range would be useful and this could be extended to the impacts of

management for thermophilous species on the populations of other butterfly species. *Polyommatus bellargus* is in a minority with regard to its recovery; 72% of UK butterfly species show a continued decline in numbers on monitored sites over the past decade and 54% show reductions in the extent of their UK distribution (Fox et al. 2011a; Thomas et al. 2004). The intensive grazing that encourages species such as *P. bellargus* and *H. comma*, can decrease the suitability of habitat for butterflies and other insect species (Davies et al. 2007; Kruess and Tscharntke 2002b; Turner et al. 2008). Research into the impacts of recent targeted habitat management on the populations other species would help in creating management objectives for habitats that accommodate the requirements of different species.

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**Chapter 3 – The role of microhabitat
preferences in defining the larval niches of
Polyommatus bellargus and *Polyommatus
coridon***

3.1 Abstract

Polyommatus bellargus and *Polyommatus coridon* share much in common, both being specialists of calcareous grassland and using the same host plant (*Hippocrepis comosa*) and ants (*Myrmica sabuleti*, *Lasius niger* and *Lasius flavus*) as larval resources, though differing in phenology and voltinism. In this chapter the role of the larval niche in differentiating the ecology of these species is explored. Data on the oviposition preference of both species, from the 1980s and the present, are used to test the hypothesis that *P. coridon* is less constrained by microhabitat in the *H. comosa* plants it selects for oviposition than *P. bellargus*. It is found for a site occupied by both species in the 1980s, that both species show a preference for *H. comosa* plants found in shorter turf and higher shelter but this is far more pronounced in *P. bellargus*. Data for 2011, collected at separate sites for each species, found that *P. bellargus* selects for *H. comosa* plants found in short turf and high shelter; whereas neither of these were of significance for *P. coridon*. Further, it is shown that the amount of host plant and bare ground are not significant determinants of oviposition for either species. Preliminary data for both species, from oviposition preferences in *P. bellargus* and larval associations in *P. coridon*, also suggest that neither species show a preference for a particular ant species. The role that the thermal requirements of *P. bellargus* and *P. coridon* over wintering phases may play in the driving these differences is discussed, along with the implications this may have for the conservation actions required for each species.

3.2 Introduction

3.2.1 Same, same but different

Polyommatus bellargus and *Polyommatus coridon* occur at the northernmost edges of their geographical distributions in the south of England (Asher et al. 2001). Molecular data suggest that they belong to two (of three) distinct clades within the *Lysandra* sub-genus, which separated around 1.45Mya (Talavera et al. 2013). Both are restricted to calcareous grasslands, and in England utilise the same (sole) larval host plant, *Hippocrepis comosa*. Also, larvae and pupae of both species are attended by mutualistic ant species (*Myrmica sabuleti*, *Lasius niger* and *Lasius flavus*). There is a large overlap in the distribution of the two species in England, though *P. coridon*'s extends further north (and within Europe the distribution of *P. bellargus* extends further south). *Polyommatus coridon*'s flight period is July – August, and it overwinters as an egg which hatches the following Spring (March-April) (Thomas and Lewington 2010). *Polyommatus bellargus* is bivoltine, having two generations a year, the first flying in April – May and the second flying August – September. The offspring of the second generation of *P. bellargus* hatch in September and overwinter in their second instar, becoming active again the following Spring (March – April). In short, these two closely related species occupy the same range and utilise the same habitat, but differ in their voltinism and phenology. This chapter will explore whether differences in the larval niche of each species serve to further separate their ecology.

3.2.2 Oviposition preferences and the larval niche

For many Lepidoptera (and phytophagous insects in general), the larval stages (at least in initial instars) show low mobility, and as such the conditions in which eggs

are laid will have fitness consequences for their larvae, and adults stages also (Garcia-Barros and Fartmann 2009; Mayhew 1997; Renwick and Chew 1994). There will then be strong selective pressure on females to oviposit in habitats that contribute to higher fitness in later stages, and therefore the oviposition preferences of female Lepidoptera can be very informative as to the habitat requirements of the larval stage. Studies of several temperate butterfly species (including *P. bellargus*) have shown that females restrict oviposition to a sub-set of host plants that fall within suitable microhabitats and microclimates for larval development or survival (Anthes et al. 2008; Anthes et al. 2003; Eilers et al. 2013; Garcia-Barros and Fartmann 2009; Thomas 1983; Thomas et al. 1986). Females may select for larger host plants, a particular growth form or nutritional content (which may be interrelated), and can have preferences for particular microhabitats and microclimates (Dolek et al. 1998; Eilers et al. 2013; Garcia-Barros and Fartmann 2009; Pennekamp et al. 2012; Rabasa et al. 2005; Wiklund 1984). Thus, from a resource-based perspective (Dennis 2010; Dennis et al. 2006) the functional resource that defines the niche of such species is not the abundance of the host plant, but host plants occurring in optimum conditions (Thomas et al. 2011). It follows that two species that use the same ecosystem and resources within it might be differentiated if they utilise a shared resource in functionally different ways. For example, two species that share the same habitat (peat bogs) and host plants in Belgium, *Lycaena helle* and *Proclossiana eunomia*, are differentiated not only by their phenologies but also by their functional use of the habitat as both larvae and adults (Turlure et al. 2009). Larvae of *P. eunomia* are found in cooler microclimates than those of *L. helle* and require higher host plant abundance, and its adults nectar only on the flowers of the larval host plant as opposed to the 24 different species used by adults of *L. helle*.

The larval niche of *P. bellargus* in the UK is defined by host plants growing in short turf and high shelter, and the abundance of host plants in optimal conditions is a major determinant of the habitat quality for this species (O'Connor et al. 2014; Roy and Thomas 2003; Thomas 1983; Thomas et al. 2001b). Here it is explored whether the microhabitats utilised by *P. bellargus* and *P. coridon*, as defined by their oviposition preferences, serve to differentiate their larval niches. Using historical and new data on oviposition preferences for both species, the hypothesis that the larval niche of *P. coridon* is less constrained by warm microhabitats is tested, along with the influence of host plant abundance. It is predicted that plants selected by *P. coridon* will be found in a broader range of turf heights, degree of shelter and less constrained by bare ground, and that both species will preferentially oviposit in areas of high *H. comosa* coverage.

Given that *P. bellargus* and *P. coridon* are both tended by ants as larvae and pupae, the species and abundance of ants may also act as an important functional resource for the larvae and so may also influence oviposition preferences. The influence of mutualistic ant partners on oviposition has been explored in a few Lycaenid species, although the majority of studies involve obligate myrmecophiles (Pierce et al. 2002). For instance, both in experiments and field observations, females of the obligate mutualist *Jalmenus evagoras* select host plants for oviposition with specific ant species (Pierce and Elgar 1985; Smiley et al. 1988). However, for *Maculinea* species, most evidence suggests the presence of ants is unimportant for host plant selection once host plant quality is accounted for (Nowicki et al. 2005; Thomas and Elmes 2001; van Dyck et al. 2000). Two studies of facultatively mutualistic species (for

which larval survivorship was positively influenced by mutualistic ant species) have been made to date; the presence of mutualistic ants had no effect on oviposition for *Glaucopsyche lygdamus*, but *Hemiargus isola* laid higher numbers of eggs on inflorescences with ants present (Pierce and Easteal 1986; Wagner and Kurina 1997). Here, preliminary data are also presented to explore whether mutualist ant species play a role in defining the larval niches of *P. bellargus* and *P. coridon*, using data on oviposition choices by the former and the occurrence of larvae with attendant ants for the latter.

3.3 Methods

All analyses were conducted using the statistical package R (R Core Development Team, 2012). Unless specified otherwise all modelling was conducted using a backwards-stepwise procedure, with the removal of the least significant terms, until the minimum adequate model was found.

3.3.1 Oviposition on Old Winchester Hill by *P. bellargus* and *P. coridon* in the 1980s

In 1982 – 1986 the oviposition preferences of both *P. bellargus* and *P. coridon* were measured at Old Winchester Hill National Nature Reserve (SU645208; Hampshire, UK). *Polyommatus coridon* is a permanent resident of this site, whereas *P. bellargus* was re-introduced in 1981. Data collection focused on a section of grazed chalk grassland where high population densities of both *P. coridon* and *P. bellargus* were established. The hillside had been divided into 16 compartments, which were subject

to different rotational grazing regimes as part of broader study investigating the effects of management on the ecology of the site (Bacon 2013).

In September of each year, thirty 25cm x 25cm quadrats were randomly chosen in each compartment and the number of eggs of both *P. bellargus* and *P. coridon* found on *H. comosa* plants were recorded, along with the height of the turf (cm) and shelter category (1-3) (see Chapter 2 for definitions). In 1982 *P. coridon* eggs were not recorded for all plants and to account for this plants lacking a record for *P. coridon* were removed from the data set.

Two separate analyses were carried out on this data set: 1) To test whether turf height and shelter influenced the number of eggs found on a host plant and 2) To examine the differences between *P. bellargus* and *P. coridon* with regard to how important turf height and shelter are in dictating the number of eggs laid on a plant.

The first analysis explored the relationship between the egg number of both species combined with turf height and shelter category. A generalized linear model (glm), with a poisson error structure was used initially, however inspection of the residuals revealed structuring and further investigation showed that an excess of zeros in the data set and overdispersion. To account for the zero inflation a hurdle model was employed, as recommended by Zuur et al. (2009), and was implemented using the VGAM package in R (Yee 2012). This is a two step modelling processes, whereby a glm with binomial errors is used to model the data set as presence-absence, to examine the cause of the zeros. A second model is then run which does not use the

zeros in the data set, utilising a zero truncated error distribution with a negative-binomial distribution to account for overdispersion.

The aim of the second analysis was to identify differences in the degree to which microhabitat influenced the preferences of each species, utilising the contingency table analysis method outlined by Crawley (1993; 2007). The dataset was first converted into a contingency table, with each plant sampled placed into one of 5 categories for turf height (A=0-1cm, B=2cm, C=3-4cm, D=5-7cm, E=8-20cm), 3 categories for shelter (1,2,3), the presence or absence of *P. bellargus* eggs (0,1), the presence or absence of *P. coridon* eggs (0, 1) and year (1982, 1983, 1984, 1985, 1986). The frequency of plants falling into each combination of these classes (60) was then calculated and used as the response variable in a glm, with a quasipoisson error structure to correct for overdispersion. First a fully saturated model was run, including the five-way interaction for all variables. A model was then run that did not contain the five-way interaction and an analysis of deviance was used to see if the change in deviance between this and the saturated model was significant. The difference was not significant and so the five-way interaction term was removed from the model. The same process was then completed for all four way and then three-way interaction terms that included the term for *P. bellargus* presence-absence, or *P. coridon* presence-absence. Significant interaction terms were retained in the model, but non-significant terms were removed. All main effects and interaction terms that did not include a species presence-absence main effect were retained in the model regardless, in order to constrain the marginal totals. The deviance explained by the interactions of the species presence-absence main effects with other terms in the model demonstrates the relative of importance of these factors in explaining the oviposition preferences of

P. bellargus and *P. coridon*.

3.3.2 Stratified sampling of habitat for field sites in 2009 and 2011

For each site sampled for oviposition, data were also collected on its overall microhabitat structure, using stratified random sampling across the site with a 50cm x 50cm quadrat. The same data were also collected for another *P. bellargus* site, Five Marys (SY7884; Dorset, UK). For each quadrat, the percentage coverage of *H. comosa* and bare ground were estimated. Within a quadrat, turf height and shelter category measurements were taken from four random selected points within a quadrat, from which average turf height and shelter category measures were derived. Due to the time it took to collect data, stratified sampling was completed in different years for different sites: Fontmell Down and Five Marys Down in 2009; and Ballard Down, Old Winchester Hill and Magdalen Hill Down in 2011.

The four variables recorded during stratified sampling – turf height, shelter, host plant cover and bare ground – were compared between sites, using data from Five Marys (n=70) and Fontmell Down (n=124) in 2009, and Ballard Down (n=145), Old Winchester Hill (n=130) and Magdalen Hill Down (n=122) in 2011. All these data were collected in July – August, to encompass the oviposition periods of *P. coridon* and the second generation of *P. bellargus*. For turf height and shelter category, a mean value was derived from each quadrat from the four records taken. For turf height an ANOVA was used for analysis, with the data log transformed to satisfy the assumption of normality. Host plant cover and bare ground were first converted from percentage to proportion of a quadrat with cover, and analysed using separate ANOVAs after an arcsine-square root transformation to satisfy the assumption of

normality. Values for shelter category were modelled using a glm with a quasipoisson distribution, which allows for the use of non-integer data.

3.3.3 Oviposition of *P. bellargus* and *P. coridon* 2009 and 2011

The original aim had been to collect data of the oviposition preferences of both *P. bellargus* and *P. coridon* at sites on which the species co-occur. However, no study site supporting both species selected had sufficient densities of both species, so data were collected for each species at separate sites.

Egg searches

Data on the oviposition preferences of *P. bellargus* were collected for two sites in Dorset: Ballard Down (SZ 023810) and Fontmell Down (SZ 023810) in August – September 2009 and again in June – July 2011. The original aim had been to collect data for both generations in both years to ascertain if there was a bottleneck in *P. bellargus* oviposition preferences, as described by Roy and Thomas (2003), but poor weather prevented this apart from on Ballard Down in 2011. From July – August 2011, data for *P. coridon* were collected from two sites in Hampshire, UK: Old Winchester Hill (the site used for data collection in the 1980s) and Magdalen Hill Down (SU505292). The grazing regime of the 1980s is no longer implemented at Old Winchester Hill, and it is noteworthy that *P. bellargus* went extinct from the site in the late 1980s, and that a subsequent re-introduction attempt in 2002 failed.

At each site egg searches were carried out on random plants, using stratified random sampling, until around 50 plants containing eggs had been located. Each plant was marked and the number of eggs counted. For eggs that were found laid on a plant

other than *H. comosa* data were collected on the host plant closest to the egg. Once a set of 50+ plants had been marked, the turf height (cm), and shelter category (1-2) of each were measured. Percentage coverage of *H. comosa* and bare ground surrounding the plant were also recorded using a 25cm x 25cm quadrat. Stratified random sampling was used to select 50 or more random plants from across the site for which the same microhabitat variables were recorded (representing the available population of host plants).

Two analyses were conducted using the expectation maximisation method outlined in chapter two, to account for the pseudo-absences in the dataset (absence plants may actually have had eggs on). The first analysis used data from August-September 2009 and June-July 2011 for both Ballard Down and Fontmell Down, to explore the effects of turf height, shelter category, *H. comosa* cover, bare ground cover and year on oviposition preferences. The second used only data collected in 2011 for Ballard Down, for both generations, to explore the effects of turf height, shelter category, *H. comosa* cover and generation on oviposition preferences.

Though data for *P. coridon* were collected with the intention of analysis using a logistic regression (modified with the expectation-maximisation method), it was found (unlike *P. bellargus*) that *P. coridon* laid a varied number of eggs on plants (1 – 7). Also, a larger percentage of those plants sampled at random had eggs present upon them compared with those sampled for *P. bellargus* (76% for Magdalen Hill Down and 26% for Old Winchester Hill, compared with a maximum of 7% in *P. bellargus*). Due to these differences two separate analyses were conducted using the *P. coridon* dataset. These are as follows

- 1) To examine the effect of microhabitat on number of eggs laid by *P. coridon* this analysis utilised only data for those plants where eggs were found. A generalised linear model with a zero truncated poisson error distribution, to account for the lack of zeros in the data set, was run using countreg package in R (Cueni et al. 2009).
- 2) This analysis was only conducted for Old Winchester Hill, given the degree of 'contamination' in the random population was so high for Magdalen Hill Down, at around 75%. A data set of turf height and shelter category records for random (pseudo absence) plants for this analysis were taken from Old Winchester Hills stratified sampling data (methods above). Only one host plant was used from each stratified sampling quadrat.

Fontmell Down host plant carbon and nitrogen ratio

Plant samples, consisting of green stems and leaves, were collected for all sites (n = 30 oviposition plants plus 30 plants sampled at random) to test whether nutrient status influenced oviposition. These samples were then oven dried at 70°C for 48 hours, or until loss of mass ceased. Owing to the expense of analyzing these samples (£5 per sample), one site (Fontmell Down) was used to conduct a preliminary analysis.

A student's t-test was used to assess if the Carbon to Nitrogen ratio of plants selected for oviposition differed significantly from those chosen at random from the population.

Adult P. bellargus 2009 – the influence of ants and other factors on first generation oviposition choices

First generation female *P. bellargus* were followed and observed for up to 30 minutes each on Ballard Down, Fontmell Down, Five Marys Down and Bindon Hill in Dorset in May – June 2009. Whenever a female landed and exhibited searching behaviour on a *H. comosa* plant it was marked and numbered. A second observer searched each plant for eggs after the female had alighted, and all marked plants were searched again after observations were finished. Observations were carried out over several days on each site, but all occurred under clear bright conditions with low wind (i.e. suitable for adult activity as specified by the Butterfly Monitoring Scheme (Pollard 1977)). For all plants, measurements were taken of turf height, shelter category, bare ground coverage, and host plant coverage (as described in chapter 2). The presence and abundance of ant species was also recorded by baiting at the base of the plant after other measurements had been taken. The ground was scuffed to expose soil and a small amount of sweet bait was placed upon the ground, and covered by an upturned plant dish (10cm diameter) to provide shading. These were then left for 30 minutes and revisited to record the species of ant (in practice *Myrmica sabuleti* or *Lasius niger*) and number present. Abundance was recorded as one of 6 classes; 0, 1-4, 5-9, 10-19, 20-29 and 30 or more workers. Data were analysed using a generalised linear mixed model (glmm) with binomial errors to model the effect of the variables described above on oviposition preferences, with site included as a random effect to control for variation between sites, as given the comparatively few samples per site compared to the previous analyses the effect of site was not of interest.

3.3.4 2012 in-field associations of *P. coridon* larvae with ants

Surveys were conducted at the Denbies Landbarn Nature reserve in Surrey, England, (TQ142503), in late June and July 2012, to identify which ant species were associated with the larvae of *P. bellargus* and *P. coridon* in the field. This site was selected as it supports both species: however it proved very difficult to find *P. bellargus* larvae and so data were collected only for *P. coridon*. The site was split into 6 compartments, which were each searched for larvae for two hours by two people. The entire compartment was searched for *H. comosa* plants with larvae, using feeding damage as an aid to locate them. When a larva was found, the species of ant tending it was recorded. A total of 70 *P. coridon* larvae were found. On July 31st each section of the site was also surveyed for ants, using 20 baits across each section (120 in total), which were examined after 30 minutes.

A chi-squared contingency table test was used to assess whether the proportion of *P. coridon* larvae found with *M. sabuleti*, *L. niger* and with no ants present was significantly different from that expected from their distributions across the site. A second chi-square test was also conducted, using only those larvae and ant baits where ants were found, to test if the proportion of larvae found with each ant species differed from expected (the proportion of ant baits with either species of ant present).

3.4 Results

3.4.1 Oviposition by *P. bellargus* and *P. coridon* on Old Winchester Hill in the 1980's

Zero Adjusted Modelling: The Presence-Absence Component

The minimum adequate model for the probability of eggs being present on a plant explained around 20% of the variation in the probability of oviposition (Adj-pseudoR2 = 0.20, $\chi^2_{(9,1646)} = 482.46$, $p < 0.001$). The probability of oviposition decreased with increasing turf height ($\beta = -0.177 \pm 0.056$, $z_{(1,1654)} = -3.158$, $p < 0.01$). The probability of oviposition for *P. coridon* and *P. bellargus* combined increased with an increase from shelter category 1 to shelter category 2 ($\beta = 0.809 \pm 0.295$, $z_{(1,1653)} = 2.748$, $p < 0.01$) and further from shelter category 1 to shelter category 3 ($\beta = 2.080 \pm 0.303$, $z_{(1,1652)} = 6.877$, $p < 0.001$). Year also had a significant effect on oviposition preferences, with a significant decrease in the probability of oviposition compared with 1982 for both 1985 ($\beta = -1.873 \pm 0.197$, $z_{(1,1649)} = -9.490$, $p < 0.001$) and 1986 ($\beta = -1.513 \pm 0.192$, $z_{(1,1648)} = -7.890$, $p < 0.001$). There was a significant interaction between turf height and shelter category, with an increase from shelter category 2 decreasing the influence of turf height ($\beta = -0.130 \pm 0.067$, $z_{(1,1647)} = -1.960$, $p = 0.05$) compared to shelter category 1, and shelter category 3 also doing so but to a greater extent ($\beta = -0.222 \pm 0.069$, $z_{(1,1646)} = -3.193$, $p < 0.01$) (Figure 3.1.).

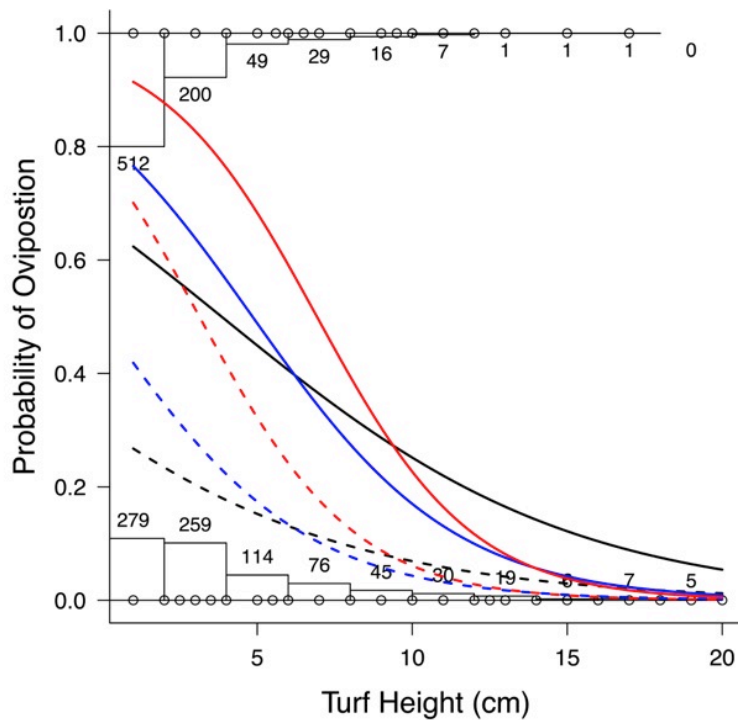


Figure 3.1 The probability of finding eggs of both *P. coridon* and *P. bellargus* combined on a plant, in response to turf height, shelter category and year, predicted by the presence-absence (logistic regression) component of the zero-inflated model for the 1980s Old Winchester Hill dataset. The solid lines are predicted values for 1982 and the dashed lines are predicted values for 1986. The black lines are predicted values for shelter category 1, the blue lines for shelter category 2 and the red lines for shelter category 3. The histogram along the bottom shows the turf height distribution for plants with no eggs present and the histogram on the top shows the turf height for plants with eggs.

Zero Adjusted Modelling: The Zero Truncated Component

The minimum adequate model for the number of eggs of both *P. bellargus* and *P. coridon* found on a plant, discounting zeros, explained significantly more variation than the null model (LogLike = -1566.7, $\chi^2_{(9, 805)} = 290.47$, $p < 0.001$). Plants in shelter category 3 had significantly more eggs than plants in shelter category 1 or 2 ($\beta = 1.757 \pm 0.296$, $z_{(1,811)} = 5.942$, $p < 0.001$). There were also significant reductions in the number of eggs laid compared to 1982 (the first year); 1983 ($\beta = -0.276 \pm 0.120$,

$z_{(1,810)} = -2.307$, $p < 0.05$), 1985 ($\beta = -0.920 \pm 0.146$, $z_{(1,808)} = -6.298$, $p < 0.001$) and 1986 ($\beta = -1.038 \pm 0.143$, $z_{(1,807)} = -7.259$, $p < 0.001$). There was a significant interaction between turf height and shelter, with a reduction in turf height significantly increasing the number of eggs found on plants in shelter category 3 ($\beta = -0.293 \pm 0.0839$, $z_{(1,805)} = -3.496$, $p < 0.001$) (Figure 3.2).

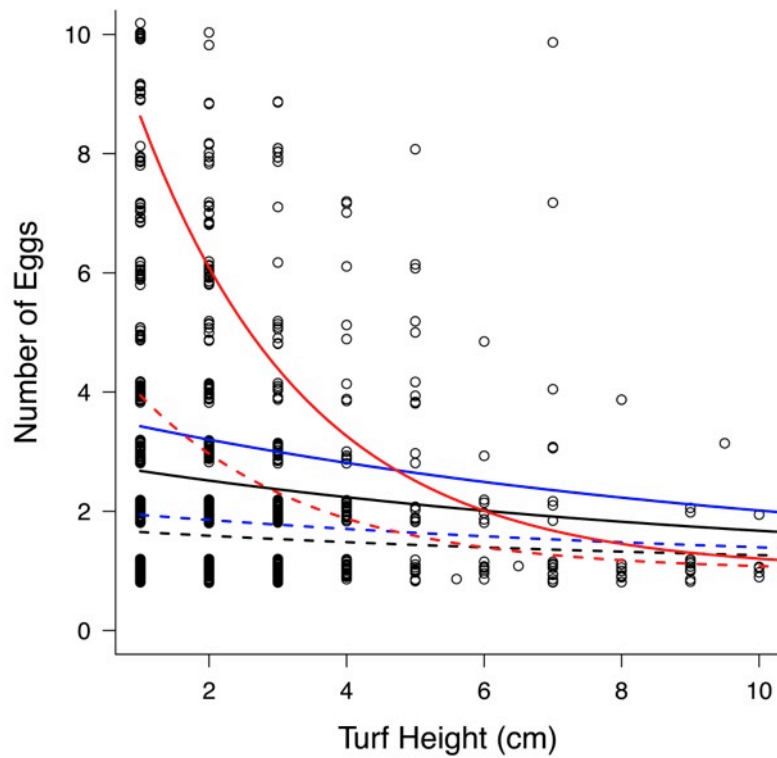


Figure 3.2 The predictions of the zero truncated (poisson regression) component of the zero-inflated model for the 1980s Old Winchester Hill dataset, showing the predicted number of eggs laid by both *P. coridon* and *P. bellargus* found on a plant based on turf height, shelter and year. The solid lines are predicted values for 1982 and the dashed lines are predicted values for 1986. The black lines are predicted values for shelter category 1, the blue lines for shelter category 2 and the red lines for shelter category 3. The points show the actual data and have been jittered to give a better impression of the density of data for each point.

Differences between species in oviposition preferences

All two-way interaction terms involving species were significant predictors of the frequency of eggs falling in to categories for turf height (1-5) and shelter (1-3) (Table 3.1). However the deviance accounted for by each term involving the presence of either species revealed important differences between the responses of each species. The interactions between the presence of eggs for *P. bellargus* and turf height accounted for nearly 6 times the amount of variation in the distribution of eggs among *H. comosa* plants than the interaction between turf height and *P. coridon* presence (see Table 3.1 and Figure 3.3). Similarly, the interaction between the presence of *P. bellargus* eggs and shelter category explained nearly 6 times the amount of variation in egg frequency than the interaction between *P. coridon* and shelter category (Figure 3.4). The significant, positive, interaction between the presence of *P. coridon* and *P. bellargus* eggs shows that the presence of eggs from one species increases the likelihood of the other, demonstrating considerable overlap in their oviposition preferences. The three-way interaction between shelter category, year and *P. coridon* presence shows that, over the recording period, the influence of shelter on *P. coridon* decreased significantly with year. The three-way interaction between turf height, year and *P. bellargus* presence shows that influence of turf height on *P. bellargus* increased significantly with year (Table 3.1).

Table 3-1 The analysis of deviance table for the minimum adequate model of the frequency of eggs falling into different categories based on butterfly species, turf height, shelter category and year. The terms of interest of those interactions containing *P. coridon* or *P. bellargus*, as a larger deviance explained by an interaction, shows that the factor with which the species term is interacting is more important in determining oviposition preferences in *P. bellargus* than in *P. coridon*.

Explanatory Variable	d.f	Deviance Explained	p(>χ^2)
Turf	4	186.77	<0.001
Shelter	2	310.60	<0.001
Year	4	197.28	<0.001
<i>P.coridon</i>	1	244.88	<0.001
<i>P. bellargus</i>	1	143.01	<0.001
Turf*Shelter	8	42.86	<0.001
Turf*Year	16	109.37	<0.001
Shelter*Year	8	118.43	<0.001
Shelter* <i>P.coridon</i>	2	34.34	<0.001
Year* <i>P.coridon</i>	4	43.74	<0.001
Turf* <i>P. bellargus</i>	4	331.08	<0.001
Year* <i>P. bellargus</i>	4	267.21	<0.001
Turf* <i>P. coridon</i>	4	57.18	<0.001
Shelter* <i>P. bellargus</i>	2	146.07	<0.001
<i>P. coridon</i> * <i>P. bellargus</i>	1	39.61	<0.001
Turf*Shelter*Year	32	73.87	<0.001
Shelter*Year* <i>P. coridon</i>	8	25.07	<0.01
Turf*Year* <i>P. bellargus</i>	16	28.39	<0.05
Residual	178	190.37	

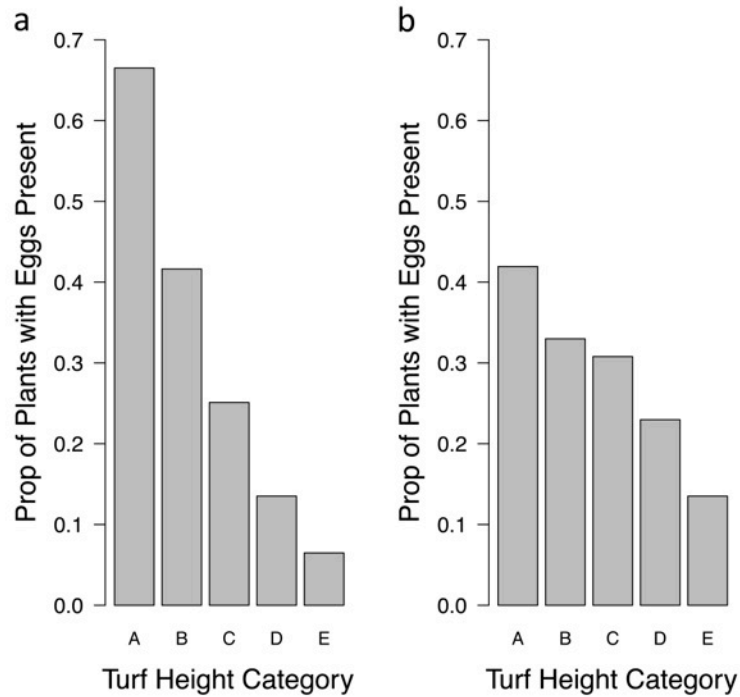


Figure 3.3 The proportion of plants falling into each turf height category that were found to have eggs present for a) *P. bellargus* and b) *P. coridon* that were found in each of the five turf height categories, A being the shortest turf and E being the longest turf on Old Winchester Hill in the 1980s.

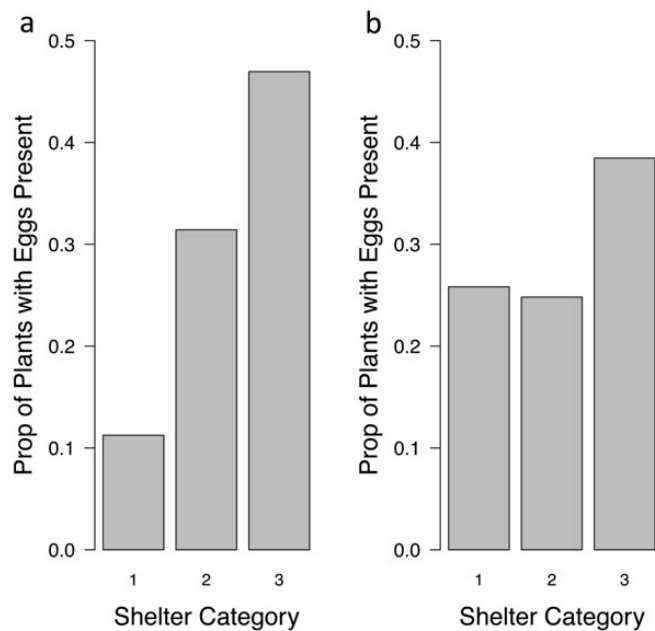


Figure 3.4 The proportion of all plants falling into in each shelter category that were found to have eggs present for a) *P. bellargus* and b) *P. coridon* on Old Winchester Hill in the 1980s. Category 3 is most sheltered.

3.4.2 Stratified sampling 2009 and 2011

Turf height

There were significant differences between the average turf height of each site ($\text{Adj}R_2 = 0.11$, $F_{(4, 586)} = 18.26$, $p < 0.001$). Post-hoc Tukey's HSD tests, at the 0.05 level, revealed that Ballard Downs' average turf height was significantly lower than all other sites, and Magdalen Hill Down had an average turf height significantly higher than all other sites (Figure 3.5). Five Marys, Fontmell Down and Old Winchester Hill were not significantly different from each other (Figure 3.5).

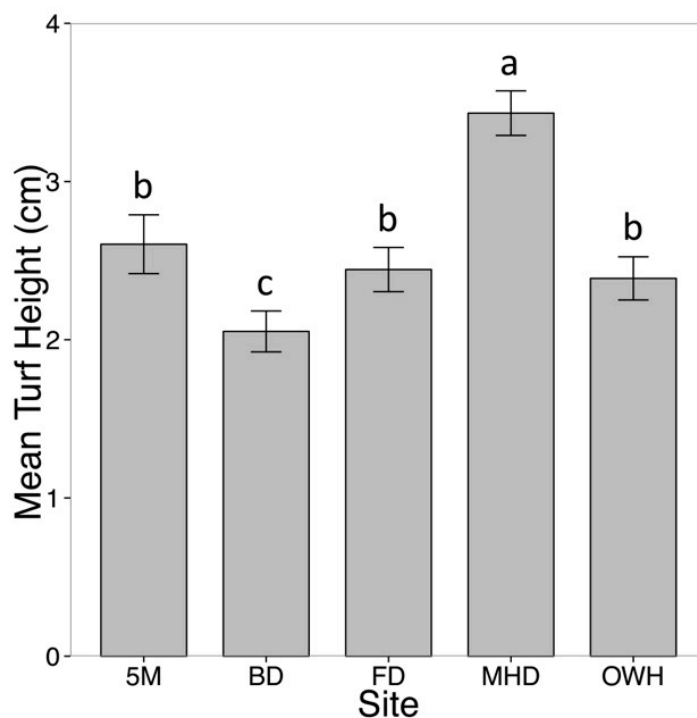


Figure 3.5 The mean turf height for five sites sampled in 2009 and 2011, as measured at four points within a 50 x 50 cm quadrat. Sites with different letters above their bar were significantly different from each other; sites with the same letter were not significantly different. 5M = Five Marys, BD = Ballard Down, FD = Fontmell Down, MHD = Magdalen Hill Down, OWH = Old Winchester Hill.

***Hippocrepis comosa* percentage cover**

The cover of *Hippocrepis comosa* differed significantly between sites ($\text{AdjR}_2 = 0.26$, $F_{(4,586)} = 53.54$, $p < 0.001$). Post-hoc Tukey's HSD tests, at the 0.05 level, revealed Ballard Down had higher *H. comosa* cover than all other sites and Magdalen Hill Down had lower coverage than all other sites (Figure 3.6). Five Marys had significantly higher cover than both Fontmell Down and Old Winchester Hill, but Old Winchester Hill and Fontmell Down were not significantly different (Figure 3.6).

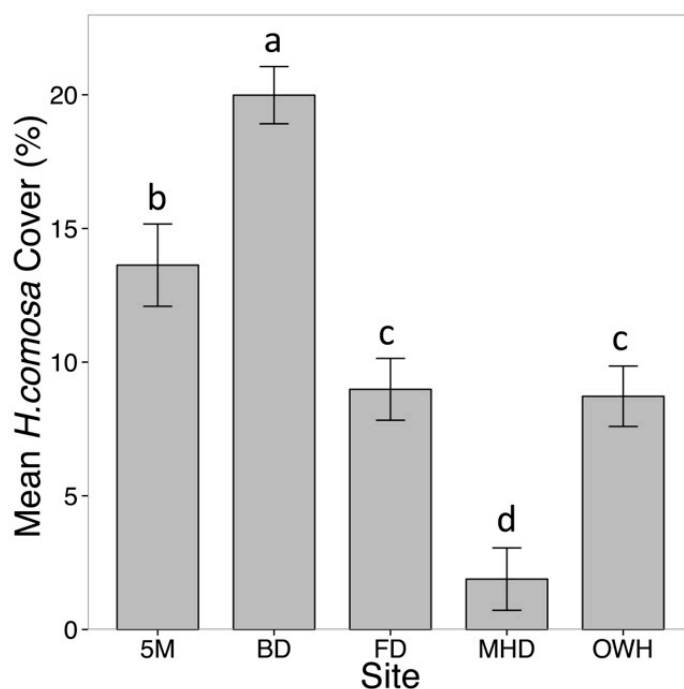


Figure 3.6 The mean percentage cover of *H. comosa* for five sites in 2009 and 2011, measured using a 50 x 50 cm quadrat. Sites with different letters above their bar were significantly different from each other; sites with the same letter were not significantly different. 5M = Five Marys, BD = Ballard Down, FD = Fontmell Down, MHD = Magdalen Hill Down, OWH = Old Winchester Hill.

Bare ground Percentage cover

Bare ground coverage differed significantly between sites ($\text{AdjR}_2 = 0.32$, $F_{(4,586)} = 76.19$, $p < 0.001$). Post-hoc Tukey's HSD tests, at the 0.05 level, revealed that Ballard Down had higher bare ground than all other sites and Old Winchester Hill had

significantly lower bare ground than all other sites (Figure 3.7). Five Marys had significantly higher coverage than Fontmell down and Magdalen Hill Down, but Fontmell Down and Magdalen Hill down were not significantly different (Figure 3.7).

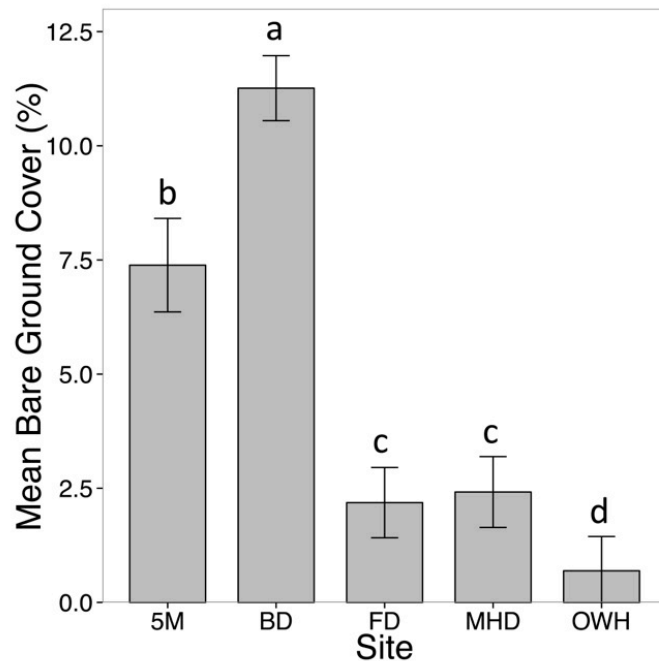


Figure 3.7 The mean percentage cover of bare ground for five sites in 2009 and 2011, measured using a 50 x 50 cm quadrat. Sites with different letters above their bar were significantly different from each other; sites with the same letter were not significantly different. 5M = Five Marys, BD = Ballard Down, FD = Fontmell Down, MHD = Magdalen Hill Down, OWH = Old Winchester Hill.

Shelter category

Average shelter category was significantly different between sites ($\text{AdjR}^2 = 0.07$, $F_{(4,586)} = 10.533$, $p < 0.001$), though this explained only a small percentage of the variation (7%). Post Hoc Tukey Tests revealed that Ballard Down and Five Marys Down had significantly higher degrees of shelter than the other sites (Figure 3.8). Fontmell Down and Magdalen Hill Down had the lowest degree of shelter, whereas Old Winchester Hills average degree of shelter was significantly higher than Fontmell Down, but not Magdalen Hill Down.

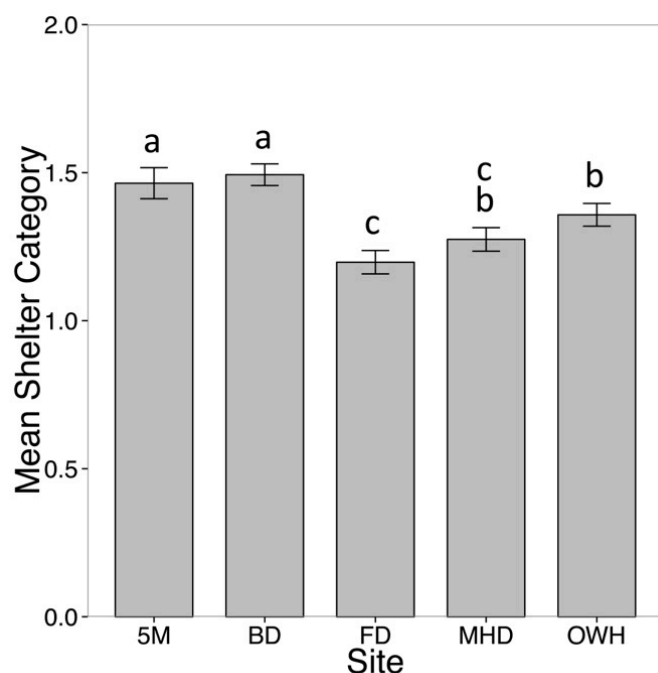


Figure 3.8 The mean shelter category for five sites as measured in 2009 and 2011, using a 50 x 50 cm quadrat. Sites with different letters above the bar were significantly different from each other; sites with the same letter were not significantly different. 5M = Five Marys, BD = Ballard Down, FD = Fontmell Down, MHD = Magdalen Hill Down, OWH = Old Winchester Hill.

3.4.3 Oviposition preferences of *P. bellargus* and *P. coridon* in 2009 and 2011

***Polyommatus bellargus* 2009 vs. 2011**

The minimum adequate model explained around 24% of the variation in oviposition preference ($\text{pseudo}R^2 = 0.236$, $\chi^2_{(5,407)} = 124.41$, $p < 0.001$), retaining terms for turf height, shelter category and their interaction with year. An increase in turf height decreased the likelihood of a plant being selected for egg laying ($\beta = -1.440 \pm 0.220$, $z_{(1,411)} = -6.543$, $p < 0.001$) and accounted for the majority of variation explained by the model ($\chi^2_{(1,411)} = 42.8$, $p < 0.0001$). The probability of oviposition decreased between years ($\beta = -1.757 \pm 0.506$, $z_{(1,410)} = -3.473$, $p < 0.001$) whilst increased shelter (from shelter category 1 to category 2) increased the probability of oviposition ($\beta = 2.999 \pm$

0.950, $z_{(1,409)} = 3.156$, $p < 0.01$). From 2009 to 2011 the effect of turf height on oviposition preferences decreased ($\beta = 0.998 \pm 0.246$, $z_{(1,408)} = 4.059$, $p < 0.001$), as did the effect of shelter category ($\beta = -2.798 \pm 1.006$, $z_{(1,408)} = -2.782$, $p < 0.01$) (Figure 3.9).

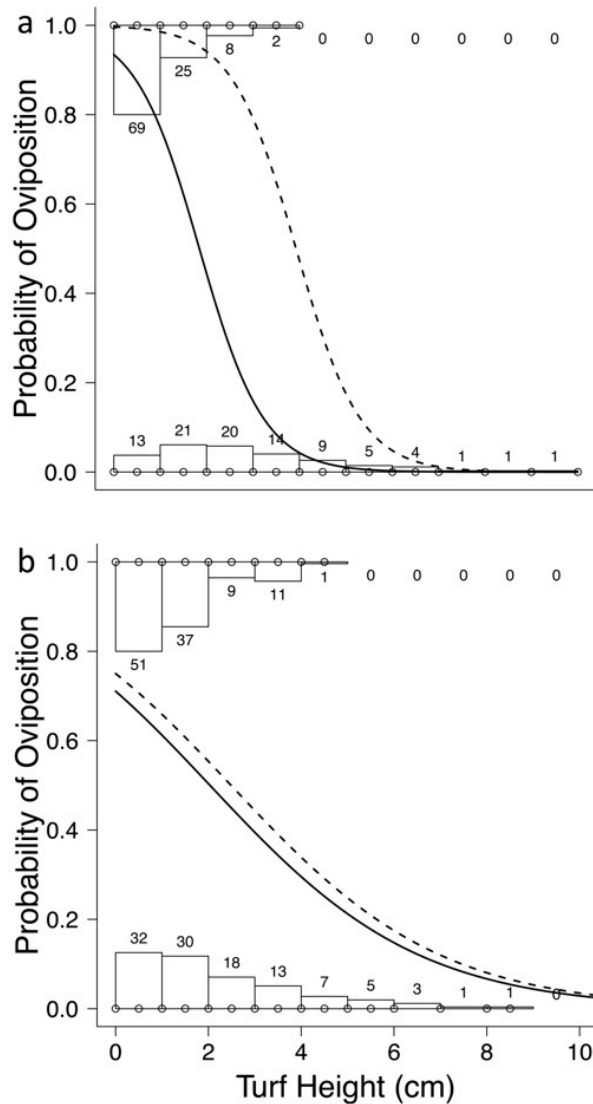


Figure 3.9 The predicted probabilities of oviposition on *H. comosa* plants by *P. bellargus* as determined by turf height and shelter category (the solid line being shelter category 1 and dashed line representing shelter category 2) in a) summer August to September 2009 and b) May-June 2011. The histogram along the bottom shows the turf height distribution for plants with no eggs present and the histogram on the top shows the turf height for plants with eggs present.

On Ballard Down in 2011 there was no significant difference between the oviposition preferences of the spring and summer generations of *P. bellargus*, with turf height being the only significant explanatory term in the model, with an increase in turf height again having a negative effect on oviposition preferences ($\beta = -0.504 \pm 0.111$, $z_{(1,197)} = -4.542$, $p < 0.001$) (pseudo $R^2 = 0.099$, $\chi^2_{(1,197)} = 24.868$, $p < 0.001$) (Figure 3.10).

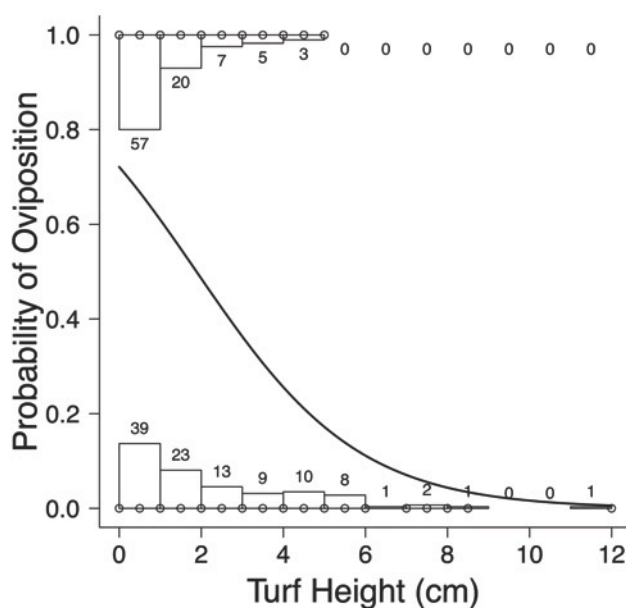


Figure 3.10 The predicted effect of turf height on the probability of oviposition for *P. bellargus* on Ballard Down in 2011, including both the May-June flying and August-September flying generations as there was no significant difference between the two. The histogram along the bottom shows the turf height distribution for plants with no eggs present and the histogram on the top shows the turf height for plants with eggs.

***Polyommatus coridon* number of eggs**

The initial model used included pairwise interaction terms for all predictor variables (turf height, shelter category, *Hippocrepis comosa* percentage cover, site). However, only site was significant in the minimum adequate model (LogLik = -171.95, $\chi^2_{(2,159)} = 7.726$, $p < 0.001$), with the number of *P. coridon* eggs per plant being significantly

higher on Magdalen Hill Down than on Old Winchester Hill ($\beta = -0.605 \pm 0.2295$, $z = -2.637$, $p < 0.01$) (Figure. 3.11).

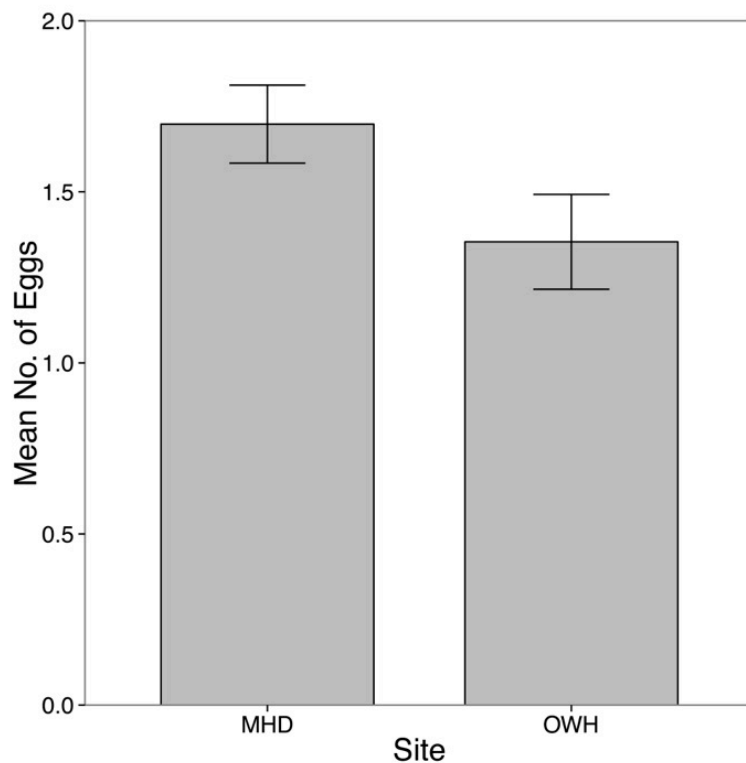


Figure 3.11 The mean number of *P. coridon* eggs found on *H. comosa* selected for oviposition on Magdalen Hill Down and Old Winchester Hill in 2011.

***Polyommatus coridon* eggs presence-absence**

Turf height and shelter category were not significant in explaining oviposition preferences in *P. coridon*, either in interaction ($\beta = 0.100 \pm 0.056$, $z_{(1,97)} = 1.779$, $p = 0.075$), or as main effects; shelter category ($\beta = -0.058 \pm 0.519$, $z_{(1,98)} = -0.112$, $p = 0.911$), turf height ($\beta = 0.031 \pm 0.020$, $z_{(1,99)} = 1.551$, $p = 0.121$).

3.4.4 Fontmell Down host plant carbon and nitrogen ratio

A two-sample t-test found that there was no significant difference in the C:N ratios ($t_{(2,58)} = 0.010$, $p = 0.992$) between plants selected for oviposition ($\bar{x} = 12.104$, $sd = 0.973$) and plants sampled at random from the population ($\bar{x} = 12.101$, $sd = 1.044$).

3.4.5 The effects of myrmecophilous ant partners

Plant selection by ovipositing *P. bellargus* females in spring 2009

The presence of either *L. niger* ($\beta = 0.116 \pm 0.7394$, $z_{(1,126)} = 0.157$, $p = 0.875$) or *M. sabuleti* ($\beta = 0.1668 \pm 0.8004$, $z_{(1,125)} = 0.208$, $p = 0.835$) was not a significant predictor of oviposition. The abundance of ants present at a host plant was on the border of significance as a determinant of oviposition choice ($\beta = 0.252 \pm 0.129$, $z_{(1,126)} = 1.950$, $p = 0.051$), but was not maintained in the final model. The probability of oviposition decreased significantly with turf height ($\beta = -1.353 \pm 0.407$, $z_{(1,129)} = -3.324$, $p < 0.001$). However, the interaction term for turf height and shelter revealed the probability of oviposition increased with turf height when a plant was found in higher shelter (shelter category 2) ($\beta = -1.571 \pm 0.464$, $z_{(1,128)} = 3.386$, $p < 0.001$).

In-field ant caterpillar associations for *P. coridon* in 2012

A total of 70 *P. coridon* larvae were found on Denbies Landbarn, 4 attended by *L. niger*, 31 attended by *M. sabuleti* and 35 not attended by any ant. Of 120 ant baits laid across the site 15 were found to have *L. niger* present, 86 were found to have *M. sabuleti* present and 19 had no ants present. A Chi-square contingency table test using the observed numbers and expected proportions of larvae found in each category (*L. niger*, *M. sabuleti* and none) found the number of larvae associated with ants was

significantly different from what would be expected given the distribution of ants across the site ($\chi^2 = 61.511$, $df = 2$, $p < 0.001$) (Figure 3.12a). A second Chi square used data only on the larvae that were found being tended by ants, and only the baits at which ants were found. This showed that the number of larvae found with each ant species ($L. niger = 0.114$, $M. sabuleti = 0.886$) did not differ significantly from what would be expected given the proportion of ant baits found with each ant species present ($L. niger = 0.149$, $M. sabuleti = 0.851$) ($\chi^2 = 0.3243$, $df = 1$, $p = 0.5691$) (Figure 3.12b).

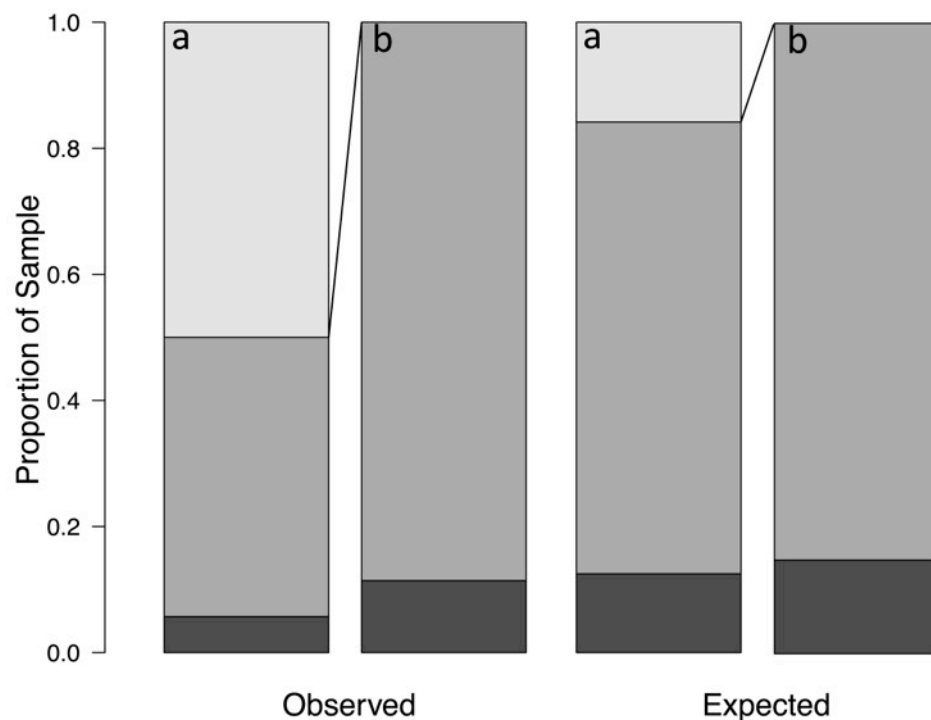


Figure 3.12 a) The observed proportion of 70 *P. coridon* caterpillars on Denbies Landbarn nature reserve found with *Lasius niger* (dark grey), *Myrmica sabuleti* (mid grey) and with no ants present (light grey), compared to the expected proportions given the proportion of 120 ant baits falling into each class collected across the site. **b)** The observed proportion of the 35 caterpillars found with ants in attendance, that were attended by either *L. niger* (dark grey), *M. sabuleti* (med grey), compared to the proportion of ant baits at which ants were present represented by each ant species.

3.5 Discussion

3.5.1 Differences in the microhabitat preferences of *P. bellargus* and *P. coridon*

In the UK populations of *P. bellargus* and *P. coridon* are separated by phenology, being bivoltine and univoltine respectively, with larval stages that show partial overlap between the dates when larvae are feeding on their foodplant, *H. comosa*. In this chapter it was addressed whether these two species show separation in their larval niche, the life-stage that determines the habitat use and population size of most temperate butterflies studied to date (Thomas et al. 2011). The results support the prediction that turf height and shelter - both factors that influence soil-level microclimate (see Chapter 2) - are less important for host plant selection in *P. coridon* than in *P. bellargus*. Oviposition measurements for both species on the same site (Old Winchester Hill) over 5 years in the 1980s, show that whilst both species had a preference for *H. comosa* plants found in shorter turf and in greater shelter, *P. bellargus* is far more constrained by these factors. The probability of oviposition and the number of eggs laid also decreased with year, and further analysis again showed this trend was much stronger for *P. bellargus*. This reflects the declines of the *P. bellargus* population on Old Winchester Hill over the recording period, eventually to local extinction (Bacon 2013).

Data collected in 2011 from multiple sites, although not for both species at the same sites, also show a distinction between the two species. In this case turf height and shelter were not significant determinants of oviposition for *P. coridon*, either in terms of their selection of host plants for oviposition or in the number of eggs laid on those

plants selected. For *P. bellargus*, however, both turf height and shelter are significant determinants of oviposition, with turf height being a stronger determinant than shelter (as also documented in Chapter 2). Host plant density or abundance did not significantly influence the probability of oviposition for either species, nor the number of eggs laid by *P. coridon*. Studies of other butterflies have found great inter-specific variation in the size of food plant selected by females for oviposition, with some species selecting the largest available individual host plants (Garcia-Barros and Fartmann 2009) and other species choosing a subset of exceptionally small plants (Thomas & Lewington 2010). Unfortunately, the sprawling growth-form of *H. comosa* meant it was often impossible to distinguish what constitutes an individual plant, making host plant cover a more meaningful measure of availability in these studies. Using this criterion, the absolute abundance of *H. comosa* was not a limiting resource for oviposition on any study site. The percentage bare ground around a host plant, which is a determinant of oviposition preferences for three other calcareous grassland butterfly species in the UK, *Aricia agestis*, *Hipparchia semele* and *Hesperia comma* (Bourn and Thomas 1992; Davies et al. 2006; Thomas et al. 1986) was also not a significant influence on oviposition for *P. bellargus* or *P. coridon*. In addition, a trial analysis of 60 plants from Fontmell Down showed that the nitrogen content of host plants selected by *P. bellargus* for oviposition did not differ from that of randomly sampled plants in the population; variation in the C to N ratio was, moreover, very low among all *H. comosa* plants sampled. This perhaps reflects the fact that as a legume possessing nitrogen-fixing nodules, the supply of nitrogen is not a constraint. Given this result, and the cost of collecting these data, no further analyses were made of the nutritional quality of selected food plants.

The only significant influence on oviposition for *P. coridon* in 2011 was ‘site’, with significantly more eggs laid on plants on MHD than on OWH. This is most likely related to the significantly lower density of *H. comosa* on MHD, which had the lowest coverage of *H. comosa* for all sites surveyed (Figure 3.7) and yet also supported the higher density *P. coridon* in 2011, with 712.5 adults ha⁻¹ compared with 218.35 adults ha⁻¹ on OWH in 2011, calculated from total count data in those years from the UK Butterfly Monitoring Scheme (2013).

Overall these results indicate that, between the decades, microhabitat has become less of a constraint to habitat usage in both species. In the 1980s, *P. coridon* was responsive to turf height and shelter category but in 2011 (on the same site) was not influenced by either. Though the effects of year and generation are confounded, data for *P. bellargus* shows that oviposition preferences of the first (May-June) generation of *P. bellargus* in 2011 was less constrained by turf height and shelter than the second (August-September) generation. However in both cases it is likely that changes in the turf height structure between years is driving this relationship (see Chapter 2). For instance, a larger proportion of host plants were available in shorter turf heights to *P. bellargus* in 2011 than in 2009 (see histograms on Figure 3.9), which would lead to an increased influence of microhabitat in 2009. This is further supported by the fact that no distinction in oviposition was found between the summer and spring generations of *P. bellargus* on Ballard Down in 2011, though turf height remains a constraint in both generations. Habitat surveys on Ballard Down showed this site had the most favourable conditions for *P. bellargus*, indicative of heavier grazing. Together these results suggest that the distinction in the oviposition preferences between alternate *P. bellargus* generations that was observed in a previous cooler decade by Roy and

Thomas (2003) is reduced once turf height is reduced to the extent that host plants found in optimal conditions are no longer a limiting resource for the second generation.

3.5.2 Site microhabitat profiles

There were significant differences in the distribution of microhabitat variables on study sites. Ballard Down and Five Marys Down, which were occupied solely by *P. bellargus*, had significantly higher values for both host plant cover and bare ground than the other sites, with Ballard Down having the highest values for all sites. However, the differences in turf height and shelter category, the two factors found to be of significance in distinguishing the larval niche of these two species, were less pronounced. The average turf height for Five Marys was closer to that of the other sites (see Figure 3.8), reflecting the terraced structure of the site which results in repeated tall strips of turf adjoining sheep-walks across the site separated by heavily grazed strips of short turf. Overall, these analyses indicate that Ballard Down and Five Marys experienced more intensive grazing than the other study sites, and this may make them more suitable for *P. bellargus*. Fontmell Down, the only site that supported both *P. bellargus* and *P. coridon*, was more similar in microhabitat structure to other sites that supported *P. coridon*.

3.5.3 The role of mutualistic ants

The results for the oviposition preferences of *P. bellargus* and the occurrence of *P. coridon* larvae with ants suggest that neither butterfly has a preference for a specific mutualistic ant species. Neither the species nor abundance of ants associated with *H. comosa* plants influenced whether female *P. bellargus* oviposited on plants on which

they landed. *Polyommatus coridon* was found more frequently with *M. sabuleti* than *L. niger* on Denbies Landbarn, but *M. sabuleti* was more widely distributed across this site, and the proportion of larvae associated with ants was actually significantly lower than would have been expected given the representation of ants across the site (Figure 3.12a). Further, considering only those caterpillars found with ants in attendance, the proportion found with each ant species was not significantly different from that expected given the representation of each species at ant baits. However, it must be emphasised that these data are preliminary for both *P. bellargus* and *P. coridon*; to be conclusive the same methods should be used across different sites and for both species, to truly identify how closely associated with ants their oviposition preferences and larvae are. For instance, it may be that the widespread occurrence of *M. sabuleti* across Denbies Landbarn is a factor making the site able to sustain a large population of *P. coridon*. In addition, these single observations provide only a snapshot of the activity of ants, and repeated observations for the same oviposition sites, or the same larvae, would capture more accurately the temporal variation in both the presence of ants and the species present. Unfortunately, a more intensive study of this sort was not feasible within the time constraints of this work programme.

3.5.4 Implications

The larval ecology of butterflies, particularly grassland specialists, can be incredibly specific; females may select for larger host plants, a particular growth form or nutritional content (which may be interrelated), and may have preference for specific microhabitats / climates (Dolek et al. 1998; Eilers et al. 2013; Garcia-Barros and Fartmann 2009; Pennekamp et al. 2012; Rabasa et al. 2005; Wiklund 1984). The results of this study show that where *P. bellargus* is restricted to *H. comosa* plants in

warmer microhabitats, *P. coridon* is (at least) far less constrained. In a study of butterfly communities in the Jura mountains, Switzerland, Balmer and Erhardt (2000) similarly found that where *P. bellargus* was associated only with early successional stages (grazed regularly or every 2-3 years) whereas *P. coridon* was equally associated with sites that had not been grazed for 10 years. This difference is most likely related to differences in the thermal requirements of each species, which may be driven by intrinsic differences in the physiological needs of each species; evidence from phylogenetics and paleoecology shows strong phylogenetic niche conservatism with regard to temperature in the genus *Polyommatus* (Vila et al. 2011). However, the more parsimonious explanation is that it relates to the requirements of the overwintering stage of each species. *Polyommatus coridon* overwinters from August to April as an egg, either on the host plant or on the soil surface. *Polyommatus bellargus* overwinters as first or second instar larvae, which feed well into October and resume in early March, and so warmer microclimates may be preferable as its larvae are feeding in these cooler periods, and must over winter as larvae. It is, however, also possible that the differences in the microhabitat preferences of each species define their phenologies and voltinism to some degree, with the selection of a warmer microclimate by *P. bellargus* being a causative agent for the hatching of its eggs before overwintering. Indeed, it has been demonstrated that exposure of *P. coridon* eggs to warmer temperatures can induce a second generation (Comont et al. 2009).

For a pair of syntopic sister butterfly species found in central Sweden, *Leptidea sinapis* and *Leptidea reali* (now named *L. juvernica*), differences in their habitat preferences (*L. sinapis* selected cooler larval habitats) were found to be driving

differences in their phenology, and developmental differences were much reduced when larvae of each species were reared under the same conditions (Friberg et al. 2008). Further work exploring the impacts of temperature on the development of *P. bellargus* and *P. coridon* would be useful to identify whether there are intrinsic differences in the thermal tolerance of each species and whether exposure to the same temperature regimes over their development causes them to develop at more similar rates.

These results suggest that the twin impacts of managing chalk grasslands for a shorter turf structure and a warming climate may result in an increased overlap in host plants utilised by each species where they co-occur. An important question is whether this will have consequences for either species. The fact that there was a positive association between the laying preferences of each species in the 1980s (Table 3.1) suggests interspecific competition between the two species is weak, which could be due to the seasonal segregation of larval feeding periods. However, it could be that a decrease in the availability of host plants falling in higher turf heights will facilitate increased competition between the two species. This would be inconsequential in years when *H. comosa* is abundant, but the dynamic could be altered should *H. comosa* become scarce, such as is possible in a drought year; a large genetic bottleneck in the UK *P. bellargus* populations has been shown to be associated with the effects of a drought of *H. comosa* population in 1976 (Harper et al. 2003).

Increased grazing, by rabbits and livestock, has been identified as a driver of the recent recoveries of *P. coridon* and *P. bellargus* in the UK (Brereton et al. 2008; Thomas et al. 2011). However, grazing will have different impacts on each species

dependent on both its intensity and the degree of spatial heterogeneity in its implementation (Davies et al. 2007; Kruess and Tscharntke 2002b). The influence of management activities such as animal grazing and fire are well recognised as being key to promoting the biodiversity of grassland invertebrate communities, as they can increase complexity and heterogeneity in vegetation structure (Joern and Laws 2013; Kruess and Tscharntke 2002a; Kruess and Tscharntke 2002b). Given that populations of *P. bellargus* and *P. coridon* have been well monitored in the UK for >35 years, a useful next step would be to examine how increased grazing pressure, and different grazing regimes, have impacted populations. Both species have increased in abundance since recording began, but between 1994 and 2004, whilst *P. bellargus* increased in overall abundance by 33%, *P. coridon* actually saw a decrease of 26% (Fox et al. 2006). Conducting an analysis of the impacts of site management on populations for different sites was something that was explored, but as there is no standardised practice between site managers for recording annual management, it is difficult to compare sites with populations of both species in this regard. Further work aimed at standardising historical records of grazing and encouraging standardised recording of current grazing regimes on nature reserves with monitored populations would be very valuable for both this purpose specifically, and for monitoring the effects of management on other species.

**Chapter 4 – Myrmecophily in *Polyommatus*
bellargus and *Polyommatus coridon*: effects of
different ant partner species on development
and behaviour**

4.1 Abstract

Within the family Lycaenidae 75% of studied species are known to interact with ants as larvae and pupae. Some of these relationships are parasitic, but around 90% of those known are mutualistic, in that larvae provide the ants with food reward secretions and the larvae receive protection from natural enemies in return. *Polyommatus bellargus* and *Polyommatus coridon* are known to be tended, as larvae and pupae, by the same species of ant in the wild, most notably *Myrmica sabuleti* and *Lasius niger*. Studies of several Lycaenid species have found that exposure to ants can have both costs and benefits to the development of larvae. Here experiments are presented which measure the developmental impacts of exposure to the ants *M. sabuleti* and *L. niger*, in larvae of both *P. bellargus* and *P. coridon*, to assess whether either species experiences differences in developmental costs or benefits depending on the species of ant it is exposed to. For *P. bellargus* behavioural interactions with each ant partner are also examined to explore if these suggest a preference for one ant partner. Results show that exposure to either ant species does not have any lasting impact on the development of either butterfly species. Behavioural interactions in *P. bellargus* also show no clear preferences for one ant partner, but do indicate that some larval behaviour may actually be employed to reduce attendance by ant workers, rather than attract ants. The results are discussed in terms of the experimental context, including the lack of natural enemies and food availability to both ants and butterflies, and areas for further research are suggested.

4.2 Introduction

4.2.1 Myrmecophily in the Lycaenidae

Of the approximately 6000 species in the family Lycaenidae, which constitute around 40% of all butterfly species, 75% of studied species are known to interact with ants as larvae and pupae (Pierce et al. 2002). These relationships span a continuum from obligate to facultative and from mutualistic to parasitic. Obligate species always associate with ants and are not observed to survive in the wild without contact for at least part of their life cycle (Fiedler et al. 1996). Facultative species are those that demonstrate irregular interactions with ants, and can apparently survive in the wild without contact, although this has been tested in very few species.

Myrmecophily has been a driving force in the evolution and diversification of the Lycaenidae, shaping their anatomy, behaviour and ecology (Fiedler 1991; Fiedler et al. 1996; Pierce et al. 2002). Larvae of many species have thick cuticles, thought to provide protection from aggressive ant behaviour (Atsatt, 1981), but three anatomical adaptations are particularly notable as their function is to facilitate interactions with ants; the pore cupola organs (PC), the tentacular organs (TO) and the dorsal nectary organ (DNO) (Fiedler et al. 1996; Kitching and Luke 1985; Pierce et al. 2002) (see Figure 4.1). The PC are single celled organs thought to secrete amino acids as well as chemicals to reduce aggressive behaviour in ants (Kitching and Luke 1985; Pierce et al. 2002). The TO are a pair of tentacle like structures located at the rear of the caterpillar that, when everted, emit volatile chemicals that are thought to be able to signal distress or simply attract ant attention. The DNO, also known as the Newcomers organ, is an adapted exocrine gland found only in species of the sub-

family *Lycaeninae* and located on the 7th abdominal segment, with the primary function of secreting a nutritious solution that attendant ants feed upon (Kitching and Luke, 1985; Pierce, 2002 (and references there-in)). Pierce (1985) suggested that the nutritional demands of producing secretions for ant partners has been a selective agent for the association of many Lycaenid species with food plants that have high nitrogen content (e.g. legumes). However Fiedler (1995a) found little support for this hypothesis, and more support for the hypothesis that selection away from myrmecophily has led some species (that he terms secondarily “myrmecoxenous”) to feed on host plants with toxic secondary metabolites.

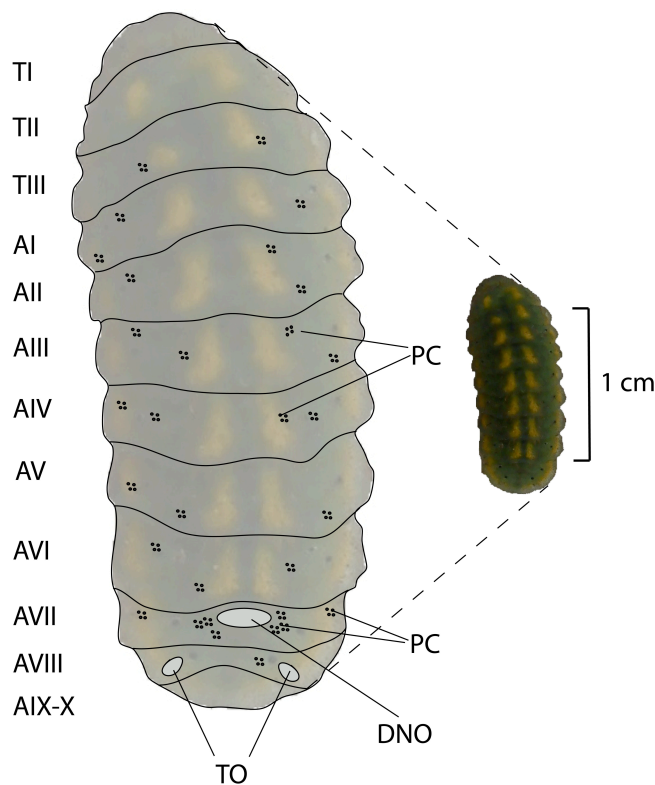


Figure 4.1 Schematic diagram of a third instar *P. coridon* larva, adapted from Kitching and Luke (1985), along with a photograph of the actual larva. There are three thoracic segments (TI-TIII) and 9-10 abdominal segments (AI-AX). TO shows the location of the tentacular organs; DNO shows the location of the dorsal nectary organ; PO represent the pore copula, which are distributed over the larva’s dorsal surface and most densely around the DNO (Kitching and Luke 1985).

4.2.2 The benefits and costs of myrmecophilous lycaenids to ants

The relationship with ants of about 90% of myrmecophilous lycaenids is considered to be mutualistic or commensal, with larvae that feed wholly on plants or similar material (e.g. lichens): the remaining 10% have evolved as specialized social parasites of ants, in at least 13 independent lines, from mutualistic ancestors (Fiedler 1998; Pierce et al. 2002). The rewards provided by mutualistic lycaenids arise from their PC and, in the case of larvae, their DNO secretions which, in the few species analysed consist largely of sugars and low concentrations of amino-acids (Daniels et al. 2005). These secretions resemble those of the near-ubiquitous trophobont aphids that are reared as domestic herds by most ant genera that interact with myrmecophilous insects, and may nevertheless provide a significant source of food for the ant colony. For example, Fiedler and Maschwitz (1988) found that nectar secretion by a *P. coridon* larvae over the duration of its larval period was worth 55-110 joules of energy. Based on this they estimated that larvae found at their observed densities of 20 per m² would provide 11 -23 % of the energy required for worker production in *Lasius alienus*. No analysis has been made of the benefits of lycaenid amino-acid secretions to ants, but their value is greater for ant larvae, which require proteins for growth, rather than workers, which require sugars for energy. It is unknown whether any mutualistic larvae impose a cost to the ants with which they interact.

Although not studied in this thesis, on current knowledge the impact of socially parasitic lycaenids is wholly detrimental to their host ants. However, knowledge derives largely from five species in the genus *Maculinea*. The pre-adoption larvae secrete minute quantities of fluids believed to contain sugars, but their main (copious)

secretions consist of cocktails of hydrocarbons that mimic those of their host ant genus, and later host species or sub-species, with increasing accuracy as the larva grows (Akino et al. 1999; Schrönrge et al. 2004; Thomas et al. 2013). In addition, the larvae and especially pupae of *Maculinea* have adapted the acoustical calls made by most lycaenids (DeVries 1990) to mimic those made by adult queens ants of their host genus (Barbero et al. 2009). Whether they are cuckoo-fed or merely predators of ant brood, larvae of lycaenid social parasites destroy, or greatly reduce the host colonies that they infest and workers in infested colonies live significantly shorter lives than those in uninfested ones, owing to the stresses imposed by feeding their ‘cuckoos’ (Wardlaw et al. 2000).

4.2.3 The benefits of ants to phytophagous lycaenids

With regard to the benefits to lycaenid larvae from myrmecophily, the main hypothesis to be tested is that ants provide protection from natural enemies, such as arthropod predators and parasitoids. Parasitoid wasps and flies in particular are known to be prolific parasitoids of Lepidoptera (Shaw 1990; Shaw et al. 2009), and associating with ants may provide larvae, prepupae and pupae with enemy free space, either through ants actively deterring natural enemies, or their presence acting as a passive deterrent (Astatt 1981).

In the passive scenario, the food secretions of larvae are seen as a gesture of appeasement, rather than actively recruiting ants for protection. Astatt (1981) claimed that the nutritional benefit of caterpillar secretions is not enough to outweigh the cost of the ants actively providing protection to the caterpillars. However, experimental studies using larvae of *P. coridon*, *P. bellargus* and *Polyommatus icarus* found that

even though DNO secretion rates sometime appear to be low, ant partners receive a significant nutritional benefit from tending larvae (Fiedler and Maschwitz 1988; Fiedler and Saam 1995). Cushman et al. (1994) also reported a 40% increase in survivorship of workers in colonies of the ant *Iridomyrmex nitidiceps* that had access to larvae of the obligate mutualist *Paralucia aurifera* compared to colonies without access.

Some studies have explicitly investigated the role ants play in providing protection from natural enemies (DeVries 1991; Pierce et al. 2002; Pierce et al. 1987; Pierce and Mead 1981; Pierce and Eastal 1986; Weeks 2003) and the limited evidence (7 out of 8 studies) indicates ants do act to reduce levels of predation and parasitism (Weeks 2003). For example Pierce and Mead (1981) found that caterpillars of *Glaucopsche lygdamus* tended by ants had a significantly lower incidence of falling prey to parasitoids. The Australian species *Jalmenus evagoras* also showed decreased rates of egg predation and increased rates of larval and pupal survivorship in the presence of its attendant ant *Iridomyrmex anceps* (Pierce et al. 1987). Most recently Weeks (2003) demonstrated that larval mortality in *Hemiargus isola* was almost twice as high (78%) when the ant attendant ants were excluded from host plants. Also, Leimar and Axén (1993) found that larvae of *Polyommatus icarus* provoked with a mock attack actively recruited higher numbers of workers of the ant *Lasius flavus* by secreting larger amounts of nectar.

Interestingly there is the potential for myrmecophily to act as a window for the evolution of new strategies of parasitism. Fiedler et al. (1992) report that larvae of several *Lycaeninae* continue to elicit ant attention, even when the larvae are dead.

Parasitoids therefore benefit from the ant protection of their deceased host, in some case until the parasitoid emerges. Mutualist ants may also reduce the search effort required by parasitoids to locate hosts, acting as a cue as to their location or by reducing competition for species of parasitoid that are not detected by ants (DeVries 1991). For example, among the socially parasitic lycaenids, the ichneumonid *Ichneumon eumerus* has evolved as a specialized parasitoid of *Maculinea rebeli* larvae, using the trail allomones laid by *Myrmica schencki* workers to detect the nest entrances of this species-specific host (Thomas and Elmes 1993).

4.2.4 The cost of ants to phytophagous lycaenids

Myrmecophilous relationships will exert energetic and resource demands on larvae, in the form of the production of secretions from the DNO and PC, although the energetic costs of the secretion of amino acids from the PC has yet to be quantified. The manifestation of these costs in the development of larvae has been the primary mode through which fitness costs associated with ants has been addressed. Studies have provided mixed results, some indicating negative impacts on development, whilst other reported positive and mixed effects (Pierce et al. 2002). For the obligate mutualist *Jalmenus evagoras*, laboratory experiments revealed that larvae tended by ants reached pupation approximately five days sooner, but have significantly lower pupal weights, higher pupal weight loss and lower weight as adults (Pierce et al. 1987). Early pupation may benefit caterpillars as it reduces the time when caterpillars are vulnerable to parasitism (Cushman et al. 1994), however, lower weights in development will be costly for fitness. Smaller females typically lay fewer eggs: interestingly the effect of myrmecophily on pupal weight was more pronounced in females, an effect echoed by Fiedler and Hölldobler (1992) who found that male

Polyommatus icarus had a significantly larger pupal mass when tended by ants, whereas the females showed significantly higher weight loss as pupae. Cushman et al. (1994) however found that larvae of the Australian *Paralucia aurifera* tended by the ant *Iridomyrmex nitidiceps* showed significantly higher larval weights (31-76%), shorter developmental times and higher weights at pupation (20%), than those larvae reared without ants. Wagner (1993) also revealed that larvae of *Hemiargus isola* tended by the ant *Formica perpilosa* resulted in significantly larger weight at pupation, although the mechanism remains unclear as subsequent studies found that this was not due to increased feeding by the larva (Wagner and Martinez 1997).

4.2.5 The facultative mutualisms of *P. bellargus* and *P. coridon*

As larvae and pupae, *Polyommatus bellargus* and *Polyommatus coridon* are known to engage with at least three ant species of two genera; *Myrmica sabuleti*, *Lasius niger* and *Lasius flavus*, all common ant species of chalk grassland (Thomas and Lewington 2010) (Plate 4.1). It is not known how specific these interactions are with regard to the different ant genera or species, to what degree the different ants coexist with each lycaenid's larval niche, or indeed whether ants serve to determine their larval niches. For example, it has been demonstrated for populations of *Plebejus argus* (the Silver Studded Blue) in Wales that *Lasius* species (*L. niger* and *L. alienus*) are more attracted to its larvae at both the 1st and 3rd instar stages than are *M. sabuleti* and *Formica fusca*, which mostly ignored its larvae (Jordano and Thomas 1992). It has also been shown that the occurrence of *P. argus* eggs, larvae and pupae is correlated with the presence of *L. niger* within field sites, as is the occurrence and abundance of *P. argus* populations across field sites (Jordano et al. 1992).



Plate 4.1 *Polyommatus coridon* larvae observed in the wild on Denbies Landbarn (National Trust) nature reserve, Surrey, being tended (at the DNO) by *Myrmica sabuleti* (left) and *Lasius niger* (right).

Just as microhabitat preferences provide a distinction in the larval niche of *P. bellargus* and *P. coridon* (see Chapter 3), it may be that a preferred species of ant with which their larvae interact as mutualistic partners are an additional, or perhaps the underlying, defining resource. For example, for the obligate mutualist *Maculinea arion* it is clear that the presence of the correct ant species (*Myrmica sabuleti*) is essential to successful larval development, and so a fundamental resource in defining this species' larval niche (Thomas 2002). Facultative species, such as *P. bellargus* and *P. coridon*, often engage with more than one ant species, but it is possible that the quality of ants as mutualistic partners varies, for instance one ant partner might provide better protection from natural enemies and so attract more resource investment. Consequently the investment of resources by larvae and the behaviour they exhibit in interactions with different ant species may differ. Axén (2000) found that the North American Lycaenid *Glaucopsyche lydamus* secreted nectar droplets at twice the rate when exposed to the ant species *Tapinoma sessile* compared with five other species of ant. Here experiments are presented that aim to test whether the larvae of both *P. bellargus* and *P. coridon* show differences in their development

when exposed to different ant species. If there are differences in the developmental effects of myrmecophily depending on the ant species they are tended by, this will indicate differential investment of resources by larvae based on the ant species available, which in turn will suggest if one ant species is a preferred mutualistic partner. It is also possible that *M. sabuleti*, being the larger of the two ant species, will extract more resources per individual and therefore have a larger effect on development (Kaminski and Rodrigues 2011). A previous study by Fiedler and Saam (1994) exposed *P. bellargus* larvae (and larvae from four other Lycaenid species) to *L. niger* or *L. flavus*, and found no significant difference between ant tended larvae and controls. However, this study did not expose larvae to a *Myrmica* species, and exposed larvae to a maximum of five worker ants with no brood present – very different from the experimental set up used here.

For *P. bellargus*, it is also tested whether larval behaviour differs in the presence of different ant species and whether the degree of attention paid to the larvae by ants differs. Behavioural observations will also be used to test whether one species of ant spends more time in contact with larvae, and whether larvae respond differently to the different ant species.

Two hypotheses are tested:

1) The developmental cost and/or benefits of interactions with ants will differ depending on the ant species with which larvae are reared. It is predicted that the growth rate of larvae, rate of weight loss in pupae and adult dry weights will differ depending on the ant species in attendance.

2) The behavioural response of *P. bellargus* larvae will differ depending on the ant species that is tending them. It is predicted that the frequency and duration of interactions between larvae and ant workers will differ depending on the ant species with which a larva is reared, indicating a preference for engaging with one ant species over another.

4.3 Methods

4.3.1 Collection of insects

Polyommatus bellargus larvae were reared from eggs laid in captivity by wild-caught *P. bellargus* females (collected with permission from the sites described in chapter 2). Females of *P. bellargus* were placed either singly on *H. comosa* plants or with a male if they appeared unmated, and fed with diluted (~5%) honey solution, in greenhouse facilities at the Centre for Ecology and Hydrology (CEH), Wallingford, UK. The butterflies were kept on the plant by covering the entire plant with a perforated plastic bag, which also kept any hatched larvae on the plant until removal. Once the females had perished, the plants were checked for eggs and, if found, were moved into a constant temperature (18°C day, 10°C night) cubicle, to ensure that larvae developed under a standardised temperatures regime. After 2-3 weeks plants were checked for larvae, which were removed and placed into communal rearing pots in a temperature control room (18°C day, 10°C night). Larvae were fed daily with fresh *H. comosa* cuttings and the pots cleaned weekly for the duration of the experiment.

In 2012 larvae of *P. coridon* were reared from eggs laid in captivity in 2011 by wild caught females. These eggs, which hibernate before hatching in spring, were kept on

their original plants in a cool greenhouse cubicle (10°C) after all females had died. From late February 2012 the plants were checked weekly for feeding damage and in April 2012 all plants with a larva were moved into the rearing chamber (18°C day, 10°C night). In May 2012 larvae were collected from each plant and placed in communal rearing pots in a controlled temperature room (18°C day, 10°C night) and fed daily with fresh *H. comosa* cuttings; pots were cleaned weekly at first and twice weekly after larvae reached their final instar.

In 2011 and 2012, colonies of *M. sabuleti* were collected from Magdalen Hill Down nature reserve (Butterfly Conservation), Winchester, UK. In 2011 6 colonies of *M. sabuleti* were collected, and 15 collected in 2012. For *L. niger* in 2011, two separate collections were made from the same large colony (from Wheatley, Oxfordshire), and in 2012 two further *L. niger* colonies were collected to create stock colonies (in Wallingford, Oxfordshire, UK), one of which was used for *P. coridon* experiments and the other for *P. bellargus*. Each stock colony was placed into a large, ventilated Perspex container (28cm x 16cm x 9cm), in which a 150mm² piece of damp cellulose cloth was placed with an upturned plastic plant pot (150mm diameter) on top. Weekly, each stock colony was provided with sucrose granules and *Drosophila melanogaster* larvae and pupae to feed on, and cleaned of dead workers and waste. Cellulose sponges were watered weekly for *M. sabuleti*, but often required watering twice weekly for *L. niger* as the sponge dried more rapidly for this species. Fluon ® (ACG Chemicals Ltd) was painted around the inside of each container to prevent ants escaping.

4.3.2 Experimental design and set up

Each experiment consisted of a set of 32 separate chambers, 12 containing *M. sabuleti* colonies, 12 containing *L. niger* colonies and 8 control chambers containing no ants. Each chamber consisted of a clear Perspex ant nest box (172mm x 113mm x 57mm) and a smaller larval box (73mm x 73mm x 80mm) connected to each other by a 100mm long, 18mm diameter perspex tube (Plate 4.2). Ventilation holes (1cm diameter) were drilled in the back of both chambers and covered with fine metal gauze. Both the ant nest and larval boxes had perspex lids and their top edges were coated with an approximately 2cm wide band of Fluon[®] to prevent workers escaping. To each ant nest box a 15cm² piece of damp cellulose cloth was added, along with an upturned plastic plant dish, to act as a shaded nesting area for the ants, adapted from the methods of (Wardlaw et al. 1998). For the 2011 *P. bellargus* experiment, each ant chamber contained a colony of 40 workers and 8 brood. I initially planned to have 20 brood per colony, but too few were collected for *M. sabuleti*. Greater success was achieved with *M. sabuleti* colonies in 2012, with the information that brood from different *M. sabuleti* colonies could be pooled (K. Schönrogge. pers comm.), allowing the number of brood to be increased to 20 in the *P. coridon* experiment. There were however problems with worker mortality in *L. niger* nests, which was likely due to 40 workers being too stressful (J. S. Pedersen pers comm.): thus in the 2012 *P. bellargus* experiment, each test colony consisted of 80 workers and 40 brood. This had the desired effect, with mortality among *L. niger* workers becoming less frequent. Once set up, all chambers were moved from the laboratory into an outdoor insectary and left for 24 hours to acclimatise. Half way through each experiment the number of workers that had died in each ant colony was counted plus a spot count of those

remaining. New workers were added to restore the number the starting number (40 or 80 depending on the experiment).

In every larval chamber, the base was covered with sterilised (autoclaved and oven dried) soil to a depth of approximately 15mm, as larvae of both *P. bellargus* and *P. coridon* are well documented to bury themselves into the soil and be buried by ants (Thomas and Lewington 2010). Four fresh stems of *H. comosa* contained in a 1.5 ml eppendorf tube (lid removed) filled with water and sealed shut with Parafilm M[®] were placed on the soil surface in the centre of the chamber (see Plate 4.2). Trials revealed that this method maintained *H. comosa* stems alive and in good condition for two weeks.



Plate 4.2 Photograph of the experimental chamber set up, showing the ant chamber on the right and the larval chamber on the left.

After 24hrs the larval boxes were attached to each ant nest box via the connecting tube. The ants were provided with a subsistence diet of sucrose granules weekly and live larvae / pupae of *Drosophila melanogaster* twice weekly, provided in separate 0.5ml Eppendorf tubes. These tubes were placed in the larval box, as opposed to the

ant nest box, to make the distinction between the nest site and the foraging area clearer to the ants and to encourage their movement into the larval box.

Colonies were then left to settle down for a further 24 hours before larvae were added. Second instar larvae were weighed and assigned to each chamber randomly. For both the 2011 and 2012 *P. bellargus* experiments, two larvae were introduced to each chamber. Each larva was tagged with either one or two dots of non toxic ink (trialled prior to the experiment) which aided in both tracking individual larvae during weighing and noting when the transition between instars occurred, as the paint was shed (Plate 4.3). Only one *P. coridon* larva was placed in each chamber due to too low numbers.

All analysis was conducted using the statistical package R (R Core Development Team, 2012). Mixed model analyses were implemented using the nlme package (Pinheiro et al. 2013).



Plate 4.3 A paint tagged 3rd instar *Polyommatus bellargus* larva being tended by *Lasius niger* workers, under experimental conditions. In this instance the larva has moved into the ant chamber. The ant to the right is tending the larva's DNO.

4.3.3 Development

Larval development

The methods outlined below were used for both the *P. bellargus* and *P. coridon* experiments. As there were two larvae in each chamber for *P. bellargus*, for each weighing interval the mean weight of the two larvae was taken.

Larvae were weighed before being placed in the larval chambers, and then removed from the chamber up to twice weekly for weighing. If in the initial 2 weeks of the experiment a larva was either found dead or missing, a new larva was added to the chamber the subsequent day. After this larvae were not replaced and instead only the weights of the remaining larvae were used in the analysis. Mortalities and replacements were all recorded. Larvae were weighed once, and when feasible twice, a week.

The average daily growth rate in each chamber was calculated in $\text{mg mg}^{-1} \text{ day}^{-1}$, for each weighing interval. Initially it had been planned to compare the growth rate within each instar, but the number of moults recorded for each larva proved too inconsistent. The data set was instead split into two phases; a growth phase and a weight loss phase. For each chamber the growth phase was taken as ended when the maximum larval weight was recorded. For both the growth phase and weight loss phases of each chamber the average rate of growth / weight loss was then calculated as the mean across all values recorded for each chamber. Figures 4.2 and 4.3 show the average growth curves for each experiment. Temperature could not be included in the analysis presented below, as it would be confounded by year, but is included in Figure

4.2. ANOVAs were then used to test the effects of ant treatment and year for *P. bellargus*, on both growth and weight loss.

Maximum weight

ANOVAs were used to analyse the data for significant difference in weight gain between ant treatments, and years in the case of *P. bellargus*.

Adult weight

On the final weighing date, all surviving pupae were removed from their box and placed in separate rearing pots. These were checked regularly for emerged adults (daily where feasible), which were removed and frozen for storage, before drying at 70°C for 76 hours and weighing using a Precisa 40SM-200A balance to determine dry mass.

A generalised linear mixed model was used to examine the effects of ant treatment, sex and year for *P. bellargus* adult dry weight, as many boxes contained both a male and female butterfly. A mixed model allowed each individual to be treated as a replicate by including a random effect term for box. Examination of the residuals in the *P. bellargus* model revealed a large degree of heteroskedasticity, due to females showing a larger degree of variation in weight than males. To correct for this, it was specified that females had a different variance structure to males. ANOVA was used to analyse the *P. coridon* data.

Table 4-1 The various classes (and types there-in) of behaviour recorded during the 15 minute observation period, along with their definition and the code when recording. DNO refers to the Dorsal Nectary Organ, a gland found on the 7th abdominal segment, which produces a nutritious solution for attendant ants. Antenation is defined as the ant repeatedly drumming the larval surface with its antennae.

Behaviour Class	Behaviour Type	Code
Ant – Larval Interactions	Tending - ant in contact with larva and antenating on the larval surface	<i>Tend</i>
	Tending at the DNO	<i>Tend with D</i>
	Neutral –contact but no antenation	<i>Neutral</i>
	Aggression – biting or stinging	<i>Attack</i>
Larval Behaviour	Eversion of the Tentacular Organs	<i>Larva, Tent</i>
	Movement any where within the chamber	<i>Start / End, Larva, Move</i>
	Feeding on host plant	<i>Start / Feeding</i>

4.3.4 Behaviour

Larvae-Ant Interactions

For each observation session, all chambers were checked for visible larvae, and observations were made for all chambers in which a larva could be seen. Each observation period lasted for 15 minutes, over which time the duration of behaviours exhibited by the larvae were recorded, along with the frequency and duration of ant worker behaviour exhibited towards the larvae (see Table 4.1). If several ants were

tending a larva at the same time, then one ant was chosen for observations until it lost contact with the larva, at which point another attendant ant was chosen.

All sessions were recorded on a digital Dictaphone (Philips Digital Voice Tracer LFH0615) and the files transferred onto a computer at the end of each session. From these files the recordings were transcribed, and the total amount of time ants spent in contact with and tending the larvae during the 15 minute recording session was calculated, along with number of TO eversions by the larva. Table 4.1 shows the behaviours that were recorded, along with their definition.

For each 15 minute observation, the number of times a larva everted its tentacular organs was recorded along with the number of seconds a larva was a) in contact with ants, b) tended by ants, and c) had its dorsal area tended by ants. Two separate analyses were conducted on this data. The first used data from both 2011 and 2012, to examine if the amount of time ants spent in contact with a larva was a function of year (as 2012 had twice the number of ant larvae and workers in each nest) and ant species. The second analysis was to examine the effect of ant species and the time spent being tended by ants on the number of tentacular eversions a larva made. A generalized linear mixed model, with a poisson error structure, was used, with box number as a random effect to account for temporal pseudo replication. For this analysis only the 2012 data set could be used, as in 2011 tentacular eversion occurred in only 3 of the 47 observations made.

Inspection of the initial models for both of the above analyses revealed overdispersion in the residuals, also evident from histograms of both the number of seconds of

contact and the number of tentacular eversions. This was addressed by adding a replicate level random effect term to the model, which provides an approximation of using a poisson-lognormal error distribution (Elston et al. 2001; Zuur et al. 2012). All mixed model R squared values were calculated using the method provided by Nakagawa et al. (2013) and are marginal, representing the variation explained by the fixed effects only.

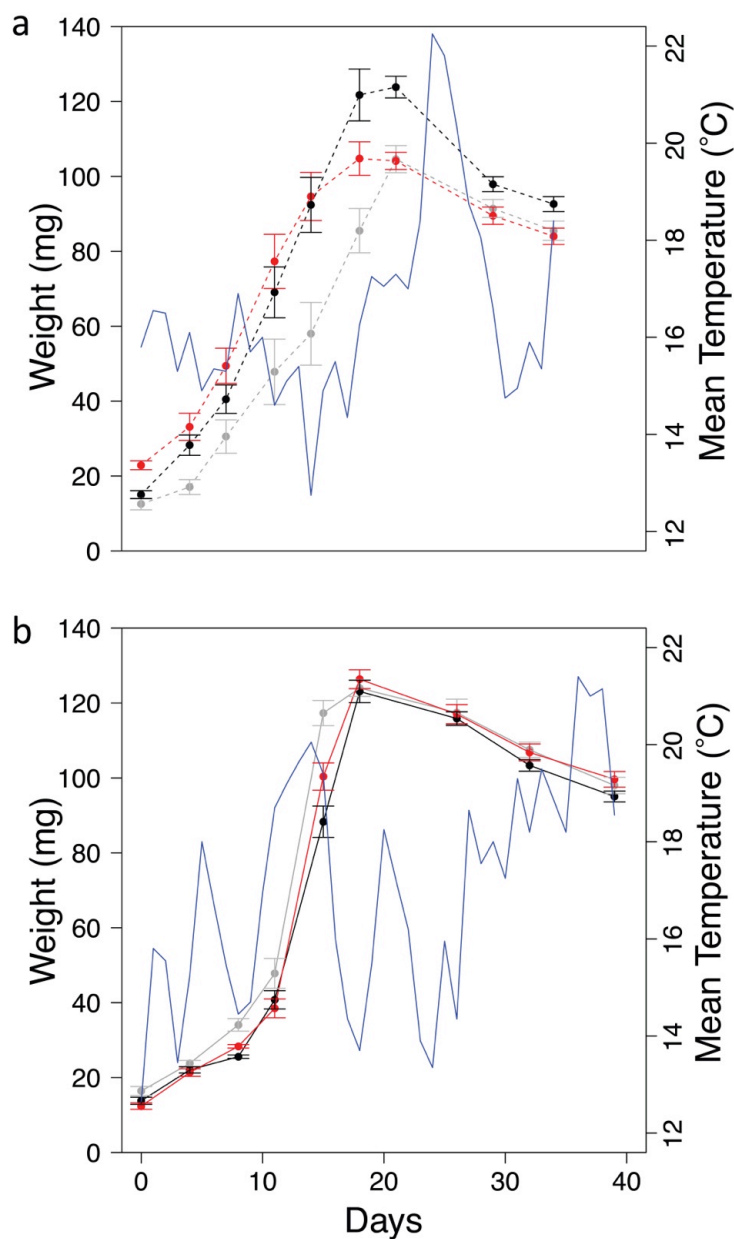


Figure 4.2 Growth curves for the a) 2011 and b) 2012 *P. bellargus* experiments. Points show the average larval weight for each ant treatment, with grey circles representing control larvae, red circles

representing larvae exposed to *M. sabuleti* and black circles representing larvae exposed to *L. niger*. The blue line shows the daily mean temperatures over the course of each experiment. The vertical dashed lines shows the division of the data into the three stages used for the analysis. Error bars show one standard error above and below the mean.

4.3.5 Temperature

Local daily mean temperature data were obtained for the duration of each experiment from the CEH Wallingford meteorological station. The blue lines in Figure 4.2, shows daily mean temperatures during each *P. bellargus* experiment.

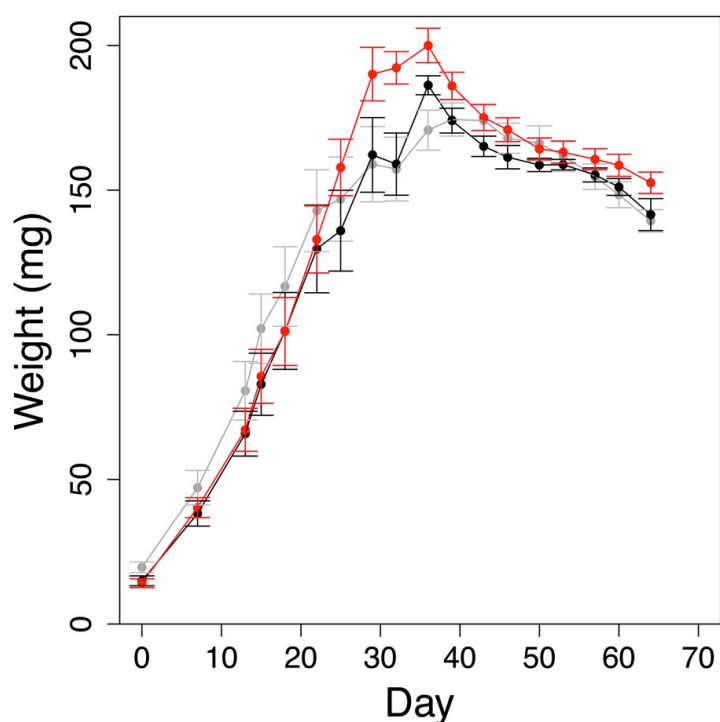


Figure 4.3 Growth curves for the *P. coridon* experiment in 2012. Points show the average larval weight for each ant treatment, with grey circles representing control larvae, red circles representing larvae exposed to *M. sabuleti* and black circles representing larvae exposed to *L. niger*. Error bars show one standard error above and below the mean.

4.4 Results

4.4.1. Mortalities and loses

Polyommatus bellargus

In 2011, five chambers (replicates) under the *M. sabuleti* treatment were lost from the experiment due to both larvae in the chamber dying. One chamber for *L. niger* was lost entirely, and a further two chambers suffered one larval mortality. However for one chamber a larva died within the first two weeks and so was replaced and used in the analysis of weight loss. Four control larvae failed to emerge as adults and therefore could not be included in the analysis of adult weights.

In 2012 two *M. sabuleti* replicates were lost, owing to both larvae in the chamber dying, and a further chamber lost one larva. A further three pupae failed to emerge, one under the control treatment and two under the *L.niger* treatment, and two *L. niger* treated individuals escaped as adults, and so these could not be used in the analysis of adult weights.

Polyommatus coridon

One larva, and so in this case one replicate, under the *L. niger* treatment was lost from the growth phase of the *P. coridon* experiment. This larva died in the first week of the experiment and was replaced and data for the replacement larva were used in the weight loss analysis. A further four *L. niger* treated and two *M. sabuleti* treated pupae failed to emerge and so these could not be used in the analysis of adult weights.

4.4.2 Larval development

Polyommatus bellargus

Growth rate was significantly lower in 2011 than 2012 ($F_{(1,52)} = 4.273$, $p = 0.044$). There was also significant interaction between ant treatment and year in explaining larval growth rate ($F_{(2,50)} = 4.758$, $p < 0.05$). Post hoc Tukey tests showed that this was the result of the growth rate for larvae exposed to *M. sabuleti* being significantly lower than that of larvae not exposed to ants ($\beta = -0.052 \pm 0.019$, $t = -2.825$, $p < 0.05$) and those exposed to *L. niger* ($\beta = -0.060 \pm 0.017$, $t = -3.493$, $p < 0.01$) (Figure 4.4a). The rate of weight loss was found to be significantly higher in 2011 than in 2012 ($F_{(1,52)} = 10.537$, $p < 0.01$). There was also a significant interaction between ant treatment and year in explaining pupal weight loss ($F_{(2,50)} = 3.820$, $p < 0.05$). Post hoc Tukey tests showed that this was due to larvae exposed to *L. niger* having a significantly higher rate of weight loss than the control in 2011 ($\beta = -0.0062 \pm 0.0023$, $t = -2.753$, $p = 0.043$). (Figure 4.4b).

For *P. bellargus*, maximum larval weights were significantly higher in 2012 than in 2011 ($F_{(1,52)} = 4.266$, $p = 0.044$), and there was a significant interaction between year and ant treatment ($F_{(2,50)} = 4.459$, $p < 0.005$). Post hoc Tukey tests showed that this was due to the maximum weight for larvae exposed to *L. niger* being significantly higher than the control in 2011 ($\beta = 24.574 \pm 8.185$, $t = 3.002$, $p < 0.05$) (Figure 4.5).

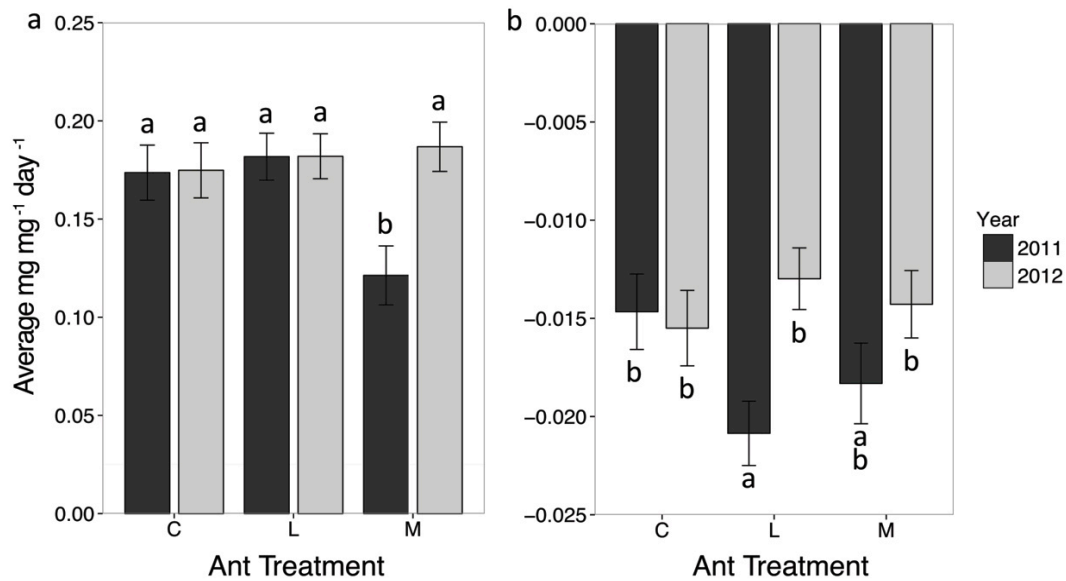


Figure 4.4 a) The average growth rates of *P. bellargus* larvae and b) the average rate of weight loss for *P. bellargus* pupae, for each ant treatment, showing rates in 2011 (black) and 2012 (grey). Treatments with a different letter above their bars are significantly different from one another. Error bars show one standard error above and below the mean. C = controls, L = larvae reared with *Lasius niger*, M = larvae reared with *Myrmica sabuleti*. (C-2011 n = 8, L-2011 n = 11, M-2011 n = 7, C-2012 n = 8, L-2012 n = 12, M-2012 n = 10).

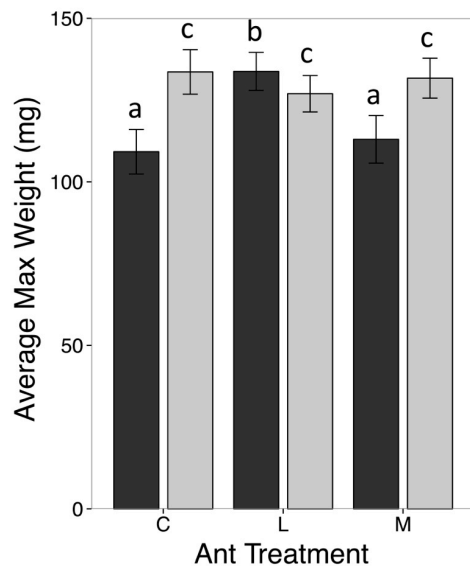


Figure 4.5 The average maximum weight of *P. bellargus* larvae under the different ant treatments, showing weights in 2011 (black) and 2012 (grey). Error bars show one standard error above and below the mean. C = controls, L = larvae reared with *Lasius niger*, M = larvae reared with *Myrmica sabuleti*. (C-2011 n = 8, L-2011 n = 11, M-2011 n = 7, C-2012 n = 8, L-2012 n = 12, M-2011 n = 10).

Polyommatus coridon

Inspection of the model residuals for the initial ANOVA of growth rate showed one data point (under the *M. sabuleti* treatment) was an extreme outlier, having a average growth rate (0.27 mg / mg /day), over a third higher than the next highest (0.19 mg /mg / day) and twice as high or higher than the majority of larva. It was decided that the highest weight for this larva may have been mis-recorded and so this replicate was removed from the analysis.

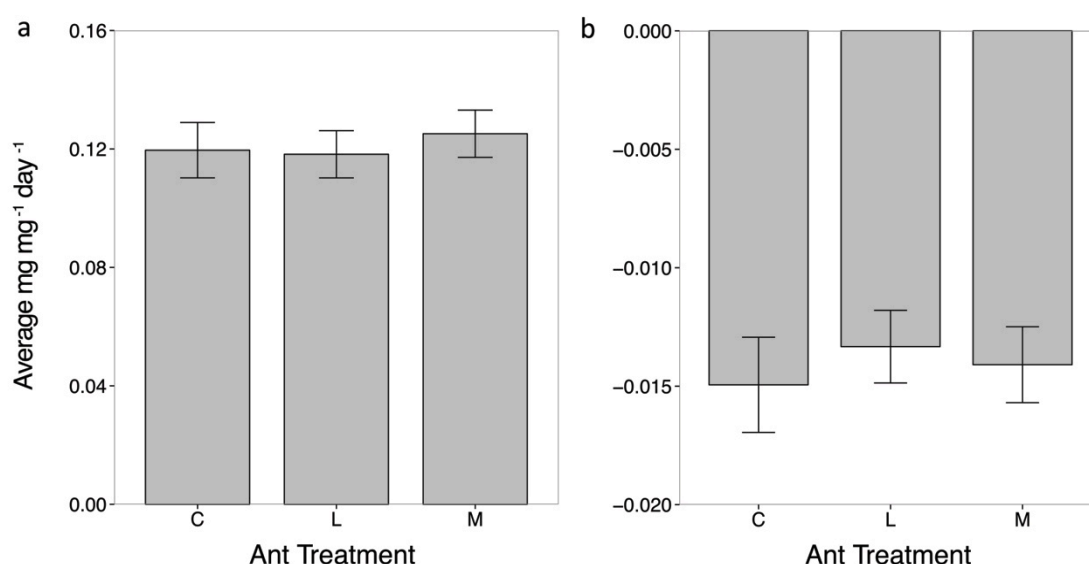


Figure 4.6 a) The average growth rates for *P. coridon* larvae and b) average rate of weigh loss of *P. coridon* pupae, for each ant treatment in 2012. Error bars show one standard error above and below the mean. C = controls, L = larvae reared with *Lasius*, M = larvae reared with *Myrmica*. (C n = 8, L n = 11, M n = 12 for growth and L n = 12 for weight loss).

There was no significant difference in growth rate for *P. coridon* larvae between ant treatments ($F_{(2,27)} = 0.196$, $p = 0.823$) (Figure 4.6a). Nor was there a significant difference in the rate of weight loss between ant treatments ($F_{(2,27)} = 0.196$, $p = 0.823$) (Figure 4.6b). Maximum weight gained also did not differ significantly between ant treatments ($F_{(2,27)} = 0.72$, $p = 0.496$) (Figure 4.7).

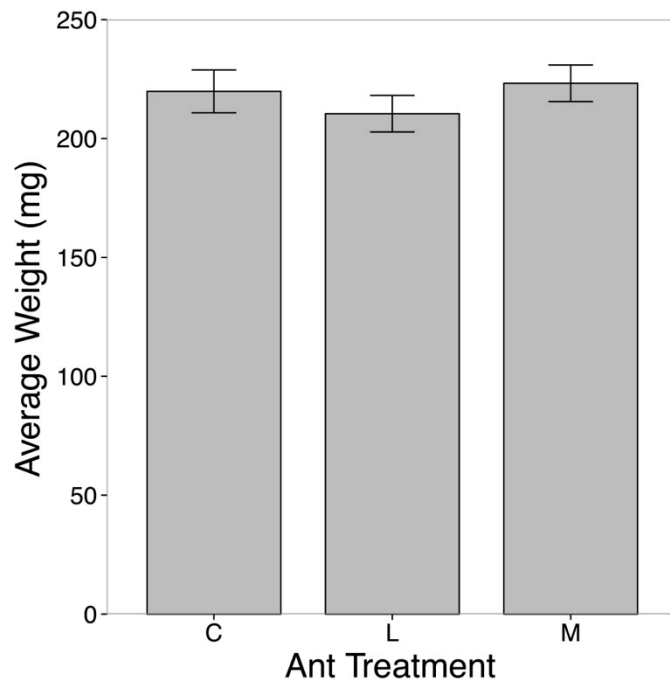


Figure 4.7 The average maximum weight of *P. coridon* larvae under each ant treatment in the 2012. Error bars show one standard error above and below the mean. C = controls, L = larvae reared with *Lasius*, M = larvae reared with *Myrmica*. (C n = 8, L n = 12, M n = 12).

4.4.3 Adult weights

Polyommatus bellargus

Year had a significant effect on adult dry weight for *P. bellargus* ($F_{(1,56)} = 33.737$, $p < 0.001$), with weights being higher in 2012 than in 2011 ($\beta = 2.690 \pm 0.475$, $p < 0.001$). Sex was also significant ($F_{(1,42)} = 27.951$, $p < 0.001$) with males being significantly lighter than females ($\beta = -2.723 \pm 0.515$, $p < 0.001$) (Figure 4.8).

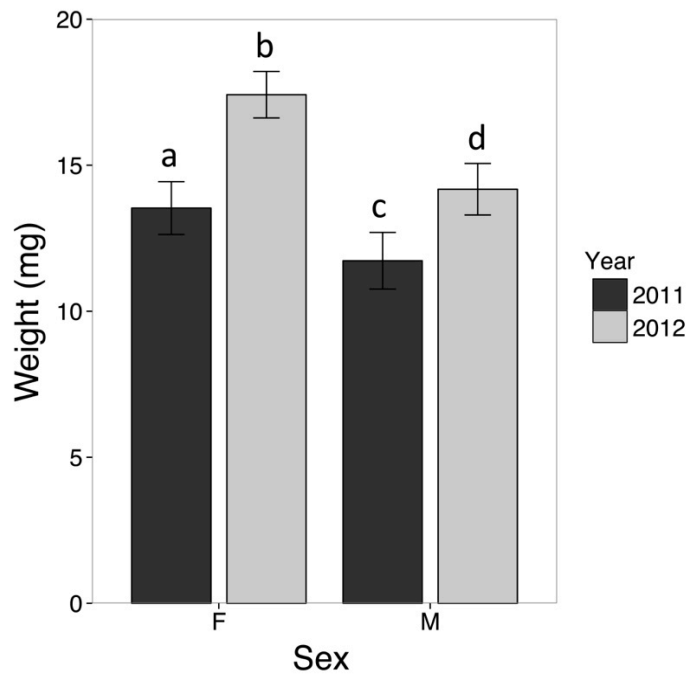


Figure 4.8 The average dry weight of *P. bellargus* adults, by year, grouped by sex. Treatment with a different letter above their bar are significantly different from one another (F-2011 n = 20, M-2011 n = 23, F-2012 n = 30, M-2012 n = 28).

Polyommatus coridon

The ANOVA for *P. coridon* found no significant difference in adult dry weights between ant treatments ($F_{(2,21)} = 0.507$, $MS = 1.034$ $p = 0.609$), nor were weights significantly different between males and females ($F_{(1,20)} = 3.224$, $MS = 20.549$, $p = 0.083$).

4.4.4 Behaviour

Ant contact in P. bellargus

The minimum adequate model of ant contact time retained terms for year and ant species, but not their interaction. The model fitted the data significantly better than the null model ($\text{loglik} = -634.027$, $\chi^2_{(5,89)} = 16.829$, $p < 0.001$), explaining around 25% of the variation in the time ants spent in contact with larvae. *Myrmica sabuleti* spent

significantly less time in contact with larvae than *L. niger* workers in both years ($\beta = -1.275 \pm 0.597$, $z_{(1,90)} = -2.136$, $p = 0.0327$), and ants spent significantly more time in contact with larvae in 2012 (when the number of workers and ant larvae was doubled) compared to 2011 ($\beta = 2.457 \pm 0.593$, $z_{(1,89)} = 4.144$, $p < 0.0001$) (Figure 4.9).

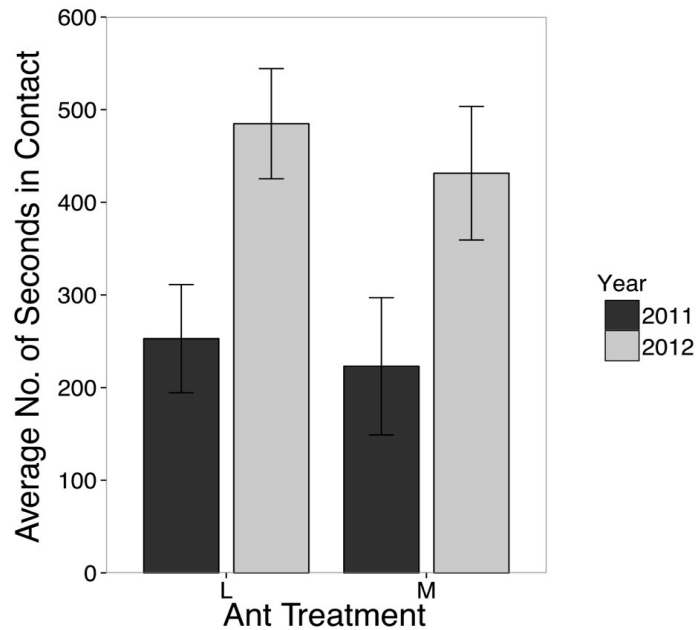


Figure 4.9 The average number of seconds for which a larva was in contact with ants for different ant treatments, *L. niger* (L) and *M. sabuleti* (M). Error bars show one standard error on the mean. (L-2011 $n = 29$, M-2011 = 18, L 2012 $n = 28$, M 2012 $n = 19$).

Larval responses to ants

The minimum adequate model of larval tentacular eversions contained terms for the number of seconds being tended and the proportion of time spent tending the DNO. The model fitted the data significantly better than the null model (loglik = -132.791, $\chi^2_{(5,42)} = 28.183$, $p = <0.0001$), and explained around 49% of the variation in tentacular eversion by the larva ($R^2 = 0.485$). Larvae that were tended for longer everted their

tentacular organs more frequently ($\beta = 0.002 \pm 0.001$, $z_{(1,43)} = 2.365$, $p < 0.05$), and the proportion of time spent tending the dorsal nectary organ also significantly increased the number of tentacular eversions ($\beta = 2.540 \pm 0.499$, $z_{(1,42)} = 5.126$, $p < 0.0001$) (Figure 4.10).

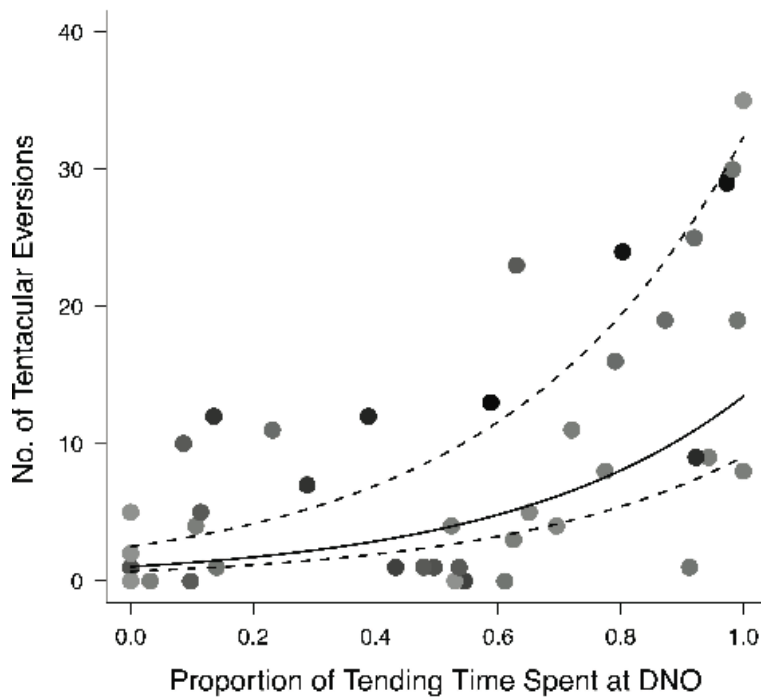


Figure 4.10 The predicted relationship between the number of tentacular eversions observed within a 15 minute recording session and the proportion of time ants spend tending a larva that was dedicated to tending the dorsal nectary organ. The darkness of the data points represents the amount of time that ants spent tending to the larva, the darker the colour the longer the amount of time spent in contact. The central, solid, line shows the predicted values when the amount of time a larvae was tended is kept at the mean across observations, with the lower and upper dashed lines showing prediction at 0.05 quantile and 0.95 quantile respectively.

4.5 Discussion

4.5.1 Myrmecophily in *P. bellargus* and *P. coridon*

It is well established that the interactions with ants can affect larval development in some mutualistic Lycaenid species (Pierce et al. 2002). Here I tested the hypothesis that ant attendance affects the development of larvae of both *P. bellargus* and *P. coridon*. For *P. coridon*, the results show that growth rate, weight loss during the pupal stage and the maximum weight of larvae were not affected by exposure to ants, nor was there a difference in the adult weights of larvae exposed to different ant species.

Results were more complicated for *P. bellargus*, as there were significant differences between ant treatments for larval growth rate, pupal weight loss and maximum larval weight in 2011, but no differences between ant treatments in the data from 2012. Larvae exposed to *M. sabuleti* in 2011 had significantly lower growth rates than both control larvae and those exposed to *L. niger*, and those larvae reared with *L. niger* had higher rates of pupal weight loss than controls. However, larvae reared with *L. niger* also had significantly higher maximum weights than control larvae in 2011. The lack of a consistent effect of ant treatment between years makes it difficult to draw conclusions on the effect of ant exposure on development in *P. bellargus*, particularly as the significant effects of ant treatment occurred in 2011 when conditions were in theory less likely to subject the butterfly larvae to significant resource demands; the ant colonies had fewer workers in 2011 (40 vs 80) and had a smaller complement of ant larvae and pupae (8 vs 40).

In 2011 larvae under *M. sabuleti* showed the highest levels of mortality and significantly lower growth rates. It is possible that the lower ant worker – ant larvae / pupa ratio in 2011 altered the behaviour of the ants during the experiment, either in the way they utilised the larva or by encouraging aggressive behaviour in the ants. However, no aggression was observed and the degree of contact between the ants and larva in 2011 was actually lower. The higher mortality rate for larvae exposed to *M. sabuleti* substantially reduced the number of replicates from 12 to 7 in 2011, so with better replication differences between treatment might change, but the fact that mortality rates were higher for *M. sabuleti* remains. The significantly higher rate of weight loss in pupae exposed to *L. niger* could suggest that this ant exacted a larger cost on *P. bellargus* during this stage and the significantly higher maximum pupal weights for these larvae may be compensatory mechanism for their higher weight loss as pupae. Ultimately, however, none of this translated into effects of adult dry weights, so it appears that any impacts ants had on development were compensated for, as was also suggested for the previous study of *P. bellargus* (Fiedler and Saam 1994). Studies of other species have also found no impact of ant treatment on adult weight, even when larval development was affected (Kaminski and Rodrigues 2011; Wagner 1993; Wagner and Kurina 1997).

Behavioural data for *P. bellargus* shows that an increase in the amount of time a larva is tended by ants and the proportion of this time that is dedicated to feeding from the DNO, increases the frequency with which larvae evert their TO. However, there was no difference in the response to different ant species. Whilst this shows that the species of ant does not influence larval behaviour, the lack of larval response observed in 2011 compared to 2012 (when the number of workers was doubled)

implies that larvae will respond more when ants are more abundant. Across years, *L. niger* spent significantly more time in contact with the larvae, which might suggest that they are more attractive to this species of ant. However, with regard to TO eversion, the larvae responded equally to both *M. sabuleti* and *L. niger* across observations.

Overall, these results suggest that their myrmecophilous life-styles do not impose a significant cost, at least in terms of development rate or ultimate biomass, on either *P. bellargus* or *P. coridon*, and that any developmental costs imposed are compensated for, for instance through increased food intake. The higher amounts of time spent in contact with *L. niger* workers in both years, the greater number of larvae observed in contact with *L. niger* and developmental impacts in 2011 suggest that *P. bellargus* may be more attractive to this species of ant. However, there was no developmental difference between ant-exposed larvae and controls in 2012 and caution must be taken when extrapolating from these results beyond the experimental conditions presented, as is discussed below.

4.5.2 Implications, caveats and future work

The primary driver of foraging in ant nests is the procurement of food for the brood (Portha et al. 2002), and though workers will also require resources, it is the number of brood within a colony that is the strongest determinant of resource requirements (G W Elmes pers comm). Therefore the number of ant brood within each experimental chamber will have moderated the use of larvae as a food resource by the ants. For example, in a comparable but inverse experiment involving larvae of *Maculinea rebeli* when reared with a variety of *Myrmica* species, workers of the non-host ant

species tolerated (and tended) the butterfly larvae when provided with abundant food, but killed them (as competitors with their own brood) when resources were scarce (Elmes et al. 2004). The numbers of brood collected with the stock colonies of ants for this experiment, particularly with *M. sabuleti*, constrained the maximum number of brood per chamber to 40 for *P. bellargus* and 20 in *P. coridon*. Further experiments to explore the consequences of rearing larvae with colonies of different sizes and with different resources would be very useful in this regard, as it may be that larger or hungrier colonies would exert more resource demands on the larvae.

The dependence of larval behaviour on environmental context could also be a crucial factor here. The facultative nature of the ant-larvae interactions of *P. bellargus* and *P. coridon* may favour plasticity in the larval responses to ants, as tending by ants may not always be beneficial. It follows that, in the larvae of generalist species, the ability to moderate the costs of interactions with ants (i.e. the amount of resources invested) would be a beneficial trait. Larvae of both the European species *Polyommatus icarus* (Common Blue) and the North American species *Plebejus acmon* have been found to significantly increase their rates of nectar secretion when subjected to simulated attacks, attracting a larger number of ants to tend them (Agrawal and Fordyce 2000; Axén et al. 1996). Nectar secretion is therefore used as an induced defense, with larvae able to alter any costs of their relationship to the context of their interaction. Conversely, for obligate species that require ants for survival, it is more likely that the degree of investment in the relationship will be fixed; this idea, however, has not been tested.

That *P. bellargus* larvae everted their TOs more frequently the more they were attended by ants could be viewed as showing TO eversions serve to attract ant workers. Axén et al. (1996) demonstrated that the TO activity in *P. icarus* (the Common Blue) was more frequent in situations where the larvae need to attract ants (i.e. when returned after isolation from ants or after simulated predator attack). However, when *P. acmon* larvae were subjected to simulated attack, they everted their TOs no more frequently than control larvae (Agrawal and Fordyce 2000). By restricting the ability of some larvae to evert their TOs, Weeks (2002) found no evidence that TO eversion functioned to attract ants in larvae of the facultative mutualist *Glaucopsyche lygdamus*, but rather served to increase aggression and agitation in those ants tending a larva. Provoking such a response in the presence of natural enemies would increase the degree of protection afforded to the caterpillar, but in the absence of natural enemies this could serve to stop ants feeding from the DNO. It is possible then that the increased TO eversion with increased amounts of DNO tending seen here in *P. bellargus* larvae was actually serving to reduce costs to development by antagonizing the ants. In this respect, further observations of the behavioural response of attending ants to the eversion of the TO would be useful.

The lack of an effect of exposure to ants on larval development is consistent with the hypothesis that larvae of *P. bellargus* and *P. coridon* can mediate their investment in mutualistic interactions with ants depending on the context in which the interaction occurs. The niche of each species may be coupled to that of these ants through some other mechanism than growth and development, for example by greater survival owing to putative protection from enemies. Imposing a cost in the form of exposure to a natural enemy would be one way of exploring this, whereby larvae are also exposed

to a parasitoid known to attack larvae in the wild. However, rearing suitable parasitoids in the laboratory would predicate such experiments. Other studies have used field experiments where plants supporting larvae are placed *in situ* and ants are excluded from half of these (Novak 1994; Pierce and Mead 1981; Pierce and Eastal 1986; Weeks 2003), but this would not provide information as to the relative importance of the attendant ant species. Another option would be to simulate a predatory attack using forceps to pinch larvae and monitor their behaviour and their nectar secretion rates (Agrawal and Fordyce 2000).

The availability of food resources to an ant colony will also influence the degree to which they utilise larvae as a food resource. If ants have a restricted diet, or their herds of trophobiont aphids fail, it may be that they utilise the secretions of butterfly larvae more heavily, and so exact a larger cost. The degree of replication, and the resultant increase in maintenance required for an experimental design in which the ants are subject to different food availability treatments (e.g. low, medium and high) was not feasible for this study, but is certainly an area for further work. Equally, variation in the food availability to the butterfly larvae might alter outcomes. For example, reduced food availability, or quality, might impair their ability to compensate for the costs incurred by myrmecophily. However, current evidence suggests it is more likely that investment in myrmecophily decreases when food quality is low (Baylis and Pierce 1991). For instance, when fed on a diet of lower nutritional quality, larvae of the facultative mutualist *Polyommatus icarus* had a corresponding reduction in the nutritional quality of the DNO secretions, and were tended less frequently by ants (Burghardt and Fiedler 1996; Fiedler 1990). Such

plasticity in the nutritional content of the DNO secretions is another route through which larvae might alter their investment in mutualistic interactions.

4.5.3 Conclusions

The results presented here suggest that larvae of *P. bellargus* and *P. coridon* do not differ in the extent to which they invest resources in myrmecophilous relationships when exposed to either *M. sabuleti* or *L. niger*. The developmental cost of engaging with either species is ultimately not significantly different from that of larvae grown without ants present. This may be surprising given that the ants were observed tending the larvae of both *P. bellargus* and *P. coridon* and that the larvae were seen to respond. However this is consistent with the results of studies of other species, in that ultimately any developmental costs were compensated for (Fraser et al. 2001; Kaminski and Rodrigues 2011; Wagner 1993). The eversion of the TO in *P. bellargus* in response to ants tending the DNO raises questions about the function of this behaviour, and whether it is used to attract (by agitating) ants or deter them from feeding on the caterpillar. The two are not mutually exclusive. Important questions remain as to how the dynamic of these mutualistic interactions might change under different conditions. Further, experiments that incorporate variation in the quality of the ant food resources, larval food resource and the size of the ant colonies to which larvae are exposed would provide useful data, as would direct measures of resource investment, such as nectar secretion rate and content. The most important next step however would be experiments to see how larval development, and behaviour is altered, in the presence of a natural enemy. This is could be the key variable that will alter the benefit of myrmecophilous interactions for larvae, and in turn alter larval investment in myrmecophily.

**Chapter 5 – The genetic structure of
Polyommatus bellargus and *Polyommatus
coridon* populations in the UK– How
fragmented are their populations?**

5.1 Abstract

Habitat loss can cause increased spatial structuring of populations as they become increasingly fragmented. The UK population of *Polyommatus bellargus* and *Polyommatus coridon* have always existed as groups of discrete subpopulations, but with declines in calcareous grasslands they have become increasingly fragmented. Previous population genetics and mark-release-recapture (MRR) studies for *P. bellargus* (in the UK) and *P. coridon* (in Germany) indicate that *P. coridon* has a better capacity for long distance dispersal and low levels of genetic structuring. Here the impacts of a fragmented population structure on the genetic structure of *P. bellargus* and *P. coridon* in the UK is explored, using microsatellite analysis. The hypothesis that *P. bellargus* is more susceptible to the effects of habitat fragmentation is addressed by assessing the degree of genetic structuring between subpopulations of each species. It is found that in the South of England *P. bellargus* exhibits a higher degree of genetic structuring than *P. coridon*, with higher levels of genetic differentiation between subpopulations based on allele frequencies. Both species, however, show evidence for low genetic diversity within subpopulations, indicating inbreeding. Isolation by distance analysis shows for both species that a larger degree of differentiation between subpopulations is related to a larger degree of geographic separation, but the landscape matrix between subpopulations may also play a role in determining connectivity. The potential causes and implications for conservation of the differences between these two species are discussed, with particular regard to the potential role the more specialised habitat requirements of *P. bellargus* might be playing.

5.2 Introduction

5.2.1 The genetic consequences of habitat fragmentation

The loss and degradation of natural habitats due to human activities is well recognised as a major threat to biodiversity at all scales (Novacek and Cleland 2001; Pimm and Raven 2000). Whilst habitat loss will decrease the density and distribution of species within landscapes, any remaining habitat will be fragmented (Fahrig 2003). The spatial arrangement and size of the remaining fragments can have important consequences for the population dynamics and persistence of a species relying on this habitat, and is consequently a major concern for ecology and conservation biology (Ewers and Didham 2006; Fahrig 2003; Harrison and Bruna 1999). Populations will be divided into spatially distinct subpopulations within patches of suitable habitat of varying size and distance from each other. The impacts of fragmentation on the population dynamics of butterflies has been extensively explored for butterflies in the context of metapopulation ecology (Hanski 1998; Hanski and Gilpin 1997). Smaller population sizes and isolation between habitat patches will leave populations more susceptible to extinction from stochastic events. Additionally fragmentation will decrease gene flow and increase genetic drift between subpopulations, moving populations away from panmixia and consequently increasing the risk of inbreeding depression (Frankham et al. 2010; Templeton et al. 1990). Ultimately this can lead to a reduction in genetic diversity and potentially reductions in the fitness of individuals in a population, increasing the risk of extinction (Reed and Frankham 2003; Saccheri et al. 1998; Vandewoestijne et al. 2008). As such, habitat fragmentation has become a major area of research for conservation genetics (Frankham 2010; Frankham et al. 2010).

5.2.2 The impacts of fragmentation on population genetics in butterflies

Given the extensively studied nature of butterflies, and in particular the impacts of fragmentation on their populations, there have been a comparatively large number of studies on the relationship between fragmentation and population genetics, particularly in Europe (Bossart and Antwi 2012; Collier et al. 2010; Nève 2009). Unsurprisingly the impacts of fragmentation have proved distinct for different species; some show genetic differentiation between habitat patches and exhibit reduced genetic variation in subpopulations (Schmitt and Seitz 2004; Williams et al. 2003), whereas for others fragmentation has little impact on population structure (Habel et al. 2010; Louy et al. 2007). Ultimately the degree to which fragmentation will alter the population structure of a species will be determined by its ability to move between habitat fragments, as this will define gene flow. This will be related to both its intrinsic ability for long distance dispersal and the degree to which the intervening landscape is permeable to movement (Dover and Settele 2009; Merckx 2005). For species that are poorer dispersers the effective fragmentation within a given landscape is likely to be higher than for a species that can move over greater distances. Indeed, for butterflies where dispersal ability has been quantified by other means, those species with low dispersal abilities are more spatially structured in fragmented habitats (Nève 2009), and this is also true for other taxa (Callens et al. 2011; Watanabe et al. 2010).

5.2.3 The effects of fragmentation of *P. bellargus* and *P. coridon*

Changes in agricultural practices and land use since the 1950s have caused severe declines in calcareous grasslands in the UK and in northern Europe, and that which remains is in a highly fragmented state (Blackstock et al. 1999; Fuller 1987; Poschlod and WallisDeVries 2002). The UK populations of both *P. bellargus* and *P. coridon* in England have always existed as a collection of discrete subpopulations restricted to fragments of chalk grassland (Asher et al. 2001), but the loss of and further fragmentation of calcareous grasslands has exacerbated this. The population genetic structure of both species has been examined previously. For *P. bellargus* a study of twenty-six populations in England, found strong evidence for genetic structuring between populations, and regions, and for an isolation by distance effect based upon the genetic similarity between populations; the further apart populations became the less similar they were (Harper et al. 2003). These results corroborate previous observations from mark-release-recapture (MRR) studies of low exchange rates of individuals between colonies of *P. bellargus* in close proximity (~100m) (Thomas 1983). Isolation was not found to influence the genetic diversity of populations, but genetic diversity was found to be lower in smaller populations. Comparisons between museum specimens and present day samples, utilising the same microsatellite loci, identified a recent and severe genetic bottleneck (loss of genetic allelic diversity) for *P. bellargus* in England, thought to be a consequence of the 1976 drought in England (Harper et al. 2003; Harper et al. 2006). Further evidence from mitochondrial DNA indicated that the current English populations originated from a small number of female migrants that crossed over from France (Harper et al. 2007).

The population genetic structure of *P. coridon* has been well studied in northern Europe using allozymes, both with regard to reconstructing historical colonisation

patterns, and estimating more localised population structure (Schmitt and Krauss 2004; Schmitt and Seitz 2001; Schmitt and Seitz 2002b). Schmitt and Seitz (2001) found that European populations could be divided into two distinct lineages, 'Western' and 'Eastern', caused by population retractions in the last glaciation event. Subsequent comparisons of populations in the 'Eastern' group (from N Germany, Hungary, Slovakia and the Czech Republic) showed low population differentiation between regions, but evidence for structuring within regions and isolation by distance between populations (Schmitt and Seitz 2002b). Two allozyme studies at a smaller scale, from western and northern Germany, have found little evidence for genetic structuring of *P. coridon* populations in fragmented landscapes, with weak differentiation and no evidence for isolation by distance (Krauss et al. 2004; Schmitt and Seitz 2002a). Further work on fragmented grassland in Germany by Krauss et al. (2005) found only a weak effect of isolation on either the occupation by, or population size of, *P. coridon*. Further, a MRR study in western Germany suggested that though inter-patch movement of *P. coridon* was relatively uncommon (5 of 157 recaptured individuals were caught in a new patch) individuals are capable of longer distance movement, with four individuals moving over 600m and one male being caught 3.7km from its natal patch (Schmitt et al. 2006).

5.2.4 Chapter aims

Polymmatius bellargus and *P. coridon* are classified in the literature as being sedentary or of low mobility (Cowley et al. 2001; Pollard and Yates 1994). However, current evidence from both observed dispersal ability and population genetics suggests that *P. coridon* may have a higher capacity for dispersal, and consequently it may experience habitat fragmentation less severely than *P. bellargus* (Schmitt et al.

2006; Thomas 1983). In this study the population genetics of fragmented populations of *P. bellargus* and *P. coridon* occurring in the south of England are examined to determine the level of genetic structuring within each species. Microsatellite loci specific to each species are used to genotype individuals from different populations found on calcareous grassland nature reserves. The hypothesis that *P. bellargus* is more susceptible to the effects of habitat fragmentation is addressed in two ways. Firstly, the degree to which genetic diversity in each species is partitioned between subpopulations will be assessed using both traditional F-statistics (and its analogues) and Bayesian clustering methods. It is predicted that genetic structuring, as measured by the distribution of microsatellite allele frequencies, will be more pronounced in *P. bellargus* than in *P. coridon*.

Secondly, the degree to which isolation by distance (IBD) is occurring between subpopulations in each species is tested, with the prediction that IBD will be higher in *P. bellargus*. Once the effects of geographic distance on the relatedness of populations has been accounted for it will also be tested whether the type of habitat found in the intervening landscape between sites, the matrix, affects how connected they are. It is hypothesised, for both species, that the nature of the matrix between sites will affect the degree of gene flow between them. It is predicated that an increased proportion of calcareous grassland in the intervening landscape will facilitate gene flow between populations, whereas coverage such as built environments, agricultural land and woodlands will present barriers to gene flow.

5.3 Methods

5.3.1 Sample collection

The sites sampled were part of 30 potential sites in the south of England selected to include sites of a range of sizes and degrees of isolation (measured in geographical distance), for the larger project of which my PhD was part. Of these 30 sites, 12 were successfully sampled for *P. bellargus* and 6 were successfully sampled for *P. coridon*. In 2012, permission to collect from four more sites was obtained, which were identified as having *P. coridon* present from the UK Butterfly Monitoring Scheme (UKBMS 2013), thereby increasing the geographical range of the sampling. For *P. bellargus* both larval and adult butterfly (male and female) samples were collected over three summers, in 2008, 2009 and 2011, utilising both generations. The samples for each site were as much as possible kept within the same year. For *P. coridon* larval and adult butterfly samples were collected in 2009 and 2010 for the 6 initial sites, and from the 4 additional sites in 2012. After collection all samples were placed in 1.5ml microcentrifuge tubes and frozen at -80°C prior to extraction. Figure 5.1. shows the location of the sites sampled for each species.

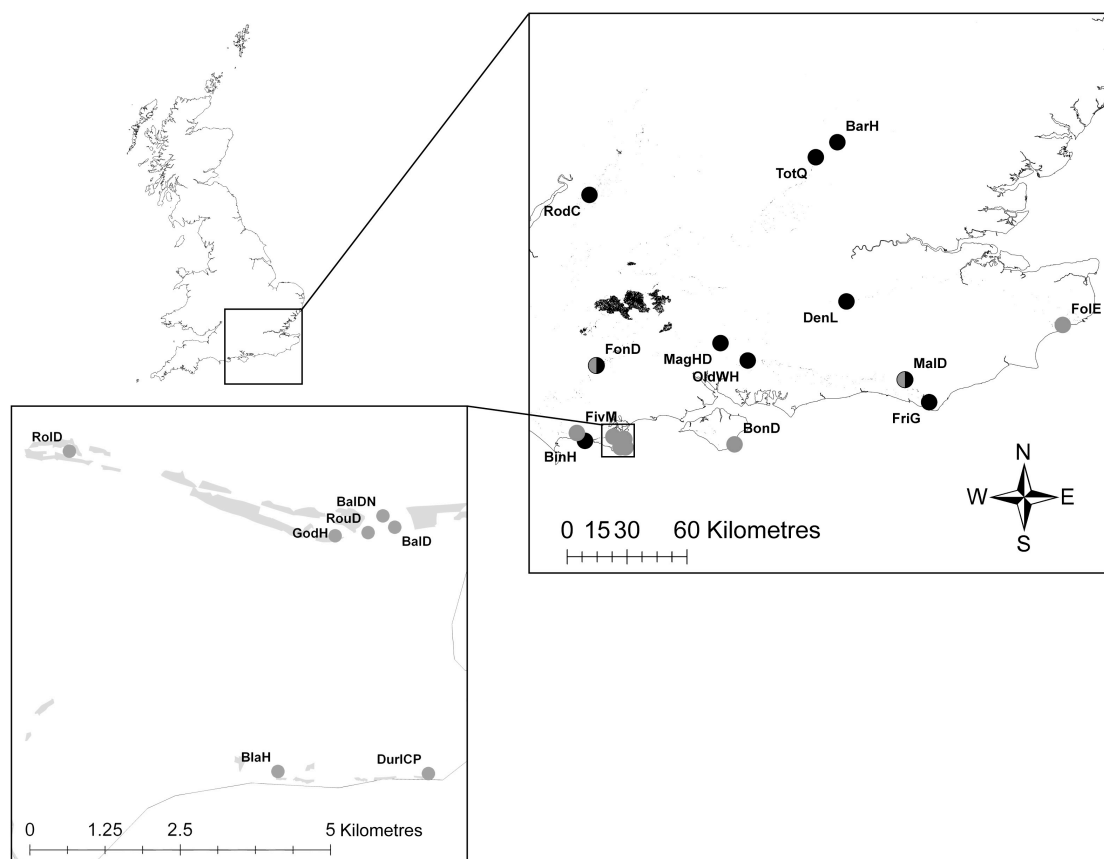


Figure 5.1 The locations of the sites used in this study from the South of England. The location of all collection sites used is shown in the first map, and the distribution of the *P. bellargus* sites found in the Swanage area of Dorset in the second. Sites marked with a grey circle were sampled for *P. bellargus*, with a black circle for *P. coridon*, and with a grey and black circle for both species. The black fragments on the main map, and grey fragments on map of the Swanage region show the distribution of calcareous grassland in the study region.

5.3.2 Sample preparation

The wings and heads were removed from every adult using separate sterilised razor blades, as the pigments can inhibit both the extraction process and subsequent PCR reactions. Each sample was then placed into a fresh microcentrifuge tube and if necessary stored again at -20°C before beginning the extraction process.

All samples were initially extracted using Qiagen DNeasy Blood and Tissue Kits (Cat no. 69506), utilising the protocols recommended for insect samples. Two methods of

homogenisation were used, with samples initially being macerated by hand, but subsequent samples being homogenised mechanically, which was found to save time and produce better homogenised samples. Methods were as follows:

i) Hand macerated samples

In a 1.5ml microcentrifuge tube, to each sample 360µl of the provided Qiagen ATL Lysis Buffer (Cat no. 19076) was added. This was then macerated with a sterile plastic pestle, a further 360µl added and the maceration completed. Eighty µl of Qiagen 600 mAU/ml Proteinase K solution (Cat no. 19131) was then added and the samples vortexed for 30 seconds to mix thoroughly. Samples were then incubated overnight at 56°C to complete lysis.

ii) Mechanically macerated samples

360µl of 1 x phosphate-buffered saline (pH 7.4, 10 mM PO_4^{3-} , 137 mM NaCl, and 2.7 mM KCl) was added to each sample (in a 1.5ml microcentrifuge tube), along with 7-8 1mm sterilised glass beads (ethanol and Bunsen flame). Samples were then lysed using a Qiagen Retch MM300 Tissue Lyser in batches of 18, being macerated at 30 beats per second for 3 minutes. Larval samples often required a further 3 minutes to sufficiently break down their thick cuticle. To each sample 400µl of Qiagen AL Lysis Buffer (Cat no. 19075) and 20µl of 600 mAU/ml Proteinase K were then added and the sample vortexed for 30 seconds to mix thoroughly. Samples were then incubated overnight at 56°C to maximise the lysis.

5.3.3 DNA extraction

i) Spin-columns and preparation

After incubation the samples were vortexed for 15 seconds and then 200 μ l of the sample pipetted into a new 1.5ml microcentrifuge tube. The remaining sample was then placed in storage at -20°C. 200 μ l of molecular grade (>99.9%) ethanol was then added to the aliquot, and after thorough mixing was pipetted on to a pre-prepared DNeasy spin column and the Qiagen protocol followed. The extracted DNA was eluted into 50 μ l of nuclease free water (Qiagen, Cat no. 129114) and the concentration in ng μ l⁻¹ measured using a Nanodrop 8 sample spectrophotometer ND-8000. An aliquot of the sample was then placed in a 0.5ml microcentrifuge tube, and diluted as necessary in nuclease free water, to achieve at an approximate concentration of 50ng μ l⁻¹ in 20 μ l. If a samples initial concentration was below this the number of μ l added to the PCR reaction was increased, to a maximum of 4 μ l, to as near as possible reach the equivalent of 50ng μ l⁻¹ DNA per PCR reaction.

ii) Phenol-chloroform extractions

Some of the samples collected in 2008 had been prepared by mechanically macerating the whole sample, including the wings and head, in sterile H₂O, which inhibited DNA extraction using the Qiagen DNeasy kit. These samples were re-extracted using the Phenol-Chloroform method outlined below. To 200 μ l of each sample, 400 μ l of the provided buffer AL (Qiagen) and 20 μ l of 600 mAU/ml proteinase K were added and each sample vortexed for 30 seconds to mix thoroughly. Samples were then incubated overnight at 56°C to complete lysis. After vortexing for 30sec 141 μ l 5M NaCl and 374 μ l polyethylene glycol (PEG) were added to each sample, which were then left to precipitate for 1 hour at 4°C. Samples were then centrifuged at 12,000 X g at 4°C for

10 min to pellet the DNA. The supernatant was removed and the pellet was resuspended in 500µl distilled H₂O, prior to the addition of 500µl 1 X tris-buffered phenol (pH8.0) and vigorous vortexing. The tubes were left to stand for 1 min at room temperature then centrifuged for 4min at 12,000 X g. The supernatant was added to fresh micro-centrifuge tubes with an equal volume of tris-buffered phenol (pH 8.0) and chloroform-isoamyl alcohol (1:1) and mixed vigorously before centrifugation at 12,000 X g for 4 minutes. The supernatant was removed into fresh microcentrifuge tubes and an equal volume of molecular grade ($\geq 99.9\%$) isopropanol (1:1), 1:10 volume of 10M ammonium acetate and 1µl of linear polyacrylamide added and mixed prior to incubation at -20°C. After 1 hour, samples were centrifuged at 12,000 X g for 10 min and the supernatant removed and discarded. The pellet was washed three times with 500µl molecular grade ethanol and dried in 45°C on a heat block for 10 min (or until all ethanol had evaporated). Pellets were re-suspended in 30µl of nuclease free water and stored at -20°C.

5.3.4 Polymerase chain reactions (PCRs)

Primer sets

Primer sets for both species, *P. bellargus* and *P. coridon*, were obtained from the Molecular Ecology Primer Database (<http://tomato.bio.trinity.edu>). *P. bellargus* primers were developed in 2000 (Harper et al. 2000), and have been used successfully in other studies on this species (Harper et al. 2003; Harper et al. 2006). Table 5.1 provides a summary of each locus used. Primers used for *P. coridon* were developed in 2010 at the University of Bayreuth (Bayreuth, Germany), though they have yet to be used in a published study. Table 5.1 provides a summary of all loci used.

Table 5-1 A summary of all microsatellite loci used in this study. Showing the species for which the locus was developed and used for, the microsatellite repeat motif, the forward and reverse primer sequences, the recommended annealing temperature (AT), the expected size range of products (Size). For the *P. bellargus* loci the GenBank Accession number and the number of alleles found when developed (NA) is also provided, but was not available for *P. coridon* loci.

Locus	Species	Repeat Motif	Forward Primer (5'–3')	Reverse Primer (5'–3')	AT	Size	GeneBank	NA
Lb141	<i>P. bellargus</i>	(GT) ₇ AGGTGA(GT) ₅ ATGAGTG C(GT) ₄	CGTGTCTGTCACCC CCTTTA	ATGACGGGTAGGG ATTAGGG	50°C	170-236	AF276048	5
Lb157	<i>P. bellargus</i>	(CA) ₁₃	TGTATCAGCAACAG CTCGGT	GGAAGCGTTCATC GGTAAAA	46°C	172-224	AF276049	4
Lb418	<i>P. bellargus</i>	(CA) ₈ GGCCCCG CCGC(T) ₉	GATACCTATGCCAG GCTCCA	CGAATGTCATACA GGTTGCG	50°C	146-153	AF276051	5
Lb419	<i>P. bellargus</i>	(GACGGT) ₁₅	AGATTAGGCATTGG CGTGTC	CTGCGATCCATTT CCGTTA	50°C	180-260	AF276051	8
LbG2	<i>P. bellargus</i>	(CA) ₁₀ GTC(CGC A) ₃ ATGC(CAGC) ₆ (GC)3TCT(CA) 12(GA) ₄	ATCAAGGTCCGCAC AGCA	CGCTCGCTTATCA GACAACA	50°C	164-182	AF276052	4
Polcor02	<i>P. coridon</i>	(AC) ₁₆ (AGAC) ₃	ATGCAGATGCAGAT	GATGAACGAAAA	56°C	136-180	–	–

			GTGGAG	CCTCAATGG				
Polcor03	<i>P. coridon</i>	(TATG) ₁₅	GTCGCCAGGATCAA	TCGGGCTTGTAGT	60°C	121-319	-	-
			CAGTG	TCCAGAG				
Polcor05	<i>P. coridon</i>	(AC) ₂₂	GACACGCACACATG	GCTTTGGAAAAGT	56°C	126-164	-	-
			CAAATAG	TCGGATG				
Polcor11	<i>P. coridon</i>	(GT) ₅ (CT) ₁₅ (GT) ₁	CGTAGGTTAGACACG	CGCCGCTATACAA	60°C	87-169	-	-
		4	TCTGTGTATATG	CGTGAG				
Polcor12	<i>P. coridon</i>	(AC) ₆ GC(AC) ₄ G	CTATCTGCTCGCGG	GAATCCGTGGAAG	60°C	184-238	-	-
		A(CA) ₁₅	ATGAAG	TGTCGTC				
Polcor13	<i>P. coridon</i>	(CA) ₄ TA(CA) ₁₄	TGAAAGATCGCGAC	CCCTCACAAACAA	56°C	135-197	-	-
			TAAATTG	TGGAATAAC				
Polcor18	<i>P. coridon</i>	(GT) ₆ (GCGT) ₂ (G	GCGTTTGGCACGTT	ATCATGGGACGAC	50°C	149-185	-	-
		T) ₄ GAAT(GT) ₈	TATCTC	GCATAC				
Polcor21	<i>P. coridon</i>	(TG) ₁₄	TTCAGGACTCCAGG	CTCAAACGTTGGC	56°C	111-187	-	-
			TTCCAC	TGCAAG				
Polcor23	<i>P. coridon</i>	(AC) ₂₀	GGTGATCGCGACAA	ACGCCGTTCTGGA	56°C	179-267	-	-
			CAATAG	AGACAC				
Polcor24	<i>P. coridon</i>	(TACA) ₉	CCGAAGATGAAGC	TTTCCTGCTGGAA	50°C	155-197	-	-
			AAACTACAAG	TACATGC				

Single PCR reactions

All single PCR reactions were carried out using 25-29 μ l, with the mix and quantities of reagents in the master mix detailed in Table 5.2. Quantities of the template DNA solution >0.5 μ l were only used when the concentration on DNA was <50ng μ l, and the quantity increased to achieve as close to 50ng of DNA as possible. The cycle used for all PCRs was as follows; 4 minutes at 94°C for activation; 35 cycles of a 94°C denaturation for 30 seconds, 30 seconds at the appropriate annealing temperature for the primer set (between 50°C and 60°C), 72°C for 120 seconds for extension; 72°C for 10 minutes.

Table 5-2 Reagent mix for single PCRs

Reagent	Quantity (μl)
PCR grade H ₂ O	19.2
10 x PCR Buffer	2.5
BSA	1.0
Forward Primer	0.5
Reverse Primer	0.5
dNTPs	0.4
Taq Polymerase	0.4
Template DNA (~50ng μ l ⁻¹)	0.5 - 4
Total	25 -29

Multiplex PCR reactions

Qiagen Multiplex PCR Kits (Cat no. 206143) was used for all multiplex PCR reactions. All reactions were carried out in 12.5-15 μ l, and the final reactions were done on 96 well plates (Table 5.3 shows the reagent mix for each reaction). Quantities

of the template DNA solution >0.5µl were only used when the concentration on DNA was <50ng µl, and the quantity increased to achieve as close to 50ng of DNA as possible. The cycle used for all multiplex PCRs was as follows: 15 minutes at 95°C for activation; then 35 cycles of a 94°C denaturation for 30 seconds, 90 seconds at 57°C for annealing; 120 seconds at 72°C for extension; 72°C for 10 minutes for the final extension.

Table 5.3 Regent mix for multiplex PCRs, using a Qiagen Multiplex PCR kit.

Reagent	Quantity (µl)
Multiplex master mix	6.25
Qiagen Q solution	2.5
PCR H2O	2.25
Primer Mix (Diluted in TE buffer (with each primer at 100 µM concentration)	0.5
Template DNA (~50ng µl ⁻¹)	0.5 - 3
Total	12- 15

Primer optimisation

All primer sets were run in single PCR reactions to check that the suggested annealing temperature was correct, that the products produced were of the expected size and that the production of non specifics was not occurring.

Polyommatus bellargus primers

Checks for primers revealed that the recommended annealing temperature produced the products of the correct size and number for all loci except Lb419 which showed a large number of non specifics. A temperature gradient was therefore run for this locus on 6 *P. bellargus* samples at 12 different annealing temperatures ranging from 48-60°C, and 53°C identified as the annealing temperature for this allele which did not produce non-specifics, confirming its optimal nature (Figure 5.2).

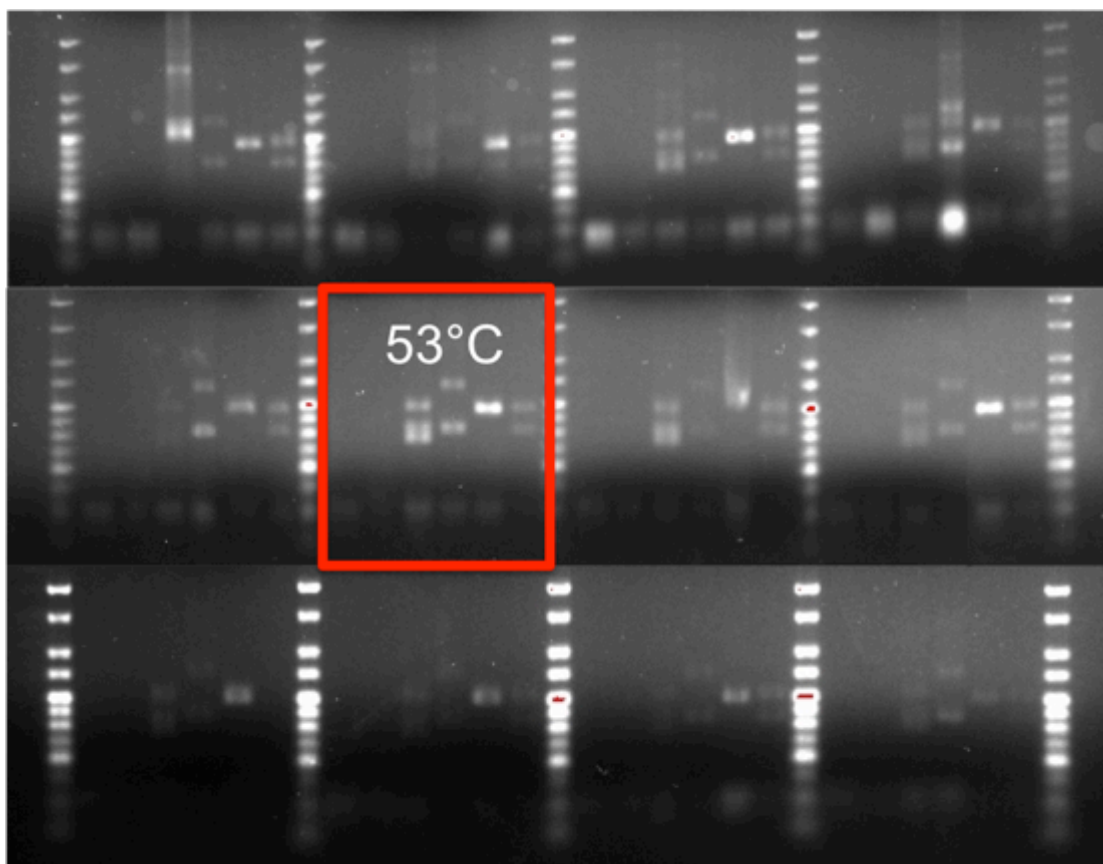


Figure 5.2 Gel electrophoresis on a 2% agarose gel, stained with ethidium bromide, of the product of temperature gradient PCRs (12 different temperatures) of the Lb419 microsatellite locus for *P. bellargus*, using 6 different samples. 53°C was identified as the optimum annealing temperature for the reaction providing the best balance of amplifying all products and minimising non-specifics. Hyper ladder 5 is shown to the left and right of each sample set, which ranges from 500 bp (top band) to 25 bp (bottom band).

***Polyommatus coridon* primers**

For all 9 of the 10 primer sets for *P. coridon* (Polcor) the correct size product, with minimal extra banding, was produced by trialling at annealing temperatures of either 50°C or 56°C. For Polcor18 a temperature gradient was used to identify 53°C as the optimal annealing temperature (Figure 5.3). However, after initial multiplex runs for the *P. coridon* primer sets, Polcor 18 was found to be producing too many non-specifics and multiple bands and so was consequently excluded from the final primer

sets used. Also, once all multiplex reactions had been completed it was decided to exclude the loci Polcor11 as it consistently produced additional peaks on the chromatogram.

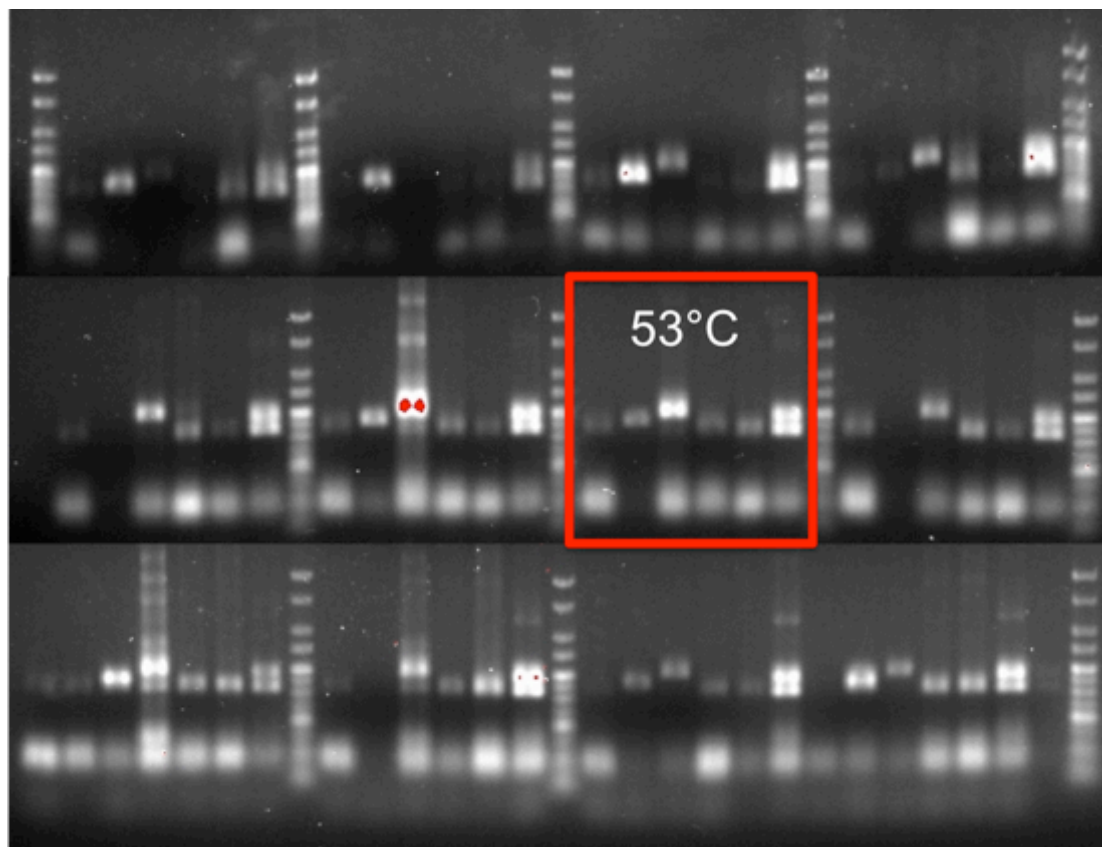


Figure 5.3 Gel electrophoresis on a 2% agarose gel, stained with ethidium bromide of the product of temperature gradient PCRs (12 different temperatures) of the Polcor18 microsatellite locus for *P. coridon*, using 6 different samples. 53°C was identified as the optimum annealing temperature for the reaction as it provided the best balance of amplifying all products and minimising non-specifics. Hyper ladder five is shown to the left and right of each sample set, which ranges from 500 bp (top band) to 25 bp (bottom band).

Multiplex PCR dyed primer sets

All multiplex primer sets contain a maximum of 5 primers with fluorescent dye 5' modifications of the forward primer (Eurofins, MWG|operon). Each primer set contained a locus labelled with ATTO 550 ($\lambda_{em} = 560$ nm, yellow), a locus labelled with 6-FAM ($\lambda_{em} = 520$ nm, blue), a locus labelled with Yakima Yellow ($\lambda_{em} = 549$

nm, Green) and two loci labelled with ATTO 565 ($\lambda_{em} = 540$ nm, red). The two loci labelled with ATTO 565 in each primer set were chosen as having the product size ranges that did not overlap. Tables 5.4 and 5.5 summarise the primer sets used for multiplex reactions.

Genotype sequencing

Prior to sequencing, the PCR products from each 96 well plate were purified using a Zymo ZR DNA 96 well clean up kit, to remove primer dimers and non-specifics. The final product for each sample was eluted in 30 μ l of DNAase and RNAase free molecular grade H₂O. For each sample a 4 x dilution was made using 2 μ l, as trials revealed that using neat product produced saturated peaks on the sequencer chromatogram. The remaining product was stored at -20°C. In a 96 well plate 1 μ l of the 4 x dilution was added to 9 μ l of Hi-DiTM Formamide, containing ABI LIZ 600 size standard for calibration, and incubated at 94°C for 4 minutes. Samples were then run on an Applied Biosystems3730 DNA Analyser.

Table 5.4 Summary of the microsatellite sets and fluorescent dyes used for genotyping *P. bellargus* specimens.

Locus	Dye
Lb1-57	6FAM
Lb1-41	ATTO 550
LbG2	Y. Yellow
Lb4-18	ATTO 565
Lb4-19	ATTO 565

Table 5.5 Summary of the microsatellite sets and fluorescent dyes used for genotyping *P. coridon* specimens. Loci highlighted in grey were not used in any the final analyses due to the production of non-specifics.

Locus	Dye
Set 1	
Polcor24	6FAM
Polcor18	ATTO 550
Polcor03	Y. Yellow
Polcor12	ATTO 565
Polcor11	ATTO 565
Set2	
Polcor21	6FAM
Polcor02	ATTO 550
Polcor13	Y. Yellow
Polcor23	ATTO 565
Polcor05	ATTO 565

Allele scoring and genotyping

All allele scoring and genotyping was carried out using the program GeneMarker ® version 1.5 (SoftGenetics), using the microsatellite calibration settings. Each sample was scored for peaks and then checked for each locus to confirm these peaks; allele calls that were flagged as being tentative were confirmed or rejected and false alleles caused by pull peaks from other loci were eliminated. At this stage it was decided that the locus Polcor11 would also be excluded from the *P. coridon* dataset as its peaks were too stuttered to be able to distinguish genuine alleles. All samples for which a particular locus had not amplified were run again in a single PCR reaction and if no product was produced, the sample was excluded from analysis for the locus in question. Samples for which the peaks were of insufficient size to be called confidently or had >2 peaks that were irresolvable were removed from the data set

either for the given problematic locus or completely. Samples for which fewer than 3 loci amplified in *P. bellargus* and 5 loci *P. coridon* were excluded from the final analysis.

5.3.5 Sequencing of PCR products

Representative PCR products for all loci were sent for sequencing to confirm that products being scored were true microsatellite alleles for the given locus and to inform about the nature of the repeat motif. For the *P. bellargus* loci Lb4-18 and Lb4-19 and *P. coridon* loci Polcor05 and Polcor23, which were included in the same multiplex sets with the same dye (ATTO 565) a larger number of samples were sent for sequencing. This was to partition alleles into their respective loci that fell on extremities of the size ranges.

Single PCR reactions were carried out for each locus, for a varied number of individuals (See Appendix A for a summary), and the products of these PCRs were then run on a 2% agarose gel with Hyperladder 5 (New England Biolabs). The products derived were cut from the gel and placed in a pre-weighed 1.5ml microcentrifuge tube for subsequent gel purification.

All gel extractions were carried out using a Qiagen QIAquick® Gel Extraction Kit (Cat no. 28704). Each sample had 3 x the gel volume of the provided QG Solubilization and Binding Buffer (Cat no. 19063) added and was incubated at 50°C for 10 minutes and vortexed for 2-3 minutes. One gel volume of molecular grade ($\geq 99.9\%$) isopropanol was then added to each sample and the spin column protocol

for the kit followed, with each sample eluted into 50µl of DNAase and RNAase free water and stored at -20°C until required.

Cloning

Ligation

For each sample 13.5µl of eluted product was added to a pre-mixed solution of 1.5µl of 10 x ligase buffer (New England Biolabs), 0.3µl of pGEM vector (Promega) and 1µl of T4 DNA ligase (New England Biolabs). Samples were then incubated at 16°C for 18 hours to complete ligation of the product into the pGEM vectors.

Transformation of competent cells

Fifty µl of chemically competent DH5α *Escherichia coli* cells, which had been stored at -80°C, were allowed to thaw on ice for 15 minutes. The ligations were added to these cells on ice and left for a further 30 minutes. The cells were heat shocked in 42°C water bath for exactly 30 seconds, then returned to ice for a further 10 minutes. 250 µl of Lysogeny Broth (LB) was added to each sample in front of a Bunsen flame to avoid contamination and the culture incubated with shaking (200rpm) at 37°C for two hours. Each sample was then spread onto an LB agar plate, supplemented with Ampicillin (100 µg/ml), Isopropyl β-D-1-thiogalactopyranoside (100 µg/ml) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (60 µg/ml) for blue white selection, and left for 30 minutes at room temperature before incubation overnight at 37°C.

Colony selection, growth and plasmid extraction

Individual white colonies were selected by picking with a sterile Gilson pipette tip and transferring to 5ml LB broth supplemented with Ampicillin (100 µg/ml) in a 15ml Falcon tube. All tubes were incubated shaking (250rpm) overnight at 37°C. Cells were collected by spinning at 300rpm for 5 minutes and the plasmid purified using a Qiagen QIAprep Spin Miniprep Kit (Cat no. 27104) according to the manufacturer's instructions. The purified plasmid DNA was sent to the Natural History Museum for Sanger sequencing using both standard M13F (5' GTAAAACGACGGCCAGT 3') and M13R primers (5' GTTTCCCAGTCACGAC 3').

Sequence editing and alignment

All sequences were initially edited using the software Sequencher version 4.6 to identify the microsatellite product within the plasmid. All sequences for each locus were aligned using Clustal W, executed using Bioedit (version 7.1.9) (Hall 2007).

5.4 Analysis methods

5.4.1 Genetic diversity, Hardy Weinberg equilibrium, linkage and null Alleles

Genetic diversity was calculated at both the global level for each locus, and for specific populations. The number of alleles, expected heterozygosity (H_e), observed heterozygosity (H_o) and F_{IS} (inbreeding coefficient) were calculated for each locus, and for each subpopulation the average number of alleles, mean H_e , mean H_o , Allelic

Richness, F_{IS} and F_{ST} were calculated using Arlequin version 3.5.1.2 (Excoffier and Lischer 2010). Allelic richness, the average number of alleles per locus, was calculated using FSTAT version 2.9.3.2 (Goudet 1995; Goudet 2001).

Tests for deviation for HWE for each locus, within each population and across all populations, and pair-wise tests for linkage between markers were carried out using Arlequin version 3.5.1.2 (Excoffier and Lischer 2010). There was evidence for deviation from HWE at all loci and so before inclusions in further analysis all loci were checked for evidence of being under selection using the programme LOSITAN (Antao et al. 2008). This evaluates the relationship between F_{ST} and expected heterozygosity for a given locus in an island model, using simulations from the dataset to identify loci with F_{ST} values that are excessively high or low given the generated expectations (Antao et al. 2008; Beaumont and Nichols 1996). Any loci identified as such are putatively under selection (not a neutral marker) and are therefore unsuitable for population genetic analysis. Using the software MICRO-CHECKER (Van Oosterhout et al. 2004) each locus was assessed for the probability of null alleles. Microsatellite null alleles are those that may not be amplified, for instance due to mutations in the primer sites, and so result in false homozygotes. This can result in deviations from HWE which are similar to those apparent from the Wahlund effects – a deviation from HWE due to population structuring causing a reduction in overall heterozygosity (Frankham et al. 2010). The frequency of null alleles for each locus was estimated using the method 3 of Brookfield (1996) (Brookfield 1 in MICRO-CHECKER), which assumes that non amplified samples could be the result of null allele homozygotes or problems with the amplification of PCR products due to DNA quality or PCR conditions.

5.4.2 Population differentiation and structure

F_{ST} Type Methods

Weir and Cockerham's F_{ST} statistic (Weir and Cockerham 1984) were calculated for each species using FSTAT version 2.9.3.2, to give an indication of the degree to which the genetic diversity was partitioned between subpopulations (Goudet 1995; Goudet 2001). For *P. bellargus* Slatkin's R_{ST} (Slatkin 1995) was also calculated, which is analogous to Weir and Cockerham's F_{ST} , but incorporates a step-wise mutation model that may make it more appropriate for analysis with microsatellites. A second analysis was conducted for *P. bellargus*, excluding Bonchurch Down (the site found on the Isle of Wight), to examine the degree to which this site influenced genetic differentiation statistics. Given the non-stepwise alleles found in *P. coridon* R_{ST} could not be estimated. With regard to measures of genetic differentiation, there has been a debate in the literature as to the effectiveness of F_{ST} and its analogue R_{ST} , due to estimates being influenced by the number of loci used and biased by loci with high mutation rates / heterozygosity. Of particular relevance to this study, this may cause problems with the comparison of F_{ST} values derived from different data sets, as when loci have more than two alleles estimates of F_{ST} are no longer bounded between 0 (no differentiation) and 1 (total differentiation). Two alternatives (among others), G'_{ST} (Hendrick 2005) and Jost's D (Jost 2008), which provide estimates bounded between 0 and 1 have been suggested and used in some studies, though F_{ST} remains the most commonly used measure of genetic differentiation and the adequacy of these alternatives is far from accepted (Holsinger and Weir 2009; Whitlock 2011). Both Hendricks G'_{ST} and Jost's D are also presented here for each data set, and were

calculated using the *diveRsity* package in R (Keenan et al. 2013; R Core Development Team 2012).

Bayesian methods

The software *STRUCTURE* (v2.3.3) was also used to estimate the number of subpopulations in each dataset, under a Bayesian statistical framework (Falush et al. 2003; Pritchard et al. 2000), as it has recently been used successfully in other studies of genetic structuring in butterflies (Habel et al. 2011b; Sielezniew and Rutkowski 2011; Smee 2011; Ugelvig et al. 2012). It uses a Bayesian, model based, clustering algorithm to assign individuals probabilistically to different clusters (populations), using the allele frequencies at available loci, whilst maximising HWE and linkage disequilibrium (Falush et al. 2003; Pritchard et al. 2000). The clustering of individuals into distinct populations (K) is explored by running separate models for successive values of K , from 1 to N (in this case the maximum value of N is the number of sampling locations for each dataset), using Markov Chain Monte Carlo (MCMC) iterations. In this study, for each value of K five independent runs of the model were executed, with 200,000 MCMC iterations, following a burn-in of 100,000 iterations, using an admixture model with correlated allele frequencies (Falush et al. 2003; Smee 2011). The ‘true’ number of clusters in each species was then identified using the ‘Evanno Method’ (Evanno et al. 2005). This uses an ad hoc statistic (Delta K) based on the change in log-likelihood between models for successive values of K to identify the most likely number of populations in a data set and was implemented using the web-based software *STRUCTURE HARVESTER* (Earl and vonHoldt 2011). The degree of correspondence between the clustering of individuals predicted by *STRUCTURE* and their geographical distribution was then visualised on a map.

Initial models for both *P. bellargus* and *P. coridon* showed the assignment of individuals into different clusters was weak. Hubisz et al. (2009) developed a method to improve the performance of STRUCTURE when the population assignment is weak and the number of loci used is low (<10), which was the case for both *P. bellargus* and *P. coridon*. It uses information on sampling location to modify the prior probability distribution for population assignment of each individual. As such a second model set was run for each species utilising this method to see if cluster assignment improved.

5.4.3 Isolation by distance

Analysis for IBD was conducted using the Isolation by Distance Web Service (Rousset 2008). Two matrices were created; one of pairwise F_{ST} values between sites and one of Euclidian geographic distances between sites, in metres. A Mantel test with 20,000 permutations was then used to determine if there is a significant correlation between Slatkin's linearized F_{ST} ($F_{ST}/(1-F_{ST})$) and log Euclidean distance between sites (Calculated using ARCMAP GIS v 10.1).

The influence of landscape cover on gene flow was also explored in the IBD framework, using the CEH Landcover Map 2007, which classifies the whole of the UK into land parcels classed as different land cover types (Morton et al. 2011). For each pairwise distance between sites a linear buffer 2km in width was created in ARCMAP GIS v 10.1. The total proportion of this buffer covered by six different land cover types was then calculated for each buffer; 1) Arable and Horticultural, 2) Buildings and Gardens, 3) Improved Grassland, 4) Calcareous Grassland, 5)

Broadleaf Woodland and 6) Sea. For every pairwise buffer the proportion of its area accounted for by each of these land cover types was calculated, to create six new pairwise matrices for each data set. For *P. bellargus* only the impact of Sea coverage was considered for the data set containing Bonchurch Down. Partial Mantel tests were then carried out to test whether there was a significant correlation between genetic distance and the proportion of a buffer accounted for by each cover type, whilst controlling for the explanatory power of geographic distance.

5.5 Results

5.5.1 Microsatellite sequencing

All but one product were confirmed as alleles of the expected microsatellite loci, and allowed for the establishment of boundaries in alleles' size range between loci for which the same fluorescent dye was used (see Appendix A). In a few instances, although the PCR product was in the expected size range it did not match the size of the corresponding peak on the chromatogram, with the most likely explanation being that the wrong sample was used for the sequencing PCR reaction (See Table A1). From the sequences two putative sources of the Polcor alleles not conforming to canonical repeats were also identified; single nucleotide insertions or deletions (indels) in the microsatellite repeat and indels in the flanking regions. For Polcor05 sequences show an instance of a 9 base pair deletion in the flanking region (Figure A8, Polcor05 05) and an instance of insertion in the microsatellite repeat motif (Figure A8, Polcor05 07). For Polcor12 there was a guanine (G) insertion in the microsatellite repeat motif (Figure A10, Polcor12 02). For Polcor21 a three base pair (ATA) insertion or deletion can be observed when comparing the flanking regions of the

products sequenced (Figure A12). Finally, for Polcor23 the variation was caused in part, if not exclusively, by a large (77bp) insertion in the flanking region, which is a large repeat of the flanking region sequence (Figure A13, Polcor23 04, 06 and 11).

A further issue encountered for the Polcor05 locus was that one product sequenced (Figure A8, Polcor05 02) was actually a non-specific, which amplified using the Polcor05 primer set, but does not contain the microsatellite repeat motif nor the same flanking region motif as the other sequences. This could be particularly problematic, as this product falls within the size range of expected alleles, and so could result in false allelic variation. Given this, for the analysis of population differentiation in *P. coridon* a set of analyses were also carried out which excluded this locus, to examine any impact it might be having on differentiation estimates.

5.5.2 Genetic diversity, Hardy Weinberg equilibrium, linkage and null alleles

Polyommatus bellargus

For *P. bellargus* all loci were found to deviate significantly from HWE at the $p < 0.001$ level (Table 5.6). The relationship between F_{ST} and H_e for all loci used indicated that they are selectively neutral, and so loci being under selection was not likely to be the cause of deviation from HWE (Figure 5.4). Pair-wise tests for linkage disequilibrium (LD) within each population revealed evidence for significant LD of loci within some populations, but the incidence rate was generally low and inconsistent among subpopulations. Checks indicated that null alleles were present for all loci, with estimates for Lb418, Lb419 and Lb417 being particularly high, though none were > 2 and so Lb419 and Lb417 were deemed as acceptable for further

analysis (Table 5.6). However, locus Lb418 had the highest estimate for null allele frequency and the lowest value for successful amplification (Table 5.6). Coupled with low observed heterozygosity and the significant deviation from HWE at several populations indicate that null alleles are potentially a problem for this locus. Due to this, it was decided that a second set of analyses would be conducted for *P. bellargus* excluding data on the Lb418 locus, to examine the impact of this locus on differentiation estimates. Across individual populations average levels of heterozygosity were generally lower than expected (Table 5.7). Bonchurch Down, the population found on the Isle of Wight has a markedly lower number of alleles than all other populations.

Table 5.6 Summary statistics for 5 microsatellite loci used to genotype *P. bellargus*. Ho, Observed heterozygosity; He, Expected heterozygosity; HWE Global, * indicates the locus was found to deviate significantly from Hardy Weinberg equilibrium when all samples were considered; HWE Sites, the number of sampling sites where loci deviated significantly from HWE, with the number once Bonferroni corrections for multiple tests were made in brackets; Null A Freq est, the estimated frequency of null alleles for a locus (Brookfield 1996); F_{IS} (inbreeding co-efficient), F_{ST} (the proportion of genetic variance found within subpopulations) and R_{ST} (and F_{ST} analogue assuming a stepwise mutation model).

Locus	No. A	N	Ho	He	HWE Global	HWE Sites	Null A Freq	F_{IS}	F_{ST}	R_{ST}
Lb418	8	390	0.34	0.67	*	9(7)	0.1825	0.453	0.079	0.046
Lb419	12	417	0.60	0.80	*	5(3)	0.1403	0.169	0.111	0.072
Lb141	8	457	0.60	0.68	*	2(0)	0.0333	0.050	0.093	0.041
Lb157	18	449	0.57	0.82	*	9(7)	0.1466	0.253	0.085	0.260
LbG2	7	453	0.52	0.65	*	4(3)	0.0314	0.118	0.093	0.072

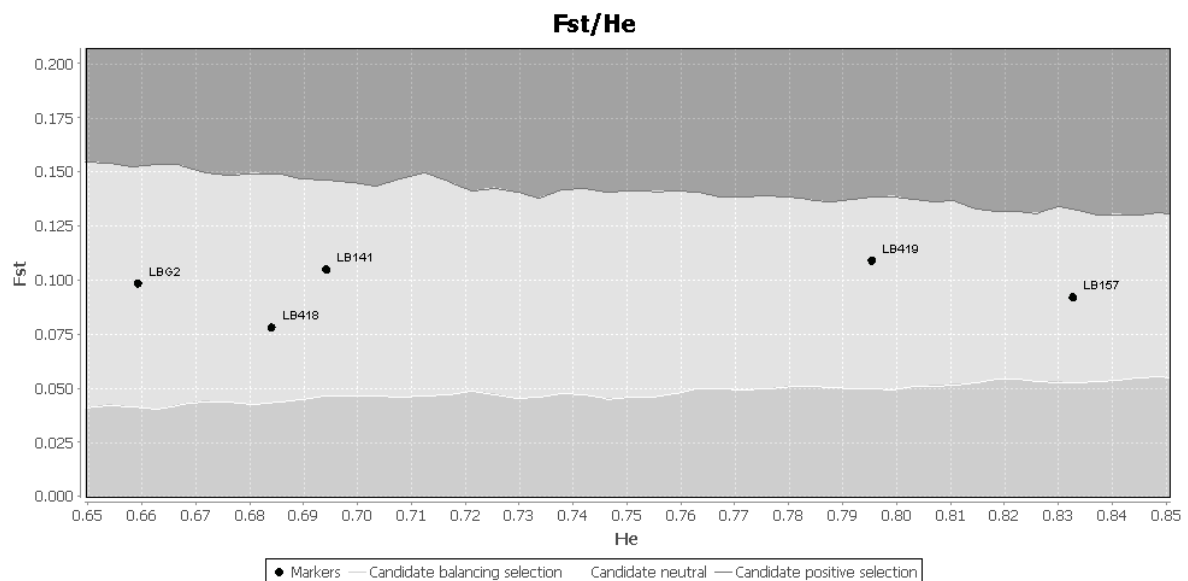


Figure 5.4 Output from 100,000 simulations using data on actual allele frequencies within the *P. bellargus* dataset by the software LOSITAN. The expected distribution of the relationship between F_{ST} and He for neutral loci is shown in light grey. The expected distribution for loci under balancing selection is shown in mid grey, in dark grey for loci under positive selection (dark grey). For all *P. bellargus* microsatellites the observed relationship between F_{ST} and He falls within the bounds expected for neutral loci..

Table 5.7 Summary statistics for each geographically defined subpopulation of *P. bellargus*. NA, the average number of alleles across the site for all loci; Ho, observed heterozygosity; He, expected heterozygosity; AR, Allelic Richness (the average number of alleles per locus in a population); F_{IS}, inbreeding co-efficient.

Site	County	Co-ordinates	N	NA $\pm$ sd	Ho $\pm$ sd	He $\pm$ sd	AR $\pm$ sd	F _{IS}
Ballard Down	Dorset	SZ023810	52	5.80 $\pm$ 2.04	0.46 $\pm$ 0.13	0.64 $\pm$ 0.12	4.09 $\pm$ 1.08	0.283
Ballard Down North	Dorset	SZ019811	36	6.40 $\pm$ 2.30	0.50 $\pm$ 0.11	0.64 $\pm$ 0.13	4.30 $\pm$ 1.13	0.216
Blackers Hole	Dorset	SZ004770	34	6.20 $\pm$ 2.16	0.60 $\pm$ 0.16	0.67 $\pm$ 0.17	4.36 $\pm$ 1.24	0.104
Bonchurch Down	Isle of Wight	SZ564785	34	3.6 $\pm$ 1.14	0.40 $\pm$ 0.27	0.37 $\pm$ 0.30	2.53 $\pm$ 0.69	-0.060
Durlston Country Park	Dorset	SZ023770	14	6.00 $\pm$ 1.41	0.60 $\pm$ 0.18	0.72 $\pm$ 0.05	4.88 $\pm$ 0.59	0.209
East Chaldon (5 Marys)	Dorset	SY785841	40	7.00 $\pm$ 2.44	0.55 $\pm$ 0.22	0.69 $\pm$ 0.04	4.28 $\pm$ 0/74	0.206
Folkestone Escarpment	Kent	TR218381	36	7.00 $\pm$ 4.06	0.56 $\pm$ 0.26	0.76 $\pm$ 0.11	5.07 $\pm$ 1.98	0.270
Fontmell Down	Shaftesbury	ST883182	50	6.20 $\pm$ 1.79	0.50 $\pm$ 0.26	0.67 $\pm$ 0.05	4.00 $\pm$ 0.71	0.261
Godlingston Hill W	Dorset	SZ010808	52	8.20 $\pm$ 2.28	0.54 $\pm$ 0.16	0.71 $\pm$ 0.11	4.84 $\pm$ 1.01	0.233
Malling Down	East Sussex	TQ430107	37	6.60 $\pm$ 2.40	0.61 $\pm$ 0.18	0.76 $\pm$ 0.05	4.92 $\pm$ 1.03	0.184
Rollington Hill	Dorset	SY970821	24	6.60 $\pm$ 1.52	0.49 $\pm$ 0.05	0.62 $\pm$ 0.09	4.26 $\pm$ 0.43	0.196
Round Down	Dorset	SZ018809	43	7.00 $\pm$ 2.00	0.59 $\pm$ 0.14	0.68 $\pm$ 0.05	4.52 $\pm$ 0.78	0.148
Total			452					

Polyommatus coridon

For *P. coridon* all loci were found to deviate from HWE at the global level (Table 5.8), the degree of deviation in HWE within different sites for each locus varied greatly (0 for Polcor23 and 10 for Polcor24). All loci were, however, found to be selectively neutral (Figure 5.5). In addition to all sites deviating from HWE for Polcor24, this locus also had a low number of alleles (Appendix B, Figure B3) a H_o much smaller than at the global level, an estimate for null allele frequency was >2 and the lowest amplification success rate, all of which indicate a high likelihood of null alleles being present (Table 5.8). Because of this Polcor24 was removed from the data set at this point and was not used in any further analysis. Polcor21 presented similar issues, particularly with regard to a lower number of samples successfully amplifying, and so as a matter of caution a set of differentiation analyses were conducted that excluded this locus, to examine its effect on estimates. Across populations there were lower levels of heterozygosity than would be expected, but no population stands out as having markedly different levels of genetic diversity (Table 5.9). It is of note that the number of alleles found for *P. coridon* loci are generally a lot higher than the numbers found for the *P. bellargus* loci (see Tables 5.6 and 5.8, and Appendix B).

Table 5.8 Summary statistics for 8 microsatellite loci used to genotype *P.coridon*. Ho, Observed heterozygosity; He, Expected heterozygosity; HWE Global, * indicates the locus was found to deviate significantly from Hardy Weinberg equilibrium when all samples were considered; HWE Sites, the number of sampling sites where loci deviated significantly from HWE, with the number once Bonferroni corrections for multiple tests were made in brackets; Null A Freq est, the estimated frequency of null alleles for a locus (Brookfield 1996); F_{IS} (inbreeding co-efficient) and F_{ST} (the proportion of genetic variance found within subpopulations).

Locus	No. Alleles	No. Samples	Ho	He	HWE Global	HWE Sites	Null A Freq	F_{IS}	F_{ST}
Polcor02	24	372	0.659	0.835	*	5(3)	0.0954	0.196	0.021
Polcor03	36	378	0.794	0.885	*	5(1)	0.0480	0.089	0.018
Polcor05	20	372	0.556	0.750	*	9(6)	0.1099	0.238	0.029
Polcor12	14	375	0.557	0.749	*	6(6)	0.1089	0.257	0.018
Polcor13	27	369	0.648	0.856	*	5(5)	0.1115	0.228	0.023
Polcor21	22	336	0.574	0.847	*	9(8)	0.1472	0.311	0.018
Polcor23	23	371	0.639	0.709	*	2(0)	0.0404	0.077	0.026
Polcor24	8	335	0.301	0.741	*	10(10)	0.2520	0.588	0.014

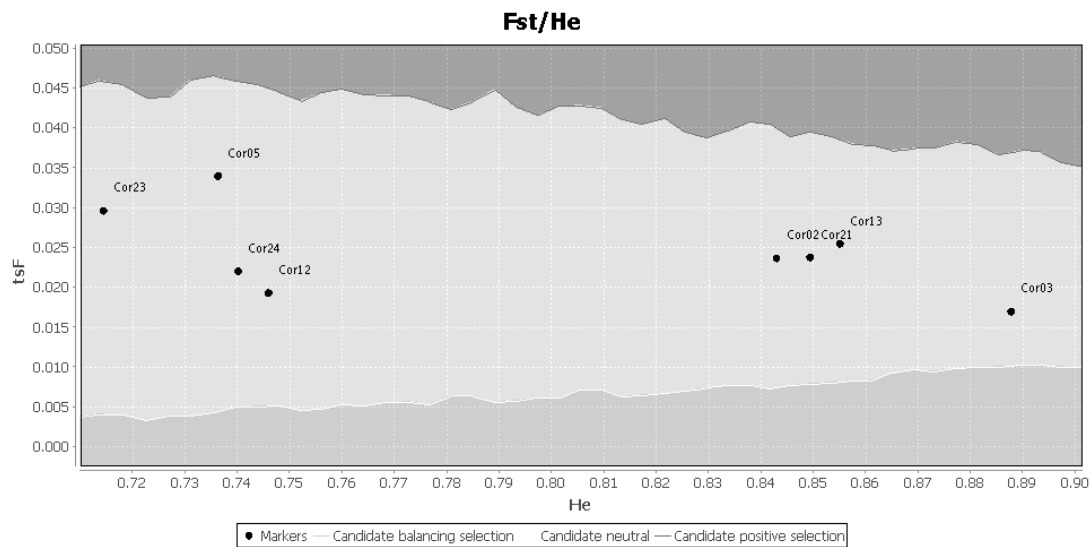


Figure 5.5 Output from 100,000 simulations using data on actual allele frequencies within the *P. coridon* dataset by the software LOSITAN. The expected distribution of the relationship between F_{ST} and H_e for neutral loci is shown in light grey. The expected distribution for loci under balancing selection is shown in mid grey, in dark grey for loci under positive selection (dark grey). For all *P. coridon* microsatellites the observed relationship between F_{ST} and H_e falls within the bounds expected for neutral loci.

Table 5.9 Summary statistics for each geographically defined subpopulation of *P. coridon*. NA, the average number of alleles across the site for all loci; Ho, observed heterozygosity; He, expected heterozygosity; AR, Allelic Richness (the average number of alleles per locus in a population); F_{IS}, inbreeding co-efficient.

Site	County	OS	N	NA ± sd	Ho ± sd	He ± sd	AR ± sd	F _{IS}
Barton Hill	Bedfordshire	TL088729	38	9.86 ± 3.60	0.59 ± 0.16	0.73 ± 0.12	7.17 ± 2.40	0.202
Bindon Hill	Dorset	SY822801	50	12.85 ± 2.41	0.59 ± 0.17	0.74 ± 0.06	8.44 ± 1.75	0.253
Denbies Landbarn	Surrey	TQ135499	32	11.14 ± 1.20	0.60 ± 0.14	0.81 ± 0.05	9.42 ± 1.16	0.255
Fontmell Down	Shaftesbury	ST 883182	16	9.71 ± 2.75	0.69 ± 0.16	0.78 ± 0.13	9.33 ± 3.04	0.116
Friston Gallops	East Sussex	TV555995	56	13.43 ± 2.37	0.68 ± 0.13	0.79 ± 0.07	8.90 ± 1.34	0.144
Magdalen Hill Down	Hampshire	SU505292	47	13.00 ± 2.82	0.62 ± 0.11	0.79 ± 0.09	8.82 ± 2.01	0.217
Malling Down	East Sussex	TQ 430107	30	10.43 ± 1.99	0.56 ± 0.14	0.75 ± 0.08	8.28 ± 1.63	0.255
Old Winchester Hill	Hampshire	SU645208	39	10.86 ± 3.19	0.60 ± 0.20	0.77 ± 0.08	8.38 ± 2.41	0.228
Rodborough C'mn	Gloucestershire	SO855010	47	12.14 ± 2.79	0.69 ± 0.13	0.81 ± 0.08	8.65 ± 2.12	0.156
Totternhoe Quarry	Bedfordshire	SP978221	35	11.14 ± 3.39	0.68 ± 0.15	0.82 ± 0.06	8.27 ± 2.55	0.164
Total			390					

5.5.3 Population differentiation

Table 5.10 A summary of the differentiation statistics provided from all analyses of the *P. bellargus* and *P. coridon* data sets, with different sets of loci and for *P. bellargus* subsets of the entire data set. Loci, shows the loci set used for each analysis with a minus sign indicating the listed loci were removed from the data set, and the number in the brackets shows the total number of loci used; K shows the number of distinct populations predicted by the Bayesian clustering analysis conducted in STRUCTURE, with prior information on sampling location provided for individuals.

Data set	Loci	F _{ST}	F _{IS}	R _{ST}	G' _{ST}	Jost's D	K
<i>P. bellargus</i>							
All Sites	All (5)	0.093	0.210	0.108	0.282	0.198	4
	- Lb418 (4)	0.096	0.154	0.108	0.300	0.2193	4
No BonD	All (5)	0.057	0.194	0.052	0.191	0.131	-
	- Lb418 (4)	0.059	0.164	0.052	0.201	0.140	-
Swanage	All (5)	0.016	0.208	0.023	0.082	0.052	-
	- Lb418 (4)	0.014	0.194	0.022	0.086	0.049	-
<i>P. coridon</i>							
	All (7)	0.021	0.198	-	0.112	0.091	2
	-Polcor05 (6)	0.021	0.192	-	0.119	0.094	2
	-Polcor21 (6)	0.022	0.178	-	0.107	0.086	2
	-Polcor05 & 21 (5)	0.021	0.167	-	0.108	0.087	2

The estimates of differentiation derived from the permutations of the *P. bellargus* and *P. coridon* datasets including different loci were generally little different from one another (Table 5.10), suggesting that the inclusion of potentially problematic loci was not biasing the results of the analysis. For this reason further discussion will focus on the analyses that used the full compliment of loci for each species. Weir and Cockerham's F_{ST} for *P. bellargus* was estimated to be 0.093 (95% C. I. from bootstrapping = 0.087 and 0.103), which suggests moderate genetic structuring between *P. bellargus* populations. The weighted estimate of R_{ST} was similar at 0.108,

though this will have been inflated by a notably large estimate for the locus Lb157 (see Table 5.6). G'_{ST} was estimated at 0.282 and Jost's D was estimated at 0.198. F_{IS} , the average inbreeding co-efficient for all populations was estimated at 0.210 (95% C. I. = 0.103 and 0.330). When Bonchurch Down was removed from the data set F_{ST} was estimated to be 0.057 (95% C. I. = 0.041 and 0.073), and R_{ST} was 0.0517 (with the estimate for Lb157 becoming much smaller and similar to estimates derived from other loci). G'_{ST} and Jost's D were similarly reduced, estimated at 0.191 and 0.131 respectively. F_{IS} was estimated to be 0.194 (95% C. I. = 0.104 and 0.354). The F_{ST} estimate when considering only the seven *P. bellargus* sites in the 'Swanage complex' was 0.016 (95% C. I. = 0.09 and 0.024), again with G_{ST} and Jost's D similarly reduced at 0.082 and 0.052 respectively and F_{IS} was estimated at 0.208 (95% C. I. = 0.094 and 0.298). Conducting all of these analyses excluding the locus Lb418 made little difference to estimates of F_{ST} and its equivalents in all analyses, but did lower the estimate of F_{IS} in all cases, particularly for the total data set and when Bonchurch down was excluded (see Table 5.10).

Models run in STRUCTURE without prior information of sampling location suggested that the most likely number of clusters within the *P. bellargus* dataset was three, with individuals from the Isle of Wight being for the majority in one cluster (Figures 5.6 and 5.7). For the other populations the structure was less clear, but the Swanage complex of sites certainly had very similar profiles to one another. When prior probabilities for cluster assignment for individuals took into account sampling site of individuals, the models suggested that the most likely number of clusters within the samples was four, which corresponded with the geographic structure of the samples

more closely (Figure 5.8 and 5.9). The exclusion of the locus Lb418 from this analysis did not change the estimation of the number of clusters (Table 5.10).

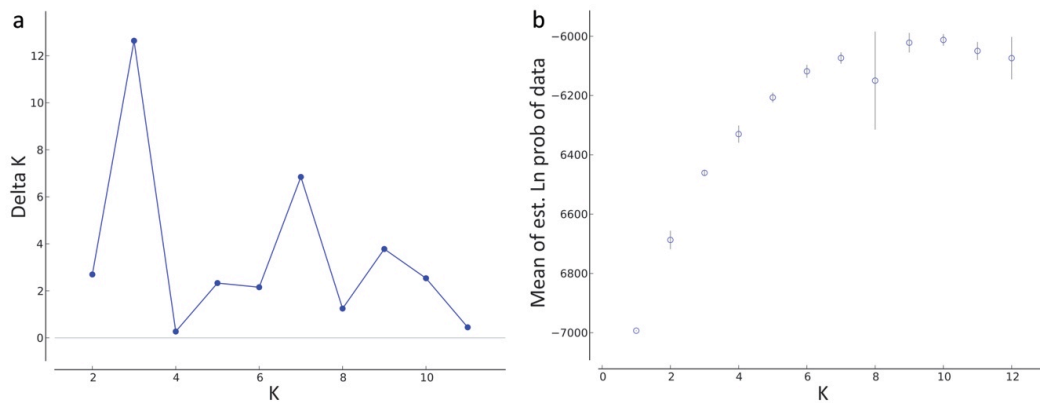


Figure 5.6 a) The estimation of Delta K (Evanno et al. 2005), the most likely number of clusters (K) within the overall population of *P. bellargus* when no information on sampling location is provided, from the second order rate of change of the log probability of data with respect to different values of K. The most likely value of K is that with the highest Delta K. b) The mean log-likelihood of the five STRUCTURE models run for each value of K. The error bars give the standard deviation.

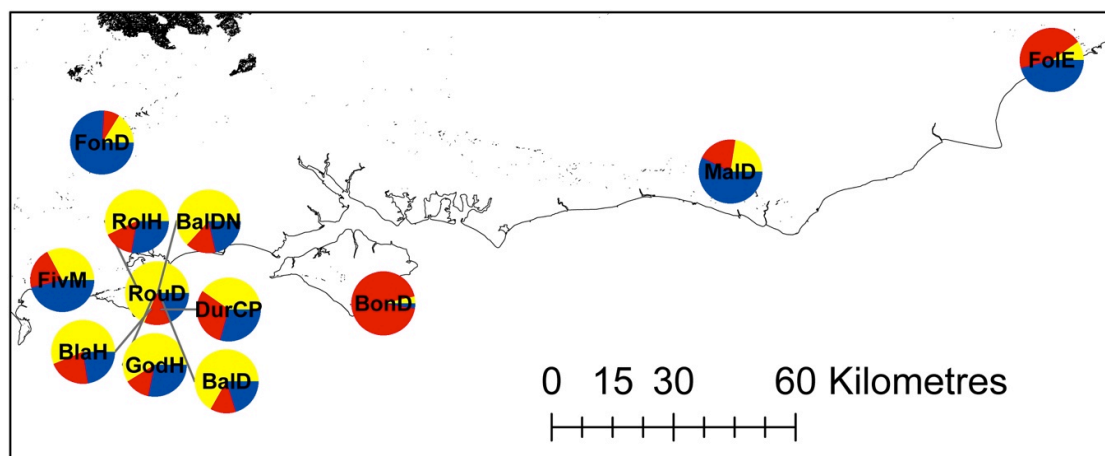


Figure 5.7 The overall clustering of *P. bellargus* subpopulations into $K = 3$, as predicted by the programme STRUCTURE without prior information of sampling location, with a different colour representing each of the 3 clusters. For each sampling location the proportion of a pie chart represents the probability that an individual drawn from it would belong to one of the three clusters identified by STRUCTURE

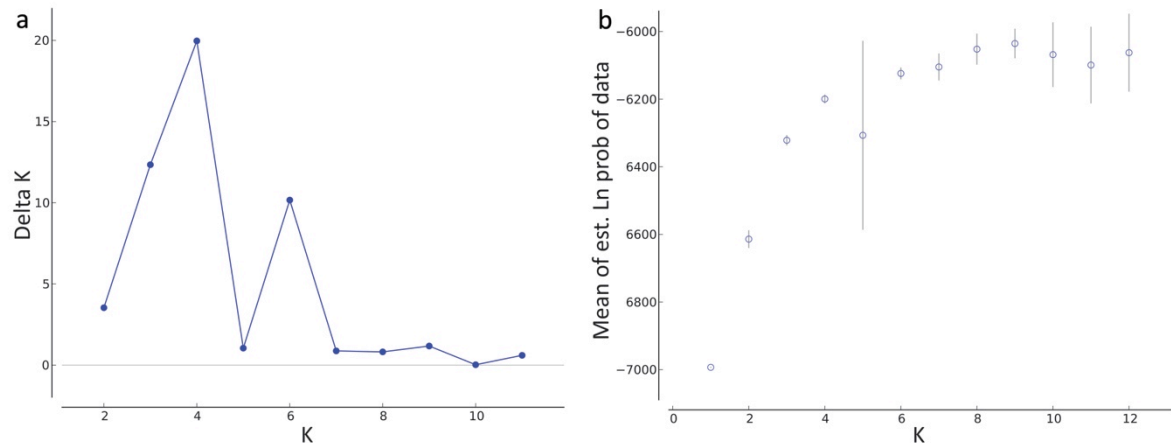


Figure 5.8 The estimation of Delta K (Evanno et al. 2005) for the *P. bellargus* when prior information is given of sampling location for each individual. The most likely value of K is that with the highest Delta K. b) The mean log-likelihood of the five STRUCTURE models run for each value of K. The error bars give the standard deviation.

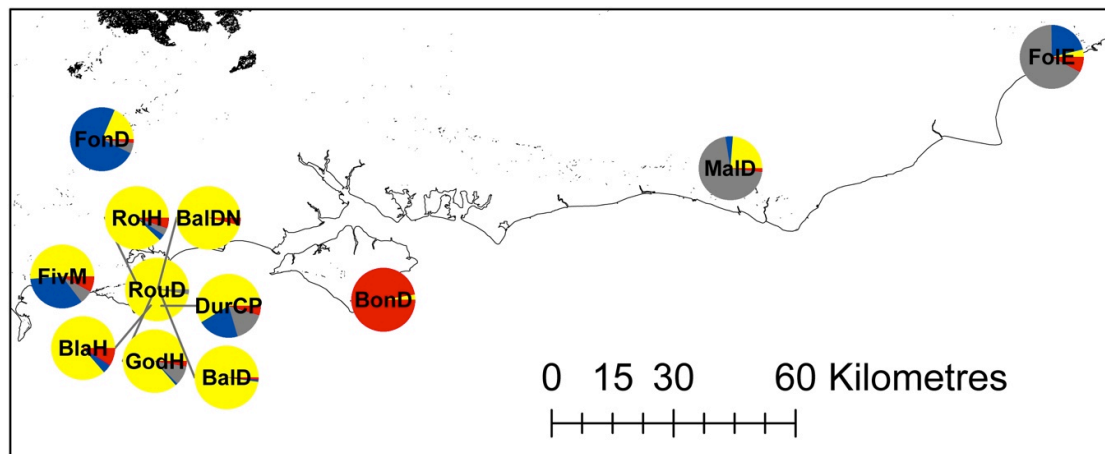


Figure 5.9 The overall clustering of *P. bellargus* subpopulations into $K = 4$, as predicted by the programme STRUCTURE when provided with prior information of sampling location, with a different colour representing each of the 4 clusters. For each sampling location the proportion of a pie chart represents the probability that an individual drawn from it would belong to one of the three clusters identified by STRUCTURE.

Weir and Cockerham's (1984) F_{ST} for *P. coridon* was estimated to be 0.021 (95% C. I. = 0.019 and 0.025), which suggests low levels of genetic structuring between the different populations, particularly compared to *P. bellargus*. G'_{ST} was estimated to be

0.112 and Jost's D at 0.091, which also suggests *P. coridon* shows lower levels of genetic differentiation than *P. bellargus*. F_{IS} was estimated at 0.198 (95% C. I. = 0.139 and 0.254). The removal of potentially problematic loci from the analysis made little difference to estimates of F_{ST} and its equivalents, but did reduce estimates of F_{IS} (Table 5.10).

Results from STRUCTURE without prior information on sampling location suggested that the global population contained three distinct genetic clusters, whose distribution among the different geographical locations gave little indication of population structuring (Figures 5.10 and 5.11). When prior information on sampling location was provided K was estimated to be two, and indicated a gradient of division between populations from east to west (Figure 5.12 and 5.13). Again, the removal of potentially problematic loci did not change the estimated of the number of clusters (Table 5.10).

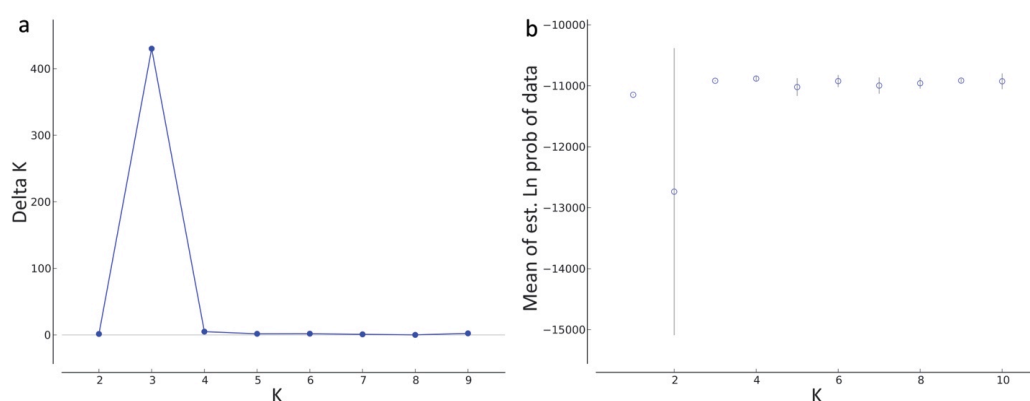


Figure 5.10 The estimation of Delta K (Evanno et al. 2005) for the *P. coridon* when no information is given of sampling location. The most likely value of K is that with the highest Delta K. b) The mean log-likelihood of the five STRUCTURE models run for each value of K . The error bars give the standard deviation.

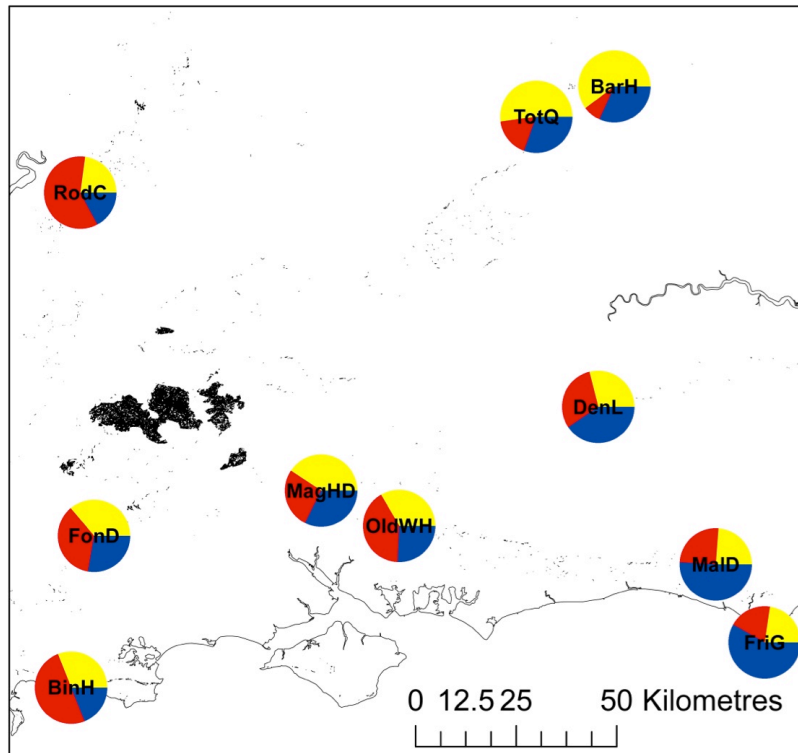


Figure 5.11 The overall clustering of all *P. coridon* samples at $K = 3$ as predicted by the programme STRUCTURE, without prior information of sampling location, with a different colour representing each of the 3 clusters. For each sampling location the proportion of a pie chart represents the probability that an individual drawn from it would belong to one of the three clusters identified by STRUCTURE.

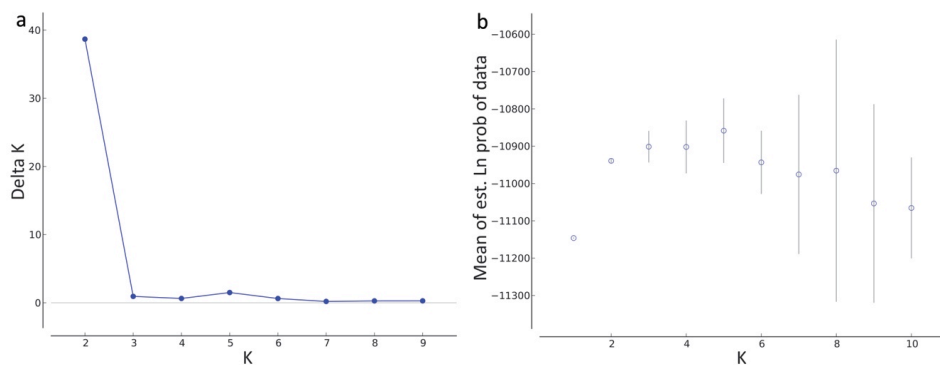


Figure 5.12 The estimation of Delta K (Evanno et al. 2005) for the *P. coridon* when prior information is given of sampling location for each individual. The most likely value of K is that with the highest Delta K. b) The mean log-likelihood of the five STRUCTURE models run for each value of K . The error bars give the standard deviation.

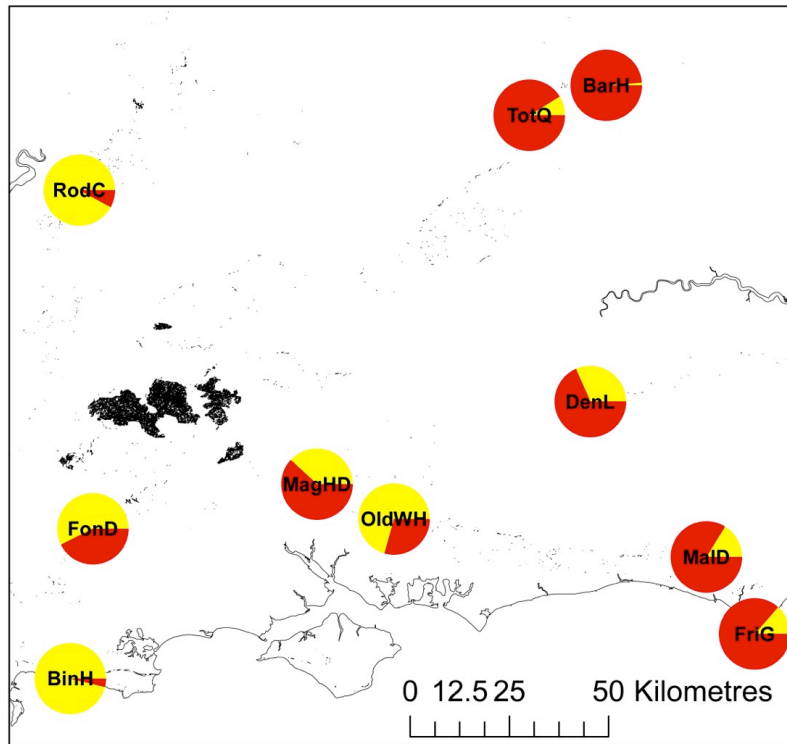


Figure 5.13 The overall clustering of all *P. coridon* samples at $K = 2$ as predicted by the programme STRUCTURE, when provided with prior information of sampling location, with a different colour representing each of the 2 clusters. For each sampling location the proportion of a pie chart represents the probability that an individual drawn from it would belong to one of the three clusters identified by STRUCTURE.

5.5.4 Isolation by distance and landscape factors

The correlation between genetic distance and log geographical distance for *P. bellargus* was not significant when considering the whole dataset ($Z = 35.935$, $r = 0.369$, $p = 0.092$, RMA $R^2 = 0.136$, intercept = -0.5891 ± 0.0831 , slope = 0.1595 ± 0.0185) (Figure 5.14a). However, as can be seen from Figure 5.14b, the relationship between genetic and geographic distance differed for Bonchurch Down (found on the Isle of Wight). When Bonchurch Down was excluded from the dataset the relationship between geographic and genetic distance was found to be significant ($Z = 15.5626$, $r = 0.5651$, $p = 0.0048$, RMA $R^2 = 0.319$, intercept = -0.1384 ± 0.0230 , slope = 0.04607 ± 0.0052). Significant isolation by distance was also found for *P.*

coridon ($Z = 4.9519$, $r = 0.3793$, $p = 0.0034$, RMA $R^2 = 0.144$, intercept = -0.1872 ± 0.0295 , slope = 0.0420 ± 0.0059) though the level of differentiation between populations was much smaller than in *P. bellargus* (Figure 5.15).

Table 5-11 Summary of results for Mantel correlation tests of isolation by distance in the genetic similarity between populations and partial Mantel correlation tests for the effect of landscape attributes on genetic similarity whilst controlling for geographic distance. * denote significance at the 0.05 level, ** at the 0.01 level and *** at the 0.001 level and ns denotes the a correlation was not significant.

Data Set	Variable	Z	r	p
<i>P. bellargus</i>	Geog Dist (log)	35.935	0.3685	0.0919
	Sea		0.7580	0.0410*
<i>P. bellargus</i> exc BonD	Geog Dist (log)	15.5626	0.5651	0.0051**
	Arable and Horticulture		0.2197	0.1698 ns
	Built and Gardens		0.2585	0.0883 ns
	Improved Grassland		-0.6208	0.0008***
	Calcareous Grassland		-0.6233	0.0006***
	Broadleaf Woodland		-0.1707	0.2034 ns
	Sea		0.2945	0.0778 ns
<i>P. coridon</i>	Geog Dist (log)	4.9519	0.3793	0.0034 **
	Arable and Horticulture		0.3569	0.0715 ns
	Built and Gardens		0.0985	0.3163 ns
	Improved Grassland		-0.2414	0.1222 ns
	Calcareous Grassland		0.1326	0.2246 ns
	Broadleaf Woodland		-0.4386	0.0199*

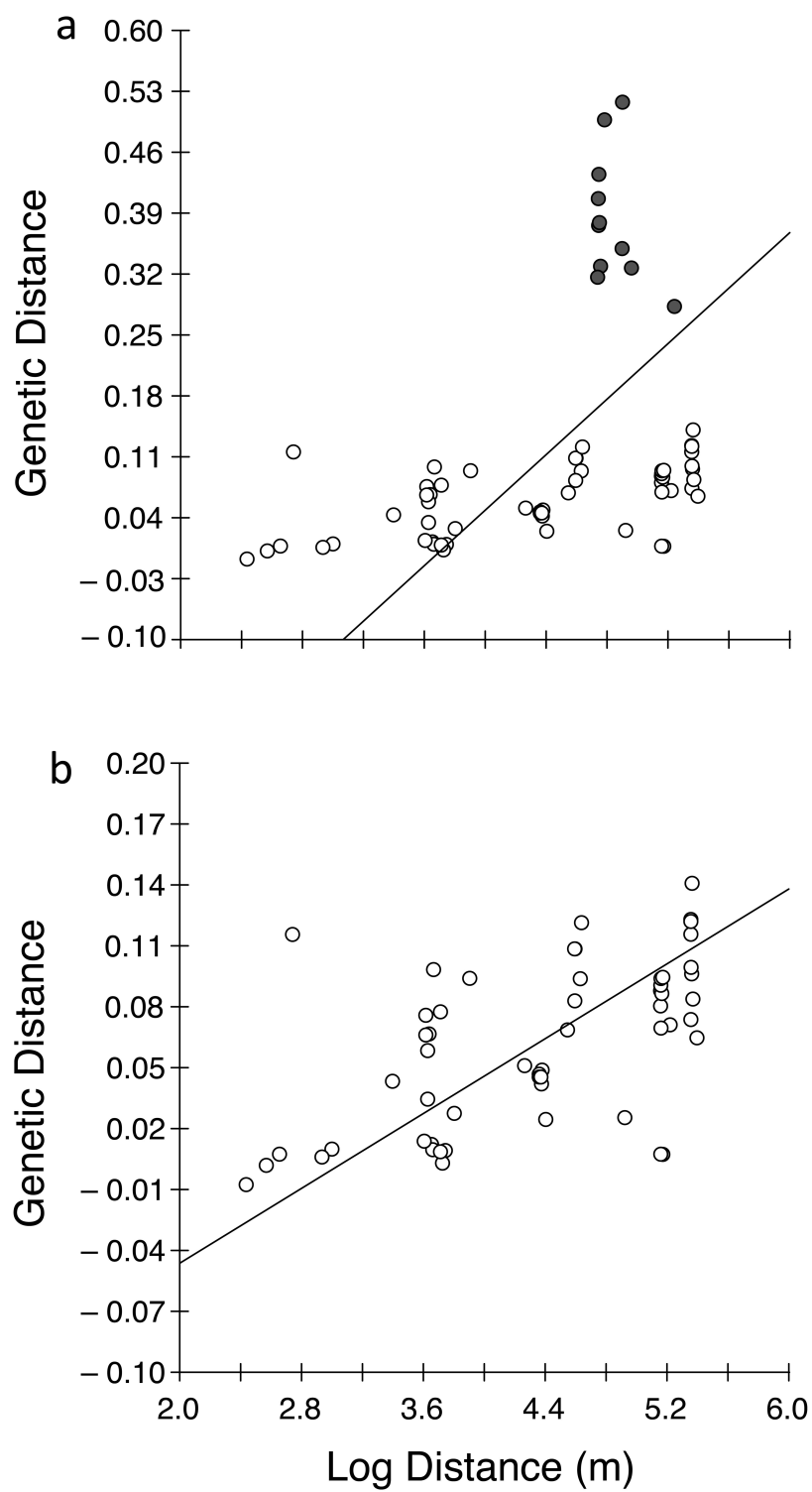


Figure 5.14 the relationship between pairwise genetic distance, measured by Slatkin's linearized F_{ST} and pairwise log geographic distance in metres for a) all *P. bellargus* subpopulations sampled b) excluding the population from Bonchurch Down, Isle of Wight. The black circles in a) are pairwise distances from Bonchurch Down.

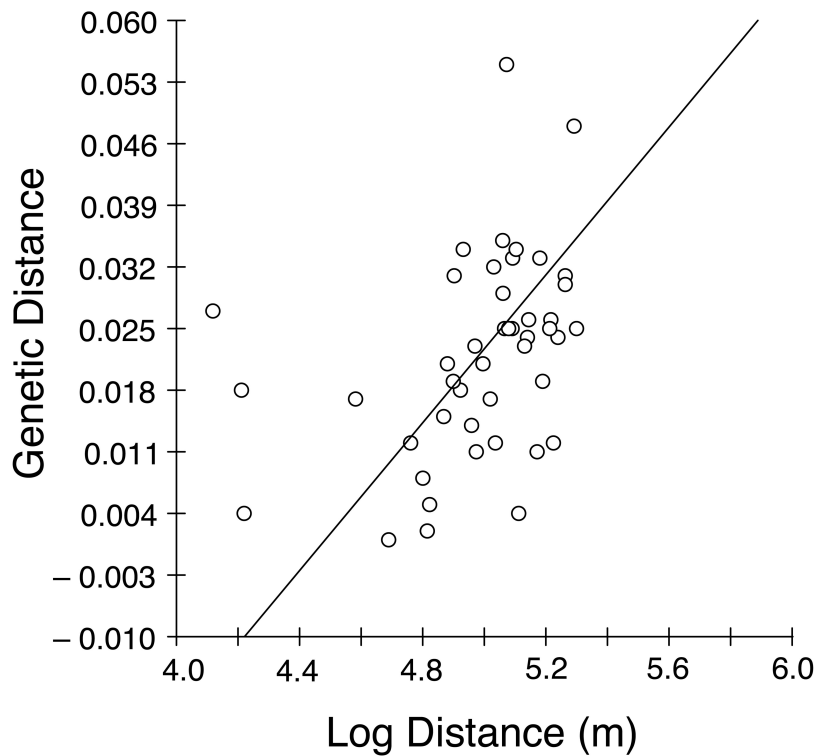


Figure 5.15 The relationship between pairwise genetic distance, measured by Slatkin's linearized F_{ST} and pairwise log geographic distance in metres for all *P. coridon* subpopulations sampled.

Partial Mantel tests, testing for an influence of land / sea cover on genetic distance, once geographic distance has been accounted for, showed that the proportion of a 2 km buffer between sites that was Sea had a positive and significant correlation with genetic distance when Bonchurch down was included in the analysis (Table 5.11). Further analysis excluding Bonchurch Down showed that Improved and Calcareous grassland coverage were significant, negative predictors of genetic distance, suggesting that an increase in the amount of grassland between sites facilitates gene flow (Table 5.11). Interestingly, for *P. coridon* there was a negative correlation between the amount of Broadleaf Woodland cover and genetic similarity, suggesting

that it increases connectivity between populations (Table 5.11). The influence of both agricultural land and built environments was not significant in any of the analyses.

5.6 Discussion

This study supports the prediction that populations of *P. bellargus* in southern England are more genetically structured than populations of *P. coridon*, suggesting that *P. bellargus* experiences habitat fragmentation more severely than *P. coridon*. These results concur with those of a previous study of *P. bellargus* in the south of England (Harper et al. 2003), and the low levels of structuring found in *P. coridon* in north and west Germany (Krauss et al. 2004; Schmitt and Seitz 2002a), but for the first time considers both species within the same landscape.

5.6.1 Genetic diversity

Between the two species the only statistic of genetic diversity for which a marked difference was found was the number of alleles per locus (Tables and Figure B1-B3). The lower number of alleles for most *P. bellargus* loci are likely in part to be a consequence of the ‘recent’ (1976) population bottleneck previously documented by (Harper et al. 2003). However, traits intrinsic to the microsatellite loci may also have served to create these differences. The presence of shorter nucleotide repeats within a locus (such as di nucleotide), will increase the potential number of alleles at a locus compared to loci with only larger repeats (such as tetra nucleotide). Given the fairly even distribution of short and long repeats between the *P. bellargus* and *P. coridon* loci this will be of little influence here. However, the non-canonical motifs observed for most Polcor loci will also have increased their potential number of alleles.

For both species, lower than expected levels of heterozygosity were found across all loci. Schmitt and Hewitt (2004) have shown that phylogeography (the history of colonisations since the last ice age that has lead to a species current distribution) impacts the genetic diversity of a species at its northern range edge. They give *P. coridon* as an example of a species whose genetic diversity and population stability both decrease on a South to North gradient through its range, representing the pattern of post-glacial expansion through Europe from the Adriatic-Mediterranean region (Schmitt and Krauss 2004). Schmitt and Hewitt (2004) conclude that 11 other species of butterfly show a similar range and pattern in decreasing population size from South to North, and are likely to show similar parallel reductions in genetic diversity. They state this is the case for *P. bellargus* though they do not present the data. The deficits in heterozygosity observed for both species in this study, at the northern edge of their geographical ranges, are in line with this prediction.

The inbreeding coefficient (F_{IS}) estimates the degree of inbreeding seen within subpopulations, or more technically the mean reduction in heterozygosity within individuals due to non-random mating. It varies from -1.0 (total heterozygosity) to 1 (total homozygosity) and so higher values indicate higher levels of inbreeding. The values for both *P. bellargus* and *P. coridon* are similar, being around 0.2 for each species, indicating that inbreeding is present within subpopulations. Compared to the levels observed in 16 grassland species within Europe this level of inbreeding is at the high end of the spectrum (Habel et al. 2013). The estimates found here for *P. coridon* are a least 4 times as high in northern Germany (0.046) (Krauss et al. 2004) and in western Germany (0.033) (Schmitt and Seitz 2002a).

A note of caution on estimates of heterozygosity is necessary. Heterozygote deficit in lepidopteran microsatellites is common, due to characteristics intrinsic to the microsatellites (Van't Hof et al. 2007). Homoplasy, where alleles are the same size but not of the same descent is a well documented limitation in microsatellites, which leads to heterozygosity being underestimated (Estoup et al. 2002). Null alleles have been also identified as a major problem for microsatellites in other studies of Lepidoptera, and have already been suggested as a potential problem for the *P. bellargus* microsatellites used in this study (Harper et al. 2003; Harper et al. 2000). Tests to estimate the frequency of null alleles for each locus were very close to 2 for some *P. bellargus* and *P. coridon* loci, the frequency above which null alleles can be problematic for an analysis (Dakin and Avise 2004) (see Table 5.6 and 5.8). Recently, however, the appropriateness of removing such loci from datasets has been questioned, as they may be providing genuine information on Wahlund effects; a heterozygosity deficit in an overall population due to genetic structuring (Dharmarajan et al. 2013). As a matter of caution the analyses presented for *P. bellargus* and *P. coridon* were also conducted after the loci with the highest null allele frequency were excluded from the dataset, but this resulted in negligible or no change in F_{ST} and other differentiation statistics.

5.6.2 Differentiation

F statistics, their analogues and Bayesian clustering all suggest that gene flow between *P. bellargus* subpopulations is more restricted than those of *P. coridon*. A larger F_{ST} (or analogous measure) indicates higher levels of genetic differentiation

between subpopulations. Variation in analogous estimates (R_{ST} , G'_{ST} and Jost's D) between *P. bellargus* and *P. coridon* were generally consistent with those for F_{ST} , and further discussion will focus on F_{ST} . For *P. bellargus* the initial value of 0.093 suggests moderate differentiation. Previously, using the same microsatellite loci as this study, Harper et al. (2003) estimated the F_{ST} for *P. bellargus* in southern England to be 0.127. The higher estimate can probably be attributed to the larger number of sites sampled (27 vs. 12), which covered a wider geographical area than sampled here. The estimate of F_{ST} drops to 0.057 when the population on the Isle of Wight is not included. This, along with the resolution of individuals from Bonchurch Down into a distinct population cluster using STRUCTURE, suggests a real island effect is occurring, whereby the sea is a stronger barrier to dispersal than geographic distance alone. When only the 7 sites found within the Swanage area of Dorset are considered together F_{ST} is reduced to 0.016, showing that with this relatively small locality gene flow between populations is high. The results of Bayesian clustering analysis, particularly when accounting for sampling location, provide further support for this scenario, with the population on the Isle of Wight representing a very distinct cluster, the 'Swanage complex' of sites representing another distinct grouping and the sites to the west and east each forming similarly distinct groups (Figure 5.8). For *P. coridon* the F_{ST} of 0.021 indicates weaker levels of differentiation between subpopulations. This is supported by the analysis with STRUCTURE (again also when incorporating sampling location), which suggests two distinct clusters, with differentiation seeming to correspond loosely with a gradient from east to west. Interestingly, though still low, this estimate is still higher than those obtained from studies of *P. coridon* in western Germany ($F_{ST} = 0.014$ (Schmitt and Seitz 2002a)) and northern Germany ($F_{ST} = 0.013$

(Krauss et al. 2004)), suggesting that *P. coridon* populations are more structured in southern England than on the continent.

For both species, the inclusion / removal of potentially problematic loci made little difference to the results and the levels of differentiation observed here for *P. bellargus* and *P. coridon* fall well within the range observed for other species of Lycaenid in Europe (Nève 2009). For instance *Polyommatus icarus* (Common Blue) has been found to show low levels of differentiation between populations across Europe ($F_{ST} = 0.0187$), and even lower when considered at the regional level of West Germany ($F_{ST} = 0.0041$) (Schmitt et al. 2003). At the other extreme, an allozyme study of *Aricia agestis* (Brown argus) samples from six regions covering its entire UK range showed a high level of differentiation between different regions ($F_{ST} = 0.1857$) (Lai and Pullin 2005).

Overall the results presented support the hypothesis that (in Southern England) *P. coridon* shows less genetic structuring between subpopulations than found in *P. bellargus*. Differentiation between populations becomes weak for *P. bellargus* when considering only sites at the localised level of the Swanage complex, whose 7 sites show minimum and maximum pairwise geographic distances of 0.25km and 8.02km respectively ($F_{ST} = 0.016$). Considering that estimated differentiation for the entire *P. coridon* population is only slightly higher when site pairwise distances range from 13.7km -199.3km ($F_{ST} = 0.021$) highlights the stronger genetic structuring in *P. bellargus*.

5.6.3 Isolation by distance and landscape

Mantel tests provide strong evidence for isolation by distance in both *P. bellargus* and *P. coridon*; the further apart sites are from one another geographically the more distinct they become genetically, but also suggest that nature of landscape between sites can modify this relationship. For *P. bellargus* the Isle of Wight population does not conform to the same pattern of isolation by distance as the other sites (Figure 5.13). The positive correlation between the amount of sea and genetic distance between *P. bellargus* subpopulations confirms that this is because English Channel acts as a stronger barrier to dispersal and gene flow than geographic distance alone. A negative correlations with genetic distance for both calcareous and improved grassland coverage, suggests contrary to predictions, that any grassland habitat can facilitate better movement between populations. For *P. coridon* it seems that, contrary to predictions, Broadleaf woodland actually facilitates gene flow between populations, rather than act as an impediment.

Though both species show clear isolation by distance, the degree of differentiation caused by distance in *P. bellargus* is generally much higher than in *P. coridon*, which shows weaker differentiation between subpopulations. This suggests that though isolation by distance occurs between the *P. coridon* subpopulations, the distance between sites is less of an impediment to gene flow between subpopulations than it is for *P. bellargus*.

5.6.4 Limitations and further work

The limitations in this study must be acknowledged before implications are considered. Microsatellites are just one of many genetic markers (such as Allozymes, Amplified Fragment Length Polymorphism (AFLPs), Randomly Amplified Polymorphic DNA (RAPD) and mitochondrial DNA) that can be used in population genetic studies, and each has its own benefits and drawbacks (Lowe et al. 2004). One of the major drawbacks of microsatellites is the expense and time involved in their isolation and optimisation, but the microsatellite sets used here had already been characterised. However, the isolation and characterisation of microsatellites in Lepidoptera is well recognised as being particularly problematic, and some of these problems might still be manifest in the primer sets used here (Nève 2009; Nève and Meglecz 2000). Some lepidopteran microsatellites have been found to have very similar primer binding sites, leaving such loci prone to cross amplification, which can result in multiple bands where a maximum of two would be expected (for diploids) (Meglecz et al. 2007; Meglecz et al. 2004; Van't Hof et al. 2007). Instability in flanking regions may also lead to null alleles and microsatellite repeat regions may be associated with transposable elements within a genome (Meglecz et al. 2007; Van't Hof et al. 2007). As discussed above, null alleles and homoplasy are also likely to present in the data sets for both butterfly species. This will result in the under-estimation of genetic diversity and so influence estimates of population differentiation, by masking genetic variation within populations (Chapuis and Estoup 2007).

With regard to *P. coridon* in particular there are reasons to believe that the loci used are not necessarily fully optimised. Two loci were excluded from the analysis due to

problems with non-specific products, which can be related to cross amplification of other loci with similar flanking regions. Further, most loci did not exhibit truly canonical repeats (step changes of three base pairs rather than the expected 2 between certain alleles) and so do not conform to the assumptions of stepwise mutation for microsatellite loci. Repeated checks and screening of samples suggested that these alleles were genuine, not an artefact of misreading in the DNA analyser or genotyping errors. Evidence, from the sequencing of PCR products, shows indels caused some of the allelic variation in Polcor05, Polcor12, Polcor21 and Polcor23. This, along with the propensity for flanking region mutations in lepidopteran microsatellites discussed above, suggests that these non-canonical alleles in the Polcor loci are likely the result of such indels. It was decided that such alleles represented genuine genetic variation and so were used for the analysis presented here, but an ideal next step would be to complete more comprehensive sequencing of the products at each locus to determine the origin of each allele.

The sites sampled here, 12 and 10 for *P. bellargus* and *P. coridon* respectively, represent only a small subset of those sites where each species are found in England. This in part reflects the difficulty of obtaining permissions to sample a species of conservation concern and in part the logistical difficulties of sampling sufficient individuals in poor weather conditions. As with any scientific study, increased replication would add statistical power and increased certainty, and it would have been preferable to sample sites in a more systematic manner. The addition of sites to each dataset would be beneficial, particularly for *P. bellargus* where 7 sites were clustered into a relatively small region (Swanage; Dorset). The sampling of multiple populations within different regions of each species range, such as achieved in the

Swanage complex, would also allow for a more detailed analysis of the genetic structuring within each species. For instance this would allow for Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992) to be conducted, to explore how genetic diversity is partitioned between regions, and populations within regions (Schmitt and Seitz 2004). This would allow for a more comprehensive picture of both how genetic variation is partitioned at different scales, and would also allow the use of Bayesian analysis to look at more regional specific structuring. The addition of a population of *P. coridon* from the Isle of Wight would also be useful for comparison with *P. bellargus* in respect to movement across bodies of water.

5.6.5 Conclusions and implications

This study has confirmed the prediction that in the south of England (at the northern edge of their geographic range), *P. bellargus* exhibits higher genetic differentiation than *P. coridon*. Both *P. bellargus* and *P. coridon* exhibit low genetic diversity, and so high levels of inbreeding. However the distribution of this genetic diversity between subpopulations is far more equitable in *P. coridon*, and so gene flow between subpopulation in this species is a more regular. The most parsimonious explanation for this is that *P. coridon* is better able to disperse effectively between habitat patches, and so maintain gene flow over larger geographical distances than *P. bellargus*. The results presented here for IBD lend support to this explanation, in that gene flow in *P. bellargus* is more constrained by distance, and IBD has been invoked to explain the degree of differentiation seen in many Lepidoptera studies (Nève 2009). Other studies that have found differences in the degree of population structuring in Lepidopteran species occupying the same fragmented calcareous grasslands in West Germany,

including another case of two Lycaenid congeners (*Arícia agestis* and *Cupido minimus*), have concluded that dispersal ability is of major importance (Habel and Schmitt 2009; Louy et al. 2007). The MRR evidence available for each species does indeed suggest that *P. coridon* has higher dispersal ability than *P. bellargus* (Asher et al. 2001; Schmitt et al. 2006; Thomas 1983). Studies exploring the influence of isolation on the occupancy habitat patches in *P. bellargus* and *P. coridon* also support the findings presented; for *P. bellargus* in the UK the degree to which a site is isolated from other habitat patches is an important determinant of patch occupancy (Thomas et al. 2001b), whereas for *P. coridon* in Germany isolation was found to be of significant, but low, importance (Krauss et al. 2005).

The nature of the habitat in the intervening landscape also seems to alter the connectivity between sites, suggesting that grassland habitats facilitate better dispersal between sites in *P. bellargus* and Broadleaf woodland for *P. coridon*. The permeability of different habitats to dispersal will vary depending on species (Dover and Settele 2009), and habitat more favourable to dispersal for a given species will increase the chance of individuals successfully moving between subpopulations (Watling et al. 2011). Given that *P. bellargus* is a grassland specialist it is not surprising given that grassland habitats promote connectivity between sites, as individuals will be better adapted to move through such habitat. Less clear is why the same increase in connectivity with grassland is not observed in *P. coridon*, and why Broadleaf woodland should promote dispersal in this species. One possibility is that the generally much shorter distances between *P. bellargus* sites increases the importance of grassland as a predictor of gene flow, but for the larger distances considered in *P. coridon* the relationship breaks down. Overall these results do suggest that increased

connectivity between *P. bellargus* populations can be facilitated by increasing the availability of suitable habitat in the landscape between sites. The implication from a conservation perspective is that the maintenance of grassland habitat in the wider landscapes should promote connectivity between subpopulations. However, caution must be taken from reading too much into these results, as they hinge on the assumption that individuals move in a linear fashion through the landscape, which is not necessarily the case.

That *P. bellargus* exhibits a more differentiated population structure undoubtedly reflects restricted dispersal between subpopulations in a fragmented landscape, and so it is tempting to suggest that promoting connectivity between existing populations should be a priority for conservation. However, it may be premature to think higher levels of differentiation are always a cause for concern. Specialist butterflies are recognised as generally exhibiting more closed population structures, being organised into discrete colonies based on patches of suitable resources with little exchange of individuals between them (Wilson and Roy 2009). This will also be played out in the genetic structure of their populations, predisposing species to lower gene flow between subpopulations and higher levels of inbreeding (Nève 2009). For a survey of 22 chalk grassland butterflies in Germany Habel et al. (2013) found higher levels of genetic structuring in specialist species than generalists. Similar studies of three species of Ghanaian forest butterflies, and another of two bee species in the same landscape also suggest that species which show a higher degree of habitat specialism show higher genetic structuring (Bossart and Antwi 2012; Zayed et al. 2006).

Polyommatus bellargus and *P. coridon* are both habitat specialists, but specialism is a continuum and, as shown in chapter 3 of this thesis, the niche of *P. bellargus* is more constrained than that of *P. coridon*. The differences in genetic structure between the two species will in part be a consequence of available habitats for *P. bellargus* having always been more patchily distributed, by virtue of its more specific habitat requirements. Broader habitat preferences in *P. coridon* mean that more calcareous grassland sites are available for populations to establish, and in a stepping stone fashion this will also promote higher connectivity between subpopulations.

High differentiation and low genetic diversity are generally perceived as a negative for species of conservation concern, being linked to decreased fitness and loss of adaptability in the face of environmental change (Frankham 2005; Frankham et al. 2010; Reed and Frankham 2003). However, a predisposition in specialist species to exist as fragmented populations may have led to populations adapted to survive under a scenario of low genetic diversity and possibly even selection against high dispersal ability in a species (Habel et al. 2011a; Habel et al. 2012; Habel and Schmitt 2012). If so, reduced genetic diversity and higher levels of population differentiation in such cases can be envisaged more as the status quo, and less of a threat to its genetic integrity. Based on this reasoning Habel and Schmitt (2012) hypothesise that fragmentation will present the greatest threat to those species intermediate on the generalist-specialist spectrum. Such species will be adapted to population structures with high rates of gene flow, but under the pressures of fragmentation will be less equipped than highly generalist species to maintain this. Interestingly, this would also correspond to the findings of (Thomas 2000) that butterfly species of intermediate

dispersal ability had experienced higher levels extinction in different regions of the UK and Europe.

The higher degree of differentiation and relatively high levels of inbreeding observed here for *P. bellargus* could reflect its more closed populations as much as contemporary habitat fragmentation. This is congruent with the previous findings of Harper et al. (2003) for *P. bellargus* in the UK, who emphasise that promoting connectivity between subpopulations may be less important than sustaining large and resilient local populations. Indeed, the results presented here suggest that there has been no change in levels of gene flow between populations of *P. bellargus* since Harper et al. (2003) study, even with the marked recovery of *P. bellargus* in the region (Fox et al. 2011a). Population genetic studies for *P. bellargus* on the European continent, where thermal constraints on its habitat needs are less, would be useful further research with regard to distinguishing the degree to which a less specialist lifestyle alters its population structure in a fragmented landscape. Studies of other Lycaenid specialists of calcareous grassland have shown that their populations are structured differently in different areas of their range where they are subject to fragmentation. Welsh populations of *Plebejus argus* (Silver Studded Blue) indicated moderate differentiation ($F_{ST} = 0.07$) (Brookes et al. 1997), a later study of Finnish and Spanish populations showed low levels of differentiation (F_{ST} 's = 0.016 and 0.015 respectively) (Péténian and Nève 2003). The remarkably high levels of differentiation in *A. agestis* across the UK ($F_{ST} = 0.1857$) (Lai and Pullin 2005) were not found when the species was studied in western Germany ($F_{ST} = 0.039$) (Habel and Schmitt 2009).

Following the reasoning of Habel and Schmitt (2012) habitat fragmentation may actually present a greater threat to the genetic integrity of *P. coridon* in the UK, it being less specialised and more widely distributed than *P. bellargus*. It is interesting that though subpopulations of *P. coridon* are well connected by gene flow, populations still exhibit lower than expected levels of genetic diversity / high levels of inbreeding. It would be useful in this instance to have historical data on population structure and genetic diversity in *P. coridon* in the UK, to see how both may have changed in response to habitat fragmentation. If conservation management in recent decades has facilitated connectivity between subpopulations of *P. coridon* this might explain why genetic diversity is low whilst connectivity is high, however, the predisposition of this species to lower genetic diversity at its northern range edge may also be at play (Schmitt and Hewitt 2004).

Chapter 6 - General Discussion

6.1 Key findings

Each chapter in this thesis is a separate study linked by the wider theme of describing and comparing the ecology of *Polyommatus bellargus* and *Polyommatus coridon* at the northern edges of their geographical ranges. Here a brief summary of the key findings is presented, followed by a discussion of broader implications and areas for further research.

In Chapter 2, sites managed for *P. bellargus* in recent decades show no evidence for an expansion in the larval niche of this thermophilous species due to climate change, when changes in microhabitat structure are accounted for. This is less surprising when it is considered that the magnitude of change in microclimate caused by reducing turf heights will have had a far greater influence in improving habitat quality than the highly variable average temperatures in the region over the past four decades (Figures 2.5 and 2.6). However, the fact that turf heights have been reduced so extensively on managed sites will have made the chances of detecting a change in microhabitat preferences more remote, so the potential expansion of the larval niche into previously suboptimal conditions cannot be excluded.

Chapter 3 demonstrates that *P. coridon*'s oviposition preferences are much broader with regard to turf height and shelter than *P. bellargus* (Figures 3.3 and 3.4). Also, current preferences in both species are less constrained by microhabitat than 30 years ago, to the extent that no effect of turf height or shelter was observed for present day preferences in *P. coridon*. This shows that the reduced turf heights that have

facilitated the recovery of *P. bellargus* have also increased the potential for overlap in habitat use between the two species. Whether this has consequences for *P. coridon* on sites where both species are present is unclear, but this is certainly an area for further research.

Field observations (chapter 3) and experimental work (chapter 4) suggest that the ant-mutualisms of both butterfly species are not specific enough to make the species of ant a defining resource of the larval niche of either butterfly. Experimental work showed that any energetic costs imposed on the larvae and pupae of both species from ant mutualisms is not sufficient to affect development, or is compensated for by the time individuals reach adulthood. Given the facultative nature of ant mutualisms for both butterflies, it is not be surprising that there is little evidence for specificity with regard to ant species. However, data from more field sites would be useful and further experimental work with varied food availability and natural enemy exposure would show how the dynamics of these mutualisms might alter: it is possible that, despite the observed lack of ant specificity, the presence of a minimum density of mutualistic ant species (e.g. *Myrmica*, *Lasius*) is necessary for a population of these butterflies to persist or thrive in the wild.

The results in chapter 5 confirm the prediction that genetic differentiation between *P. bellargus* subpopulations in the south of England is higher than levels in *P. coridon*. Two (interrelated) factors are likely at play here: Firstly, *P. coridon* has a better capacity for long distance dispersal events and less dependent on the intervening landscape, and so gene flow between subpopulations will be more frequent. Secondly, the more specialised habitat requirements of *P. bellargus* compared to *P. coridon* (as

described in chapter 3) will serve to impose a more fragmented structure on its populations, especially during its ‘bottleneck years’ in the UK of the mid 1950s to 1990s. This predisposition to a fragmented population structure may mean *P. bellargus* is adapted to be more resilient to the genetic consequences of fragmentation (low genetic diversity and inbreeding). Further work with *P. bellargus* on the European continent would help tease apart the relative influence of specialisms and habitat loss on this species population structure.

6.2 Discussion

6.2.1 Conservation in a changing climate

Chapter 2 shows that changes to the management of calcareous grasslands (and other habitats) need to be accounted for when exploring how climate change has altered habitat use in insects of conservation concern. Climate driven changes in habitat use are possible: for instance where grazing has been less extensive, in particularly warm years, or with future warming. However, its relative importance as a driver of the recovery of *P. bellargus* is likely to be low. This also emphasises the importance of considering the impacts of a climate change in the context of other (often localised) elements of environmental change. As highlighted by Oliver and Morecroft (2014) and in recent reviews of the causes of insect pollinator declines (González-Varo et al. 2013; Vanbergen et al. 2013), the interaction of climate change (both synergistically and antagonistically) with other components of global change (e.g. land use, pollution, eutrophication) will be key to how it impacts species, their habitats and the services

they provide. This has been best explored in the context of habitat fragmentation disrupting climate driven range expansion (Travis 2003; Warren et al. 2001). In an example more specific to changes in microclimatic conditions, WallisDevries and Van Swaay (2006) have suggested that average temperature increases may be promoting cooler microclimatic conditions, rather than warming ground level microclimates. A combination of extended growing seasons (Sparks and Menzel 2002) with increased fertility from nitrogen loading will result in taller swards and so a cooling of ground level microclimates in semi natural habitats. Further, they suggest that in the Netherlands this is already resulting in the decline of species that overwinter as eggs and larvae (WallisDevries and Van Swaay 2006). If this holds it may be that grazing management to maintain early successional stages becomes all the more important for thermophilous species.

Climate change is associated with an increased frequency of extreme weather events, such as unusually heavy rain or summer drought. These events can have important impacts on vulnerable populations (Parmesan 2006; Parmesan et al. 2000). Extreme weather, such as particularly wet summers and winters or summer heatwaves, are known to cause declines in abundance of some butterfly species, though there is variation between species, with specialists being generally more susceptible (Dennis and Sparks 2007; Morecroft et al. 2002; Roy et al. 2001). A severe heat wave in Europe in the summer of 2003 brought drought that caused significant declines in the abundance of butterfly species found in drought-susceptible habitats (such as calcareous grassland) (Piessens et al. 2009; WallisDeVries et al. 2011). Evidence suggests habitat fragmentation serves to exacerbate these effects, with reduced size and connectivity between populations putting them at higher risk of extinction (Oliver

et al. 2013; Thomas et al. 1996). In the future, the impacts of increased frequency of extreme events on the already vulnerable and fragmented butterfly species of the UK could be just as important, if not more so, than changes due to average temperature increase (Bauerfeind and Fischer 2013).

Climate change presents challenges to insect conservation, potentially altering the ecology and habitats of species in multiple ways, interacting with other anthropogenic pressures. In the face of this uncertainty, promoting large and resilient local populations will remain critical to the persistence of vulnerable and fragmented species. The case study of the recovery of *P. bellargus* in England, including the results presented in chapter 2, demonstrates that effective management of habitat is fundamental for the conservation of vulnerable insect specialists. Basic ecological studies of the type presented in this thesis are essential for quantifying the habitat requirements of species of conservation concern. This, alongside improved monitoring of species' responses to interacting global change pressures, will inform effective actions for insect conservation.

6.2.2 Larval habitat requirements and conservation action

Differences in the larval niches of *P. bellargus* and *P. coridon* explain in part how these similar species coexist when utilising the same habitat and host plant. This is also of applied importance with regard to how calcareous grasslands can be managed to support both species. In the past three decades *P. bellargus* and *P. coridon* have served as flagship species for the conservation of calcareous grassland in the south of

England and their presence is seen as a key indicator of ‘good health’ for this habitat in Europe (van Swaay et al. 2013). In the original UK BAP, *P. bellargus* and *P. coridon* were classed as a priority species and a species of conservation concern respectively, but neither species featured on the most recent BAP, due to their recoveries (Anon 2014). However, managing grasslands based on the requirements of one species will not necessarily be optimal for both.

Polyommatus coridon is less restricted in its habitat preferences than *P. bellargus* (chapter 3). This explains why it occupies a larger range and number of sites than *P. bellargus* in England (see Figure 1.1); it is less constrained by microclimate and so a broader suite of *H. comosa* plants are available to it. Research to explore the use of adult resources, such as nectaring and roosting preferences, might also reveal differences in these two species, and would be a useful addition to the research presented here. What is not clear is whether there are consequences for *P. coridon* if grasslands are managed to optimise larval habitat availability for *P. bellargus*. For a given site, this will serve to decrease the number of *H. comosa* plants exclusively available to *P. coridon*. The recovery of *P. bellargus* in the UK is a success story for single species conservation (Merckx et al. 2013; Thomas et al. 2011), and better grazing has also been identified as a driver of the recovery of *P. coridon*, as well as their foodplant *H. comosa* (Brereton et al. 2008). However, *P. coridon*’s recovery has not been as marked as that of *P. bellargus* and from 1995 – 2009 it actually declined in overall abundance in the UK (Fox et al. 2011a). Further work to quantify how management regimes (especially grazing intensity) have influenced the populations of these species on sites where they occur together and alone could provide an indication of how *P. coridon* fares on sites managed to optimise the habitat for *P. bellargus*.

This is part of a broader question about the consequences of prescribing grazing regimes that favour thermophilous species of butterflies and other insects with contrasting habitat requirements. Even more broadly, it raises questions about the limitations of the species focussed approach to insect conservation (Stewart 2012). A key example of the potential for conflict comes from the butterfly *Thymelicus acteon* (the Lulworth Skipper), another occupant of calcareous grassland with an exceedingly restricted range in the south of England (Asher et al. 2001). As opposed to the short turf requirements of *P. bellargus*, *T. acteon* requires tall swards (>10cm) of its host grass *Brachypodium pinnatum*. Between 1995 – 2009 when the abundance of *P. bellargus* increased by 30 % the already low abundance of *T. acteon* in England decreased by 52 % (Bourn et al. 2000; Bourn and Thomas 2002; Fox et al. 2011a). This is particularly pertinent as *T. acteon* is now a UK BAP priority species and classified as Vulnerable and Near Threatened in Europe, based on IUCN Redlist criteria for abundance and distribution, respectively. In contrast *P. bellargus* is no longer a UK BAP species and was last assessed as Least Concern on the IUCN Redlist (Anon 2014; van Swaay et al. 2011).

Across several European studies exploring the impact of grazing intensity on butterflies and other insects of calcareous grasslands, levels of diversity are highest when low intensity grazing is implemented (Balmer and Erhardt 2000; Dolek and Geyer 1997; Kruess and Tscharntke 2002a; Kruess and Tscharntke 2002b; Pöyry et al. 2006). Whilst effective in conserving populations of *P. bellargus* and other thermophilous species at their northern most range edge, short-turf grazing regimes may be having detrimental impacts on other species. Recent examples of the

butterflies *Melitaea cinixia* (Noordwijk et al. 2012) and *Colias myrmidone* (Konvicka et al. 2007) in Europe, have shown how overgrazing can cause serious declines in their populations by increasing larval mortality. It is likely that rotational and compartmentalised grazing regimes will promote the heterogeneity of habitat favourable to a diverse range of species (Dolek and Geyer 1997; Kruess and Tschardt 2002b). Future research into how different management regimes support different species would be helpful in building an evidence base to inform conservation practice (Rosin et al. 2011).

Although the single species conservation approach has been incredibly successful for *P. bellargus* in the UK, the majority of butterfly species continue to experience declines (Fox et al. 2011a), a trend also seen in moths and other insect groups (Burns et al. 2013; Conrad et al. 2006). The European Grassland Butterfly Indicator, created using population trends in 17 European Grassland butterfly species (7 widespread, 10 specialists), shows that since 1990 grassland butterfly abundance in Europe has declined by 50% (van Swaay et al. 2013). This may in part be related to current conservation measures not adequately integrating the needs of multiple species. Davies et al. (2007) show that whilst the management of Sites of Special Scientific Interest in UK to promote “favourable” conditions benefits some species of conservation concern (including *P. bellargus*), other species actually require habitat conditions that would be classed as “unfavorable”. In Sweden the over application of intensive grazing regimes under the EU Common Agricultural Policy (CAP) has recently been identified as a driver of declines in the diversity of butterflies and burnet moths (Nilsson et al. 2013). The approach to the conservation of calcareous grassland and other semi-natural habitats in northern Europe may need to start integrating the

needs of different species. However, research into the requirements of individual species will still play a fundamental and invaluable role in informing insect conservation practices, particularly given that these requirements will be different in different regions of species' range. Even with the most effective management of protected areas, fragmentation and the landscape surrounding these sites is of major importance; there will continue to be declines as long as nature reserves are surrounded by, and encroached upon, by an inhospitable landscape (Rösch et al. 2013; Slancarova et al. 2013; Wenzel et al. 2006). Indeed, results in Chapter 5 show that an increase in the amount of grassland habitat between *P. bellargus* colonies increases gene flow between them, suggesting that this increases the permeability of the landscape for dispersal in this species.

Monitoring management?

The UK Butterfly Monitoring Scheme collects the majority of its data from nature reserves across the UK (UKBMS 2013), many of which will have specific management plans. One way of exploring the effects of different habitat management techniques on the presence and abundance of different species would be the collection of standardised habitat management data. As discussed in chapter 3, an analysis to examine the impacts of different management regimes on both *P. bellargus* and *P. coridon* was considered for this thesis, but a lack of standardised (or any) recording of management between reserves made this impossible. If some form of standardised records of management activity were kept and collated for different sites (such as species / type of animal used, the number and grazing periods of animals used, whether grazing is rotational and other management activities carried out) this would

allow for a large-scale analysis of the effects of different management practices on different species. Further, this could serve to create an evidence base that would allow the integration of habitat management data into statistical models, such as those examining range expansion or metapopulation dynamics.

6.2.3 Population fragmentation – from neutral to adaptive change

Improving the quality of habitats for species is only part of the solution for conserving insects in fragmented ecosystems; maintaining connectivity between patches of habitat is also essential (Lawton et al. 2010; Rösch et al. 2013; Wenzel et al. 2006) (though as discussed in chapter 1, increasing habitat quality within a system may facilitate connectivity by increasing the frequency of dispersal). Population genetics quantifies the connectivity between populations, based on the degree of gene flow between them. However, as discussed in chapter 5, estimates of gene flow must be interpreted not just with habitat fragmentation and land use in mind, but also with how a species' ecology determines the spatial structure of populations. Specialist species, such as *P. bellargus* and *P. coridon*, exhibit a more 'closed' population structure than generalist species, being organised into discrete colonies based on patches of suitable resources with little exchange of individuals between them (Wilson and Roy 2009). This will also be played out in the genetic structure of their populations (Nève 2009), and results in chapter 5 suggest that *P. bellargus* has a more fragmented population structure than *P. coridon*.

The partitioning of neutral genetic variation shown in this thesis could be supplemented by further work on the impacts of habitat fragmentation and a warming

climate on genetic variation of adaptive significance. As Hill et al. (2011) discuss, where species have an opportunity to expand their range due to warmer climates, the individuals that colonise new habitat will not be a random subset of the entire population, but those best adapted to dispersal into new environments. This in turn will create conditions for assortative mating and the potential for evolutionary change. For *Aricia agestis* (the Brown argus), evolutionary change at its high-latitude range margins has been documented in the UK, where northern populations have been shown to diverge from southern populations in their host plant choice, which appears to have a genetic basis (Thomas et al. 2001a). Recent work comparing populations at long established sites with recently colonised sites suggests that there has been evolutionary divergence in this species at its range edge (Buckley et al. 2013). Much like the process of post-glacial expansion discussed in chapter 5, such change can facilitate the utilisation of new habitats at a species range edge but also has the potential to reduce genetic diversity in populations (Hill et al. 2006).

The enzyme Phosphoglucose isomerase (Pgi) is an important component of the metabolic pathway for glycolysis (the conversion of sugars in pyruvate to form ATP). The impact of genetic variation in Pgi on flight ability, mating success and longevity in a butterfly were first characterised in the genus *Colias* (Watt 1992). Extensive studies of a Finnish metapopulation of *Melitea cinixia* have subsequently shown that genetic variation in Pgi influences several life history traits (e.g. dispersal rate, flight metabolic rate, clutch size and lifespan) and is under selection (Hanski 2011; Saastamoinen et al. 2009). Haag et al. (2005) found that individuals from newly colonised, isolated sites had a higher frequency of a particular allozyme allele for Pgi (*Pgi-f*), and that females with *Pgi-f* allele had higher flight metabolic rates and egg

clutch sizes. Higher population growth rates in small, isolated populations have also been found to be associated with an increased frequency of the *Pgi-f* allele (Hanski and Saccheri 2006). For the willow beetle (*Chrysomela aeneicollis*) at its southern range edge in the USA, the fitness advantage of Pgi genotypes has been found to vary with temperature, with different genotypes favoured in warmer locations, and so it may also be of adaptive significance under climate change (Dahlhoff et al. 2008). Pgi's strong influence on traits of adaptive significance led Wheat (2010) to suggest that it could be adopted as an adaptive marker for exploring questions of conservation relevance across arthropods, such as finding gene-environment interactions, locally adapted populations and identifying dispersive phenotypes. However, recent work by Smee (2011) on variation in Pgi in populations of the Marsh Fritillary (*Euphydryas aurinia*) across Spain and the UK found no evidence of heterozygote selective advantage in different areas of its range, nor any pattern in geographical distribution of alleles.

Exploring whether there is evidence for evolutionary change in *P. bellargus* may be a useful way to discern whether the population structuring observed in chapter 5 has been influenced by contemporary habitat fragmentation. The comparative framework in which the ecology of *P. bellargus* and *P. coridon* has been explored in this thesis could be extended further using Pgi (and other adaptive markers if identified) to study if there is selection on traits such as dispersal capacity and longevity in these two species. For example, it may be that habitat fragmentation results in different evolutionary responses in each species – *P. coridon*'s capacity for longer distance dispersal might exert selection for an even higher dispersal ability where habitat fragments are isolated from one another. Conversely the low dispersal ability and

higher fragmentation of *P. bellargus* may result in selection working in the opposite direction, against high dispersal ability, as long distance dispersal events will be associated with high mortality or a failure to lay eggs (Bonelli et al. 2013; Schtickzelle et al. 2006).

6.2.4 The role of myrmecophily – are pathogens the neglected natural enemies?

With regard to myrmecophily in *P. bellargus* and *P. coridon*, and more generally the evolution and function of myrmecophily in facultative Lycaenid species, an interesting area for further research will be to consider how this dynamic alters when larvae / pupae are exposed to natural enemies. Protection from predation and parasitism have been considered as a function of ant mutualists before (Agrawal and Fordyce 2000; Astatt 1981; Kaminski et al. 2010; Pierce and Mead 1981; Pierce and Eastal 1986; Weeks 2003), but no study to date has explored the potential of ants to provide protection from pathogens.

Only one paper, a general note, has addressed the role of pathogens in ant-butterfly mutualisms, and on the contrary suggests that ant mutualisms might be providing a route for fungal pathogens to infect larvae (Fiedler 1995b). Larvae of *P. bellargus* and *Polyommatus icarus* reared in the laboratory were found to have higher rates of fungal infection of the DNO when reared in the presence of ants, though this difference was not significant. Fiedler (1995b) suggests that the opening in the cuticle provided by the DNO represents a cost to Lycaenid larvae, as its secretions produced by the DNO (in response to ants) provided the substrate for the infection to

become established. On the other hand, it has long been known among entomologists who rear lycaenid larvae that those species that produce the most secretions (e.g. *Maculinea* spp., *Plebejus argus*) frequently die of mould unless they are regularly washed or kept with ants (J A Thomas pers. comm.).

The larvae of *P. bellargus* and *P. coridon* spend much of their time in contact with the soil surface beneath their host plant, and as later instars (3-4) are both invariably found buried in the soil at its roots (pers obs), behaviour which will result in exposure to soil-borne pathogens. Pathogens are known to be a large source of mortality in insect populations (Roy et al. 2009), and Lepidopteran caterpillars are vulnerable to pathogenic bacteria, fungi and viral infections. The tending behaviour exhibited by ants towards Lycaenid larvae might provide an opportunity for the removal or inhibition of microbes and pathogenic spores. Ants from many families are known to secrete antimicrobial compounds, from their metapleural gland in particular, that are spread onto the mouth and cuticle by grooming, and which inhibit and kill bacterial pathogens and inhibit fungal growth (Brough 1983; Fernandez-Marin et al. 2006; Schluns and Crozier 2009). For instance the Japanese ant *Lasius japonicus* increases self grooming and allogrooming (grooming of nestmates) when exposed to the entomopathogenic fungus *Metarhizium anisopliae*, and this increases survivorship in nests (Okuno et al. 2011).

As part of the work presented in chapter 4 it was initially planned to explore the potential of ants to provide protection from entomopathogens. An additional set of replicates was attempted for the *P. bellargus* experiment in which the soil was inoculated with a fungal isolate extracted from wild *P. bellargus* larvae. In a bioassay,

this has been shown to infect and kill laboratory-reared larvae (unpublished data). These additional replicates aimed to test the hypothesis that attendance of *P. bellargus* larvae by ants can provide protection from a fungal entomopathogen. However, the need for moisture in the soil inoculated with fungus caused complications. As such this part of the experiments was curtailed, but refining an effective technique for pathogen exposure would be a priority should future work be possible. Such research could provide important insights into the potential role of pathogens in the evolution of myrmecophily in the Lycaenidae.

Conclusions

The discussion above has emphasised that the themes explored in the separate chapters of this thesis (habitat requirements, climate change and population structure) are interlinked and that their impacts on different species will be contingent on specific aspects of their ecology. Detailed study of the ecology of specialist butterflies provides both species-specific insights that can be of great importance for their conservation, but also contributes to our general understanding of how other specialists might respond to the pressures of global change. An important challenge for insect conservation in temperate climates is to integrate the contrasting needs of different species in increasingly fragmented landscapes, in order to create resilient populations in the face of environmental change.

Appendix A. Microsatellite product sequencing

Below is a summary of the sequencing results from 53 different microsatellite-containing ss, where possible homozygous individuals were assessed.

Table A1 correlates amplicon size derived from sequence data compared with size given from the ABI trace files

Figures A1 – A13 show sequences alignments for each locus and in the case of *Polyommatus bellargus* loci their alignment with the reference sequences from GenBank.

Product sizes for Lb418 and Lb419, as well as Polcor05 and Polcor23, were used to calibrate the peak scoring as each pair utilised the same colour dye within their respective multiplex set.

Table A 1 Summary of microsatellite sequence information, showing the size expected from the peak profile obtained from multiplex PCRs run on the Applied Biosystems 3730 DNA Analyser and the size of products obtained from the cloning and sequencing of amplicons of individual loci. The match column indicates whether the size of the product from sequencing matched the size estimated from the chromatogram peak with an error of four base pairs allowed.

Sample	Barcode	Amplicon	Chromatogram	Correct	
		Size	Size	Repeat	Match
Lb141 01	8023	201	201.2	y	y
Lb141 02	8022	229	232	y	y
Lb157 01	8095	177	173	y	y
Lb157 02	8084	209	205	y	y
Lb418 01	8007	152	150	y	y
Lb418 02	8048	149	149.2	y	y
Lb418 03	8049	148	149.2	y	y
Lb418 04	8194	143	147.9	y	n
Lb418 05	8100	148	149.2	y	y

Lb419 01	8007	221	222.7	y	y
Lb419 02	8007	161	162.7	y	y
Lb419 03	8048	191	193.2	y	y
Lb419 04	8100	221	222.6	y	y
Lb419 05	8100	155	162.6	y	n
Lb419 06	8049	191	193.2	y	y
Lb419 07	8194	209	210.4	y	y
LbG2 01	8041	178	170	y	n
LbG2 02	8180	169	162.7	y	n
Polcor02 01	8417	144	149	y	n
Polcor02 02	11016	156	159	y	y
Polcor03 01	8792	165	163	y	y
Polcor03 02	8816	177	179.2	y	y
Polcor03 03	11001	177	174.8	y	y
Polcor05 01	8838	129	127.6	y	y
Polcor05 02	8299	146	146.1	y	y
Polcor05 03	8939	159	156.1	y	y
Polcor05 04	8915	137	135	y	y
Polcor05 05	8747	128	128	y	y
Polcor05 06	8971	161	156	y	y
Polcor05 07	11014	136	136.6	y	y
Polcor05 08	8754	139	136.3	y	y
Polcor11 01	8783	113	122	y	n
Polcor11 02	8966	137	140	y	y
Polcor12 01	8966	192	189.1	y	y
Polcor12 02	8955	231	227.6	y	y
Polcor12 03	8965	198	197	y	y
Polcor13 01	8981	166	146.8	y	n
Polcor13 02	8391	154	142.2	y	n
Polcor13 03	8922	154	155	y	y
Polcor21 01	11119	143	142	y	y
Polcor21 02	8996	200	112	y	n

Polcor23 01	8838	185	173.2	y	n
Polcor23 02	8939	187	178 or 197	y	n
Polcor23 03	8915	185	184.7	y	y
Polcor23 04	8740	250	247.4	y	y
Polcor23 05	8740	175	173.7	y	y
Polcor23 06	8747	262	245	y	n
Polcor23 07	8971	181	179	y	y
Polcor23 08	8392	181	184.7	y	y
Polcor23 09	11014	183	184.7	y	y
Polcor23 10	8754	173	172	y	y
Polcor23 11	11037	262	259.5	y	y
Polcor23 12	11037	173	172.6	y	y

		10	20	30	40	*	50
Lb141ref	230	<u>CGTGTCTGTC</u>	<u>ACCCCCCTTTA</u>	<u>GTGTGTGAGT</u>	<u>CTGTCTGTGG</u>	TTT	GTGTGTG
Lb141 01	201	<u>CGTGTCTGTC</u>	<u>ACCCCCCTTTA</u>	<u>GTGTGTGAGT</u>	<u>CTGTCTGTGG</u>	TTT	GT -----
Lb141 02	229	<u>CGTGTCTGTC</u>	<u>ACCCCCCTTTA</u>	<u>GTGTGTGAGT</u>	<u>CTGTCTGTGG</u>	TTT	GCGTGTG
		60	70	80*	90		100
Lb141ref	230	TGTGTGT AGG	TGAG TGTGTG TGT	ATGAGTG	GTGTGTGT T	TGGTATACTA	
Lb141 01	201	-----	-----	---ATGAGTG	TGTGTGTGT T	TGGTATACTA	
Lb141 02	229	TGTGTGT AGG	TGAG TGTGTG TGT	ATGAGTG	TGTGTGTGT T	TGGTATACTA	
		110	120	130	140		150
Lb141ref	230	TTCTGGTATC	GCAATTAATT	TTTCAGTGGT	CTGAGGCCAC	GATGAATGAG	
Lb141 01	201	TTCTGGTATC	GCAATTAATT	TTTCAGTGGT	CTGAGGCCAC	GATGAATGAG	
Lb141 02	229	TTCTGGTATC	GCAATTAATT	TTTCAGTGGT	CTGAGGCCAC	GATGAATGAG	
		160	170	180	190		200
Lb141ref	230	CTGTAGCCTA	TAGTGCACAA	CCATGGACAA	TAGACAAC T A	GATAC CG TAC	
Lb141 01	201	CTGTAGCCTA	TAGTGCACAA	CCATGGACAA	TAGACAAC T A	GATAC CG TAC	
Lb141 02	229	CTGTAGCCTA	TAGTGCACAA	CCATGGACAA	TAGACAAC T A	GATAC CG TAC	
		210	220	230			
Lb141ref	230	TTATTCCATT	<u>CCCTAATCCC</u>	<u>TACCCGTCAT</u>			
Lb141 01	201	TTATTCCATT	<u>CCCTAATCCC</u>	<u>TACCCGTCAT</u>			
Lb141 02	229	TTATTCCATT	<u>CCCTAATCCC</u>	<u>TACCCGTCAT</u>			

Repeat motif - (GT)₇AGGTGA(GT)₅ATGAGTGC(GT)₄

Figure A 1 Aligned sequences of *P. bellargus* Lb141 amplified product. The first sample shows the reference sequence taken from GenBank. The microsatellite repeat motif is highlighted in yellow (reference) and in blue (this study). An asterisk denotes the occurrence of a substitution polymorphism and an insertion is shown in bold font. Primer sequences are underlined.

		10	20	30	40	50
Lb157ref	177	<u>TGTATCAGCA</u>	<u>ACAGCTCGGT</u>	GATATTTTGA	AGCAAAGAGA	GCATTTTGCG
Lb157 01	177	<u>TGTATCAGCA</u>	<u>ACAGCTCGGT</u>	GATATTTTGA	AGCAAAGAGA	GCATTTTGCG
Lb157 02	209	<u>TGTATCAGCA</u>	<u>ACAGCTCGGT</u>	GATATTTTGA	AGCAAAGAGA	GCATTTTGCG
		60	70	80	90	100
Lb157ref	177	ATAACTCGTG	ATGCTATGCT	TGTCGCTATA	CTGGAGCTCG	TACT CACACA
Lb157 01	177	ATAACTCGTG	ATGCTATGCT	TGTCGCTATA	CTGGAGCTCG	TACT CACACA
Lb157 02	209	ATATCTCGTG	ATGTTATGCT	TGTCGCTATA	CTGGAGCTCG	TACTCACACA
		110	120	130	140	150
Lb157ref	177	CACACACACA	CACACACACA	-----	-----	-----
Lb157 01	177	CACACACACA	CACACACACA	-----	-----	-----
Lb157 02	209	CACACACACA	CACACACACA	CACACACACA	CACACACACA	CACACACACA
		160	170	180	190	200
Lb157ref	177	--AGTGTCG	CTAGGCGTCG	CTAGTGTCAA	TCCCACTATA	<u>TTTTACCGATG</u>
Lb157 01	177	--AGTGTCG	CTAGGCGTCG	CTAGTGTCAA	TCCCACTATA	<u>TTTTACCGATG</u>
Lb157 02	209	CA AGTGTCG	CTAGGCGTCG	CTAGTGTCAA	TCCCACTATA	<u>TTTTACCGATG</u>
		210				
Lb157ref	177	<u>AACGCTTCC</u>				
Lb157 01	177	<u>AACGCTTCC</u>				
Lb157 02	209	<u>AACGCTTCC</u>				

Repeat motif - (CA)₁₃

Figure A 2 Aligned sequences for products of PCR reactions using the *P. bellargus* Lb157 primer set. The first sample shows the reference sequence taken from GenBank. The microsatellite repeat motif is highlighted in yellow (reference) and in blue (this study). An insertion is shown in bold font. Primer sequences are underlined.

		10	20	30	40	50
Lb418ref	150	<u>GATACCTATG</u>	<u>CCAGGCTCCA</u>	<u>TACAAAAAGG</u>	<u>TTCGAACCCC</u>	<u>TTTATATATT</u>
Lb418 01	152	<u>GATACCTATG</u>	<u>CCAGGCTCCA</u>	<u>TACAAAAATG</u>	<u>TTCGAACCCC</u>	<u>TTTATATATT</u>
Lb418 02	149	<u>GATACCTATG</u>	<u>CCAGGCTCCA</u>	<u>TACAAAAATG</u>	<u>TTCGAACCCC</u>	<u>TTTATATATT</u>
Lb418 03	148	<u>GATACCTATG</u>	<u>CCAGGCTCCA</u>	<u>TACAAAAATG</u>	<u>TTCGAACCCC</u>	<u>TTTATATATT</u>
Lb418 04	143	<u>GATACCTATG</u>	<u>CCAGGCTCCA</u>	<u>TACAAAAATG</u>	<u>TTCGAACCCC</u>	<u>TTTATATATT</u>
Lb418 05	148	<u>GATACCTATG</u>	<u>CCAGGCTCCA</u>	<u>TACAAAAATG</u>	<u>TTCGAACCCC</u>	<u>TTTATATATT</u>
		60	70	80	90	100
Lb418ref	150	TGCATTTTAC	ACACAGAATC	AGCCGGCGGC	TTCCTTATAC	ACACACACAC
Lb418 01	152	TGCATTTTAC	ACACAGAATC	AGCCGGCGGC	TTCCTTATAC	ACACACACAC
Lb418 02	149	TGCATTTTAC	ACACAGAATC	AGCCGGCGGC	TTCCTTATAC	ACACACACAC
Lb418 03	148	TGCATTTTAC	ACACAGAATC	AGCCGGCGGC	TTCCTTATAC	ACACACACAC
Lb418 04	143	TGCATTTTAC	ACACAGAATC	AGCCGGCGGC	TTCCTTATAC	ACACACACAC
Lb418 05	148	TGCATTTTAC	ACACAGAATC	AGCCGGCGGC	TTCCTTATAC	ACACACACAC
		110	120	130	140	150
Lb418ref	150	ACACA--GGC	CCCGCCGCTT	TTTTTTTCTT	GTCGCAACCT	GTATGACATT
Lb418 01	152	ACACACAGGC	CCCGCCGCTT	TTTTTTTCTT	GTCGCAACCT	GTATGACATT
Lb418 02	149	ACACA--GGC	CCCGCTGCTT	TTTTTTT-CTT	GTCGCAACCT	GTATGACATT
Lb418 03	148	ACA----GGC	CCCGCCGCTT	TTTTTTTCTT	GTCGCAACCT	GTATGACATT
Lb418 04	143	-----GGC	CTCGCTGCTT	TTTTTTT-CTT	GTCGCAACCT	GTATGACATT
Lb418 05	148	ACA----GGC	CCCGCCGCTT	TTTTTTTCTT	GTCGCAACCT	GTATGACATT
Lb418ref	150	<u>CG</u>				
Lb418 01	152	<u>CG</u>				
Lb418 02	149	<u>CG</u>				
Lb418 03	148	<u>CG</u>				
Lb418 04	143	<u>CG</u>				
Lb418 05	148	<u>CG</u>				

Repeat motif - (CA)₈GGCCCCGCCGC(T)₉

Figure A 3 Aligned sequences for products of PCR reactions using the *P. bellargus* Lb418 primer set. The first sample shows the reference sequence taken from GenBank. Microsatellite repeat motifs for the reference sequence are highlighted with different colours for different repeat components and repeat motifs in sequences from this study are highlighted in blue. Primer sequences are underlined

		10	20	30	40	50
Lb419ref	161	<u>AGATTAGGCA</u>	<u>TTGGCGTGTC</u>	<u>CTTCCCGATG</u>	<u>CGTCATGTAG</u>	<u>TGATAGT</u> GAC
Lb419	01 221	<u>AGATTAGGCA</u>	<u>TTGGCGTGTC</u>	<u>CTTCCCGATG</u>	<u>CGTCATGTAG</u>	<u>TGATAGT</u> GAC
Lb419	02 161	<u>AGATTAGGCA</u>	<u>TTGGCGTGTC</u>	<u>CTTCCCGATG</u>	<u>CGTCATGTAG</u>	<u>TGATAGT</u> GAC
Lb419	03 191	<u>AGATTAGGCA</u>	<u>TTGGCGTGTC</u>	<u>CTTCCCGATG</u>	<u>CGTCATGTAG</u>	<u>TGATAGT</u> GAC
Lb419	04 221	<u>AGATTAGGCA</u>	<u>TTGGCGTGTC</u>	<u>CTTCCCGATG</u>	<u>CGTCATGTAG</u>	<u>TGATAGT</u> GAC
Lb419	05 155	<u>AGATTAGGCA</u>	<u>TTGGCGTGTC</u>	<u>CTTCCCGATG</u>	<u>CGTCATGTAG</u>	<u>TGATAGT</u> GGC
Lb419	06 191	<u>AGATTAGGCA</u>	<u>TTGGCGTGTC</u>	<u>CTTCCCGATG</u>	<u>CGTCATGTAG</u>	<u>TGATAGT</u> GAC
Lb419	07 209	<u>AGATTAGGCA</u>	<u>TTGGCGTGTC</u>	<u>CTTCCCGATG</u>	<u>CGTCATGTAG</u>	<u>TGATAGT</u> GAC
		60	70	80	90	100
Lb419ref	161	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GGTGACGGTG	ATGTTGACGG
Lb419	01 221	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GTTGACGGTG	ACGGTGACGG
Lb419	02 161	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG
Lb419	03 191	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG
Lb419	04 221	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GTTGACGGTG	ACGGTGACGG
Lb419	05 155	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG
Lb419	06 191	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG
Lb419	07 209	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GTTGACGGTG	ACGGTGACGG
		110	120	*	130	* 140 150
Lb419ref	161	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG	TAACGGTAAC	GG -----
Lb419	01 221	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GGTGACGGTG
Lb419	02 161	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG	TAACGGTAAC	GG -----
Lb419	03 191	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GGTGACGGTG
Lb419	04 221	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GGTGACGGTG
Lb419	05 155	TGACGGTGAC	GGTGACGGTG	ACGGTAACGG	TAACGG ----	-----
Lb419	06 191	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GGTGACGGTG
Lb419	07 209	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG	TGACGGTGAC	GGTGACGGTG
		*	160	*	170	180 190 200
Lb419ref	161	-----	-----	-----	-----	-----
Lb419	01 221	ACGGTAACGG	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG	TAACGGTAAC
Lb419	02 161	-----	-----	-----	-----	-----
Lb419	03 191	ACGGTGACGG	TAACGGTAAC	GG -----	-----	-----
Lb419	04 221	ACGGTAACGG	TGACGGTGAC	GGTGACGGTG	ACGGTGACGG	TAACGGTAAC
Lb419	05 155	-----	-----	-----	-----	-----
Lb419	06 191	ACGGTGACGG	TAACGGTAAC	GG -----	-----	-----
Lb419	07 209	ACGGTGACGG	TGACGGTGAC	GGTGACGGTA	ACGGTAACGG	-----
		210	220			
Lb419ref	161	--TAACGGAA	ATGGATCGCA	G		
Lb419	01 221	GG TAACGGAA	ATGGATCGCA	G		
Lb419	02 161	--TAACGGAA	ATGGATCGCA	G		
Lb419	03 191	--TAACGGAA	ATGGATCGCA	G		
Lb419	04 221	GG TAACGGAA	ATGGATCGCA	G		
Lb419	05 155	--TAACGGAA	ATGGATCGCA	G		
Lb419	06 191	--TAACGGAA	ATGGATCGCA	G		
Lb419	07 209	--TAACGGAA	ATGGATCGCA	G		

Repeat motif - (GACGGT)₁₅

Figure A 4 Aligned sequences for products of PCR reactions using the *P. bellargus* Lb419 primer set. The first sample shows the reference sequence taken from GenBank. Microsatellite repeat motifs are shown highlighted in yellow (reference) and blue (this study). An asterisk denotes the occurrence of a substitution polymorphism. Primer sequences are underlined.

		10	20	30	40*	50
LbG2ref	180	ATCAAGGTCC	GCACAGCACA	CACACACACA	CACA	AACACG TGCGCACGCA
LbG2 01	178	ATCAAGGTCC	GCACAGCACA	CACACACACA	CACA	AACACG TGCGCACGCA
LbG2 02	170	ATCAAGGTCC	GCACAGCACA	CACACACACA	CA	--AACACG CGCGCACGCA

		60	* 70	80	90	100
LbG2ref	180	CGCAACGCAT	GCACGCACGC	ACGCACGCAC	GGCGCG	ATTC TTA
LbG2 01	178	CGCAACGCAT	GCACGCACGC	ACGCACGCAT	GGCGCG	ATCC T
LbG2 02	169	CGCA-----	-CACGCATGC	ACGCACGCAC	GGCGCG	ATCC T

		*				
		110	120	130	140	150
LbG2ref	180	ACACACACAC	ACACAGAGAG	AGAAAACGAG	GGGGATAGTC	AGAATGACGA
LbG2 01	178	ACACACACAC	ACACAGAGAG	AGAA--CGAG	GGGGATAGTC	AGAATGACGA
LbG2 02	169	ACACACACAT	ACAGAGAGAG	AGAA--CGAG	GGGGATAGTC	AGAATGACGA

		160	170	180
LbG2ref	180	TAGACCAAGG	TGTTGTCTGA	TAAGCGAGCG
LbG2 01	178	TAGACCAAGG	TGTTGTCTGA	TAAGCGAGCG
LbG2 02	169	TAGACCAAGG	TGTTGTCTGA	TAAGCGAGCG

Repeat motif - (CA)₉ AACACGTG (CGCA)₃A(CGCA)T(GCAC)₅ (GC)₃ATTCTTA(CA)₁₁(GA)₄

Figure A 5 Aligned sequences for products of PCR reactions using the *P. bellargus* LbG2 primer set. The first sample shows the reference sequence taken from GenBank. Microsatellite repeat motifs for the reference sequence are highlighted with different colours for different repeat components and repeat motifs in sequences from this study are highlighted in blue. An asterisk denotes the occurrence of a substitution polymorphism. Primer sequences are underlined.

			10	20	30	40	50
Polcor02 01 144			<u>ATGCAGATGC</u>	<u>AGATGTGGAG</u>	CAGACAACAA	AGACGGCTAA	ATCACCCGGC
Polcor02 02 156			<u>ATGCAGATGC</u>	<u>AGATGTGGAG</u>	CAGACAACAA	AGACGGCTAA	ATCACCCGGC
			60	70	80	90	100
Polcor02 01 144			GAGACGGTTA	CCGAATGTAA	CACACACACA	CACACACACA	-----
Polcor02 02 156			GAGACGGTTA	CCGAATGTAA	CACACACACA	CACACACACA	CACACACACA
			110	120	130	140	150
Polcor02 01 144			--GACAGACA	GACACCTCAG	TTTTGTAAAT	AATTTCCATT	GAGGTTTTTCG
Polcor02 02 156			CAGACAGACA	GACACCTCAG	TTTTGTAAAT	AATTTCCATT	GAGGTTTTTCG
Polcor02 01 144			<u>TTCATC</u>				
Polcor02 02 156			<u>TTCATC</u>				

Repeat motif - (AC)₁₆(AGAC)₃

Figure A 6 Aligned sequences for products of PCR reactions using the *P. coridon* Polcor 02 primer set. Microsatellite repeat motifs are shown highlighted in colour, with a different colour for each repeat component. Primer sequences are underlined.

			10	20	30	40	50
Polcor03	01	165	<u>GTCGCCAGGA</u>	<u>TCAACAGTGG</u>	TAACCAACCA	CGAAAACCCT	TTGATATGAT
Polcor03	02	177	<u>GTCGCCAGGA</u>	<u>TCAACAGTGG</u>	TAACCAACCA	CGAAAACCCT	TTGATATGAT
Polcor03	03	177	<u>GTCGCCAGGA</u>	<u>TCAACAGTGG</u>	TAACCAACCA	CGAAAACCCT	TTGATATGAT
			60	70	80	90	100
Polcor03	01	165	CTCAGGGATC	AGGGATATAA	TATGTATGTA	TGTATGTATG	TATGTATGTA
Polcor03	02	177	CTCAGGGATC	AGGGATATAA	TATGTATGTA	TGTATGTATG	TATGTATGTA
Polcor03	03	177	CTCAGGGATC	AGGGATATAA	TATGTATGTA	TGTATGTATG	TATGTATGTA
					**		
			110	120	**130	140	* 150
Polcor03	01	165	TGTATGTATG	TATGTATGTA	TG-----	----TTTTTA	TGTTTGTGCA
Polcor03	02	177	TGTATGTATG	TATGTATGTA	TGTATGTTTT	TATGTTTTTA	TGTTTGTGCA
Polcor03	03	177	TGTATGTATG	TATGTATGTA	TGTATGTATG	TATGTTTTTA	TGTTTTTGCA
			160	170			
Polcor03	01	165	CGCATATCTC	TGGAACCTACA	AGCCCGA		
Polcor03	02	177	CGCATATCTC	TGGAACCTACA	AGCCCGA		
Polcor03	03	177	CGCATATCTC	TGGAACCTACA	AGCCCGA		

Repeat motif - (TATG)₁₅

Figure A 7 Aligned sequences for products of PCR reactions using the *P. coridon* Polcor 03 primer set. The microsatellite repeat motif is highlighted in blue. An asterisk denotes the occurrence of a substitution polymorphism. Primer sequences are underlined.

			10	20	30	40	*	50
Polcor05	01	129	GACACGCACA	CATGCAAATA	GATATACTGA	CACACACACA	CACACA	----
Polcor05	03	159	GACACGCACA	CATGCAAATA	GATATACTGA	CACACACACA	CACACACACA	
Polcor05	04	137	GACACGCACA	CATGCAAATA	GATATACTGA	CACACACACA	CACACACACA	
Polcor05	05	128	GACACGCACA	CATGCAAATA	G-----	CACACACACA	CACACACACA	
Polcor05	06	161	GACACGCACA	CATGCAAATA	GATATACTGA	CACACACACA	CAAACACACA	
Polcor05	07	136	GACACGCACA	CATGCAAATA	GATATACTGA	CACACACACA	ACAAACACAC	
Polcor05	08	139	GACACGCACA	CATGCAAATA	GATATACTGA	CACACACACA	CACACACACA	
Polcor05	02	146	GACACGCACA	CATGCAAATA	G T ACTACAA	CCCGCTCTCA	TTGTGCATAG	
			60	70	80	90		100
Polcor05	01	129	-----	-----	-----CC	GATTGCGGAT	TGACACATCA	
Polcor05	03	159	CACACACACA	CACACACACA	CACACA--	CC GATTGCGGAT	TGACACATCA	
Polcor05	04	137	CACA-----	-----	-----CC	GATTGCGGAT	TGACACATCA	
Polcor05	05	128	CACA-----	-----	-----CC	GATTGCGGAT	TGACACATCA	
Polcor05	06	161	CACACGCACA	CACACACACA	CACACACA	CC GATTGCGGAT	TGACACATCA	
Polcor05	07	136	ACA-----	-----	-----CC	GATTGCGGAT	TGACACATCA	
Polcor05	08	139	CACA-----	-----	-----CC	GATTGCGGAT	TGACACATCA	
Polcor05	02	146	AGTGACGGCG	CCAATGACAC	GCACACATCC	TACACGGGCG	GCCATTACTG	
			110	120	130	140		150
Polcor05	01	129	TTAAATGTAA	ACATTCTCGT	TTCCACGACA	AAGGAACATT	CCATCCGAAC	
Polcor05	03	159	TTAAATGTAA	ACATTCTCGT	TTCCACGACA	AAGGAACATT	CCATCCGAAC	
Polcor05	04	137	TTAAATGTAA	ACATTCTCGT	TTCCACGACA	AAGGAACATT	CCATCCGAAC	
Polcor05	05	128	TTAAATGTAA	ACATTCTCGT	TTCCACGACA	AAGGAACATT	CCATCCGAAC	
Polcor05	06	161	TTAAATGTAA	ACATTCTCGT	TTCCACGACA	AAGGAACATT	CCATCCGAAC	
Polcor05	07	136	TTAAATGTAA	ACATTCTCGT	TTCCACGACA	AAGGAACATT	CCATCCGAAC	
Polcor05	08	139	TTAAATGTAA	ACATTCTCGT	TTCCACGACA	AAGGAACATT	CCATCTTTAC	
Polcor05	02	146	TGCTTCACTC	GGGATTAGAC	AA-----	-----GTTT	ACATCCGAAC	
			160					
Polcor05	01	129	-TTTTCCAAA	GC				
Polcor05	03	159	-TTTTCCAAA	GC				
Polcor05	04	137	-TTTTCCAAA	GC				
Polcor05	05	128	-TTTTCCAAA	GC				
Polcor05	06	161	-TTTTCCAAA	GC				
Polcor05	07	136	-TTTTCCAAA	GC				
Polcor05	08	139	CTTTTCCAAA	GC				
Polcor05	02	146	--TTTCCAAA	GC				

Repeat motif - (AC)₂₂

Figure A 8 Aligned sequences for products of PCR reactions using the *P. coridon* Polcor 05 primer set. The microsatellite repeat motif is highlighted in blue. An asterisk denotes the occurrence of a substitution polymorphism. An insertion is shown in bold font. Primer sequences are underlined. The final sequence, highlighted in purple, shows a non specific product which does not contain the microsatellite repeat motif.

			10	20	30	40	50
Polcor11	01	113	<u>CGTAGGTAGA</u>	<u>CACGTCTGTG</u>	<u>TATATGTG</u>	TG TGTGCGTGTG	CGTGT-----
Polcor11	02	137	<u>CGTAGGTAGA</u>	<u>CACGTCTGTG</u>	<u>TATATGTG</u>	TG TATGTGTGTG	TGTGTCTCTC
			60	70	80	90	100
Polcor11	01	113	-----	-----GTGTG	TGTGTGT---	-ATACGTATA	CGTATGTCGT
Polcor11	02	137	TCTCTCTCTC	TCTCTGTGTG	TGTGTGTGTG	TATGCGTATA	CGTATGTCGT
			110	120	130		
Polcor11	01	113	<u>GACTCGTGCA</u>	<u>GTGCGTTACT</u>	<u>CACGTTGTAT</u>	<u>AGCGGCG</u>	
Polcor11	02	137	<u>GACTCGTGCA</u>	<u>GTGCGTTACT</u>	<u>CACGTTGTAT</u>	<u>AGCGGCG</u>	

Repeat motif - (GT)₅(CT)₁₅(GT)₁₄

Figure A 9 Aligned sequences for products of PCR reactions using the *P. coridon* Polcor 11 primer set. Microsatellite repeat motifs are shown highlighted in colour, with a different colour for each motif component. Primer sequences are underlined

			10	20	30	40	50
Pocl _{or} 12	01	192	<u>CTATCTGCTC</u>	<u>GCGGATGAAG</u>	GGTTGAGTCG	CGGAACAGGT	CACCCGTCTA
Pocl _{or} 12	02	231	<u>CTATCTGCTC</u>	<u>GCGGATGAAG</u>	GGTTGAGTCG	CGGAACAGGT	CACCCGTCTA
Pocl _{or} 12	03	198	<u>CTATCTGCTC</u>	<u>GCGGATGAAG</u>	GGTTGAGTCG	CGGAACAGGT	CACCCGTCTA
			60	70	80	90	100
Pocl _{or} 12	01	192	CACACACACA	CGCACACACA	CGACACAC-A	CACAC-ACAC	AC-ACACACA
Pocl _{or} 12	02	231	CACACACACA	CGCACACACA	CAACACACGA	CACACGACAC	ACGACACACA
Pocl _{or} 12	03	198	CACACACACA	CGCACACACA	CGACACAC-A	CACAC-ACAC	AC-ACACACA
			110	120	130	140	150
Pocl _{or} 12	01	192	CACACA	----	-----	-----	--GGCGCCAG
Pocl _{or} 12	02	231	CACACACACA	CACACTCACA	CACACACACA	CACACACACA	CA
Pocl _{or} 12	03	198	CACACACACA	CA	-----	-----	--GGCGCCAG
			160	170	180	190	200
Pocl _{or} 12	01	192	TTAACGCGAC	TCATATTTGT	ATTTGTGATT	GCCTTATCGA	AATATAACTT
Pocl _{or} 12	02	231	TTAACGCGAC	TCATATTTGT	ATTTGTGATT	GCCTTATCGA	AATATAACTT
Pocl _{or} 12	03	198	TTAACGCGAC	TCATATTTGT	ATTTGTGATT	GCCTTATCGA	AATATAACTT
			210	220	230		
Pocl _{or} 12	01	192	TATGTGTTGT	<u>CGACGACACT</u>	<u>TCCACGGATT</u>	C	
Pocl _{or} 12	02	231	TATGTGTTGT	<u>CGACGACACT</u>	<u>TCCACGGATT</u>	C	
Pocl _{or} 12	03	198	TATGTGTTGT	<u>CGACGACACT</u>	<u>TCCACGGATT</u>	C	

Repeat motif - (AC)₆GC(AC)₄GA(CA)₁₅

Figure A 10 Aligned sequences for products of PCR reactions using the *P. coridon* Polcor 12 primer set. Microsatellite repeat motifs are shown highlighted in colour, with a different colour for each motif component. Insertions are shown in bold font. Primer sequences are underlined.

			10	20	30	40	50
Polcor13 01 166			TGAAAGATCG	CGACTAAATT	GATATAAGAC	ACACATACAC	ACACACACAC
Polcor13 02 154			TGAAAGATCG	CGACTAAATT	GATATAAGAC	ACACATACAC	ACACACACAC
Polcor13 03 154			TGAAAGATCG	CGACTAAATT	GATATAAGAC	ACACATACAC	ACACACACAC
			*				
			60	70	80	90	100
Polcor13 01 166			AAACACACAC	ACACACACAC	ACCCACAGGA	AACCATTATT	ATGTAGTTAA
Polcor13 02 154			ACACACACAC	ACACA-----	-----GGA	AACCATTATT	ATGTAGTTAA
Polcor13 03 154			ACACACACAC	ACACA-----	-----GGA	AACCATTATT	ATGTAGTTAA
			110	120	130	140	150
Polcor13 01 166			GTTAAATGTA	AGAATAAATA	GTTCTAGATA	TTAGTCATTA	CTTTGTTAGT
Polcor13 02 154			GTTAAATGTA	AGAATAAATA	GTTCTAGATA	TTAGTCATTA	CTTTGTTAGT
Polcor13 03 154			GTTAAATGTA	AGAATAAATA	GTTCTAGATA	TTAGTCATTA	CTTTGTTAGT
			160				
Polcor13 01 166			<u>CCATTGTTTG</u>	<u>TGAGGG</u>			
Polcor13 02 154			<u>CCATTGTTTG</u>	<u>TGAGGG</u>			
Polcor13 03 154			<u>CCATTGTTTG</u>	<u>TGAGGG</u>			

Repeat motif - (CA)₄TA(CA)₁₄

Figure A 11 Aligned sequences for products of PCR reactions using the *P. coridon* Polcor 13 primer set. Microsatellite repeat motifs are shown highlighted in colour, with a different colour for each motif component. An asterisk denotes the occurrence of a substitution polymorphism. Primer sequences are underlined

			10	20	30	40
50						
Polcor21 01 143	<u>TTCAGGACTC</u>	<u>CAGGTTCCAC</u>	CCGCAGATTA	GAGAAGAACT	TAGGTAAATA	
Polcor21 02 200	<u>TTCAGGACTC</u>	<u>CAGGTTCCAC</u>	CCGCAGATTA	GAGAAGAACT	TAGGTAAATA	
	60	70	80	90		
100						
Polcor21 01 143	TGAGATTTTA	TTAAAAATAT	AAGAATTGTG	T--ATATATT	ATGTGTGTGT	
Polcor21 02 200	TGAGATTTTA	TTAAAAATAT	AAGAATTGTG	TGT ATATATT	ATGTGTGTGT	
	110	120	130	140		
150						
Polcor21 01 143	GT-----	-----	-----	-----	-----	
Polcor21 02 200	GTGTGTGTGT	GTGTGTGTGT	GTGTGTGTGT	GTGTGTGTGT	GTGTGTGTGT	
	*160	170	180	190		
200						
Polcor21 01 143	-----AAA	AGTATAAAGC	GTATACTTTG	CTTGCAGCCA	ACGTTTTGAG	
Polcor21 02 200	GTGT ATA TAA	AGTATAAAGC	GTATACTTTG	CTTGCAGCCA	ACGTTTTGAG	

Repeat motif - (TG)₁₄

Figure A 12 Aligned sequences for products of PCR reactions using the *P. coridon* Polcor 21 primer set. The microsatellite repeat motif is highlighted in blue. An insertion is shown in bold font. Primer sequences are underlined.

		10	20	30	40	50
Polcor23	01	173	GGTGATCGCG ACAACAATAG	AATACATTTC	CGAGAATCTC	CGATCGATGG
Polcor23	02	187	GGTGATCGCG ACAACAATAG	AATACATTTC	CGAGAATCTC	CGATCGATGG
Polcor23	03	185	GGTGATCGCG ACAACAATAG	AATACATTTC	CGAGAATCTC	CGATCGATGG
Polcor23	04	250	GGTGATCGCG ACAACAATAG	AATACATTTC	CGAGAATCTC	CGATCGAAGG
Polcor23	05	175	GGTGATCGCG ACAACAATAG	AATACATTTC	CGAGAATCTC	CGATCGATGG
Polcor23	06	262	GGTGATCGCG ACAACAATAG	AATACATTTC	CGAGAATCTC	CGATCGAAGG
Polcor23	07	181	GGTGATCGCG ACAACAATAG	AATACATTTC	CGAGAATCTC	CGATCGATGG
Polcor23	08	181	GGTGATCGCG ACAACAATAG	AATACATTTC	CGAGAATCTC	CGATCGATGG
Polcor23	09	183	GGTGATCGCG ACAACAATAG	AATACATTTC	CGAGAATCTC	CGATCGATGG
Polcor23	10	173	GGTGATCGCG ACAACAATAG	AATACATTTC	CGAGAATCTC	CGATCGATGG
Polcor23	11	262	GGTGATCGCG ACAACAATAG	AATACATTTC	CGAGAATCTC	CGATCGAAGG
Polcor23	12	173	GGTGATCGCG ACAACAATAG	AATACATTTC	CGAGAATCTC	CGATCGATGG
		60	70	80	90	100
Polcor23	01	173	ATTAAATTAA CGTTCCTTTA	TCAAAACTAT	TAAACGTGCC	GACACACATA
Polcor23	02	187	ATTAAATTAA CGTTCCTTTA	TCAAAACTAT	TAAACGTGCC	GACACACATA
Polcor23	03	185	ATTAAATTAA CGTTCCTTTA	TCAAAACTAT	TAAACGTGCC	GACACACATA
Polcor23	04	250	ATTAAATTAA CGTTCCTTTA	TCAAAACTAT	TAAACGTGCC	GACACACATA
Polcor23	05	175	ATTAAATTAA CGTTCCTTTA	TCAAAACTAT	TAAACGTGCC	GACACACATA
Polcor23	06	262	ATTAAATTAA CGTTCCTTTA	TCAAAACTAT	TAAACGTGCC	GACACACATA
Polcor23	07	181	ATTAAATTAA CGTTCCTTTA	TCAAAACTAT	TAAACGTGCC	GACACACATA
Polcor23	08	181	ATTAAATTAA CGTTCCTTTA	TCAAAACTAT	TAAACGTGCC	GACACACATA
Polcor23	09	183	ATTAAATTAA CGTTCCTTTA	TCAAAACTAT	TAAACGTGCC	GACACACATA
Polcor23	10	173	ATTAAATTAA CGTTCCTTTA	TCAAAACTAT	TAAACGTGCC	GACACACATA
Polcor23	11	262	ATTAAATTAA CGTTCCTTTA	TCAAAACTAT	TAAACGTGCC	GACACACATA
Polcor23	12	173	ATTAAATTAA CGTTCCTTTA	TCAAAACTAT	TAAACGTGCC	GACACGCATA
		110	120	130	140	150
Polcor23	01	173	CACACGCACA CACACACACA	CAC-----	-----	-----
Polcor23	02	187	CACACGCACA CACACACACA	CACACACACA CACACAC	-----	-----
Polcor23	03	185	CACACGCACA CACACACACA	CACACACACA CACAC	-----	-----
Polcor23	04	250	CACACGCACA CACACACACA	CAC-----	TTACA	GTGGACTTAA
Polcor23	05	175	CACACGCACA CACACACACA	CACAC-----	-----	-----
Polcor23	06	262	CACACGCACA CACACACACA	CACACACACA CACAC	TTACA	GTGGACTTAA
Polcor23	07	181	CACACGCACA CACACACACA	CACACACACA C	-----	-----
Polcor23	08	181	CACACGCACA CACACACACA	CACACACACA C	-----	-----
Polcor23	09	183	CACACGCACA CACACACACA	CACACACACA CAC	-----	-----
Polcor23	10	173	CACACGCACA CACACACACA	CAC-----	-----	-----
Polcor23	11	262	CACACGCACA CACACACACA	CACACACACA CACAC	TTACA	GTGGACTTAA
Polcor23	12	173	CACACGCACA CACACACACA	CAC-----	-----	-----
		160	170	180	190	200
Polcor23	01	173	-----	-----	-----	-----
Polcor23	02	187	-----	-----	-----	-----
Polcor23	03	185	-----	-----	-----	-----
Polcor23	04	250	CGCATGTAAA CGTTAGTAAA	ACAATTGTAT	CTTCCAAAAT	GTGCCAACAC
Polcor23	05	175	-----	-----	-----	-----
Polcor23	06	262	CGCATGTAAA CGTTAGTAAA	ACAATTGTAT	CTTCCAAAAT	GTGCCAACAC
Polcor23	07	181	-----	-----	-----	-----
Polcor23	08	181	-----	-----	-----	-----
Polcor23	09	183	-----	-----	-----	-----
Polcor23	10	173	-----	-----	-----	-----
Polcor23	11	262	CGCATGTAAA CGTTAGTAAA	ACAATTGTAT	CTTCCAAAAT	GTGCCAACAC
Polcor23	12	173	-----	-----	-----	-----
		210	220	230	240	250
Polcor23	01	173	-----	--TTACAGTG	GATTTAACGT	TAGTAAAATA
						ATTGTGTCTT

Polcor23	02	187	-----	--TTACAGTG	GATTTAACGT	TAGTAAAATA	ATTGTGTCTT
Polcor23	03	185	-----	--TTACAGTG	GATTTAACGT	TAGTAAAATA	ATTGTGTCTT
Polcor23	04	250	CGATACACAC	ACTTACAGCG	GATTTAACGT	TAGTAAAATA	ATTGTGTCTT
Polcor23	05	175	-----	--TTACAGTG	GATTTAACGT	TAGTAAAATA	ATTGTGTCTT
Polcor23	06	262	CAATACACAC	ACTTACAGCG	GATTTAACGT	TAGTAAAATA	ATTGTGTCTT
Polcor23	07	181	-----	--TTACAGTG	GATTTAACGT	TAGTAAAATA	ATTGTGTCTT
Polcor23	08	181	-----	--TTACAGTG	GATTTAACGT	TAGTAAAATA	ATTGTGTCTT
Polcor23	09	183	-----	--TTACAGTG	GATTTAACGT	TAGTAAAATA	ATTGTGTCTT
Polcor23	10	173	-----	--TTACAGTG	GATTTAACGT	TAGTAAAATA	ATTGTGTCTT
Polcor23	11	262	CAATACACAC	ACTTACAGCG	GATTTAACGT	TAGTAAAATA	ATTGTGTCTT
Polcor23	12	173	-----	--TTACAGTG	GATTTAACGT	TAGTAAAATA	ATTGTGTCTT

			260
Polcor23	01	173	<u>CCAGAACGGC</u> GT
Polcor23	02	187	<u>CCAGAACGGC</u> GT
Polcor23	03	185	<u>CCAGAACGGC</u> GT
Polcor23	04	250	<u>CCAGAACGGC</u> GT
Polcor23	05	175	<u>CCAGAACGGC</u> GT
Polcor23	06	262	<u>CCAGAACGGC</u> GT
Polcor23	07	181	<u>CCAGAACGGC</u> GT
Polcor23	08	181	<u>CCAGAACGGC</u> GT
Polcor23	09	183	<u>CCAGAACGGC</u> GT
Polcor23	10	173	<u>CCAGAACGGC</u> GT
Polcor23	11	262	<u>CCAGAACGGC</u> GT
Polcor23	12	173	<u>CCAGAACGGC</u> GT

Repeat motif - (AC)₂₀

Figure A 13 Aligned sequences for products of PCR reactions using the *P. coridon* Polcor 23 primer set. The microsatellite repeat motif is highlighted in blue. Insertions are shown in bold font. Primer sequences are underlined.

Appendix B. Allele frequency distributions

The figures below show the allele frequency distributions for loci used to genotype *Polyommatus bellargus* (Figure B1) and *Polyommatus coridon* (Figures B2 and B3), within the total sample set for each species. For ease of presentation the loci for *P. coridon* have been split between two figures and are presented in the numerical order of their names.

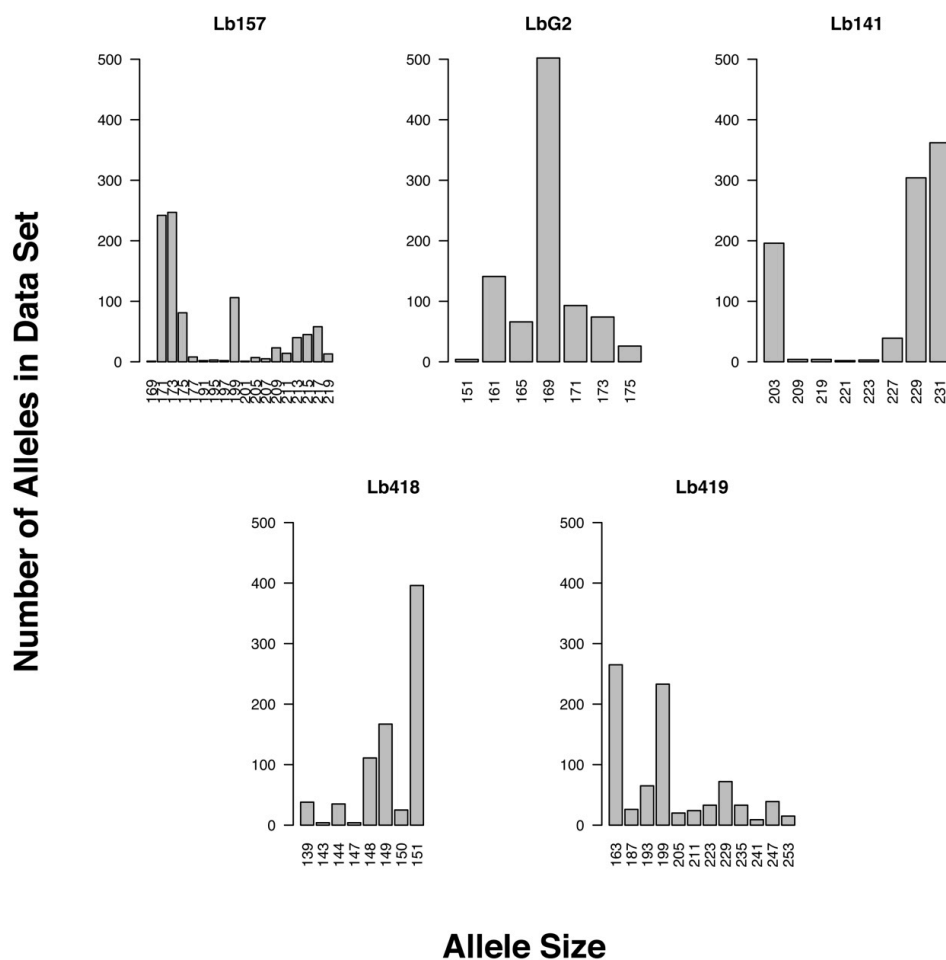


Figure B 1 For each microsatellite locus used to genotype *P. bellargus* the number of copies for each allele found within the entire *P. bellargus* sample set is shown. The name of the locus is shown above each graph.

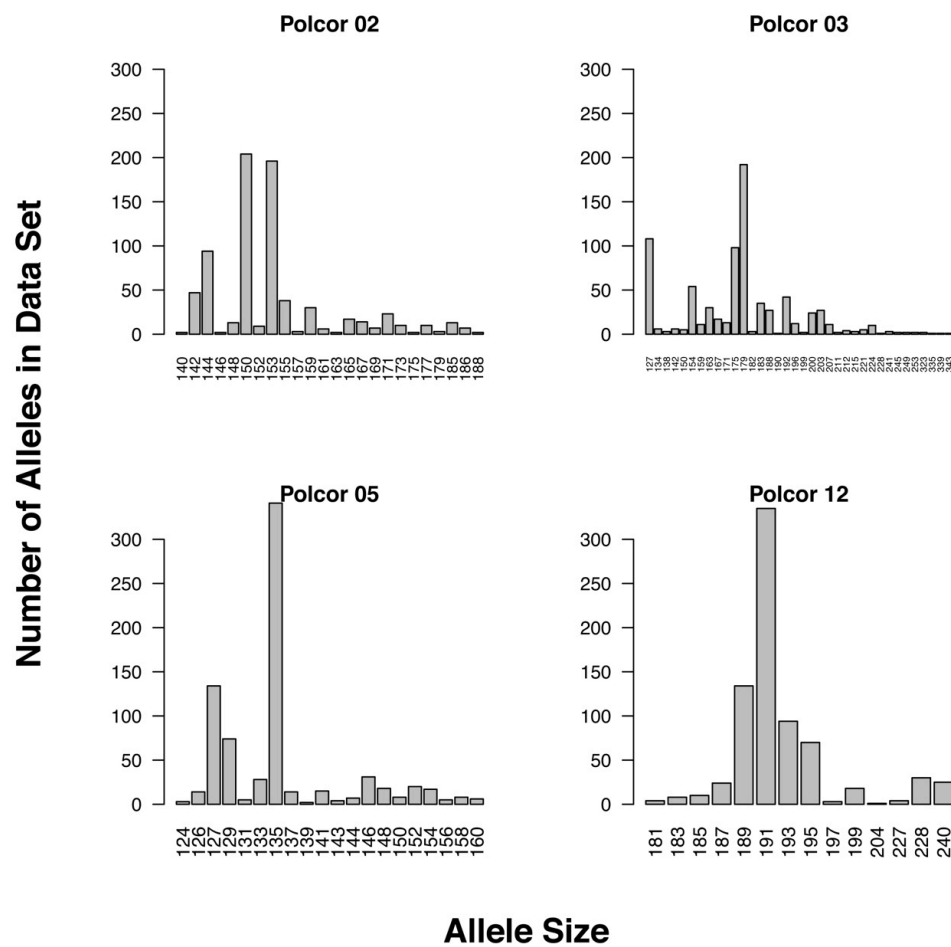
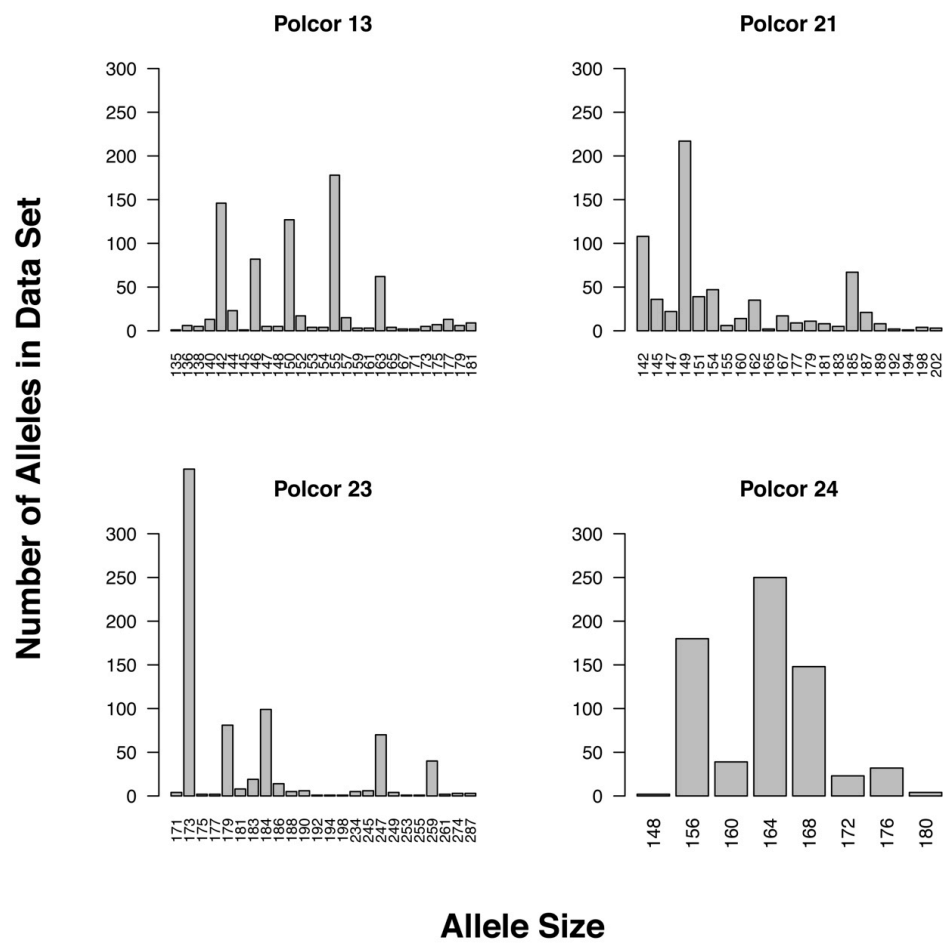


Figure B 2 For each of the first four microsatellite loci use to genotype *P. coridon* the number of copies of each allele found within the whole *P. coridon* sample set is shown. The name of the locus is shown above each graph.



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