Spatial structuring in trait variation in *Polyommatus icarus* in a functional context

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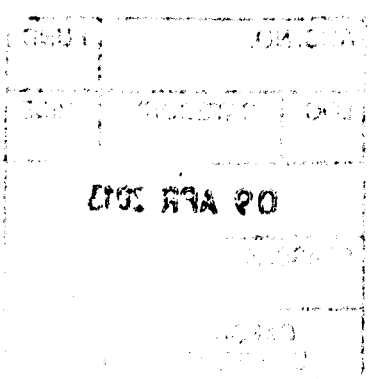

THE ILLUSTRATIONS ON PAGES 29 AND 32 HAVE BEEN REDACTED AT THE REQUEST OF THE UNIVERSITY

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Right then, here we are. The end of a long road. The end of a rocky road. It certainly was not easy, but fortunately I had many people who were helping me to push through and reach my final goal.

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Abstract

The Common blue butterfly, *Polyommatus icarus* (Rott.), is widespread throughout its Palaearctic distribution and persists in areas with differing climatic conditions. It is known to be a highly variable butterfly with marked within and between population variation in morphology, thermal biology, and voltinism. These traits together with allozymes and a neutral DNA marker (AFLP) variation are studied here to understand how geographic trait variation is related to environmental variation. The approach adopted here is to study this along a latitudinal cline of temperature and photoperiod, using four populations from south to north within mainland Britain.

AFLP differences, but not allozyme variation, indicate genetic structuring, with an isolation by distance effect. Enzyme diversity of *P. icarus* butterflies in the British Isles is lower than on mainland Europe, indicative of a past bottleneck. This, combined with selection on, or drift in, the allozymes could cause for a lack of population structure in this marker. Despite high levels of gene flow between populations, local adaptation is possible, as differentiation in certain allozyme loci was found (PGM and PGI). Populations differed in their response to developmental cues. Northern populations have an obligate diapause strategy and southern populations' development times differ in response to temperature, indicating local variation in response to environmental conditions.

Populations differed in wing morphology (size, shape and melanisation) but this was not related to latitude. Experimental determination of heating rates in different basking positions and thoracic temperature at take-off revealed no strong relationships of the morphological characteristics with heating or cooling rates and an indication of relationship with PGI alleles. It is suggested that in comparison to larger butterflies morphological variation is unconstrained by thermal requirements.

The persistence and widespread occurrence of this butterfly may be a consequence of the variability of traits within this butterfly.

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Chapter 1 Background

1.1 INTRODUCTION

The distribution and persistence of organisms depends on a number of interacting biological and environmental factors which operate over a range of spatial and temporal scales (Bunnell & Huggard, 1999; Maurer & Taper, 2002; Dennis *et al.*, 2003). Variation in these factors can result in within and between population variation. The nature of this variation can be used to infer past evolutionary events and colonisation history (Hewitt, 1996; Hoffmann & Merilä, 1999; Thompson, 1999), but it is also of fundamental importance to organismal responses to future environmental changes (Skelly *et al.*, 2007; Bolnick *et al.*, 2011). Within a location, developmental responses to environmental conditions, physiological, morphological and behavioural tolerances of individuals and the overall variation within populations underlies how individuals respond and have responded to varying environments (Gordon, 1991; Lande & Shannon, 1996; Kelly *et al.*, 2011; Thomassen *et al.*, 2011). Specifically for adult butterflies, those traits that influence searching for food, mates and oviposition sites and the ability to evade or escape predators are important (Shreeve, 1992) and are the ones upon which selection could operate. Butterflies are ectotherms and their activities depend on the accumulation of heat from external sources in order to reach a suitable body temperature (Watt, 1968). Trait variation in these animals is therefore very likely to be tightly correlated with latitude (and habitat temperature). Organisms can track climate change and expand their distribution accordingly as a response (Davis, 1986; Graham & Grimm, 1990; Hewitt, 1996; Hill *et al.*, 2002). Increasing habitat fragmentation however, makes movements between fragments more difficult and the ability of species to express an evolutionary response to changing environments will become more important in the future persistence of a species (Opdam & Wascher, 2004; Chevin *et al.*, 2010).

Currently many butterfly species are in decline (Channell & Lomolino, 2000; Ceballos & Ehrlich, 2002; Thomas *et al.*, 2004; Fox *et al.*, 2011) and the focus of much species related ecological research is on the conservation of declining and/or vulnerable species (Warren, 1991; Brereton *et al.*, 2008; Thomas *et al.*, 2009; Smee *et al.*, 2011). However, there are many widespread species that are also in decline (Conrad *et al.*, 2006; Van Dyck *et al.*, 2009; Kadlec *et al.*, 2010) and the status of species can change from abundant to rare and *vice-versa* (e.g. Shreeve *et al.*, 1996). At issue is the response of individual populations to past extinction-colonisation dynamics on the one hand and to the threats of current and future environmental change on the other. For many species the spatial scale of a population unit is not well defined, even though these may operate as independent evolutionary units and an understanding of the way that these units respond to change and the causal mechanisms of these responses is essential in order to maintain biodiversity.

The focus of this thesis is on the variation in a common butterfly species *Polyommatus icarus* (Rott.) (Common blue) within the British Isles. This species is used to investigate genetic, morphological, enzyme and life-cycle variation over a latitudinal (=climatic) gradient to describe a species' response to past and future environmental changes. Current latitudinal patterns are the results of past selection pressures and it is in this respect that this information can be used to reflect the post-glacial history of this butterfly. Analysis of within and between population variation may be an indication of the potential for future persistence of this butterfly over the British Isles.

Polyommatus icarus is widespread in the British Isles and is univoltine in its northern range (NW Scotland: one generation per year) and multivoltine south of Yorkshire (Emmet & Heath, 1989; Asher *et al.*, 2001). The voltinism pattern is the product of development rate and the timing of diapause, but it is uncertain which factor is regulating this pattern and whether this is completely plastic trait or not. Previous quantitative work on variation in *P. icarus* within the British Isles has

characterised differences in morphology, behaviour and thermoregulation between a single central-southern England population and a population in northwest Scotland (Howe, 2004; Howe *et al.*, 2007). These differences have been related to selection in relation to mate-attraction, requirements for flight capacity and potentially predator avoidance (Howe, 2004). This research builds on this earlier work by examining the nature of variability along an environmental gradient within the British Isles. This allows for an interpretation in terms of adaptation and variation in morphology and enzyme variation against a background of differences between populations using neutral molecular markers.

1.2 MORPHOLOGY

The size and shape of butterfly wings may influence flight performance, and hence the general capability to find mates, egg-laying sites, other adult resources and to escape from predators (Betts & Wootton, 1988; Chai & Srygley, 1990; Kingsolver, 1995; Dudley, 2000). The degree of wing melanisation can affect rates of heat gain and have an important function in thermoregulation and therefore in activity patterns (Watt, 1968; Kingsolver, 1996; Clusella-Trullas *et al.*, 2007). In some species, the wings may also have complex colour patterning on the upper- or underside and this pattern influences mate signalling and/or conspicuousness to predators (Dennis & Shreeve, 1989; Brakefield & Shreeve, 1992).

1.2.1 Butterfly wings in thermoregulation

Most butterflies have to elevate their body temperature above ambient temperature before they can become active (Watt, 1968; Roland, 1982; Kingsolver, 1983). Although achieving a suitable body temperature can be greatly influenced by butterfly behaviour, including posture and selection of specific microclimatic conditions, between-individual variation in melanisation of the wings has been related to differences of activity because of the effects of melanisation on heating rates (Watt, 1968; Kingsolver, 1996; Clusella-Trullas *et al.*, 2007). The wings are too thin to conduct heat to the body over large distances, therefore melanisation of the basal wing part (i.e. closest to the body) is most important in heat absorption (Wasserthal, 1975). Three main basking mechanisms for butterflies have been described: dorsal basking, in which the dorsal wing surfaces are exposed to the sun and heat absorbed by the wings is conducted to the body; lateral or ventral basking in which the wing undersides are exposed to the sun and heat is conducted to the body (Clench, 1966; Kingsolver, 1985a; Heinrich, 1986b) and reflectance basking in which solar radiation is reflected onto the body from the open dorsal wing surfaces (Kingsolver 1985a; Kingsolver 1985b). Butterflies that adopt a dorsal basking strategy

(opened wings) can absorb more heat with darker basal upper forewings (Wasserthal, 1975; Kingsolver, 1987; Van Dyck *et al.*, 1997b), whereas lateral baskers (wings closed) benefit most from a higher degree of underside hindwing melanisation (Watt, 1968; Roland, 1982; Kingsolver, 1996; Stoehr & Goux, 2008). In those species that use the third mechanism (reflectance basking) the colour of the whole upper wing surface is of importance and a brighter coloured wing will provide faster heating (Kingsolver, 1985b).

In several butterfly species a relationship between melanisation of the wings and temperature is found along latitudinal or altitudinal gradients (Watt, 1968; Guppy, 1986; Ellers & Boggs, 2002; Ellers & Boggs, 2004; Roland, 2006; Clusella-Trullas *et al.*, 2007). The general pattern for absorbance baskers is that, in colder climates, butterflies with darker coloured wings will have an advantage, as they can reach a suitable body temperature faster. Alternatively, in warmer locations, more lightly coloured wings might help in avoiding overheating. For multivoltine species occurring in locations with seasonally varying temperatures, cues during development have to be interpreted in order for an appropriate adult phenotype to appear (seasonal polyphenism; Shapiro, 1976). Butterflies eclosing in spring, when temperatures are low, benefit from darker coloured wings, whereas in summer, lighter coloured individuals have an advantage in avoiding overheating (Kingsolver & Wiernasz, 1991; Kingsolver, 1995; Stoehr & Goux, 2008). For reflectance baskers the pattern is generally reversed. The melanisation of the adult wings is usually determined by the developmental conditions in the larval stage (Brakefield, 1996; Nijhout, 1999). The majority of studies on wing colouration have been conducted on large or moderate sized species of the butterflies from the Pieridae and Nymphalidae. For smaller species, such as *P. icarus,* the thermal consequences of variation in melanisation are relatively understudied. *Polyommatus icarus* is mainly a dorsal basker, but is also suspected of adopting a reflectance basking strategy (Shreeve, 1992). It is therefore expected that this butterfly will have darker basal wing parts in colder

locations (i.e. going further north, or at high elevation) or colder seasons (i.e. spring generation).

Compared to females, males have more active flight and are more prone to convective cooling and consequently may spend a larger part of the day basking (Gilchrist, 1990). Absorbed heat is more readily conserved in individuals with a larger body (Heinrich, 1986a), but they have a longer response time than small bodied individuals to both heating and cooling (Kemp & Krockenberger, 2004). In most butterfly species males are usually the smaller sex and will exchange heat with the environment faster than females. It is therefore not surprising that sexual differences in the plastic response for melanisation have been found for several butterfly species (Pivnick & McNeil, 1986; Van Dyck & Wiklund, 2002; Davis *et al.*, 2005; Stoehr & Goux, 2008).

1.2.2 Body size, wing size and wing shape

The size of a butterfly is usually dependent on developmental conditions in the larval stage (e.g. temperature, daylength, host plant quality (Gotthard 2008; Leimar 1996; Nylin 1992)) and a longer development time usually allows growth to a larger size (see Chapter 6). Development time is inversely related to temperature and in cool conditions individuals are therefore expected to grow larger than under warm conditions (Stearns & Koella, 1986; Nylin & Gotthard, 1998). This is true for some species in seasonal systems, where warm temperatures in spring allow for a faster development and smaller summer butterflies (Van Dyck & Wiklund, 2002). Similarly, the relationship between developmental temperature and final size serves as an explanation for the commonly observed Bergmann clines with latitude (Atkinson & Sibly, 1997; Blackenhorn & Demont, 2004). Higher latitudes imply colder temperatures, where the size of ectotherms can be larger. However, size can also decrease along a latitudinal or altitudinal cline (converse Bergmann rule) (Mousseau, 1997; Blackenhorn & Demont, 2004). This indicates that there is another important factor, namely season length, which determines development time and final adult size

(Nylin & Svard, 1991). For species with a shift in voltinism (see section 1.5 – Voltinism), the graph of development time or size with latitude will show a "saw-tooth-pattern" (Figure 1.1), because development time (and therefore size) per generation is limited at the northern limit of the bivoltine strategy, but less constrained by time at the southern edge of the univoltine strategy (Roff, 1980; Roff, 1983). At lower latitudes or altitudes, the favourable season is longer, and this allows for a longer development time.

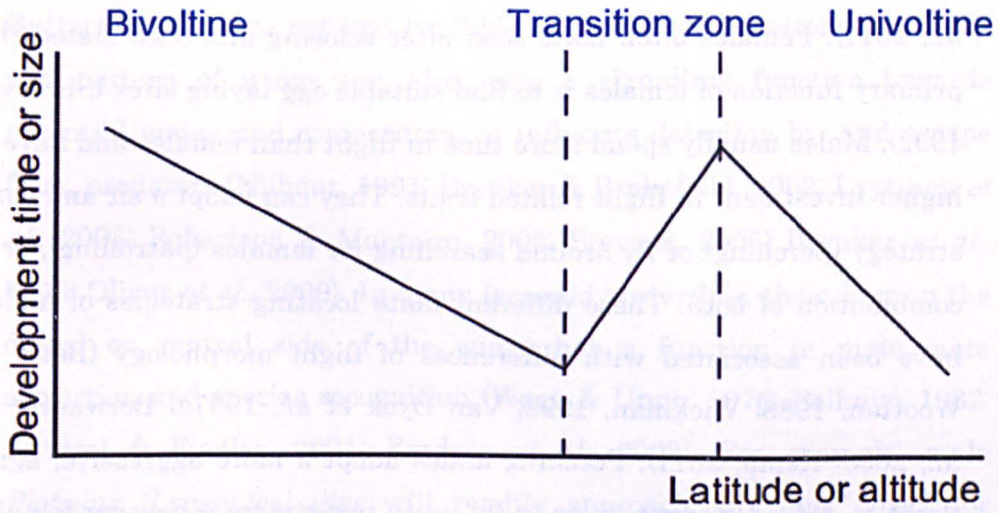

Figure 1.1 Schematic representation of a "saw tooth" cline for insects with a shift in voltinism. Modified from Roff (1983).

As larger adults generally have a higher fecundity (Wiklund & Karlsson, 1988; Karlsson & Wickman, 1990), the available time is readily used for development. However, for males the pattern is not always this straightforward. In butterflies with discrete generations, males can increase the probability of mating by emerging before virgin females eclose, resulting in a smaller size. In some species, emerging early can also assist in establishing a territory before other males. This results in a trade-off between emerging early (i.e. before females = protandry) and being large (Wiklund *et al.*, 1991; Zonneveld, 1996). This trade-off is influenced by the mating system: generally there is positive correlation between selection for large size and female polygamy (Wiklund & Forsberg, 1991). If females only mate once, early male emergence will increase the likelihood of more matings for a male. However, if females are polygamous, the size of the males will influence

how many matings they can secure. Selection will therefore be in favour of large size for males in species with polygamous females and *vice versa*. *Polyommatus icarus* females are thought to only mate once (Drummond, 1984), and are therefore expected to be the larger sex.

Differences between male and female wing morphology have been related to the different needs of the sexes (Dudley, 2000; Norberg & Leimar, 2002; Berwaerts *et al.*, 2006; Breuker *et al.*, 2007; DeVries *et al.*, 2011). Females often mate soon after eclosing and once mated the primary function of females is to find suitable egg-laying sites (Shreeve, 1992). Males usually spend more time in flight than females and have a higher investment in flight related traits. They can adopt a sit-and-wait strategy (perching) or fly around searching for females (patrolling), or a combination of both. These different mate locating strategies of males have been associated with differences of flight morphology (Betts & Wootton, 1988; Wickman, 1992; Van Dyck *et al.*, 1997a; Berwaerts *et al.*, 2008; Kemp, 2011). Perching males adopt a more aggressive, agile flight to defend a territory or to fly up to investigate a passing female. This is enhanced by relatively more pointed wings. Patrolling butterflies benefit from more rounded wings, which allow for an energetically less costly, more aerodynamic flight (Betts & Wootton, 1988). *Polyommatus icarus* butterflies in Scotland generally have more rounded wings compared to butterflies in the south of England (Howe, 2004). The former shape is associated with slower, more prolonged flight in comparison with angular wings (Chai & Srygley, 1990) and supports the idea (Howe, 2004) that more rounded wings facilitate flight in Scotland where higher wind speeds prevail.

Variation in wing morphology in *P. icarus* has been identified before in two populations at the extremes of a cline within the UK (Howe, 2004; Howe *et al.*, 2007). The environmental conditions (mainly temperature) vary along a south-north cline within the UK and, because of the importance of flight, variation in wing size and shape is expected. Both males and females in Scotland are larger than butterflies in south-

central England. Additionally, in museum specimens of *P. icarus* in Sweden, a negative size cline with latitude has been found (Nygren *et al.*, 2008). This cline exhibits a "saw-tooth" pattern where the butterfly shifts from bivoltine to univoltine. However, this study pooled the individuals across generations and years at each latitude, possibly excluding important environmentally fluctuating parameters.

1.2.3 Signalling

Butterfly wings are not just for flight and thermoregulation, the colour and pattern of wings can also have a signalling function towards potential mates and competitors, or influence detection by, and escape from, predators (Nijhout, 1991; Breuker & Brakefield, 2002; Lyytinen *et al.*, 2003; Robertson & Monteiro, 2005; Stevens, 2005; Breuker *et al.*, 2006; Oliver *et al.*, 2009). In many lycaenid butterflies, the colour on the dorsal or ventral side of the wings has a function in male mate attraction and species recognition (Wago & Unno, 1976; Pellmyr, 1982; Knuttel & Fiedler, 2001; Fordyce *et al.*, 2002). For example, male *Plebejus (Lycaeides) idas* will readily approach any blue object for inspection (Pellmyr, 1982). Females that have just emerged, often rest on high parts of vegetation, exposing the ventral hindwing (or the dorsal forewing while basking). Males of *P. icarus* for example, prefer females with a relatively high ultraviolet (UV) absorbance of the ventral wing area (Burghardt *et al.*, 2000; Knuttel & Fiedler, 2001). This intraspecific communication via UV reflectance was also found in *Bicyclus anynana* (Robertson & Monteiro, 2005) and in some pierid butterflies (Brunton & Majerus, 1995). At least for lycaenids, the UV-absorbance of the wings is dependent on the uptake of flavonoid plant components in the caterpillar stage and it can therefore act as an indication of individual quality (Burghardt *et al.*, 2000; Knuttel & Fiedler, 2001).

In order to avoid being detected by a predator (i.e. primary defence), butterflies can mimic an inedible prey item - Batesian (Bates, 1862) or Müllerian (Müller, 1879) mimicry - or they can camouflage (crypsis) (Endler, 1978) (see also review by Mallet & Joron (1999). Once detected,

some species deflect predator attacks from the body by means of deflective wing markings (secondary defence) (Wourms & Wasserman, 1985; Lyytinen *et al.*, 2004; Stevens, 2005; Stevens *et al.*, 2008) of which the success rate is dependent on the environmental conditions (Olofsson *et al.*, 2010; Vallin *et al.*, 2011). Cryptic lepidoptera that can "surprise" predators with an appearing eyespot (e.g. *Inachis io*; *Smerinthus ocellatus*) may, by startling the predator, gain time to escape (Blest, 1957; Vallin *et al.*, 2005). A few studies showed that the "surprise effect" or the conspicuousness, rather than mimicking an actual vertebrate eye, is more important than specific resemblance in escaping a predator (Vallin *et al.*, 2006; Stevens *et al.*, 2008). The situation is different for butterflies with marginal eyespots, a pattern very commonly seen in satyrine butterflies, which deflect predator attacks away from the body. The forewings of a butterfly are the most important for flying (Dennis *et al.*, 1984) and the hind wings are fragile, therefore an attack directed to the hindwing will give butterflies a higher chance of escape and future flight capacity (DeVries, 2002). In seasonal systems variation in butterfly activity levels and colour of the vegetation may be a driver of seasonal wing pattern variation related to predation (Brakefield & Larsen, 1984; Wiklund & Tullberg, 2004; Joiris *et al.*, 2010; Vallin *et al.*, 2011). For example, in the wet season when *Bicyclus anynana* is most active, it has large eyespots on the underside of the wings to deflect predator attacks but in the dry season, which the butterfly tries to bridge in a more cryptic state, the eyespots are very small (Brakefield & Larsen, 1984; Brakefield & Reitsma, 1991; Brakefield, 1998). However, in both seasons, this butterfly shows large eyespots on the dorsal side of the wings as the size of these influences mating success (Breuker & Brakefield, 2002; Robertson & Monteiro, 2005). A slightly different strategy is used in several lycaenid butterflies. The combination of wing markings and wing shape resembles in a "false head" at the end of the body, again directing predators towards the hindwings, rather than the head, increasing chances for survival (Robbins, 1980; Robbins, 1981; Wourms & Wasserman, 1985).

Generally, the ventral side is primarily devoted to primary and secondary defence mechanisms and the dorsal side can have the additional function of intra-specific communication. However, the underside pattern in Lycaenidae can also function as a species recognition signal (Fordyce *et al.*, 2002). Because the formation of the different wing elements (e.g. lunules, eyespots, major pattern elements, dorsal and ventral elements) can in some cases be developmentally uncoupled, conflicting selection pressures can act on each element separately (Schwanwitsch, 1948; Nijhout, 1985; Beldade *et al.*, 2002; Oliver *et al.*, 2009).

1.3 THERMOREGULATION

Insects are ectotherms and mainly rely on external heat sources to accumulate heat in order to reach a body temperature suitable for activity (Watt, 1968). This body temperature of active or potentially active individuals is limited around a narrow optimal range and thermoregulation helps an individual to reach and maintain this temperature.

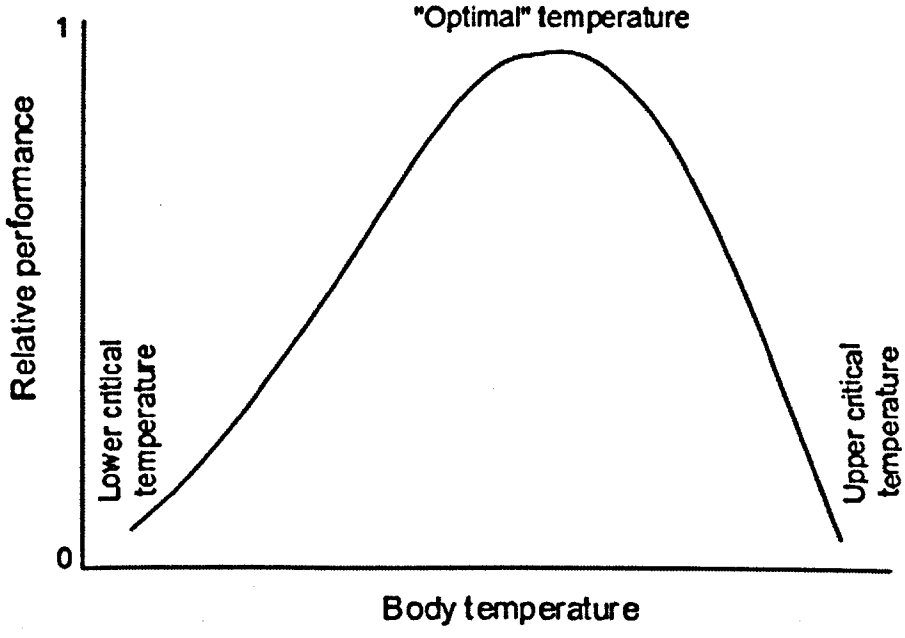

Figure 1.2 Thermal performance curve (Huey & Stevenson, 1979). The relative performance of an individual is highest at the optimal temperature and will decrease with the body temperature moving away from the optimum.

This process is a balance between heating up till a suitable temperature is reached and avoiding overheating. The performance of an individual will be highest at the thermal optimum and will decrease at higher and lower body temperatures (Figure 1.2). Individuals that are able to regulate their body temperatures more efficiently within close range of the optimum will have higher activity levels, which may eventually translate to higher fitness (e.g. more efficient search for food, for mates and oviposition sites and higher abilities to escape predators (Shreeve, 1992)). Activity levels under varying temperatures can be maintained

by 1) allocating a greater fraction of the resources to a physiological function at the expense of another, 2) spending more time gathering resources with increased risk of predation or 3) specialising for temperatures that are most often encountered (Angilletta *et al.*, 2003).

In small insects, wing-beat frequency, and hence flight performance, is directly related to ambient temperature (Unwin & Corbet, 1984). In cooler conditions butterflies need to invest relatively more energy in flight performance. Hence, flight performance for butterflies in cool conditions – expressed as lift capacity – could be improved by increasing relative flight muscle mass (Berrigan, 1991), increased wing loading and increased wing aspect ratio (Betts & Wootton, 1988). The explanation for the latter is that longer wings can move air with a larger relative speed and generate higher aerodynamic forces per area-unit. It is now known that for some species (e.g. *Colias* sp., *Lycaena tityrus*) the interaction between glycolytic enzyme types (principally PGI) and temperature influences metabolic efficiency at different thoracic temperatures (Watt, 1977; Karl *et al.*, 2008; Kallioniemi & Hanski, 2011). Different enzymes have different thermal properties and stabilities and the genotype of individuals may influence the temperature range at which they are maximally efficient (Watt, 1977; Watt, 1983; Wheat *et al.*, 2005; Karl *et al.*, 2008).

1.3.1 Behavioural mechanisms

Butterflies achieve their body temperature by appropriate microhabitat usage, basking postures and orientation relative to the sun (Kammer & Bracchi, 1973; Kingsolver, 1985a; Dennis & Shreeve, 1989; Shreeve, 1992). In general, these behavioural mechanisms will be balanced by the associated costs (time and energy) or risks (predation) of temperature regulation (Huey & Slatkin, 1976; Huey & Stevenson, 1979).

Not all butterflies solely rely on ectothermic mechanisms to elevate body temperature. Some species are known to produce endothermic heat by

shivering (rapid contraction of the flight muscles; Clench, 1966), although this process is more common within moths (Kingsolver, 1985a), which is probably an energetically expensive mechanism for rapid warming. A few butterflies in which shivering has been observed are *Danaus plexippus, Papilio polyxenes, Aglais urticae, Pararge aegeria, Lasiommata megera, Hipparchia semele, Vanessa cardui, Inachis io, Vanessa atalanta* (Kammer & Bracchi, 1973; Rawlins, 1980; Shreeve, 1992; Maier & Shreeve, 1996). In addition, temperature acclimation can allow butterflies to fly at lower body temperatures than preferred (Heinrich, 1986b), but possibly with a high energetic cost.

1.3.2 Enzymes

The temperature required by flight muscles can be influenced physiologically by enzyme systems with different thermal optima (Watt, 1977). Variation in thermal characteristics of glycolytic enzymes is therefore likely to be of importance as flying insects have the highest known mass-specific rates of energy consumption (Suarez, 2000). Enzymes with a lower thermal optimum can confer an advantage in colder environments by allowing for a longer time span for activity or can have a direct influence on survival, for instance in escaping a predator attack early in the day (Watt, 1977). However, while these cold-optimum enzymes function more effectively at lower temperatures, they unfold more readily at high temperatures and may thus be disadvantageous on warm conditions (Watt, 1977; Watt, 1983; Dahlhoff & Somero, 1993). Variation in allelic frequencies in *Colias* butterflies at the PGI-locus has previously been related to habitat temperature (Watt, 1977; Watt, 1983), suggesting possible selection pressures on this enzyme (Dahlhoff & Rank, 2000; Wheat *et al.*, 2005). Variation in glycolytic enzymes has been identified in several other butterfly species (Goulson, 1993; Haag *et al.*, 2005; Hanski & Saccheri, 2006; Saastamoinen, 2007; Karl *et al.*, 2008; Saastamoinen & Hanski, 2008; Vandewoestijne & Van Dyck, 2010; Kallioniemi & Hanski, 2011) and most of the variable enzymes are located at the top of the glycolytic pathway (Eanes, 2011). However, at least for some enzymes the effects of the variation are not entirely understood. For example, even at

activity levels as low as 17% for PGI or PGM, no reduction in flight activity was found in *Drosophila melanogaster* (Eanes *et al.*, 2006). An additional problem is the multiple functions that (glycolytic) enzymes can fulfil in the body (Sun *et al.*, 1999; Kim & Dang, 2005) complicating investigation of selective responses on enzyme variation. A first step to identify candidate loci on which selection can act is to compare the allozyme pattern with the population genetic structure. Further experimental tests should then aim to identify the fitness response of enzyme variation.

1.4 POPULATION GENETIC STRUCTURE

Population structure can be defined as the way different population units are distributed over a geographic or temporal scale. It is the result of a balance between the forces that increase genetic differentiation (mutation, drift, selection) and those that tend to produce genetic homogeneity (gene flow) (Slatkin, 1987; Frankham *et al.*, 2002). Only few species exist as a large panmictic population, and therefore most species are genetically structured (Beebee & Rowe, 2004). This can be as a consequence of barriers restricting gene flow between groups of individuals, but even without barriers differentiation can occur as a consequence of isolation by distance (Wright, 1943). This is a pattern commonly found in widespread species in which the geographic range is larger than the distance one individual can cover during its lifetime (Peterson & Denno, 1998). The genetic distance between two populations can thus indirectly give information about the dispersal abilities of a species, a characteristic which is often difficult to study in the field. Regardless of the possible difficulties in interpretation of the data within analyses of genetic distances, they are very useful as indirect measures of dispersal (Bohonak, 1999; Miller *et al.*, 2002; Schmitt *et al.*, 2002; Schmitt *et al.*, 2003; Vandewoestijne & Baguette, 2004). Genetic information will only give information about successful dispersal (i.e. reproduction after dispersal), and it is thus mainly of use for studies of population structure, but selection may introduce a bias if the marker(s) used to characterise differentiation are not neutral to selection.

Intricately linked to population structuring is the concept of metapopulations (Levins, 1970; Hanski, 1998) in which individual population units are geographically isolated, but connected by infrequent movements of individuals between population units and where extinction and colonization of the different populations occur regularly over time. The most common approach to investigating how

populations are structured is to quantify the genetic diversity within populations and compare this between populations (see section 1.4.3.1).

1.4.1 Mutation, drift and selection

All genetic variation is originally caused by mutations (gene mutations and chromosome mutations), and variation is essential in order for evolution to take place (Stearns & Hoekstra, 2005). Most mutations have no effect (neutral mutations) because they do not result in a phenotypic change (no change in amino acid or the amino acid substitution does not alter the protein function; Beebee & Rowe, 2004). Furthermore, eukaryote organisms possess a repair mechanism to correct for mutations in the coding DNA (Friedberg *et al.*, 1995). The increase of variation by mutation is counterbalanced by drift, a stochastic process that leads to fixation of random alleles (and hence the loss of variation). Without influx from neighbouring populations, this process of allelic extinction is irreversible and will impoverish the gene pool of that population (Saccheri *et al.*, 1998; Nieminen *et al.*, 2001). As a result of mutation and drift, populations which are isolated may diverge and it is more likely that individuals within populations have more similar DNA compared to individuals of other populations, because the variation will be inherited and there is lower exchange of DNA (low gene flow) between populations than within populations.

Population differentiation through selection can occur when selection pressures differ between populations (Mayr, 1963; Maynard Smith, 1966). This may be the result of continuous different selection regimes or the result of intense transient selection pressures. In these situations, different sets of genes will provide a higher fitness in each location and will be relatively more represented in the next generation. Higher fitness will result from traits that improve reproduction in the broad sense (e.g. higher survival rate of adults through more efficient feeding or escape from predators, or higher reproductive output). In

order for (local) adaptation through selection to take place, these phenotypic traits must have a heritable basis (Darwin, 1859). In combination with isolation, selection can cause rapid microevolution (e.g. pesticide resistance in arthropods e.g. Labbe *et al.*, 2007; Jansen *et al.*, 2011). High gene flow can be an impediment for local adaptation (Mayr, 1963), but complete lack of gene flow will make populations isolated and more vulnerable. Intermediate levels of gene flow will increase and maintain high levels of genetic variance, allowing for adaptation (Wright, 1931; Endler, 1973; Bossart & Scriber, 1995; Gomulkiewicz *et al.*, 1999; Lenormand, 2002).

1.4.2 Gene flow

Gene flow (the movement of genes between populations) can homogenise the genetic composition of populations (Mayr, 1963; Lenormand, 2002). If gene flow is high, populations will be prevented from becoming genetically distinct. Theoretical models predict that only one individual per generation can be sufficient to impede population differentiation (Hartl & Clark, 2007), although if different strong selection pressures exist in two populations, the effects of gene flow can be overcome and adaptive genetic differences can still be maintained (Endler, 1973; Karhu *et al.*, 1996; Freeland *et al.*, 2010). A high genetic similarity of two populations (short genetic distance) can therefore have three different explanations: populations are connected by dispersing individuals, they have only recently separated or they experience similar selection pressures.

1.4.3 Measuring population differentiation

Estimations of genetic differentiation are most commonly conducted using F-statistics, particularly F_{st} (Wright, 1951) and G_{st} (Nei, 1973) which are measures of population differentiation. These values range from 1 (complete fixation of different alleles in two populations) to 0 (homogenous population). Usually, the pattern of genetic differentiation is measured by comparing the genetic variation within sites with the variation between sites. In differences between populations there is an

expectation for between-site variation to be larger than within-site variation and that the further away two sites are in geographic distance, the bigger their between-site variation will be.

1.4.3.1 Wright's F_{ST} & Nei's G_{ST}

Wright's F-statistics (Wright, 1951) are fixation indices which measure the partitioning of total genetic variation within (F_{IS}) and among (F_{ST}) subpopulations. It is estimated using:

$$F_{ST} = (H_T - H_S)/ H_T,$$

where Hs is the mean expected heterozygosity of an individual in a subpopulation and H_T the expected heterozygosity of an individual in the total population. High values ($F_{ST} > 0.15$) indicate high population differentiation and low migration rates between populations. Because of the strong relationship between F_{ST} and gene flow, the migration rate can be estimated as follows:

$$Nm \approx ¼ (1/ F_{ST} - 1)$$

(Slatkin, 1993)

with m the migration rate and N the effective population size.

This model however, assumes there is no selection, there is no mutation, all populations host the same number of individuals and contribute equally to the migration pool, migration is random (i.e. irrespective of the distance between populations) and the system is at equilibrium (Whitlock & McCauley, 1999). In natural populations these assumptions will usually be violated, but F_{ST} values are robust enough to compare among population migration within and between species (Bohonak, 1999). Nei (1973) suggested a variation on the F-statistic that is less sensitive to selection and the reproductive method and is independent of the number of alleles. This measure, G_{ST}, is the coefficient of gene variation and measures the proportion of total genetic variation distributed among and within populations (Nei, 1973). With two alleles at a locus this measure, G_{ST}, is essentially the same as Wright's F_{ST}.

1.4.3.2 Analytical methods

Different techniques have been developed to study genetic distances between populations, each with their advantages and disadvantages. The most frequently used methods are protein electrophoresis (Hubby & Lewontin, 1966; Lewontin & Hubby, 1966), mitochondrial DNA sequencing (Anderson *et al.*, 1981), Random Amplified Polymorphic DNA (RAPD) (Williams *et al.*, 1990) and Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.*, 1995), and microsatellites (Litt & Luty, 1989).

Protein electrophoresis is probably the oldest method and relies on the different forms of a protein (allozymes) migrating at different rates, dependent on their size and charges along an electrical charge gradient, usually on a starch or cellulose acetate plate or polyacrylamide gel. As a result, the separated allozymes can be visualised as individual bands on the gel. If the enzyme composition of two populations differs, the banding pattern will show these differences. Although this method is a relatively cheap and easy method, it has a major drawback for estimating genetic distance as proteins have functions and can be under selection pressures so that the result of a protein electrophoresis will not necessarily reflect a species' true population structure (Bohonak, 1999). Additionally the estimate of divergence between populations may be dependent on the relationship between the number of enzyme systems studied and the strength of selection on these different systems.

To avoid the uncertainties of allozyme electrophoresis, more neutral DNA markers can be used. All eukaryote organisms possess mitochondria and thus mitochondrial DNA (mtDNA). MtDNA is a small, circular (or linear for lower eukaryotes), extra-chromosomal, double-stranded DNA molecule that has an equal or higher mutation rate than nuclear DNA (Brown *et al.*, 1979). The most studied parts of the mitochondrial DNA are the control region (CR) and cytochrome oxidase I or II (COI and COII). The selected region can be amplified by PCR and the pattern can then be compared between individuals. In this

method it is also assumed that mtDNA is not subject to selection, but according to Gerber *et al.* (2001) this is not always the case.

One big advantage of the next two methods, Randomly Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP), is that they do not require *a priori* information about the DNA sequence of the organism under study. The short primers (ca. 10 bp) consist of a random sequence of nucleotides and they are commercially available in 'RAPD' kits. These primers are short enough to anneal to a matching sequence on the DNA purely by chance and are long enough not to amplify too many fragments. The primer and the DNA together undergo a PCR and the resulting banding pattern can differ between individuals due to mutations in the primer sites, causing presence or absence of bands. A major drawback is that the results of RAPD are very sensitive to laboratory conditions and contamination.

To avoid the drawbacks of RAPD, AFLP has been developed (Vos *et al.*, 1995; Figure 1.3). In this process, sample DNA is first digested with two restriction enzymes producing different sticky ends (Müller & Wolfenbarger, 1999). After adding double-stranded 'linkers' with compatible sticky ends, a PCR is run with primers corresponding to the linkers' sequences. A series of bands are then produced of the amplified DNA regions. This method is slightly more laborious than RAPD, but has a major advantage of being more reproducible.

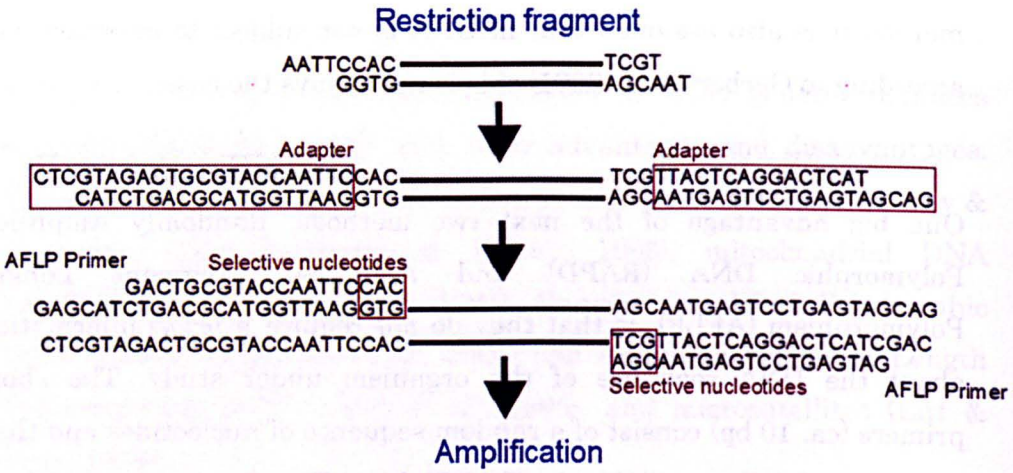

Figure 1.3 Schematic representation of the AFLP technique. Top: fragment with sticky ends produced by restriction enzymes. Centre: the same fragment with the corresponding 'linkers'. Bottom: the two strands both with their AFLP primers. After Vos *et al.* (1995).

Another technique frequently used in population genetic studies is microsatellite analysis. Microsatellites are neutral markers that consist of short tandem repeats (i.e. 1 to 4 bp motifs), such as ACACACAC. These microsatellite loci can be found in all eukaryotes, most commonly in non-coding DNA and are less likely to be subject to selection. They have high mutation rates that change the repeating array length, causing high polymorphism. This makes this marker a successful tool in population genetics although its neutrality is not always guaranteed: microsatellites can hitch-hike to a gene under selection or sometimes they are even under selection themselves (Estoup & Cornuet, 1999). Microsatellite analysis is also a PCR-based method and requires primers that are complementary to the two flanking regions of the microsatellite locus. Identification of the microsatellite and the design of the primers are the most time-consuming tasks and have to be repeated for every new species. However, primers of closely related species can be used as a starting point for developing species specific microsatellite primers and this has been done with varying success. A drawback of this method is the possible occurrence of "null-alleles" which arise from mutations in the flanking regions, causing a lack of amplification of this allele. Nevertheless, if time and money to develop the primers is not an issue, this is the preferred technique for population genetic studies.

1.4.3.3 Recent developments

Recently, the geographic pattern of allozymes has been used to detect loci under selection (Goulson, 1993; Dhuyvetter *et al.*, 2004; Haag *et al.*, 2005; Karl *et al.*, 2009; Vandewoestijne & Van Dyck, 2010). Candidate loci can be discovered if their pattern deviates from the general allozyme pattern, which is then assumed to be neutral. Alternatively the allozyme pattern can be compared with that of a more neutral DNA marker to detect candidate loci. Both these approaches assume that deviations from the 'neutral' pattern are the result of selection. Once such a locus is discovered, experiments can then further investigate the selective value of the allelic variants (Watt *et al.*, 1983; Watt *et al.*, 1985; Goulson, 1993; Hanski & Saccheri, 2006; Wheat *et al.*, 2006; Dahlhoff *et al.*, 2008; Saastamoinen & Hanski, 2008; Niitepõld *et al.*, 2009; Orsini *et al.*, 2009; Karl *et al.*, 2010).

1.5 VOLTINISM

In seasonal systems, suitable times for growth, development and reproduction are followed by unfavourable periods that are often bridged with a diapause stage (Danilevskii, 1965; Roff, 1983; Stearns, 1992). For insects living in such systems it is therefore necessary for them to regulate their life cycle and use reliable cues about the environment and translate them into a development strategy (Gotthard & Nylin, 1995). There often is a relationship between the length of the favourable period and the number of generations (= voltinism) (Roff, 1983; Stearns, 1992). In such temperate climates, butterflies can overwinter either as larvae, pupae or adults, and the stage adopted is usually species specific, so it is important to ensure that the appropriate development stage coincides with the beginning of winter (Roff, 1983). Developing larvae are therefore expected to use reliable environmental predictors to 'calculate' the time left until the unfavourable season and 'choose' between direct development to yield a new generation or to spend the unfavourable season in a resting state, diapause (Danilevskii, 1965). The most reliable end-of-season cue for butterflies in seasonal environments resulting in faster development or diapause is photoperiod (Nylin *et al.*, 1989; Leimar, 1996; Burke *et al.*, 2005). Often a combination of photoperiod and temperature signals will induce diapause: short photoperiods and low temperatures generally induce diapause and long photoperiods and high temperatures promote direct development (Danilevskii, 1965; Burke *et al.*, 2005).

The northernmost and southernmost part of a species range will differ in daylength and temperatures and thus in seasonal length. Cues for development speed and timing of diapause are therefore expected to differ within a species' range, with direct consequences for voltinism with a tendency for two or more generations in the south and only one generation in the north. This is a consequence of a decrease in season length with latitude, only allowing for one generation in the more northern latitudes. Generally there will then be a decrease in

development time (speeded development) with latitude, resulting in a decrease in size (Roff, 1980; Roff, 1983). However, because of a shift in voltinism, at latitudes where a second generation is only just possible, the available time for only a single generation is much higher. As a consequence, a plot of development time along a gradient of season length from south to north will, corresponding to life-history models, produce a 'saw-tooth' pattern (Roff, 1983; Nylin & Svard, 1991). The generally strong correlation between development time and body size results in a similar pattern when size is plotted against latitude (see 1.2.2). This theory has some support from studies of butterflies (Nylin & Svard, 1991; Burke *et al.*, 2005) and crickets (Masaki, 1978; Mousseau & Roff, 1989b).

The Common blue (*P. icarus*) occurs with more than one generation (multi-voltine) over its whole range, except for the northernmost part, where it is univoltine (Emmet & Heath, 1989; Asher *et al.*, 2001). In the southern border of their distribution, Common blue butterflies are at least trivoltine where a large proportion bridge the hot periods of the year in a diapause state as eggs or larvae (aestivation) (Tolman & Lewington, 1997). These voltinism patterns are not fixed however, as in climatically favourable years, *P. icarus* can produce a partial third generation in the south of its distribution within the British Isles and up to two in the north (Brakefield & Shreeve, 1992). This suggests that voltinism is plastic rather than being genetically determined, in which case the transition zone for single and double brooded populations may shift northwards in response to climate change (Asher *et al.*, 2001).

1.6 SUMMARY AND GENERAL AIMS

Current trait variation in organisms is the result of past selection pressures and colonisation events. Using a common and widespread butterfly species, *Polyommatus icarus*, allows for investigating general patterns of species' responses to environmental change. This species is generally very common, but their numbers can locally fluctuate from year to year (pers. obs.). Whether or not a species is common can therefore quickly change (Shreeve *et al.*, 1996), and multiple bottlenecks could leave a very common species genetically quite impoverished. In an increasingly fragmented landscape, organisms will have more difficulties tracking environmental change and the amount of variation (evolutionary potential) becomes more important. Maintenance of (genetic) variation therefore determines a species' persistence in the light of current environmental change.

Polyommatus icarus is an extremely variable species which occurs in a large variety of environments and is distributed across most of the Palaearctic (Frohawk, 1934; Emmet & Heath, 1989; Asher *et al.*, 2001). The importance of morphology, thermoregulation and interpretation of developmental cues means that these traits are expected to vary between populations. Within the British Isles, variation between two populations on the extremes of a latitudinal cline has previously been identified and related to selection in relation to mate-attraction, requirements for flight and potentially predator avoidance (Howe, 2004; Howe *et al.*, 2007). However, with only two populations the interpretation of these differences is rather uncertain. Studying variation in traits along a latitudinal cline allows for an interpretation in relation to environment (Kraushaar *et al.*, 2002; Demont & Blanckenhorn, 2008; Nygren *et al.*, 2008). Further experimental work on the variation in these traits allows for a functional interpretation and can place the field variation in context. The recent finding of strong selection on allozymes in several butterfly species (Watt *et al.*, 1985; Carter & Watt, 1988; Goulson, 1993; Hanski & Saccheri, 2006; Karl *et al.*, 2008; Vandewoestijne & Van Dyck, 2010) and the high level of

enzyme polymorphisms in Common blue butterflies (Schmitt *et al.*, 2003), indicates that there are possibilities for clinal variation in allozyme loci in *P. icarus*.

This project will therefore investigate for *P. icarus* in the British Isles:

- Clinal variation in wing morphology in the field, with special attention to size, shape and melanisation (Chapter 3).
- Influence of wing characteristics, behavioural traits and allozyme variation on thermal characteristics (i.e. heating and cooling rate; take-off temperature) (Chapter 4).
- Latitudinal variation in allozymes along with a DNA marker to detect candidate loci for selection (Chapter 5).
- Variation in developmental response to photoperiod and temperature cues between populations (Chapter 6).

Chapter 2 General methods

2.1 THE COMMON BLUE BUTTERFLY *Polyommatus icarus* (Rott.)

The Common blue butterfly was first described by Rottenburg (1775) as *Papilio icarus*, a grassland butterfly with a pronounced sexual dimorphism. Nearly thirty years later *Papilio icarus* was added to the Polyommatinae and renamed *Polyommatus icarus* by Latreille (1802). In 1973, Elliot proposed, on the basis of the male genitalia, to add the *Polyommatus* section in his higher classification of the Lycaenidae. Bálint and Johnson (1997) made the classification of the *Polyommatus* genus more stable by integrating female genitalia and shared wing characters (Figure 2.1).

Figure 2.1 Phylogeny of butterflies and skippers with indication of the *Polyommatus* genus (blue) (Redrawn from Wahlberg *et al.*, 2005).

The most typical of these shared wing characteristics is the amount of blue wing colouring, which occurs in all Lycaenid butterflies except for *Aricia* and *Agrodiaetus* species. The species under study, *Polyommatus icarus*, shows considerable variation within and between populations. This variation has led to descriptions of two subspecies within the British Isles: *P. icarus icarus* in most of Britain and subsp. *marsicolore* in Ireland (Kane 1893), but the latter also being described as occurring in northern and western Scotland (Thomson, 1980). This distinction is not, however, generally accepted (Higgins & Riley, 1980) and its basis is poorly defined.

Male *P. icarus* generally have a blue upperside wing colouring while the upper wing colour of females can vary from deep dusky-brown to completely blue (Frohawk, 1934; Figure 2.2). The spotting pattern on the underside of the wings is subject to extreme variation, with some individuals totally lacking spots except for the outer marginal spots. This pattern seems to act as a species recognition signal (Fordyce *et al.*, 2002) and the enormous variation probably has influenced the species-richness of the group of the Lycaenidae (Robbins, 1982).

Polyommatus icarus is a relatively small butterfly species with a wingspan between 29 and 36mm (Emmet & Heath, 1989). First generation butterflies tend to be larger than second generation butterflies (Tolman & Lewington, 1997) and within the British Isles, the more northern populations in Scotland and Ireland comprise larger individuals compared to the south (Frohawk, 1934).

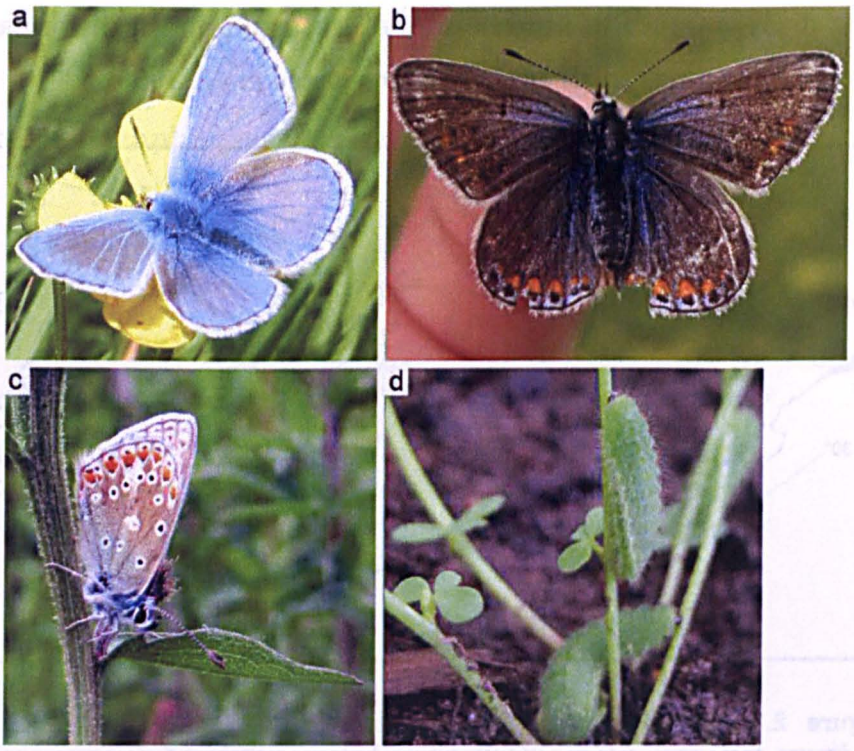

Figure 2.2 ***Polyommatus icarus*****: a) male; b) female; c) male underside; d) larvae**

Larvae of *P. icarus* (Figure 2.2) feed on different plants of the Fabaceae family with Bird's-foot trefoil *Lotus corniculatus* being the major food plant (Emmet & Heath, 1989). The eggs are circular, only 0.6 mm in diameter and are laid singularly on young shoots of the chosen food plant (Frohawk, 1934) or on or close to the flowers (Janz *et al.*, 2005). Directly developing larvae reach pupation within about 45 days, while those that overwinter live for 270 days (Frohawk, 1934). Overwintering larvae hibernate on the lower stems of their food plants or at ground level amongst litter (Frohawk, 1934; Asher *et al.*, 2001).

The distribution of *P. icarus* covers nearly the whole of the Palaearctic region from sea level to up to 2700m (Tolman & Lewington, 1997; Figure 2.3), where it usually occurs in rough, flowery grasslands (Frohawk, 1934). It tends to live in discrete colonies that can locally reach high numbers (Emmet & Heath, 1989; Asher *et al.*, 2001). Like most butterflies, *P. icarus* is in decline (Fox *et al.*, 2006), mainly caused

by a disappearance of semi-improved grassland and transformation of coastal dune areas to other uses (Leon-Cortes *et al.*, 1999).

Figure 2.3 Distribution of Polyommatus icarus in the Palearctic region (redrawn from Artem'eva, 2006). The phylogeography of *P. icarus* and close relatives is unresolved and different subspecies have been described across the whole range based on morphology (for details see Artem'eva, 2006). Indicated in grey is the area were the subspecies icarus occurs.

2.2 MAIN STUDY SITES

Most field sampling was conducted in four sites (Figure 2.4).

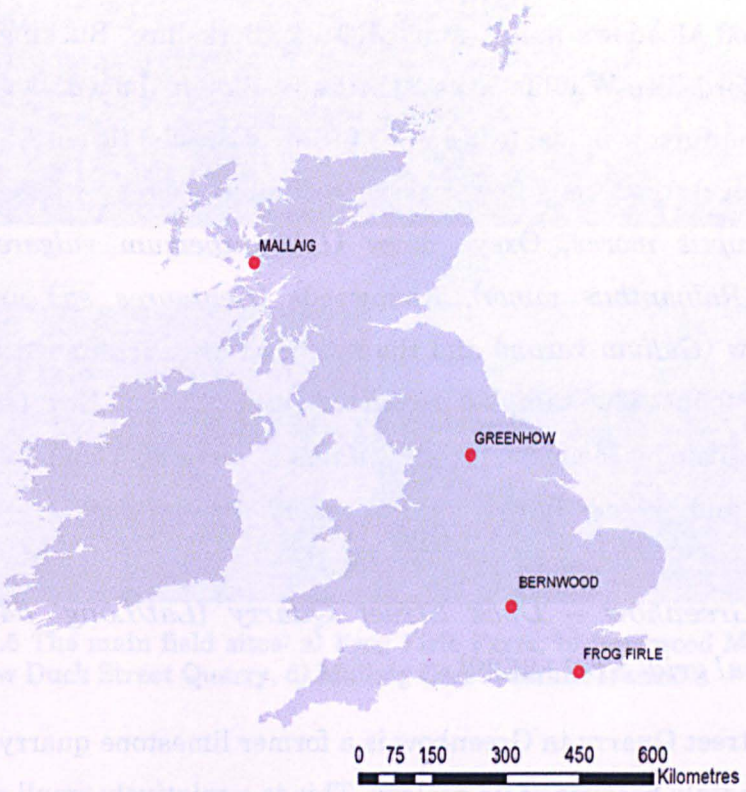

Figure 2.4 The location of the four major field sites used in this project.

2.2.1 Frog Firle Farm (Lat/Long: 50.79/0.14; National grid: TQ 508014)

Frog Firle Farm is a National Trust site of 50 ha located in East Sussex on the South Downs. This is part of the large chalk ridge that stretches from Eastbourne to Winchester and is characterised by typical chalk grassland vegetation including Musk orchid (*Herminium monorchis*), Honeysuckle (*Lonicera caprifolium*) and Marjoram (*Origanum majorana*) (Figure 2.5a). Key Lepidoptera species on this site are Silver-spotted skipper (*Hesperia comma*), Adonis blue (*Polyommatus bellargus*) and Chalk carpet moth (*Scotopteryx bipunctaria*) (Kemp, 2006).

2.2.2 Bernwood Meadows (Lat/Long: 51.80/-1.12; National grid: SP 606111)

Bernwood Meadows make up a BBOWT (Berkshire, Buckinghamshire and Oxfordshire Wildlife Trust) nature reserve managed as traditional ridge-and-furrow fields. It is an SSSI (Site of Special Scientific Interest). The vegetation is flower-rich including Green-winged orchid (*Anacamptis morio*), Oxeye daisy (*Leucanthemum vulgare*), Yellow rattle (*Rhinanthus minor*), Knapweeds (*Centaurea sp.*) and Lady's bedstraw (*Galium verum*) and the meadows are surrounded by hedges (Figure 2.5b). The sampled meadows cover 3.5 ha. Key Lepidoptera species include Meadow brown (*Maniola jurtina*), Common blue (*P. icarus*), and Six-spot burnet moth (*Zygaena filipendulae*).

2.2.3 Greenhow – Duck Street Quarry (Lat/Long: 54.07/-1.83; National grid: SE 113639)

Duck Street Quarry in Greenhow is a former limestone quarry and is an SSSI, mainly because of its geology. This is a relatively small site (5 ha), located in North-Yorkshire at a high altitude (at it highest point it is 428 m above sea level) and is characterised by typical limestone vegetation with Sheep's fescue (*Festuca ovina*) and Spring sandwort (*Minuartia verna*) (Figure 2.5c). Key Lepidoptera species include Common blue (*P. icarus*), Meadow brown (*Maniola jurtina*) and Ringlet (*Aphantopus hyperantus*) (Barnham, 2006).

2.2.4 Mallaig – Camusdarach dunes (Lat/Long: 56.96/-5.85; National grid: NM 661916)

These dunes are located on the west coast of north Scotland and are characterised by a dense vegetation of Marram grass (*Ammophila arenaria*) including also Harebell (*Campanula rotundifolia*) and Gorse (*Ulex europaeus*) (Figure 2.5d). The part of the dunes that was sampled for this study was a short narrow stretch of ca. 1.5 ha. Key lepidoptera species include Scotch argus (*Erebia aethiops*), Grayling (*Hipparchia semele*) and Speckled wood (*Pararge aegeria*).

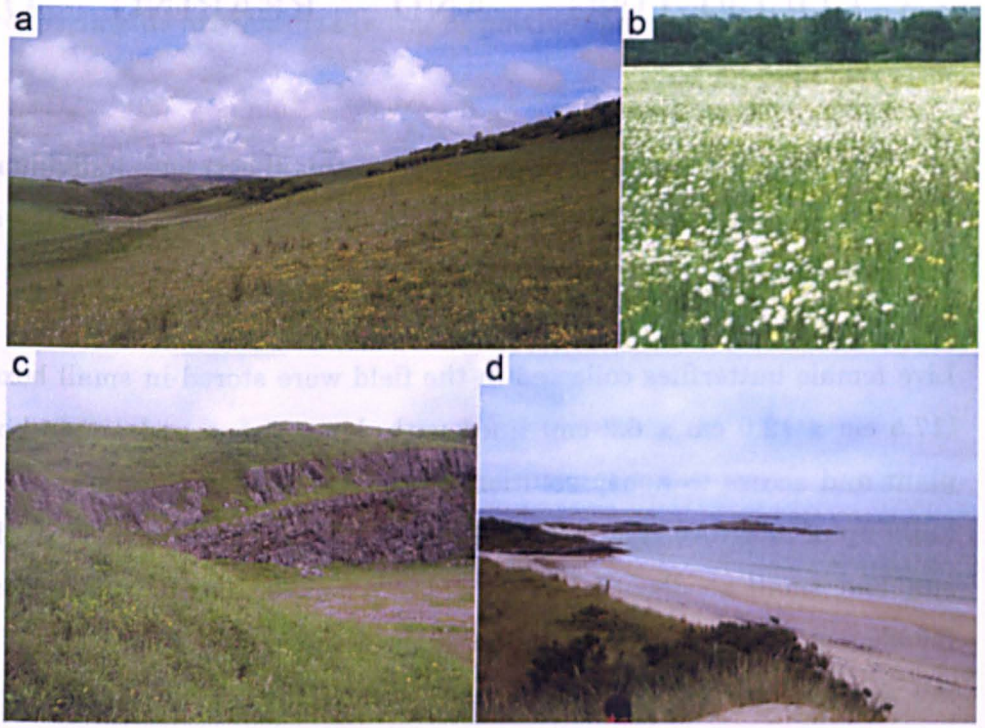

Figure 2.5 The main field sites: a) Frog Firle Farm, b) Bernwood Meadows, c) Greenhow Duck Street Quarry, d) Mallaig Camusdarach Dunes.

2.3 COLLECTING AND REARING OF INDIVIDUALS

Most of the experimental work presented in this thesis uses individuals reared from females collected in the field and a standard protocol was developed for this procedure.

Live female butterflies collected in the field were stored in small boxes (17.5 cm x 12.0 cm x 6.2 cm) lined with damp tissue and ample host plant and access to honey solution. Nets were used as lids to prevent build up of moisture (Figure 2.6). Boxes were placed in the field in the sun and small cardboard lids provided a shaded shelter place. One female was placed in each box and the butterflies were allowed 2 days to lay eggs on the available host plant, which was collected after this period. Females were then frozen at -80°C for further enzyme analysis. The eggs were isolated from the majority of plant material and stored in petri dishes with a damp filter paper to avoid desiccation of the eggs.

Figure 2.6 Egg-laying boxes with female *P. icarus* butterflies in one of the field sites.

As soon as the eggs hatched, the first instar larvae were transferred with a tiny paintbrush onto a single potted *Lotus corniculatus* host plant. Rearing temperature and photoperiod were specific for each experiment and details are given in the relevant chapters. Plants were checked regularly and replaced when necessary to provide ample food for the larvae.

The precise methods and data analysis procedures for the different experiments are detailed in the respective chapters.

Chapter 3 Morphological variation of *Polyommatus icarus* butterflies in the British Isles

3.1 INTRODUCTION

Wing shape, size and colour are key characteristics that influence apparency to conspecifics, conspicuousness to predators and thermoregulation in butterflies (Dennis & Shreeve, 1989; Brakefield & Shreeve, 1992). Variation in wing size and shape can influence flight characteristics (Betts & Wootton, 1988; Dudley, 2000) and the degree of wing melanisation strongly influences rates of heat gain and loss (Watt, 1968; Kingsolver, 1996; Clusella-Trullas *et al.*, 2007). Morphological variation in these wing characteristics is therefore expected between different (selective) environments. Reactions to these environmental selection pressures can be genetic (local adaptation) or flexible (plasticity) (Via & Lande, 1985). Plasticity in traits would be promoted in variable environments; fixed environmental conditions could lead to local adaptation.

Rounder wings may provide more sustained and faster flight than angular wings, with the latter providing greater acceleration, but more energy expensive flight (Betts & Wootton, 1988). The study species, *Polyommatus icarus*, has more rounded wings in Scotland than in the south of England (Howe, 2004). These differences were described by Howe (2004) as being consistent with the need for more prolonged flights for mate location and resource finding in lower density populations and/or cooler conditions in the north. *Polyommatus icarus* butterflies also show a negative size cline with latitude in Sweden (Nygren *et al.*, 2008) presumably as a result of shortening seasons with latitude (Nylin & Svard, 1991; Blackenhorn & Demont, 2004). Shorter seasons reduce the available development time often resulting in smaller individuals (Atkinson *et al.*, 1994; Davidowitz & Nijhout, 2004).

Where there is a shift from a bivoltine to univoltine strategy there is an increase in size according to a "saw-tooth-pattern" because development time per generation is limited at the northern limit of the bivoltine strategy, but less constrained by time at the southern edge of the univoltine strategy (Roff, 1980; Roff, 1983). At their most northern latitudes in both the British Isles and Sweden, *P. icarus* is significantly larger than in more southern populations (Howe, 2004; Nygren *et al.*, 2008). It has also been suggested that this increase in size in the north may also be the result of a two-year life cycle (Johansson, 2003; Wipking & Mengelkoch, 1994; Nygren *et al.*, 2008).

As well as latitudinal differences in size, there are often size differences between generations in bivoltine populations. Higher temperatures usually promote faster development and smaller adults (Atkinson *et al.*, 1994; Atkinson & Sibly, 1997; Kingsolver *et al.*, 2004), which explains why second generation butterflies, which develop over the warm summer period are often smaller than spring butterflies which develop during the autumn-spring period. However, in some species the opposite pattern has been found and the differences have been related to different dispersal strategies between generations (Windig & Lammar, 1999; Fric & Konvicka, 2002; Fric *et al.*, 2006). Second generation *P. icarus* butterflies are usually smaller than those of the spring generation (Howe, 2004).

The degree of wing melanisation can affect rates of heat gain and have an important function in thermoregulation and therefore in the activity patterns of butterflies (Watt, 1968; Kingsolver, 1996; Clusella-Trullas *et al.*, 2007). Previously, patterns in wing melanisation have been related to latitudinal and seasonal thermal differences in several butterfly species and are related to the basking strategy (see Chapter 1). *Polyommatus icarus* uses a combination of basking strategies (Shreeve, 1992) and the influence of melanisation on thermal characteristics is therefore not entirely clear.

Male *P. icarus* generally have a blue upperside wing colouring while the upper wing colour of females can vary from deep dusky-brown to completely blue (Frohawk, 1934). In colder locations and seasons females, but not males, of this butterfly generally have brighter coloured wings (Howe, 2004), which was interpreted as an indication that this butterfly is a reflectance basker, with cooler northern conditions selecting for traits that increase heating rates (Howe, 2004).

In the Lycaenidae there are two scale types, basal scales and cover scales which are responsible for their colour, which is the total reflected light spectrum (Ingram & Parker, 2008). Cover scales can give rise to iridescent colours, which is often referred to as a structural colour. Basal scales are melanised and lack the physical structure to reflect blue light and over time this melanin degrades due to exposure to UV. Thus the visible colour of Lycaenids appears to become less intense over time as the capacity of the basal scales to prevent back reflection of light of a range of wavelengths decreases over time (Ghiradella, 1998); therefore the colour reflected by newly emerged specimens is of narrower wavelength range than that produced by older specimens (i.e. it is purer).

Cover scales are also variable in Lyceanids and they differ in their nano-structure. Cover scales have ridges, and cross members between the ridges and can have a complex internal structure, often termed a pepper-pot structure, which may be multilayered, acting as a photonic crystal which can reflect short wavelength light (e.g. blue) (Bálint *et al.*, 2004; Kertész *et al.*, 2006). The size of the holes and the architecture of the pepper-pot structure determine which wavelengths are reflected – this can vary from orange/red to uv/blue across the Lycaenids. Those scales that lack the pepper-pot structure do not reflect blue light and light is absorbed by melanin within the scales (Bálint *et al.*, 2004). Increased proportions of scales lacking this pepper-pot structure is of thermoregulatory significance, with the proportion of scales lacking this pepper-pot structure increasing in some Lycaenids with altitude and latitude (Biró *et al.*, 2003).

When examined under a light microscope the wings of *P. icarus* are covered by 'blue' cover scales and 'brown' basal scales and the proportion of these varies between individuals and the sexes (Howe, 2004). To date there has been no detailed study of variation in the architecture of scales in *P. icarus* and there is no available information on variation in melanin deposition within scales, but for the sake of argument increasing proportions of brown scales is termed melanisation within this study. This terminology is the same as that adopted by Watt (1968) who termed an increase in the proportion of dark scales in *Colias* butterflies as melanisation.

Polyommatus icarus is geographically and seasonally variable in wing shape, colouration and size and it is sexually dimorphic (Frohawk, 1934; Emmet & Heath, 1989; Asher *et al.*, 2001; Howe, 2004). Studies of wing morphology on other species have related such differences to the environment experienced during development and to selection pressures (Watt, 1968; Guppy, 1986; Ellers & Boggs, 2002; Ellers & Boggs, 2004; Roland, 2006; Clusella-Trullas *et al.*, 2007; Kingsolver & Wiernasz, 1991; Kingsolver, 1995; Atkinson & Sibly, 1997; Blackenhorn & Demont, 2004; Stoehr & Goux, 2008). Morphological variation in *P. icarus* has been identified before in two populations at the extremes of a cline within the UK (Howe, 2004; Howe *et al.*, 2007). The environmental conditions vary along a south-north cline and seasonally within the UK and therefore, because of the importance of flight, variation in wing size, shape and melanisation is expected (see below). Further rearing experiments (Chapter 6) will investigate plastic responses and a study of the population genetic structure (Chapter 5) will identify the degree of isolation of the different populations and thus the potential for local adaptation.

This chapter will therefore investigate in *P. icarus*

(1) whether latitudinal variation in size is in accordance with the predictions of a saw-tooth cline for a species with a latitudinal shift in voltinism.

(2) whether shape differences vary along a latitudinal cline and whether these differences can be related to variation in environmental parameters along this cline

(3) whether there is an increase in melanisation along a latitudinal cline in accordance with the butterfly being an absorbance basker

(4) seasonal variation: Are summer butterflies smaller because of the shorter development time? Are spring butterflies more melanised because of colder temperatures? Do the more active summer butterflies have more pointed wings for more agile flight?

3.2 MATERIAL & METHODS

3.2.1 Sample collection and preparation

Field samples were collected during the flight peaks of *P. icarus* in each of four locations (Table 3.1). The four main study sites were used (see Chapter 2) and in the sites with bivoltine populations (Frog Firle and Bernwood) samples were taken both in spring and summer, to enable a comparison between these two generations. During each sampling period butterflies were collected in a range of weather conditions and times of day to encompass variation in the population and reduce the chance of sampling being biased towards specific phenotypes if weather related activity is linked to phenotype. Individuals were kept alive in envelopes until they were transferred to a freezer (-80°C). Subsequently, wings were removed (bodies were retained for molecular studies: see Chapter 5) and photographed in a dark room with the specimen illuminated with a fibre optic ring light (90 Watt). Images were taken with a Nikon D1 (ISO 200, F8, exp: 1/320s) at a fixed focal length of 58 cm. All samples were photographed on a black and white background to allow for standardising colour measurements.

Table 3.1 Sample sites, latitudes, acronyms, sampled dates and sample sizes (n).

Site	Latitude	Acronym	Date collected	n (males)	n (females)
Frog Firle	50.791	FROG1	7-9 June 2010	34	24
		FROG2	9 August 2010	39	25
Bernwood	51.795	BERN1	14-16 June 2010	36	22
		BERN2	5-8 August 2010	56	31
Greenhow	54.071	GREE1	13-14 July 2010	30	22
Mallaig	56.958	MALL1	22-25 July 2010	19	15

3.2.2 Quantifying shape and size variation and melanisation in butterfly wings

Shape and size

Differences in shape were quantified using geometric morphometrics (Goodall, 1991; Klingenberg & McIntyre, 1998; Breuker *et al.*, 2010). The advantage of this method is that wing shape is treated as a multivariate trait, rather than a univariate trait such as wing length or aspect ratio. This allows for the detection of all aspects of shape variation. Centroid size was used as a measure for wing size and this is calculated as the square root of the sum of squared distances from a set of landmarks to the centroid (i.e. mean x and y coordinate of a set of landmarks per individual; see Klingenberg & McIntyre, 1998). As asymmetry *per se* is not of interest in this study, both wing size and shape were determined as the average of the left and right wing.

Eight landmarks were digitised on the ventral wing surface (see Figure 3.1) in ImageJ v 1.43 (Rasband, 1997-2011). Landmarks were chosen in such a way that an overall outline of the wing shape could be obtained (Breuker *et al.*, 2010). To remove all non-shape variation, the landmark configurations of several individuals were then superimposed. This was done in four steps to remove all non-shape variation as follows: 1) reflection of either left or right configurations (i.e. so left and right were orientated the same way), 2) scaling to unit centroid size (to remove size and shape associations), 3) superimposing the centroids of all configurations, and finally 4) rotation of the configurations around their centroid to obtain the optimal alignment.

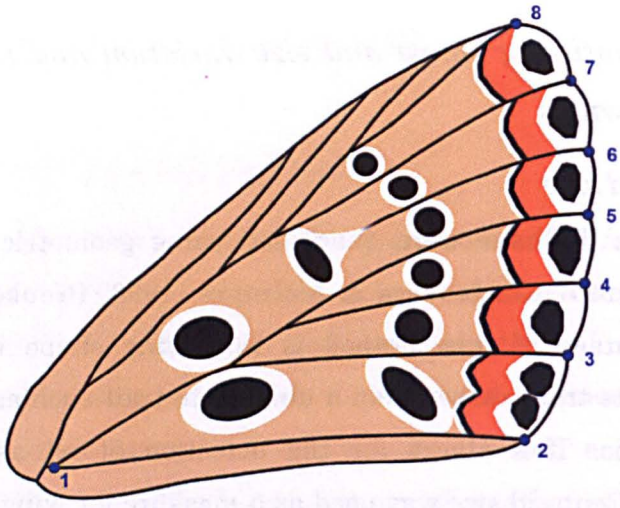

Figure 3.1 Schematic representation of the positions of the 8 landmarks on forewings

Basal melanisation

The brightness of the basal part of both upper forewings was used as a measure for basal melanisation (Talloen *et al.*, 2004; Figure 3.2), as this area is most likely to be important in thermoregulation (Wasserthal, 1975). The grey value (the sum of the grey values of all the pixels in the selection divided by the number of pixels) of an area of 100 pixels was measured. To standardise between the photos, the colour (RGB) was first rescaled in Adobe Photoshop (CS5) using the "match colour" command (which does not affect brightness), adjusting the colour of samples by matching to a standard area of background. To further standardise brightness values between samples, a melanisation index was calculated as:

$$1\text{-}(\text{Wing} - \text{Black})/(\text{White} - \text{Black})$$

with Wing = wing measurement, Black = measurement of the black background, White = measurement of the white background. All measurements were on brightness scale of 0 (black) to 255 (white).

This adjustment converts absolute values to a proportionate value of the total brightness scale for each image and reduces the chance of any small changes in lighting conditions affecting brightness estimates of wing melanisation. The average index value of left and right wing was then taken as a melanisation value for the individual.

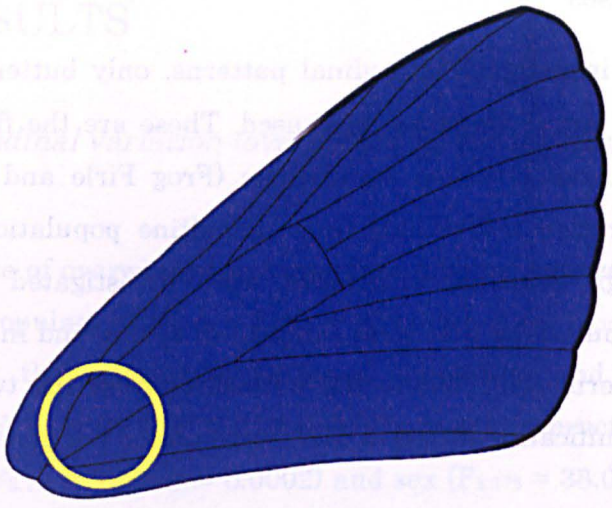

Figure 3.2 Schematic representation of the forewing of a male *P. icarus*. The colour of the basal part of the wing is measured as the mean grey value of the area in the yellow circle (selection brush size 100 pixels).

Error estimation

To estimate measurement error the left and right wings of 31 individuals were photographed and digitised twice (resulting in 4 photos or 8 measurements per individual). Measurement error was negligible as the variation between individuals is much larger than between imaging or digitising ($P < 0.0001$; Table 3.2).

Table 3.2 Error analysis for centroid size, shape and melanisation for 31 repeated individuals. Imaging error is the error of photographing the wings and residual error is the error due to digitising the landmarks.

SIZE					
Source	SS	MS ($x10^2$)	df	F-ratios	P-values
Individual	116.953	389.84	30	67.21	<0.0001
Imaging error	1.798	5.8	31	5.76	<0.0001
Residual	1.873	1.01	186		
SHAPE					
Source	SS	MS ($x10^6$)	df	F-ratios	P-values
Individual	0.1326	368.27	360	43.52	<0.0001
Imaging error	0.0032	8.46	372	0.7	1
Residual	0.0269	12.05	2232		
MELANISATION					
Source	SS	MS ($x10^3$)	df	F-ratios	P-values
Individual	3.45963	115.32	30	31.488	<0.0001
Imaging error	0.16117	5.2	31	1.42	0.082
Residual	0.6812	3.66	186		

3.2.3 Analyses

In order to investigate latitudinal patterns, only butterflies that had experienced larval diapause were used. These are the first generation individuals from bivoltine populations (Frog Firle and Bernwood) or single generation individuals from univoltine populations (Greenhow and Mallaig). Seasonal differences were investigated with the two bivoltine populations that were sampled in spring and in summer (Frog Firle and Bernwood). Where the butterflies from the two populations were not significantly different, they were pooled per generation.

The factors explaining variation in forewing size and melanisation were analysed using general linear models (GLM). Population, generation and sex were used as fixed factors. Significant effects were further examined with Tukey post hoc tests. To investigate the expected latitudinal trends, the analyses were repeated with latitude treated as a continuous predictor. For example, at the northern latitudes a smaller size and a higher degree of melanisation were expected. All data were checked for normality using the Shapiro-Wilk's normality test in R 2.11.1 (R Development Core Team, 2010).

Wing shape differences between populations, generations and sexes were quantified with Procrustes Distances. This measure is calculated as the square root of the sum of squared distances between corresponding landmarks of two wing shapes (Klingenberg & McIntyre, 1998). Discriminant Function (DFA) and Canonical Variate Analyses (CVA) were performed in MophoJ v 1.02d (Klingenberg, 2011) to investigate differences in wing shape between the sexes, populations and seasons. There is often a relationship between size and shape of body parts in animals (Huxley, 1932; Gould, 1966), therefore analyses were executed with the residuals of the regression of shape on centroid size to remove effects of allometry (Monteiro, 1999).

3.3 RESULTS

3.3.1 Latitudinal variation (overwintering butterflies)

Size

Forewing size of overwintering butterflies differed between populations and sexes (Population: $F_{3,170} = 8.40$, $P < 0.0001$; Sex: $F_{3,170} = 34.89$, $P < 0.0001$), but the interaction between population and sex was not significant ($F_{3,170} = 2.17$, $P = 0.094$). Without the interaction term both population ($F_{3,173} = 7.08$, $P = 0.0002$) and sex ($F_{1,173} = 38.02$, $P < 0.0001$) remained significant. *Post hoc* tests for pairwise differences between the populations showed that butterflies from Mallaig had significantly larger forewings compared to butterflies from the other three populations (Tukey HSD: Bernwood: $P = 0.0003$; Frog Firle: $P = 0.0019$; Greenhow: $P = 0.01$; Figure 3.3a), but all other populations did not differ ($P > 0.5$). With latitude treated as a continuous variable, the interaction effect between sex and latitude was significant ($F_{1,174} = 4.33$; $P = 0.039$) as well as the main effects of latitude ($F_{1,174} = 18.78$, $P < 0.0001$) and sex ($F_{1,174} = 5.46$, $P < 0.021$). Females within populations were smaller than males (Frog Firle: $F_{1,51} = 14.22$, $P < 0.0004$; Bernwood: $F_{1,43} = 17.92$, $P = 0.0001$; Greenhow: $F_{1,46} = 8.84$, $P = 0.0047$), apart from Mallaig ($F_{1,30} = 1.07$, $P = 0.308$). There was a general increase in size with latitude ($r^2 = 0.063$, $P = 0.0007$, Figure 3.3a), but with the sexes separated (Figure 3.3b), there was no significant relationship for males ($r^2 = 0.029$, $P = 0.0777$), but there was for females ($r^2 = 0.196$, $P = 0.0001$).

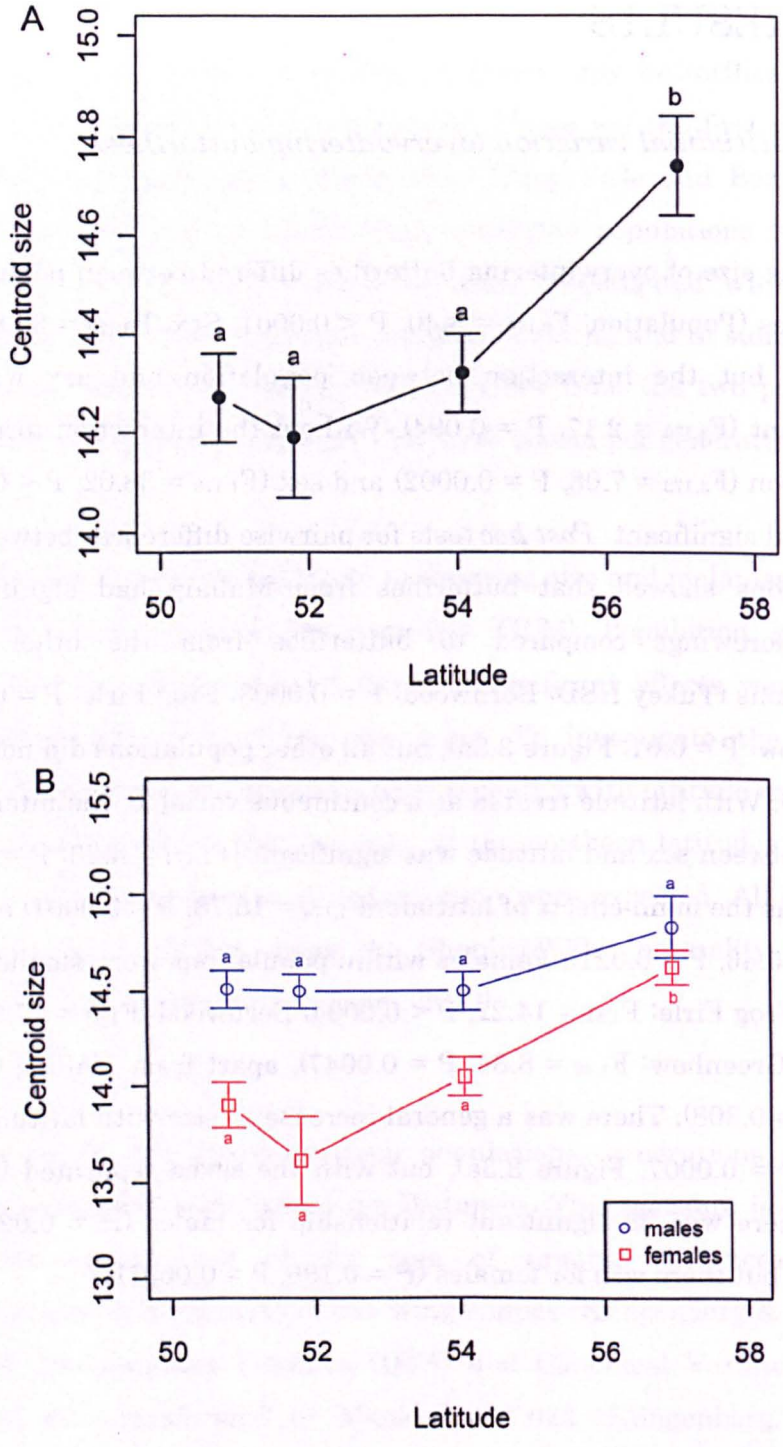

Figure 3.3 Relationship between size and latitude with (A) the sexes together and (B) separated. Plot shows means and standard errors for males (blue) and females (red). Populations are (from south to north): Frog Firle (*Lat: 50.79*), Bernwood (*Lat: 51.80*), Greenhow (*Lat: 54.07*), Mallaig (*Lat: 56.96*).

Shape

The differences in forewing shape between males and females in each population are small, but significant (DFA for each population: $P < 0.01$

after 1000 permutation runs). Differences are mainly associated with landmark 1 and 8, with males having a slightly shorter and pointier wing than females (Figure 3.4).

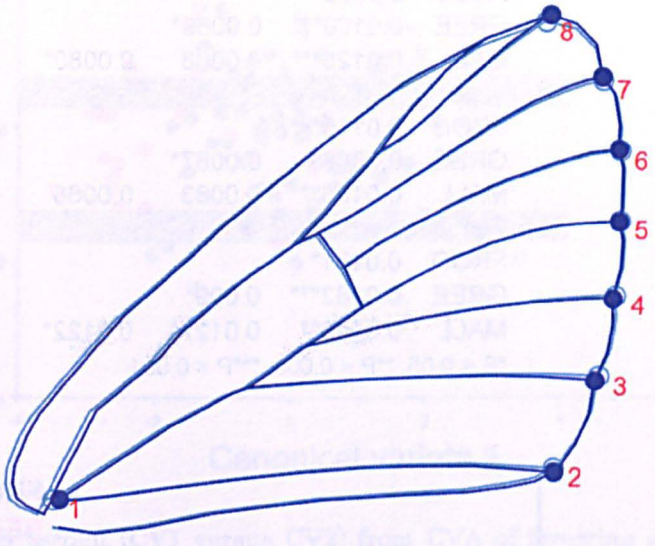

Figure 3.4 Shape differences between males and females. The light-blue shape is the average female wing and the dark blue is the average male wing. Differences in the internal vein structure are not entirely accurate as no internal landmarks were digitised.

The results of the CVA indicate that there are consistent differences in forewing shape between most populations, but that there was no difference between butterflies from Mallaig and Frog Firle (Table 3.3). The CVA was run with the sexes separately. For both sexes the differences between populations were small (Figure 3.5 and 3.6), but most pairs were significantly different (Table 3.3). For both Canonical Variates, most of the variation in shape was in the wing base and wing tip (landmarks 1 and 8; Figure 3.7). The extremes of the shape variation along each CV-axis are visualised in Figure 3.7.

Table 3.3 Procrustes distances of forewing shape between populations for males and females sampled only in spring. The P-values were calculated using 10000 random permutations.

		BERN	FROG	GREE
a	Both sexes combined			
	FROG	0.0092**		
	GREE	0.0100**	0.0069*	
	MALL	0.0128***	0.0068	0.0080*
b	Males			
	FROG	0.0110***		
	GREE	0.0086*	0.0087*	
	MALL	0.0136***	0.0083	0.0068
c	Females			
	FROG	0.0131*		
	GREE	0.0162***	0.009	
	MALL	0.0149**	0.0127*	0.0122*

*P < 0.05, **P < 0.005, ***P < 0.001

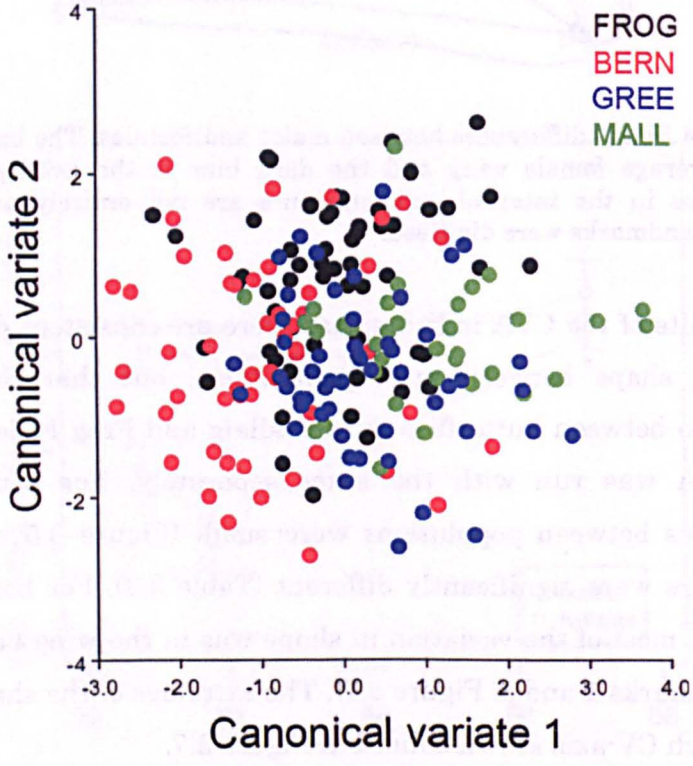

Figure 3.5 Scatterplot (CV1 versus CV2) from CVA of forewing shape of first generation males from the four sampled populations.

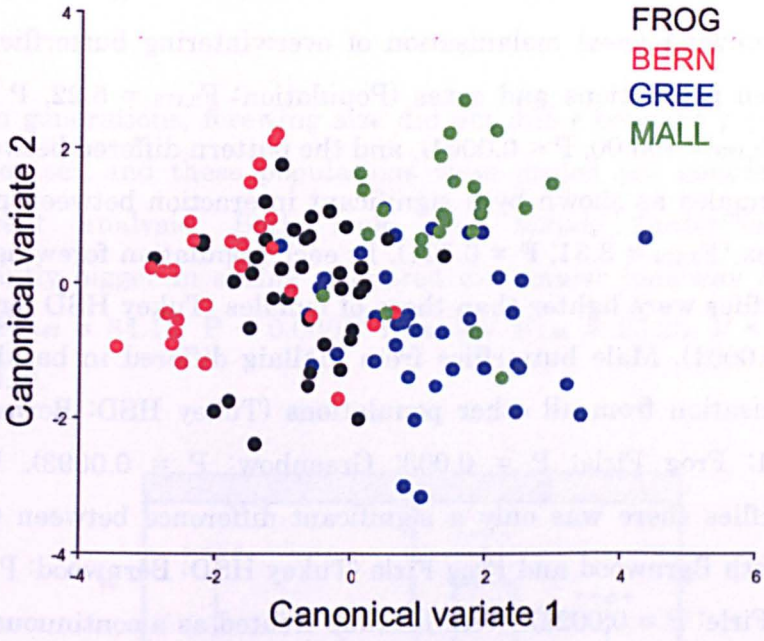

Figure 3.6 Scatterplot (CV1 versus CV2) from CVA of forewing shape of first generation females from the four sampled populations.

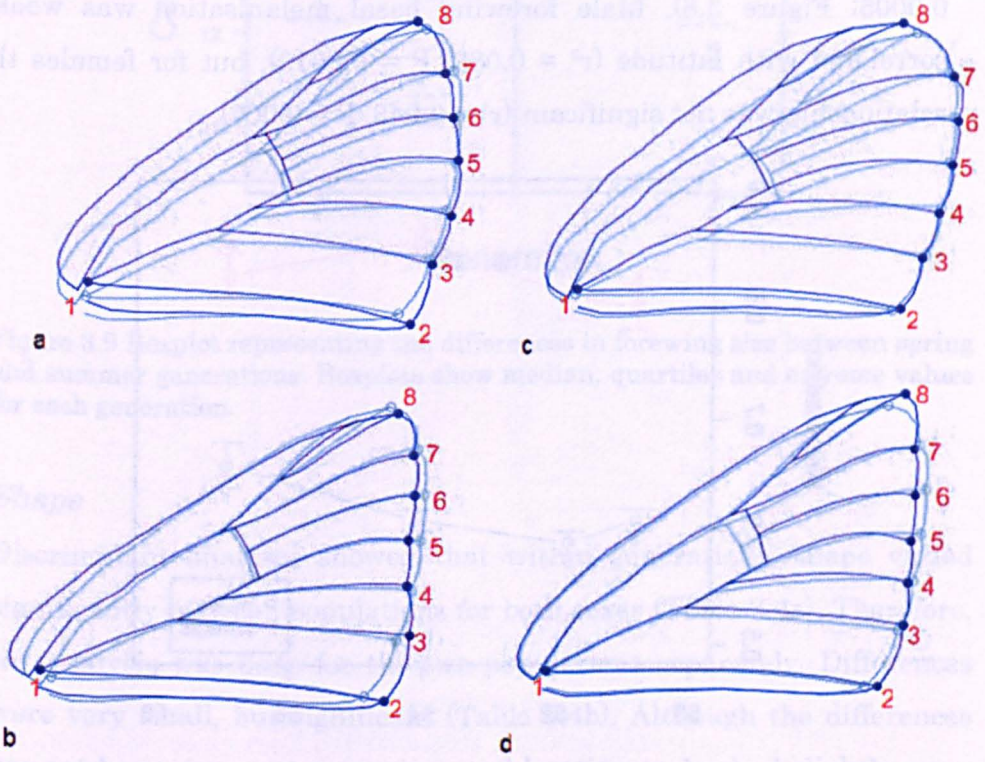

Figure 3.7 Differences in forewing shape associated with CV1 (a and c) and CV2 (b and d) for the four populations sampled in spring for males (a and b) and females (c and d). The difference between the light-blue and dark-blue shape indicate the shape differences along the CV.

Basal melanisation

The forewing basal melanisation of overwintering butterflies differed between populations and sexes (Population: $F_{3,179} = 5.22$, $P = 0.0018$; Sex: $F_{1,179} = 493.00$, $P < 0.0001$), and the pattern differed between males and females as shown by a significant interaction between population and sex ($F_{3,179} = 3.31$, $P = 0.021$). In each population forewings of male butterflies were lighter than those of females (Tukey HSD for all pairs: $P < 0.0001$). Male butterflies from Mallaig differed in basal forewing melanisation from all other populations (Tukey HSD: Bernwood: $P < 0.0001$; Frog Firle: $P = 0.008$; Greenhow: $P = 0.0093$). In female butterflies there was only a significant difference between Greenhow and both Bernwood and Frog Firle (Tukey HSD: Bernwood: $P = 0.0374$; Frog Firle: $P = 0.0026$). With latitude treated as a continuous variable, the interaction with sex was not significant ($F_{1,183} = 0.192$, $P = 0.662$). With the interaction removed, both sex and latitude were highly significant (Sex: $F_{1,184} = 514.57$, $P < 0.0001$; Latitude: $F_{1,184} = 12.60$, $P = 0.0005$; Figure 3.8). Male forewing basal melanisation was weakly correlated with latitude ($r^2 = 0.089$, $P = 0.0019$), but for females this relationship was not significant ($r^2 = 0.043$, $P = 0.065$).

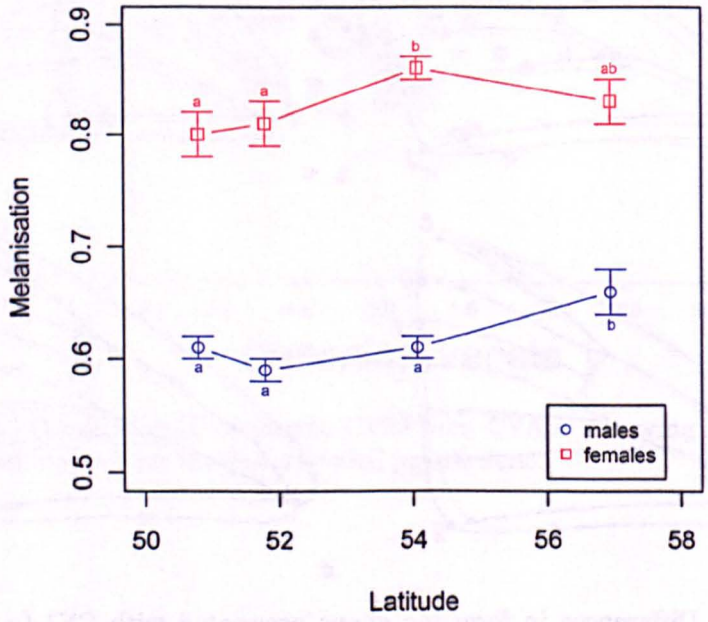

Figure 3.8 Variation in melanisation of forewings along a latitudinal cline. The mean melanisation index for both males (blue) and females (red) is shown with standard errors. Populations are (from south to north): Frog Firle (*Lat: 50.79*), Bernwood (*Lat: 51.80*), Greenhow (*Lat: 54.07*), Mallaig (*Lat: 56.96*).

3.3.2 Between generations

Size

Between generations, forewing size did not differ between populations for either sex and these populations were pooled per generation for subsequent analysis. Both male and female butterflies were significantly bigger in spring compared to summer (one-way ANOVA, Males: $F_{1,147} = 84.16$, $P < 0.0001$; Females: $F_{1,88} = 27.35$, $P < 0.0001$; Figure 3.9).

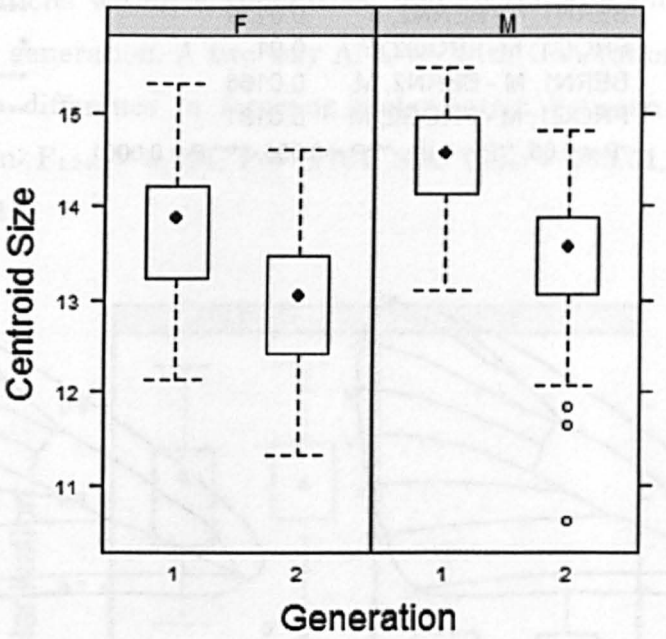

Figure 3.9 Boxplot representing the differences in forewing size between spring and summer generations. Boxplots show median, quartiles and extreme values for each generation.

Shape

Discriminant analyses showed that within generations, shape varied significantly between populations for both sexes (Table 3.4a). Therefore, the analysis was done for the two populations separately. Differences were very small, but significant (Table 3.4b). Although the differences are not large, in every generation and location males had slightly more angular wings than females, this angularity being most pronounced at the wing apex. At the two sites individuals of each sex also had narrower wings in the first generation than the second. Individuals of

both sexes from Bernwood also had slightly more angular wings than corresponding individuals from Frog Firle (Figure 3.10).

Table 3.4 Forewing shape differences for spring and summer generation butterflies as expressed by Procrustes distances. The P-values were calculated using 10000 random permutations.

	Pair	Procrustes distance	P
a	Differences within a generation between populations		
	FROG1, F - BERN1, F	0.0126	*
	FROG2, F - BERN2, F	0.0077	0.075
	FROG1, M - BERN1, M	0.011	***
	FROG2, M - BERN2, M	0.0083	****
b	Differences between generations within a population		
	BERN1, F - BERN2, F	0.0139	****
	FROG1, F - FROG2, F	0.01	*
	BERN1, M - BERN2, M	0.0166	****
	FROG1, M - FROG2, M	0.0131	****

*P < 0.05, **P < 0.01, ***P < 0.005, **** P < 0.0001

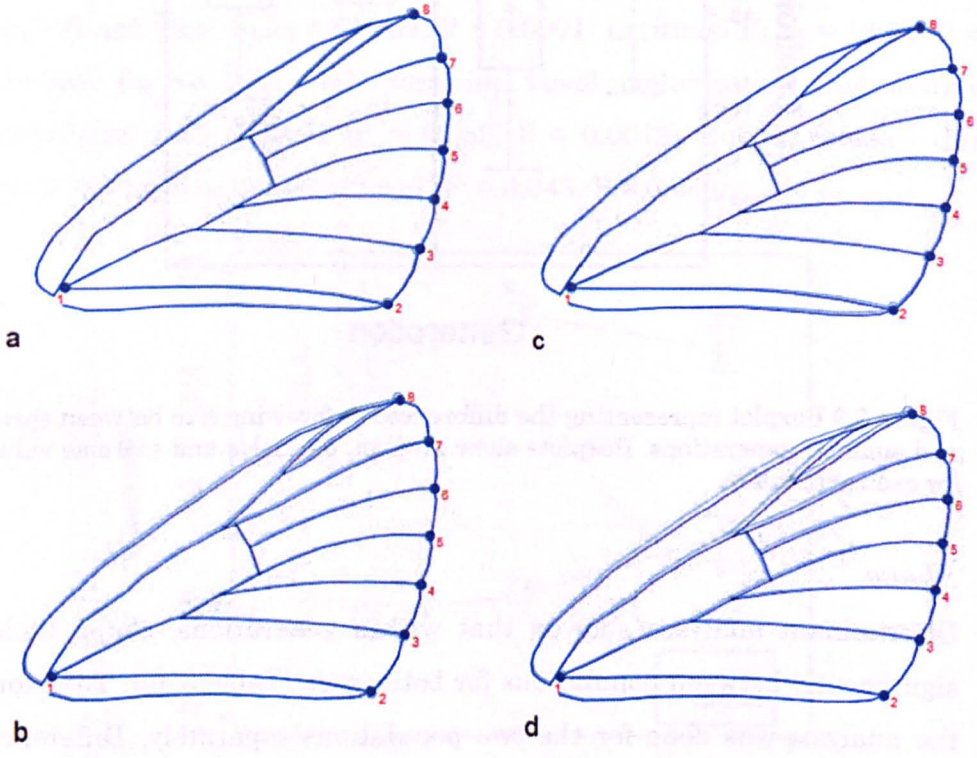

Figure 3.10 Differences in forewing shape for the DFA analysis between first and second generation males (a and b) and females (c and d) for Frog Firle (a and c) and Bernwood (b and d). The dark-blue shape is the average shape of spring generation butterflies; the light-blue shape represents the average shape of summer generation butterflies.

Melanisation

There was a significant effect of sex, but not of population, on basal melanisation (Two-way ANOVA, Sex: $F_{1,240} = 581.06$, $P < 0.0001$; Population: $F_{3,240} = 0.38$, $P = 0.765$). In both generations and populations males were lighter than females (Tukey HSD: $P < 0.0001$, Figure 3.11). Spring males from Bernwood had lower forewing basal melanisation (brighter) compared to summer males (Tukey HSD: $P < 0.05$), but this difference was not found in Frog Firle or for females from either population ($P > 0.05$). There was no difference in melanisation between the populations within a generation, and therefore populations were pooled per generation. A two-way ANOVA with Generation as a factor showed no difference in forewing melanisation between generations (Generation: $F_{1,240} = 0.091$, $P = 0.764$; Sex: $F_{1,240} = 585.51$, $P < 0.0001$; Figure 3.11).

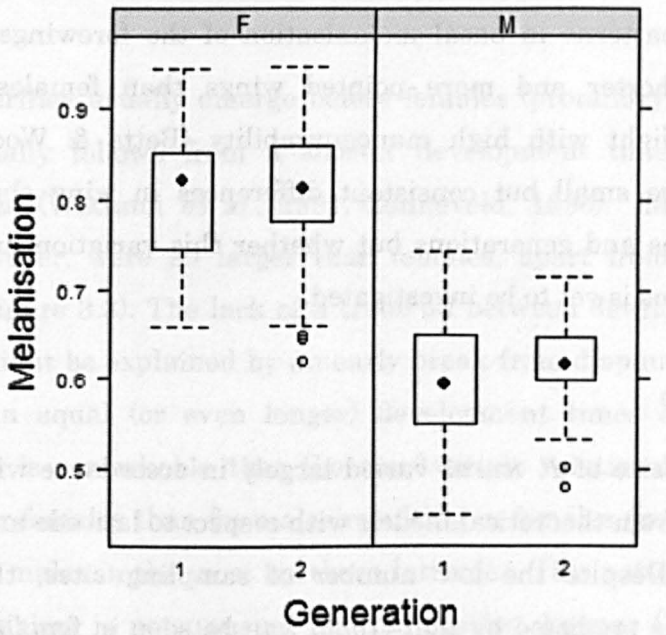

Figure 3.11 Boxplot representing the differences in melanisation between the generations for two populations sampled in spring and summer. Boxplots show median, quartiles and extreme values for each generation.

3.4 DISCUSSION

There were different patterns for males and females in the latitudinal variation of size and melanisation. Despite the small number of populations, the latitudinal "saw-tooth" pattern in size (See Figure 1.1 from Roff, 1983) was found for females, but not for males. However, in the most northern latitudes, in Scotland, butterflies were larger than in any of the more southern populations. Males were the overall larger sex and butterflies that have experienced larval diapause are larger than directly developing butterflies, which agrees with earlier observations (Tolman & Lewington, 1997). There was a trend of increasing basal melanisation of the forewings with latitude for males, with Scottish male butterflies being darker than the other populations, but not females. Females in a north-English population (Greenhow) were darker than the two southern populations. Overall, there were no seasonal patterns in basal melanisation of the forewings. Males had slightly shorter and more pointed wings than females, combining efficient flight with high manoeuvrability (Betts & Wootton, 1988). There were small but consistent differences in wing shape between populations and generations but whether this variation has functional implications is yet to be investigated.

3.4.1 Size

Forewing size of *P. icarus* varied largely in accordance with what was expected from theoretical models with respect to latitude and generation number. Despite the low number of sampling sites, the saw-tooth pattern, as predicted by Roff (1983), can be seen in females, but not in males. The shift from a bivoltine to a univoltine life-cycle in the British Isles is located around Greenhow (latitude: 54°), where this species is univoltine above 300 m and bivoltine in an adjacent valley below 60 m. For logistic reasons, the lowland bivoltine populations could not be sampled. The pattern found in this study is similar to that found by Nygren *et al.* (2008), but the shift in voltinism in Britain is a few degrees in latitude lower than in Sweden, where it is around 60°. This

shift might be related to overall energy inputs. The British Isles have a more cloudy Atlantic climate than Sweden and thus at any given latitude average solar radiation loads will be lower in the former. This could reduce overall temperature and effectively shift latitudinal gradients southwards. As a result of the negative relationship of season length with latitude (see Chapter 1), butterflies in the most northern population (the shortest season) were expected to be the smallest. However, female butterflies were much larger than those in the other three populations and for males there was a non-significant trend for larger size in Scotland (Figure 3.3). This increase in size in extreme northern latitudes was also found by Nygren *et al.* (2008). In line with the saw-tooth pattern, this could be the result of a 2-year life-cycle (Nylin & Svard, 1991; Wipking & Mengelkoch, 1994; Johansson, 2003). Another possibility is that the warm gulfstream in NW Scotland produces a longer growing season.

Male butterflies usually emerge before females (protandry), a strategy which usually follows from a shorter development time and hence smaller size (Wiklund *et al.*, 1991; Zonneveld, 1996). Males in this study, however, were all larger than females, apart from those from Mallaig (Figure 3.3). The lack of a trade-off between development time and size might be explained by an early break from diapause by males, resulting in equal (or even longer) development times compared to females. It is remarkable that the size/latitude relationship is much stronger for females than for males resulting in females growing almost as large as males in the most northern latitudes. This pattern of sexual size dimorphism is not unusual for this species. Leimar (1996), using laboratory stocks of this species reared in different photoperiods, found that males, not females, were the more variable sex. Males grew to a smaller size with shorter development times, but females had no variation in their final adult size. In female butterflies there is usually a strong relationship between size and fecundity (Karlsson & Wickman, 1990), therefore it can be expected that there might be selection against rapid female development. This study is based on field samples, where

different environmental factors interplay. The fact that females are smaller and more variable in their size suggests that they are less capable of dealing with the interaction of photoperiod, temperature and other environmental variables compared to males. However, body size (or weight) was not taken into account in this study. In the case that there is no allometric relationship between wing size and body weight and female butterflies develop similar body sizes throughout the British Isles, then the increase in wing size implies a lower wing loading. This could mean an advantage in colder climates, as a lower wing-beat frequency is needed with a lower wing loading and this allows for a more efficient flight at low temperatures (Casey & Joos, 1983).

Seasonal variation in size was considerable. Both males and females were smaller in summer, compared to butterflies of the first generation (Figure 3.9), a pattern previously found in this and other species (Tolman & Lewington, 1997; Van Dyck & Wiklund, 2002; Fric *et al.*, 2006). This pattern could be a consequence of the combination of end-of-season cues (Leimar, 1996) and higher development temperatures, promoting a faster growth and therefore a smaller size of summer emerging butterflies (Stearns & Koella, 1986; Nylin & Gotthard, 1998). Alternatively, larger spring butterflies could be linked with a relatively more important dispersal strategy of the first generation butterflies (Windig & Lammar, 1999; Fric & Konvicka, 2002; Fric *et al.*, 2006). In *Araschnia levana* for instance, summer generation butterflies are heavier and have larger wings and this has been linked experimentally with dispersal (Fric & Konvicka, 2002). Although *P. icarus* is not generally described as a mobile species (Emmet & Heath, 1989; Asher *et al.*, 2001), it quickly colonises newly created habitat. If flight capacity is related to size, then in bivoltine populations it may be the spring emerging butterflies which play the greatest role in dispersal.

3.4.2 Shape

The differences in forewing shape between males and females across all sites most probably are linked to general sexual differences in

behaviour. Males are more active and the shorter, more pointed wings allow for a more agile flight combined with high efficiency (Betts & Wootton, 1988). Females usually fly more steadily with longer flights, looking for suitable egg-laying sites (Berwaerts *et al.*, 2008). This kind of flight is promoted by longer, less pointed wings, as was found for females in this study. Similar wing shape differences between sexes have previously been linked with behaviour in other butterfly species (e.g. Van Dyck & Wiklund, 2002 for *P. aegeria*; Breuker *et al.*, 2007 for *M. cinxia*).

Spring generation butterflies had slightly narrower wings, which are generally associated with butterflies that fly longer distances, a behaviour often termed 'patrolling' (Betts & Wootton, 1988; Van Dyck *et al.*, 1997a). In a previous study, *P. icarus* was found to fly in longer flight bouts in spring compared to summer butterflies (Howe, 2004). This study now demonstrates that these behavioural differences might be related to wing morphological differences. However, the wing shape differences found in this study are extremely small and the theoretical interpretations of wing shape variation do not always translate very easily to differences in mobility in the field (Baguette *et al.*, 2000; Merckx & Van Dyck, 2002). Whether small wing shape variation in *P. icarus* can influence its flight still remains to be experimentally tested.

3.4.3 Melanisation

The basal melanisation of females is always greater than of males because of the colour dimorphism in this butterfly (males are blue, most females are brown). An increase in melanisation with latitude was as expected on the basis of previous studies (Watt, 1968; Guppy, 1986; Ellers & Boggs, 2002; Ellers & Boggs, 2004; Roland, 2006; Clusella-Trullas *et al.*, 2007). This relationship was found for males, but was weak. In the most northern population (Mallaig), there was a significantly higher degree of basal melanisation compared to the more southern populations. This population is close to the coast, which implies stronger winds and potentially more convective cooling than inland. Additionally the higher activity levels of males may cause even

more convective cooling and therefore males may spend a large proportion of the day basking (Gilchrist, 1990). Darker coloured wings can be an advantage in these situations, because of the faster heating rate of individuals with greater wing melanisation (Watt, 1968; Kingsolver, 1996; Clusella-Trullas *et al.*, 2007). However, because *P. icarus* may also adopt a reflectance basking strategy (Shreeve, 1992) and because of the relatively weak relationship of basal melanisation with latitude for both males and females, the function of melanisation in this butterfly is not entirely clear. Mechanistic studies of heating rates (see Chapter 4) suggest that basal melanisation is not important for thermoregulation in *P. icarus*.

The seasonal pattern does not follow the general trend known for other butterflies (Kingsolver & Wiernasz, 1991; Kingsolver, 1995; Stoehr & Goux, 2008). There was no difference in wing melanisation between spring and summer generation butterflies. This is surprising considering the predictable weather differences between spring and summer (Howe, 2004; Howe *et al.*, 2007). It is possible that other factors that have not been measured vary seasonally in this butterfly. The number of hairs for insulation or the colour of the thorax for instance has previously been linked to thermoregulation (Van Dyck *et al.*, 1998) and these traits could vary seasonally. Another possibility again is that, because of the small size of the butterfly, variation in melanisation does not have a major effect on heating rates (see Chapter 4).

3.5 INTEGRATION

Polyommatus icarus shows considerable variation within and between populations in wing size, shape and colour. There is a sexual dimorphism in the three traits measured: females are usually smaller than males, have longer wings and a higher degree of basal melanisation compared to male butterflies. This could be related to behavioural differences between the sexes: males usually bask for shorter periods than females and spend longer times on the wing (Howe, 2004). Large bright wings could facilitate reflectance basking for males; a smaller size and darker colour is beneficial for fast heating in dorsal basking females. However, because of the lack of seasonal variation in basal melanisation it is possible that in a small butterfly, like *P. icarus*, basal wing colour is not extremely important for thermoregulation (see Chapter 4). Butterflies from the Lycaenidae have previously also been described as body baskers (Kingsolver, 1985a), and this also could explain the relatively weak geographic pattern in melanisation. The wings then merely serve as a means to minimise convective cooling by shielding the body from the wind (Heinrich, 1990).

Chapter 4 Thermal characteristics of *Polyommatus icarus* butterflies

4.1 INTRODUCTION

Butterflies in temperate climates usually have to elevate their body temperatures above ambient temperature in order to become active (e.g. flying to search for food, mates and escape predators; Clench, 1966; Kingsolver, 1983; Roland, 1982; Watt, 1968). The suitable body temperature is usually limited around a narrow optimal range and usually lies between 30°C and 40°C (e.g. Kingsolver, 1983; Shreeve, 1984; Kingsolver, 1985b; Rutowski *et al.*, 1994) and thermoregulation helps an individual to reach and maintain this temperature (Huey & Stevenson, 1979). This suitable temperature is often lower for male butterflies than for females (Gilchrist, 1990; Berwaerts & Van Dyck, 2004; Merckx *et al.*, 2006), which is probably related to sexual differences in flight behaviour: males usually show a more active flight than females and whether they perch or patrol for flight their chance of finding a mate will be enhanced if flight ability is maximised. One way of achieving this is to have a low optimal temperature.

Butterflies can achieve their required body temperature by selecting a sunlit patch and orienting the body in a particular direction to the sun while adopting a specific wing posture (basking) (Kammer & Bracchi, 1973; Kingsolver, 1985a; Dennis & Shreeve, 1989). Basking posture is related to basking method (see Chapter 1). Heating and cooling processes can further be influenced by the physical properties of the wings (Wasserthal, 1975; Van Dyck & Matthysen, 1998; Ellers & Boggs, 2004; Roland, 2006 and also see Chapter 1). Generally, absorbance basking (dorsal and lateral) benefits from darker coloured wings to heat up faster; reflectance baskers heat up faster with lighter coloured wings. Darker coloured males of *Pararge aegeria* heat up faster than lighter coloured individuals, but their take-off temperature does not differ (Van Dyck & Matthysen, 1998). This suggests that adaptations in terms of

heating and cooling only help obtaining or maintaining the optimal body temperature, but do not necessarily influence the activity threshold temperature. Within the Lycaenidae dorsal basking is the most dominant strategy, but reflectance basking (Shreeve, 1992) and also body basking (Kingsolver, 1985a) have been described.

The maximum temperature reached is a balance between heat gained from basking and endothermic heat production and heat loss through convection (Kingsolver, 1985a). Because of the lower surface to volume ratio, larger bodies will suffer less from convective cooling and can reach a higher body temperature. Smaller butterflies on the other hand, can heat up faster, but they are less thermally stable (Heinrich, 1986a; Kemp & Krockenberger, 2004). This means that small butterflies have to build up a heat-reserve in order to prolong flight duration (Heinrich, 1986b). However, by choosing appropriate microclimate sites and behavioural strategies, the constraints of body size on activity and body temperature can be minimised (Angilletta *et al.*, 2003; Gilchrist, 1990; Heinrich, 1986b; Howe *et al.*, 2007).

Common blue (*Polyommatus icarus*) butterflies differ in the body temperature they need in order to become active. Populations in a colder climate (northern Scotland) initiate flight when their body temperature reaches on average 25.6°C (Howe *et al.*, 2007). These body temperatures at flight initiation are much lower than for individuals from a more southern population (i.e. 30°C - 32°C; central England) (Howe *et al.*, 2007). However, these differences are strongly correlated with the temperature at the basking site (Howe, 2004), suggesting that this variation might be the consequence of the available ambient temperatures.

This chapter investigates whether basking position, wing colour differences and body size (weight) influence heating and cooling. Investigating the effects of basking postures and physical properties in this butterfly on the heating and cooling processes will then allow

placing the behavioural (Howe, 2004) and morphological (Chapter 3; Howe, 2004) variation of *P. icarus* in the field in an ecological context. Behavioural and morphological mechanisms to reduce cooling can help an individual to maintain a suitable body temperature during cloudy spells. To investigate whether butterflies have additional mechanisms (e.g. physiological mechanisms, not determined by the physical properties) to enhance heating or reduce cooling, heating and cooling rates of live and dead specimens are compared. The take-off temperature of an individual is defined here as the body temperature at which an individual voluntarily takes off (Howe, 2004). This is an important trait as it determines how long an individual needs to bask for before this temperature is reached.

This chapter tests three specific hypotheses:

(1) Individuals of *P. icarus* with an open wing basking posture will heat up faster than individuals with the wings closed. Cooling rates on the other hand will be fastest in individuals with open wings as they may be the most liable to convective cooling.

(2) Individuals with darker coloured wings will heat faster than lighter coloured individuals. Because of the sexual colour dimorphism, females are expected to have a faster heating rate than males.

(3) Males will take-off with a lower body temperature than females, because the suitable body temperature for activity is generally lower in male butterflies.

4.2 MATERIAL & METHODS

4.2.1 Heating and cooling with dead specimens

Butterflies were reared in the lab from eggs collected in different field sites in the UK (Frog Firle, Bernwood, Greenhow and Mallaig; see Chapter 2 for details of the sites and of rearing conditions). Because there is no behavioural or genetic component in this experiment, butterflies were treated as originating from one large population. Freshly thawed dead individuals (killed after wing drying on eclosion) were weighed to the nearest 0.001 g prior to testing (HR-120-EC, A&D Instruments). Heating and cooling rates were measured by inserting specimens in a frame in which the butterfly body could be held in place with nylon lines (Figure 4.2a). This frame allowed manipulation of different basking positions (basking position1: 0° = closed wings, representing non-basking; basking position 2: 90° = partially opened wings representing 'reflectance basking'; basking position 3: 180° = fully opened wings, representing dorsal basking; Figure 4.2b).

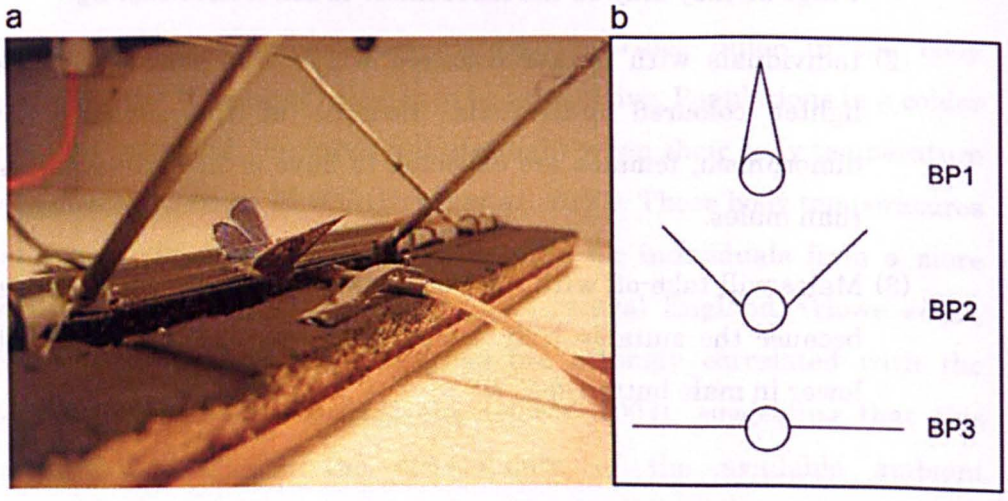

Figure 4.1 Frame with Common blue male with partially opened wings (basking position 2). The thermoprobe (front) is inserted in the thorax and held in place by a strap (a). Schematic representation of the three basking positions (b).

The frame with the butterfly was then placed under 2 halogen lamps (Halogen 500 Watt, NR10461, Philips Lighting, 5600VB Eindhoven, The Netherlands) placed 0.8 m above the frame in a constant temperature

room kept at 20 (±1) °C. The lighting conditions resulted in an average radiation load of 400 Wm^{-2}, measured at body height. Body temperature of the butterflies was measured by inserting a thermocouple (Type K, 0.04 mm diameter) into the thorax, attached to a Physitemp BAT-12 digital thermometer. The initial temperature was read and further readings were made every 5 seconds for a period of 5 minutes. This time was chosen because most basking durations in the field for this butterfly are between 1 and 300s (Howe, 2004). The initial body temperature was as close to 20°C as possible before transferring the butterfly from the shade to the lamp-lit area. To exclude the error caused by transferring the frame with the butterfly from the shaded area to the area under the lamp, the first temperature reading was excluded. After measurements of warming, the frame with the butterfly was placed in the shade and the cooling rate was measured by recording the body temperature every 5 seconds for 5 minutes. Heating and cooling rates of 45 individuals (21 females, 23 males) were measured in the three different wing positions (in random order) for every individual in one sequence in order to avoid increasing the measurement error and damage to the specimen caused by inserting the thermocouple three different times. Ambient temperature and light intensity was measured with a Datahog2 data logger (Skye Instruments Ltd.). Under the lights, temperature was 23.9 (± 0.8) °C and in the shade it was 19.8 (± 0.5) °C. Light intensity was on average 405.2 (± 6.7) W/m^2. After testing, the wings of the butterflies were removed and photographed and the melanisation of the basal part of the forewing was measured. Details of the photographic techniques and methods of recording wing melanisation are given in Chapter 3.

4.2.2 Heating and cooling with live specimens

Live butterflies were used from the Frog Firle stock population reared at 20°C (L16D8). Within 24 hours of eclosing, adult butterflies were transferred to paper envelopes and weighed. The protocol is similar to that of dead butterflies, except that specimens were only tested in 2 basking positions (BP1 and BP3; Figure 2b) and that body temperature was monitored for only 2 minutes, as most of the heating process was found to happen in this period from studies of dead butterflies. Wing

measurements for these samples could not be used because of the relatively high degree of wing damage after testing live individuals. 17 individuals were tested (12 females, 5 males).

4.2.3 Take-off experiment

Female butterflies were collected in spring in two successive years in Frog Firle (2009 and 2010) and Bernwood (2010) (see Chapter 2 for details of sites) and allowed to lay eggs on potted host plants in the lab. Caterpillars were reared at 20°C (±1) °C and 16:8LD in 2009 and at 20°C (±1) °C and 19:5LD in 2010. Every morning, butterflies were transferred to paper envelopes and weighed within 24h of eclosing. On the same day, they were cooled down to 4°C for 20 minutes prior to testing and kept cool till the start of the experiment. The specimen was then transferred, without touching the butterfly itself, to a matt green surface (to prevent reflection from the lab bench surface) beneath 2 halogen lamps (see above). At take-off, the butterfly was immediately captured with a small net and body temperature was measured by inserting a thermocouple (Type K, 0.04 mm diameter) through the net into the thorax without manual handling of the individual. For consistency of the results, only readings made within 3s after netting were retained (Heinrich, 1986a). After testing, 36 male butterflies were frozen (-80°C) for enzyme analysis. Allozyme electrophoresis and scoring was performed as described in Chapter 5. Three glycolytic enzymes were analysed (G6PDH, PGI, PGM) and these were labelled with either presence or absence of each common allele. In 2010, the ambient temperature next to the butterfly body was also read together with the body temperature at take off. In 2009, 31 males and 18 females from one population (Frog Firle) were tested. In 2010, butterflies from 2 populations (Frog Firle (12 males, 9 females) and Bernwood (7 males, 3 females) were tested.

4.2.4 Analyses

Heating and cooling rate were taken from the slope of the regression between log (time) and body temperature for individual butterflies (Van

Dyck & Matthysen 1998). Maximum body temperature, heating and cooling rates were analysed using general linear models (GLM). The effect of sex was investigated by fitting a model including sex and individual nested within sex, using the latter as the error term. The effect of basking position was investigated by fitting a GLM model with sex, individual nested within sex and basking position using the interaction between basking position and individual as the error term. The influence of the measured individual traits was investigated by fitting a GLM with sex and basking position and either weight, wing size or wing melanisation as covariates. Quantitative comparison of the models showed whether any of these traits explained more variation than individual.

Take-off temperatures were investigated using GLMs with sex, weight and population of origin as variables. The dataset was collected over different years (2009, 2010) and analyses were done on the datasets separately first before combining the data. In the 2010 dataset ambient temperature next to the butterfly was measured and incorporated as covariate in the model. The subset for enzyme analyses was taken across these two datasets and for the GLM one allele of each enzyme was incorporated separately together with weight.

All data were checked for normality using the Shapiro-Wilk's normality test in R 2.11.1 (R Development Core Team, 2010).

4.3 RESULTS

4.3.1 Maximum body temperature of dead specimens

Male butterflies reach a higher body temperature in all three basking positions than female butterflies ($F_{1,42}$ = 13.1, P = 0.04). There was a highly significant effect of basking position on the maximum body temperature butterflies could reach ($F_{2,86}$ = 998.8, P < 0.0001). The highest maximum temperature was reached with fully opened wings and the lowest with closed wings (Figure 4.2).

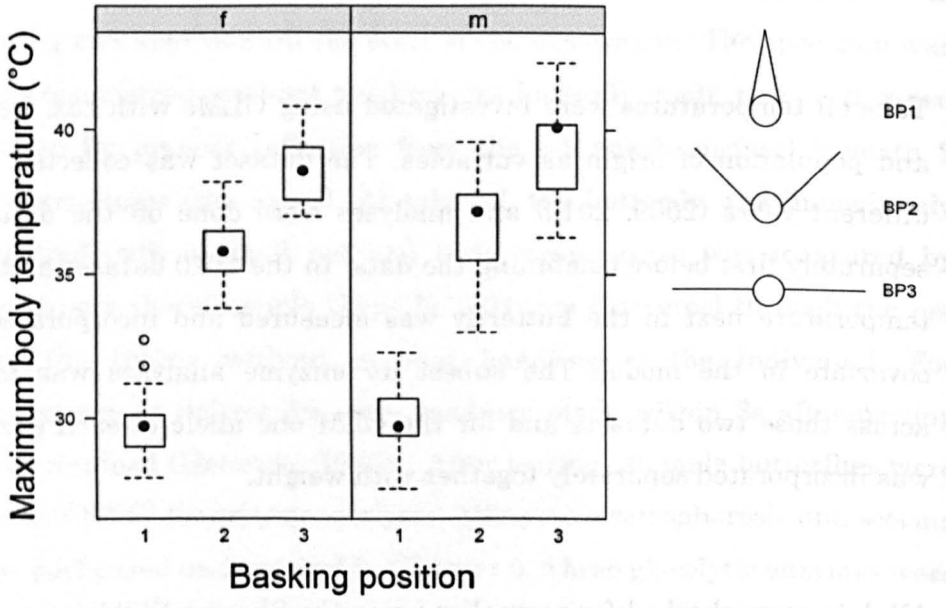

Figure 4.2 Maximum body temperatures for the three different basking positions for males and females. Boxplots show median, quartiles and extreme values for each basking position.

Three separate models with sex and basking position and each covariate were tested. Weight and wing size had a significant positive effect on maximum body temperature (weight: $F_{1,127}$ = 14.17, P = 0.005; wing size: $F_{1,127}$ = 31.40, P < 0.0001), but not wing melanisation ($F_{1,127}$ = 0.31, P = 0.683).

However, the model with only individual and basking position (model 1) explained significantly more variation than the model with any of the covariates (model 2) (Table 4.1). This means that the variation in maximum body temperature reached after 5 minutes is not accounted for by any of these three measured traits alone, but could be due to a combination of these factors.

Table 4.1 Comparison of model 1 with a model including sex, basking position and one of the measured individual traits (weight, size or melanisation).

Trait	SS (model 1)	SS (model 2)	df 1	df 2	df (den.)	F diff.	P
Weight	2175.5	2022.29	45	4	86	2.87	<0.0001
Size	2175.5	2030.05	45	4	86	2.73	<0.0001
Melanisation	2175.5	2001.21	45	4	86	3.27	<0.0001

4.3.2 Heating rates of dead specimens

There were no differences between the sexes in heating rates for dead individuals ($F_{1,42}$ = 7.41, P = 0.081). Basking position was highly significant ($F_{2,86}$ = 414.15, P < 0.0001) with a higher heating rate the more opened the wings were (Figure 4.3).

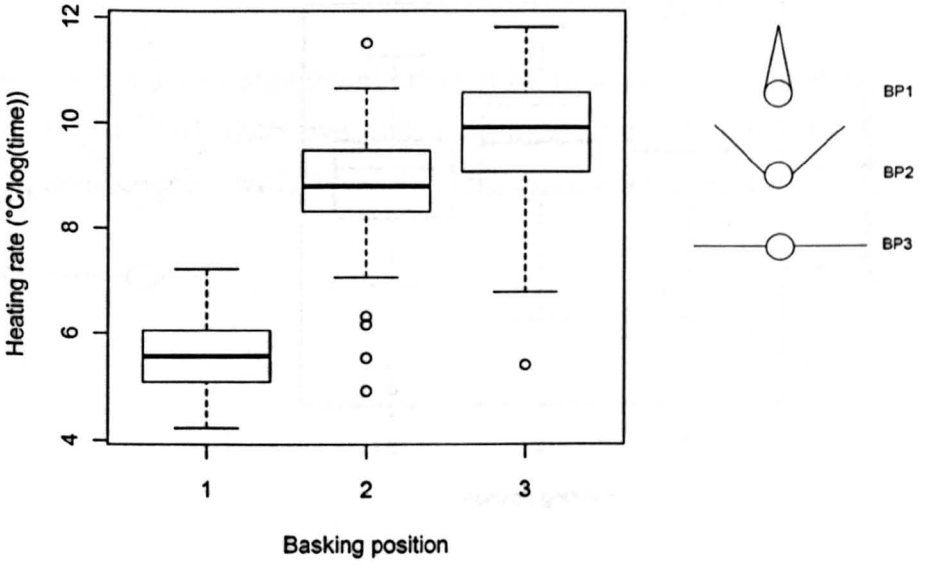

Figure 4.3 Heating rates of butterflies in the three basking positions. Boxplots show median, quartiles and extreme values for each basking position.

Of the individual traits, only wing size had a significant effect on heating rates ($F_{1,127}$ = 4.74, P = 0.042). None of the three models with the individual traits explained more variation than the model with only ID (Table 4.2). This means that the variation in heating rates is not down to any of these three measured traits alone, but that the individual variation (the combination of traits) is of a larger influence.

Table 4.2 Comparison of model 1 of heating rates (Table 5) with a model including sex, basking position and one of the measured individual traits (weight, size or melanisation).

Trait	SS (model 1)	SS (model 2)	df 1	df 2	df (den.)	F diff.	P
Weight	518.69	422.43	45	4	86	3.98	<.0001
Size	518.69	421.46	45	4	86	4.02	<.0001
Melanisation	518.69	415.79	45	4	86	4.25	<.0001

4.3.3 Cooling rates

There was no difference between the sexes in cooling rates ($F_{1,42}$ = 4.37, P = 0.284). Wing position was significant ($F_{2,86}$ = 4.74, P = 0.011). Post hoc Tukey tests showed that the only significant difference was between basking position 2 and 3 (Figure 4.4).

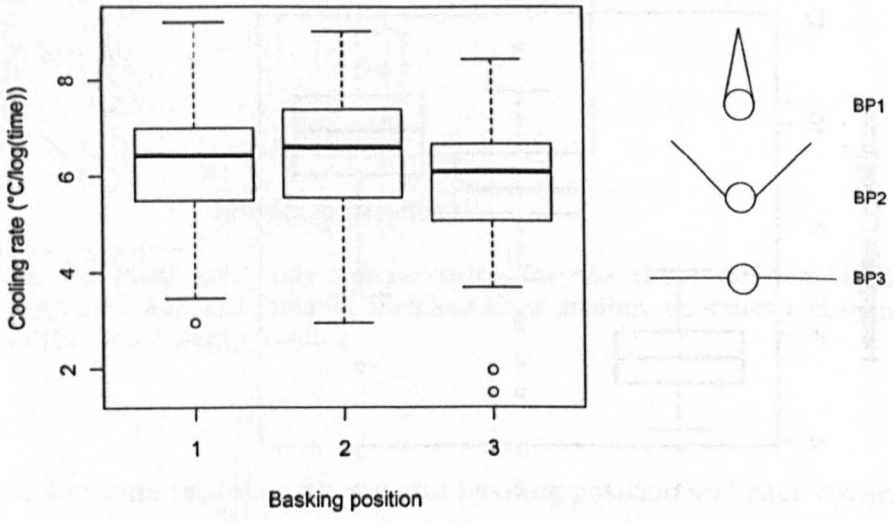

Figure 4.4 Differences in cooling rates between the three basking positions. Boxplots show median, quartiles and extreme values for each position.

The comparison of the model including only individual and basking position with a model including the individual trait (weight, wing size or

melanisation), none of these traits explained more variation than the model which takes all three into account (Table 4.3). This means that the variation in cooling rates is not due to any one of these three measured traits alone.

Table 4.3 Comparison of model 1 of cooling rates (Table 5) with a model including sex, basking position and one of the measured individual traits (weight, size or melanisation).

Trait	SS (model 1)	SS (model 2)	df 1	df 2	df (den.)	F diff.	P
Weight	169.93	14.17	45	4	86	3.80	<.0001
Size	169.93	12.72	45	4	86	3.83	<.0001
Melanisation	169.93	20.41	45	4	86	3.65	<.0001

4.3.4 Live and dead butterflies: comparison - Maximum body temperature

There was a significant effect of status (alive or dead) on maximum body temperature reached ($F_{1,59}$ = 950.85, $P < 0.0001$). There was no significant effect of sex in the complete dataset (live and dead individuals together) ($F_{1,59}$ = 2.72, $P = 0.105$). Dead butterflies reached a much higher temperature than live ones (Figure 4.5).

There was a highly significant effect of basking position ($F_{1,60}$ = 1561.02, $P < 0.0001$) with both live and dead butterflies reaching the highest body temperatures with their wings fully spread (Figure 4.5).

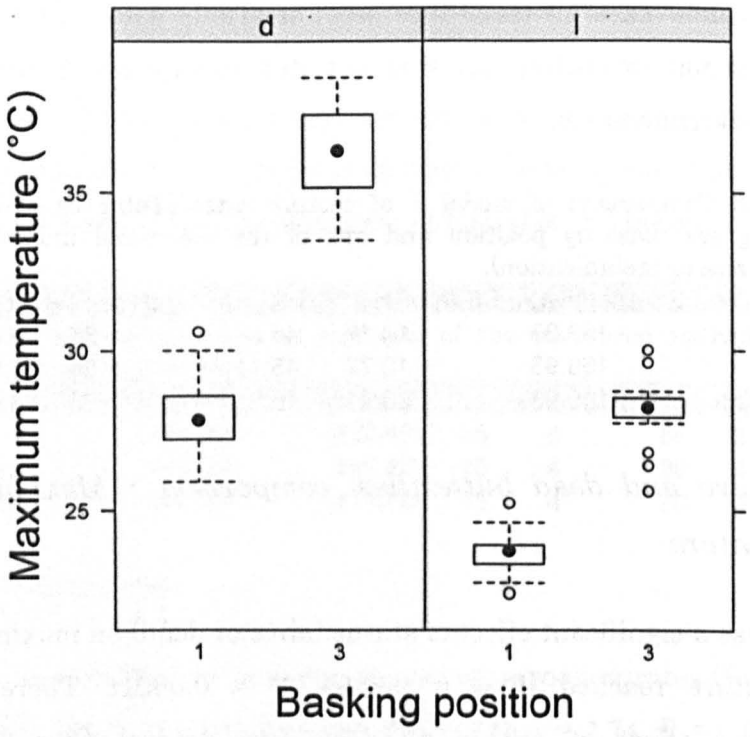

Figure 4.5 Maximum temperatures for dead (d) and live (l) butterflies with wings closed (1) and open (3). Boxplots show median, quartiles and extreme values for each position.

4.3.5 Live and dead butterflies: comparison - Heating and cooling rates

There was no significant effect of sex on either heating or cooling rates (Heating: $F_{1,59} = 2.56$, $P = 0.115$; Cooling: $F_{1,59} = 0.67$, $P = 0.418$). Status (alive or dead) had a significant effect on heating rates ($F_{1,59} = 210.45$, $P < 0.0001$), but not on cooling rates ($F_{1,59} = 0.06$, $P = 0.808$): dead butterflies heat up faster than live ones (Figure 4.6).

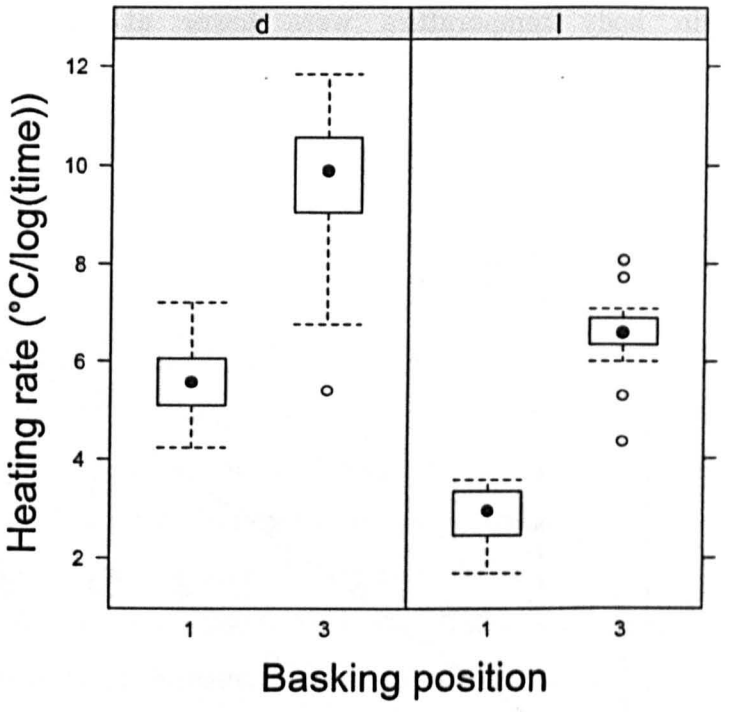

Figure 4.6 Heating rates for dead (d) and live (l) butterflies with wings closed (1) and open (3). Boxplots show median, quartiles and extreme values for each position.

There was a significant effect of basking position on heating rates ($F_{1,60}$ = 494.28, P < 0.0001), but not on cooling rates ($F_{1,60}$ = 0.46, P = 0.498). Butterflies with their wings fully opened (basking position 3) had higher heating rates than butterflies with their wings closed (Figure 4.6).

4.3.6 Take-off experiment

There was no effect of sex, weight or population on take-off temperature in either the 2009 or 2010 dataset when analysed separately. Butterflies reared in 2009 were heavier than butterflies from 2010 ($F_{1,100}$ = 4.58, P < 0.05; see also Chapter 6) and with the two datasets together there was a significant effect of weight on take-off temperature: heavier butterflies took off with a lower body temperature ($F_{1,100}$ = 4.87, P < 0.05; R^2 = 0.046). In the 2010 dataset there was a strong positive correlation between take-off temperature and ambient temperature (R^2 = 0.356, P < 0.0001). This correlation differed from an isothermal correlation, i.e.

excesses in body temperature were lower at higher ambient temperature, suggesting thermoregulation before take-off (Figure 4.7).

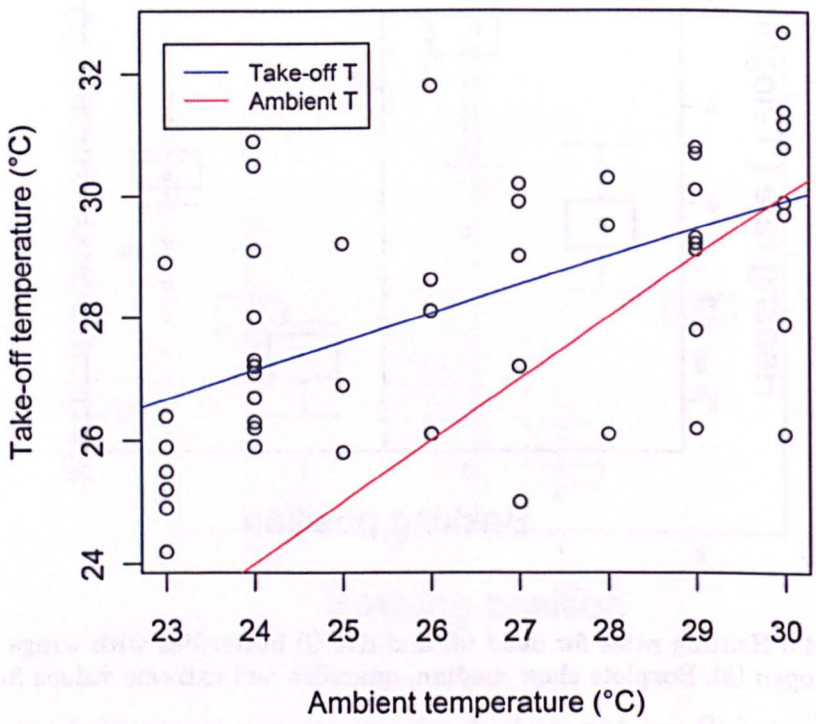

Figure 4.7 Relationship between ambient temperature and take-off temperature for butterflies tested in 2010.

In the subset with the enzymes there was a significant effect of both butterfly weight and PGI allele b (See Chapter 5 for allele details) on take-off temperature (weight: $F_{1, 33} = 10.22$, $P < 0.01$; PGI b: $F_{1, 33} = 5.51$, $P < 0.05$). Individuals with allele b (4 individuals in this dataset) took off with a higher body temperature; heavier individuals had a lower take-off temperature. No other alleles had any effect on take-off temperature ($P > 0.05$ in all cases).

4.4 DISCUSSION

Both male and female *P. icarus* can reach higher body temperatures the more opened the wings are, as was expected. In *P. icarus* there is a sexual colour dimorphism: males have blue upper wing colouring and females can vary from brown to nearly completely blue upper wing colouring (Emmet & Heath, 1989; Frohawk, 1934; Howe, 2004). The darker coloured females, which have more basal melanisation (see Chapter 3) were therefore expected to reach a higher body temperature as increased wing melanisation in butterflies generally is related to higher body temperatures (Kingsolver, 1996; Ellers & Boggs, 2004; Clusella-Trullas *et al.*, 2007). However, males reach a higher final body temperature than females. This suggests that wing melanisation does not play a large role in the maximum body temperature this butterfly can obtain. Male *P. icarus* butterflies generally are larger than females (Chapter 3; Howe, 2004) and the higher temperature reached by males could therefore be a mere consequence of their larger size. This is further confirmed by a lack of effect of wing melanisation on maximum temperature, but a significant effect of both weight and wing size. However, none of the measured traits explained more of the variation in maximum temperature than the model that treated the characters together.

The fastest heating rates and highest maximum body temperatures were reached with wings fully spread. There was no influence of individual morphological characteristics in this basking posture, suggesting that fully opening the wings is only a means to expose the body to solar radiation. Because of the small size of *P. icarus* butterflies, it is possible that their bodies very readily heat up without additional mechanisms. Indeed, previously it has been documented that body temperature in this species in the field is tightly correlated with the ambient temperature of the basking spot (Howe *et al.*, 2007), making microsite choice for basking most important for reaching an optimal

body temperature. Because of the smaller size, these butterflies can heat up quickly, but will also cool down more rapidly (higher exchange rate of heat with environment for smaller bodies). Behavioural mechanisms could therefore be of more importance than the physical properties of *P. icarus* and the wings may simply serve as convection-reducing devices in the thermoregulation process. This is further supported by lower cooling rates with the wings fully spread than with the wings closed or half-open. The most likely explanation is that opening the wings keeps warm air trapped between the basking substrate and the wings and body, thereby slowing down the cooling process. Trapping hot air under the wings has previously been identified as a thermal mechanism (Kemp & Krockenberger, 2002), and this is provides another explanation for the weak relationship between melanisation and heating rate. In order to heat up or stay warm, *P. icarus* keep their wings fully spread; to cool down or avoid overheating, they should rest with their wings closed. This closed wing posture has been observed in the field at high temperatures (Howe, 2004).

There were no strong differences in the heating and cooling rates of live and dead individuals. Dead individuals had an overall faster heating rate and higher body temperature, which is possibly a mere consequence of the more dehydrated state of these specimens compared to live butterflies. Wasserthal (1975) observed a similar pattern in dried butterflies compared with fresh specimens. These results suggest that *P. icarus* butterflies do not posses additional physiological mechanisms to enhance heating or reduce cooling. The lack of physiological mechanisms is further supported by the enzyme analysis: only one rare allele in PGI affected the take-off temperature and the presence of this allele in the field is not related to habitat temperature (see Chapter 5). Therefore, as in most butterflies, behavioural thermoregulation is the most important factor in reaching a suitable body temperature in this butterfly.

Males are usually expected to be able to fly at lower temperatures because of their lower wing loading compared to females (Berwaerts & Van Dyck, 2004; Gilchrist, 1990; Merckx *et al.*, 2006). However, the

sexual differences in ability to fly at lower temperatures are not reflected in the take-off temperatures. In the field there are no sexual differences in take-off temperatures in *P. icarus* (Howe, 2004) and they did not appear in this lab experiment either. There was a strong relationship of take-off temperature and temperature next to the body, again suggesting that microhabitat choice is the most important factor in determining the body temperature of small butterflies. The air temperature in the room was kept constant at 20 (± 1) °C, but temperature next to the butterfly reached 26.4 (± 2.5) °C. It is possible that in this experimental set-up, with high ambient temperatures and minimal airflow, individual differences in take-off temperature are minimised.

The trait measured here was voluntary flight and this differs from flight at sub-optimal temperatures. Even if males are able to start flying at lower temperatures, they might not do so if there is no incentive to initiate flying. It is possible that sexual differences arise in other flight performance traits such as flight at sub-optimal temperatures or flight duration (Gilchrist, 1990; Merckx *et al.*, 2006). Butterflies reared in the different years differed in weight because of the difference in photoperiod (see Chapter 6). There was a difference in take-off temperature between the two years with heavier butterflies taking off with a lower body temperature than smaller butterflies. Small butterflies do not produce heat during flight and are very subjective to convective cooling (Heinrich, 1986a). It is therefore possible that smaller butterflies have to reach a higher body temperature in order to fly for a same period than a larger butterfly. This finding of larger individuals taking off with lower body temperatures provides some support for the observations of take off temperatures in northwest Scotland and southern England (Howe, 2004; Howe *et al.*, 2007). Lower take-off temperatures in Scotland may be associated with larger body size (as identified in Chapter 3)

4.5 INTEGRATION

Reaching a suitable body temperature for activity is important for flight in butterflies. In most butterflies heat gain is obtained by behavioural basking assisted by physical adaptations of the wings or body (Heinrich, 1986a) and several different basking mechanisms and strategies have been described (Kemp & Krockenberger, 2002; Kingsolver, 1985b; Kingsolver & Koehl, 1985). Many previous studies have found positive relationships between melanisation of the wings and heating rate or habitat temperature (Clusella-Trullas *et al.*, 2007; Kingsolver, 1996; Watt, 1968). However, the results of this study suggest that in *P. icarus* the colour and size of the wings play a minor role in thermoregulation. Appropriate microhabitat choice is then of prime importance for this species to become active. Butterflies in which a relationship of melanisation with thermoregulation has been found are generally much larger than the species under study. The minor importance of wing colour in *P. icarus* may then relax selection pressures from thermoregulation on wing colouring and patterning, possibly facilitating variation in wing morphology in response to other factors in this, and maybe other small butterflies.

Chapter 5 Enzyme variation in *Polyommatus icarus* and population genetic structure in the UK

5.1 INTRODUCTION

Allozymes have been used in many biogeographical studies to investigate patterns of postglacial expansion and population genetic structure (Hewitt, 2000; Schmitt *et al.*, 2002; Schmitt *et al.*, 2003). The interpretation of the results of these studies is based on the assumption that allozymes are selectively neutral and thus that allele frequencies in populations are primarily affected by drift and gene flow. However, part of the variation in allozyme loci frequencies can be the result of selection as neutrality is not always guaranteed (Eanes, 1999). One way to discover candidate loci under selection (and therefore not neutral) is to compare the geographic pattern of the allozymes with the pattern obtained from a more neutral marker, such as AFLPs. The AFLP marker (amplified fragment length polymorphism) (Vos *et al.*, 1995) is a DNA-marker and is based on restriction sites that are randomly distributed across the whole genome, and therefore provides excellent opportunities as a neutral marker (Bensch & Åkesson, 2005). Deviations in the geographic pattern of one or several of the allozyme loci from that of the neutral marker suggest a possible influence of selection.

The Common blue butterfly *Polyommatus icarus* is distributed across the whole Palearctic region and occurs in a wide range of environmental conditions (Tolman & Lewington, 1997). It is characterised by a high allelic diversity (Schmitt *et al.*, 2003), which makes it a perfect study organism to investigate candidate loci. Several studies on other butterflies have identified a relationship between allelic variation in glycolytic enzymes and performance (Goulson, 1993; Haag *et al.*, 2005; Hanski & Saccheri, 2006; Wheat *et al.*, 2006; Karl *et al.*, 2008; Karl *et al.*, 2009; Niitepõld *et al.*, 2009; Orsini *et al.*, 2009; Saastamoinen *et al.*, 2009; Vandewoestijne & Van Dyck, 2010). The best studied example is

probably the allelic variation in phosphoglucose isomerase (PGI) in *Colias* butterflies (Watt, 1977; Wheat *et al.*, 2006). The alleles of this enzyme have different thermal and kinetic optima (Watt, 1977; Watt, 1983) and the frequency of the alleles varies with habitat temperature (Watt *et al.*, 2003). Furthermore, butterflies with an allelic variant with a low thermal optimum can be active at earlier times of the day and/or in cooler locations than those with other alleles. At high temperatures or in warm locations however, these cold-optimum enzymes will more readily unfold and may thus be disadvantageous under these conditions (Watt, 1977; Watt, 1983). In *P. icarus* butterflies there is behavioural variation which has previously been related to habitat temperature: butterflies in northwest Scotland generally start flying with a much lower body temperature than butterflies in the southeast of England (Howe *et al.*, 2007). The mechanistic basis for these differences has not yet been investigated, but it is possible that this is caused by allelic variation in certain glycolytic enzymes.

Despite some loci possibly being under selection, when a large number of loci are applied, biogeographical patterns and population genetic structure can be reliably inferred from allozyme data (Besold *et al.*, 2008; Habel & Schmitt, 2009; Joyce *et al.*, 2009). The study species *P. icarus* was probably widespread in the Mediterranean area during the last ice-age and has spread across Europe without a major reduction in genetic diversity (Schmitt *et al.*, 2003). The British Isles however, are thus far unstudied and because of its somewhat isolated position from mainland Europe, the genetic configuration is likely to differ substantially from that of the continental populations. Comparison of allozyme data from the British Isles with the existing dataset from mainland Europe allows for inferences about post-glacial colonisation history of *P. icarus*. Furthermore, this butterfly is relatively common in meadows in the UK and is characterised by a colonial population structure (Emmet & Heath, 1989; Asher *et al.*, 2001). The population genetic structure of this butterfly is therefore expected to show an isolation-by-distance effect, as has been found for this species in

mainland Europe, where there was also low differentiation between populations (Schmitt *et al.*, 2003).

In this study, data from allozymes and AFLPs of nine populations of *P. icarus* along a latitudinal cline in the UK were compared. The main aims were to:

(1) investigate the population genetic structure of *P. icarus* within the British isles both with allozymes and AFLP as a marker. Is there an isolation-by-distance effect (Wright, 1943) across the UK and do the markers differ in their pattern? Are the populations genetically separated, allowing for local adaptation?

(2) explore patterns of colonisation of *P. icarus* in the UK by comparing the allozyme dataset with that from mainland Europe (Schmitt *et al.*, 2003). A lower allelic diversity in the UK than on mainland Europe would suggest a bottleneck during the colonisation of the British Isles.

(3) discover candidate allozyme loci under selection which vary in relation to thermal gradients, by comparing the variation in three loci (PGI, PGM, G6PDH) with the pattern of the neutral marker.

5.2 MATERIAL & METHODS

5.2.1 Sample collection

Adult butterflies were collected during the spring and summer of 2010 in nine field sites across the UK (Table 5.1, Figure 5.1), frozen alive and stored at -80°C. On four locations (Frog Firle, Bernwood, Greenhow, Mallaig), light intensity, windspeed and three temperature measures (ground, vegetation, ambient) were recorded using a Datahog2 data logger (Skye Instruments Ltd.) together with the time of capturing of the insect. 17 different enzymes were studied using cellulose acetate electrophoresis. AFLP (amplified fragment length polymorphisms) was used as a neutral marker.

Table 5.1 Details of the sample sites and codes, dates of sampling and sample sizes for allozyme and AFLP analyses.

Site	Latitude	Acronym	Date collected	n (males)	n (females)
Friston	50.77	FRIS	09/08/2010	14	1
Frog Firle	50.79	FROG	7-9/06/2010	34	24
			09/08/2010	39	25
Bedelands Farm	50.97	BEDE	09/08/2010	19	6
Aston Rowant	51.66	ASTO	08/08/2010	29	1
Grangelands	51.74	GRAN	08/08/2010	23	17
Bernwood	51.80	BERN	14-16/06/2010	36	22
			5-8/08/2010	56	31
Otmoor	51.82	OTMO	08/05/2010	10	1
Greenhow	54.07	GREE	13-14/07/2010	30	22
Mallaig	56.96	MALL	22-25/07/2010	19	15

Figure 5.1 Sample site locations for allozyme and AFLP analyses. Details of sites are given in Table 5.1.

5.2.2 Enzyme electrophoresis

Previously frozen (-80°C) butterflies collected in the field were transferred to a cooled working surface (ice blocks) to prevent thawing. Half of the abdomens of individuals were each placed in 80 µL animal buffer (Harris & Hopkinson, 1976) in a 2 mL Eppendorf tube. The rest of the samples was transferred back to the freezer (-80°C). Abdomens were homogenised with a supersonic needle (drHielscher Ultraschall prozessor) and then centrifuged for 3 minutes at 8000 rpm.

The samples were then transferred onto a cellulose acetate plate (Hebert & Beaton, 1993) that had been saturated in the buffer appropriate for each enzyme (Table 3.2). The 17 enzymes studied are 6-phosphogluconate dehydrogenase (6PGDH), pyruvate/creatine kinase (PK/CK), fumarate hydratase (FUM), glucose-6-phosphate dehydrogenase (G6PDH), glyceraldehyde-3-phosphate dehydrogenase (G3PDH), glutamate oxaloacetate transaminase (GOT1 & GOT2), glycerol-3-phosphate dehydrogenase (GPDH), β-hydroxybutyrate dehydrogenase (HBDH), isocitrate dehydrogenase (IDH1 & IDH2),

malate dehydrogenase (MDH1 & MDH2), malate enzyme (ME), phosphoglucose isomerase (PGI), phosphoglucomutase (PGM) and peptidase with Phe-Pro substrate (PPP). For the 17 enzyme systems studied, 4 different buffer systems were used: Tris-citrate pH 8,2 (Richardson et al. 1986), Tris-glycine pH 8.5 (Hebert & Beaton, 1993), Tris-maleate pH 7.0 (adapted from T-M pH 7.8 (Richardson *et al.* 1986)) and Tris-borate pH 89 (adapted from T-B pH 7.0 (Shaw & Prasad, 1970)). The plate with the samples applied was then transferred onto an electrophoresis tank (Helena Biosciences) which contained the appropriate buffer. 200 V current was applied to the tank and running times differ for each enzyme system (Table 5.2).

Table 5.2 Enzymes, buffers and running times for the 17 enzymes under study.

Enzyme	Buffer	Running time (min)
IDH1	TM	45
IDH2	TM	45
MDH1	TC	40
MDH2	TC	40
6PGDH	TM	45
G6PDH	TC	50
PGI	TG	40
PGM	TG	40
GOT1	TG	40
GOT2	TG	40
GPDH	TM	45
FUM	TC	45
ME	TB	40
PEP(Phe-Pro)	TM	40
HBDH	TG	30 (reversed polarity)
PK/CK	TM	45
GAPHD	TC	30

TM: Tris-maleate; TC: Tris-citrate;
TG: Tris-glycine; TB: Tris-borate

After running, the plates they were stained by mixing the appropriate reagents with agar (1.80 g/100 mL) following the protocols of (Hebert & Beaton, 1993). After staining in the dark, the reagents are washed off the plate which was then allowed to dry for storage. The allozymes were scored visually and labelled by comparison with previous plates from European *P. icarus* populations (Schmitt *et al.*, 2003).

5.2.3 DNA extraction and AFLP

DNA of frozen samples was extracted using a Qiagen kit (DNEasy Blood & Tissue). The head and legs of specimens were mechanically homogenised in 250 µL phosphate buffered saline (PBS). 20 µL of proteinase K was added to 180 µL of this homogenised sample together with 200 µL of lysis buffer (Qiagen AL) buffer. This mix was vortexed and then incubated in a shaker at 55°C for 1.5 hours. 200 µL of 90% ethanol was then added and the mixture was centrifuged at 8000 rpm for 1 min and the liquid that had gone through the membrane discarded. 500 µL of wash buffer (Qiagen AW1) was then added, centrifuged at 8000 rpm for 1 min and the liquid again discarded. 500 µL of wash buffer (Qiagen AW2), was then added and the sample centrifuged at 13000 rpm for 3 mins. The flow-through liquid was again discarded and the sample washed off the membrane by adding 200 µL of elution buffer (Qiagen AE), and centrifuged at 8000 rpm for 1 min after incubation for 1 min at room temperature.

The protocol for the AFLPs was modified from Vos *et al.* (1995). Firstly, two adapters were prepared by incubating two oligonucleotides at 95°C for 5 minutes and then letting them cool down at room temperature (Table 3.3). The restriction of the source DNA was performed in a 40 µL reaction with 250 ng of genomic DNA (the source DNA was quantified with a spectrophotometer (NanoDrop ND-1000)), 0.5 µL of EcoRI (20U/µL), 1 µL of MseI (10U/µL), 0.25 µL bovine serum albumin (BSA), 4 µL 10x NEB (New England Biolabs) buffer Number 4 was added and the solution made up to a final volume of 40 µL with mp H_2O. This was then incubated at 37°C for 3 hours and the restriction enzymes were then heat-inactivated at 65°C for 20 minutes. After the restriction mix had cooled, 1 µL of MseI-adapter and EcoRI adapter (1:10) were added together with 1 µL NEB buffer Number 4 (10x dilution), 0.5 µL T4-Ligase (Promega), 1 µL ATP (10 mM), 5.5 µL mp H_2O. The ligation mix was homogenised using a vortex and left to ligate on the bench at room temperature overnight (c.15 hours).

The digested DNA fragments were amplified using a preselective amplification, in which primers with just one selective nucleotide were added in the reaction (Table 5.3). The reaction consisted of 1 μL of EcoRI-pre primer (10 pmol/μL) and 1 μL of MseI-pre primer (10 pmol/μL), 2 μL 10x PCR buffer with $MgCl_2$ (Sigma), 0.25 μL dNTP-mix (25 mM each dNTP), 1 μL of template DNA with linkers, 0.2 μL of Taq polymerase (5U/μL)(Sigma) and 14.55 μL mp H_2O. The PCR conditions were as follows: the 3' recesses were allowed to fill in by heating to 72°C for 2 minutes, then the mix went through 20 cycles at 94°C for 30s, 56°C for 30s, and 72°C for 2 minutes. At the end, the reaction was allowed to clean up for 10 minutes at 72°C and was then cooled down to 8°C.

In order to reduce the amount of bands produced by the AFLP, a selective amplification step with primers with three selective nucleotides at the 3' end was performed. The EcoRI primers were labelled with fluorescein at the 5' end. The two combinations that gave the highest number of bands were used: EcoRI-AGC_FAM/MseI-CAA and EcoRI-ACA_FAM/MseI-CAT. In this reaction 1 μL of the 1:10 diluted preamplification product was added to a mix of 0.25 μL dNTP-mix (25mM each dNTP), 2.5 μL $MgCl_2$ (50mM), 2 μL 10x PCR buffer with $MgCl_2$ (Sigma) 0,2 μL Taq polymerase (5U/μL)(Sigma), 1 μL MseI-CAA or MseI-CAT primer (10 pmol/μL) and 1 μL of labelled EcoRI-ACG or EcoRI-ACA primer (10 pmol/μL) and 12.05 μL mp H_2O. This mix was placed under the following PCR conditions: 94°C for 2 min (initial denaturation), then 10 cycles at 94°C for 30 s, 66°C (dropping by 1°C per cycle) for 30 s, 72°C for 2 min, then 25 cycles at 94°C for 30 s, 56°C for 30 s, followed by 72°C for 2 min. This mix was then cooled down to 8°C.

Table 5.3 Structure of adapters and primers. The NN at the 3' end of the selective primers stands for the two added extra selective nucleotides.

EcoRI adapter	5'-CTCGTAGACTGCGTACC-3' 3'-CATCTGACGCATGGTTAA-5'
MseI adapter	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
EcoRI - preselective primer	5'-GACTGCGTACCAATTCA-3'
MseI - preselective primer	5'-GATGAGTCCTGAGTAAC-3'
EcoRI - selective primer	FAM-5'-GACTGCGTACCAATTCANN-3'
MseI - selective primer	5'-GATGAGTCCTGAGTAACNN-3'

An internal size standard (GeneScan 1200LIZ, Applied Biosystems (ABI)) was required for each sample. To prepare the size standard, 0.5 µl of size standard was mixed with 9 µls of High dye formamide (ABI). This mix was denatured by incubation at 95°C for 5 min. Immediately after this, 9.5 µl of the denatured size standards were added to 0.5 µl of processed sample on ice (to prevent renaturing). The samples were run on a DNA Analyzer (ABI 3730) with the following settings: pre-run voltage: 15kV, pre-run time: 180 sec, injection voltage: 1.6kV, injection time: 15 sec, run time: 7000 seconds, run voltage: 8kV, dye set: G5.

The output was analysed with GeneMapper Software V4.0 (ABI), which automatically assigns bin sets to peaks between 100 and 999 base pairs and a cut off relative fluorescence unit (rfu) of 80. The bin sets were inspected visually and bins that were off-centre were manually corrected. Those that could not be unambiguously scored were removed.

To investigate which loci (peaks) would provide the lowest error rates, an error analysis was carried out. This was done following the protocol of Whitlock *et al.*, (2008) and using the program AFLPScore v.1.4a. This method calculates mismatch error rates for a range of user-specified locus and phenotype selection thresholds. The mismatch error rate is the percentage of differences in phenotype (peaks pattern) amongst replicated samples (i.e. repeat analyses of single samples). The selection of thresholds was based on the lowest mismatch error percentage.

5.2.4 Allozyme analyses

Euclidian geographic distances between the nine populations were calculated in ArcGIS (ESRI, 2011). Percentage polymorphic loci, observed and expected heterozygosity, F_{IS} and F_{ST} values were calculated and Mantel tests executed using G-stat v.3.2 (Siegismund, 1997). The number of alleles was calculated with Popgene v.1.31 (Yeh *et al.*, 1997). Relationships between allele presence of three loci (PGI, PGM, G6PDH) and time of activity or any of the weather variables was tested with general linear models. Enzymes were labelled with either presence or absence of an allele for each common allele. Only data of

active (flying) individuals was used. Data were checked for normality using the Shapiro-Wilk's normality test in R 2.11.1 (R Development Core Team, 2010).

5.2.5 Population genetic structure

The resulting binary matrix was analysed using AFLPSurv v.1.0 (Vekemans *et al.*, 2002). Allelic frequencies were estimated with a Bayesian method with non-uniform prior distribution (Zhivotovsky, 1999). Statistics of genetic diversity and population genetic structure were calculated following the protocol of Lynch & Milligan (1994). The number of random permutations for the calculation of F_{ST} was set to 10000. The relationships between the nine populations were visualised with the program PHYLIP v.3.69 (Felsenstein, 2004). The relationship between genetic and geographic distances (isolation-by-distance) was investigated with a Mantel test in G-stat v.3.2 (Siegismund, 1997).

5.3 RESULTS

5.3.1 Enzyme analyses

Across all populations 14 of the 17 enzymes studied were polymorphic. FUM, GPDH and IDH1 were monomorphic across all populations. True polymorphisms, where the commonest allele does not exceed a frequency of 0.95, were found in G6PDH, GOT1, GOT2, PGI, PGM and $PEP_{(Phe\text{-}Pro)}$ (Table 5.4). This means that in these enzymes the different alleles are not just rare ones, but that different alleles coexist. PGM had four different alleles that occurred in relatively high frequencies and this was the most polymorphic enzyme (Table 5.4). PGI had one allele present in a higher frequency at Mallaig compared to the other populations; ME had one allele that was more abundant at Bernwood compared to the other populations (Table 5.5). At Greenhow both PGM and G6PDH had allele frequencies that were slightly different from the other populations (Table 5.5). The percentage of polymorphic loci across all populations was 64.7%. Across all populations there was a tendency for homozygote excess (the mean observed heterozygosity (H_o) was 12.4%; the mean expected heterozygosity (H_e) was 15.9%). Also, for most loci there was a tendency for a heterozygote deficiency, except for PGI (Table 5.6). The most common homozygotes in this enzyme were at lower frequencies than expected (Table 5.7). There were no significant deviations from Hardy-Weinberg equilibrium and no consistent linkage disequilibria were found.

Table 5.4 Allele frequencies of the 14 polymorphic loci across the nine study populations. The labelling of the alleles is in accordance with the labelling of European populations of *P. icarus* (Schmitt *et al.*, 2003).

6PGDH		CK		G6PDH		GAP	
b	0.9808	c	0.0187	0	0.0032	b	0.9953
c	0.0176	d	0.9781	a	0.2636	c	0.0047
x	0.0016	e	0.0016	b	0.7204		
		x	0.0016	c	0.0128		

GOT1		GOT2		HBDH		IDH2	
c	0.4602	0	0.0775	b	0.0068	a	0.0016
d	0.5398	a	0.0016	c	0.9796	b	0.9953
		b	0.0206	d	0.0136	c	0.0031
		c	0.8892				
		d	0.0032				
		y	0.0032				
		z	0.0047				

MDH1		MDH2		ME		PGI	
b	0.9984	b	0.9627	a	0.0078	0	0.0047
c	0.0016	c	0.0373	b	0.9656	b	0.0717
				c	0.0266	c	0.7212
						d	0.1495
						e	0.0405
						h	0.0031
						u	0.0031
						v	0.0047
						y	0.0016

PGM		PPP	
0	0.0879	a	0.0482
a	0.0016	b	0.0032
b	0.2834	c	0.9003
d	0.443	d	0.045
e	0.0049	x	0.0016
f	0.1743	z	0.0016
g	0.0033		
z	0.0016		

Table 5.5 Within population allele frequencies for a selection of loci. Remarkable allele frequencies are highlighted.

	Friston	Frog Firle	Bedelands Farm	Aston Rowant	Grangelands	Bernwood	Otmoor	Greenhow	Mallaig
PGI									
b	5	3	2	7	9	9	2	7	1
c	18	50	38	57	75	76	16	68	65
d	5	11	7	10	19	12	3	21	8
e	0	3	2	2	3	0	1	0	15
h	0	0	0	0	0	0	0	0	0
o	0	0	0	3	0	0	0	0	0
u	1	0	0	1	0	0	0	0	0
v	1	1	0	0	0	0	0	0	1
y	0	0	1	0	0	0	0	0	0
PGM									
a	0	0	0	0	0	1	0	0	0
b	7	17	16	27	17	36	8	20	26
d	15	25	24	37	51	32	11	33	44
e	0	0	1	0	0	0	0	0	2
f	2	11	3	12	23	11	2	30	13
g	1	0	0	1	0	0	0	0	0
o	5	12	6	3	7	12	1	7	1
z	0	1	0	0	0	0	0	0	0
G6PDH									
a	8	13	2	7	35	33	4	47	14
b	21	52	47	70	70	56	18	44	73
c	0	1	1	3	1	0	0	2	0
o	1	0	0	0	0	1	0	0	0

Table 5.5 *continued* Within population allele frequencies for a selection of loci

	Friston	Frog Firle	Bedelands Farm	Aston Rowant	Grangelands	Bernwood	Otmoor	Greenhow	Mallaig
ME									
a	1	0	0	0	1	0	0	0	2
b	28	65	50	79	105	86	22	95	88
c	1	1	0	1	0	12	0	1	0
GOT1									
c	1	46	13	2	16	74	3	70	41
d	29	12	35	78	74	4	19	12	48
GOT2									
a	0	0	0	0	0	1	0	0	0
b	5	2	3	2	0	0	1	0	0
c	24	60	43	70	96	87	20	83	79
d	0	0	0	0	0	2	0	0	0
o	1	6	3	6	8	4	1	11	9
y	0	0	0	0	0	0	0	2	0
z	0	0	1	2	0	0	0	0	0

Table 5.6 Observed and Nei's (1973) expected heterozygosities for all 17 loci.

Locus	Observed heterozygosity	Nei's (1973) expected heterozygosity
6PGDH (86)	0.000	0.045
CK/PK (88)	0.046	0.087
FUM (90)	0.000	0.000
G6PDH (88)	0.296	0.283
GAPDH (86)	0.070	0.067
GOT1 (90)	0.022	0.496
GOT2 (88)	0.159	0.184
GPDH (90)	0.000	0.000
HBDH (70)	0.057	0.056
IDH1 (90)	0.000	0.000
IDH2 (90)	0.000	0.000
MDH1 (90)	0.000	0.000
MDH2 (90)	0.067	0.064
ME (90)	0.044	0.044
PGI (90)	0.511	0.443
PGM (86)	0.488	0.623
Pphe-pro (82)	0.171	0.160
Mean (± St. Dev.)	0.114 ± 0.166	0.150 ± 0.195

Table 5.7 Observed and expected frequencies of the different PGI genotypes across all populations.

Genotypes	Obs. (O)	Exp. (E)	2*O*Ln(O/E)
(b, b)	2	1.6147	0.856
(c, b)	33	33.2262	-0.451
(c, c)	163	166.8534	-7.617
(d, b)	9	6.8892	4.811
(d, c)	74	69.3417	9.623
(d, d)	4	7.1139	-4.606
(e, c)	20	18.78	2.518
(e, d)	5	3.8939	2.500
(h, c)	2	1.4446	1.301
(o, c)	3	2.1669	1.952
(u, c)	2	1.4446	1.301
(v, c)	3	2.1669	1.952
(y, e)	1	0.0406	6.410

The nine populations are not differentiated for the enzymes studied (> 93% similarity; Table 5.8) and there is a low F_{ST} = 0.121, which is slightly lower than the within population variation (F_{IS} = 0.126).

Table 5.8 Nei's (1978) unbiased measures of genetic distance and genetic identity. Genetic identities above diagonal, genetic distances below.

	FRIS	FROG	BEDE	ASTO	GRAN	BERN	OTMO	GREE	MALL
FRIS	****	0.959	0.992	0.996	0.995	0.937	0.997	0.946	0.983
FROG	0.042	****	0.980	0.959	0.971	0.995	0.970	0.992	0.990
BEDE	0.008	0.020	****	0.995	0.991	0.959	0.998	0.959	0.996
ASTO	0.004	0.042	0.005	****	0.993	0.935	0.999	0.940	0.986
GRAN	0.005	0.029	0.009	0.007	****	0.955	0.997	0.964	0.991
BERN	0.065	0.005	0.042	0.067	0.046	****	0.952	0.994	0.977
OTMO	0.003	0.030	0.002	0.001	0.003	0.050	****	0.954	0.993
GREE	0.056	0.008	0.042	0.062	0.036	0.006	0.047	****	0.977
MALL	0.017	0.010	0.004	0.014	0.009	0.024	0.007	0.023	****

There was no correlation between pairwise genetic and geographical distances (Mantel test: $r = -0.184$; $P > 0.05$). This lack of correlation remained when the monomorphic enzymes were removed from the analysis (Mantel test: $r = -0.166$; $P > 0.05$) or with only the truly polymorphic enzymes included (Mantel test: $r = -0.131$; $P > 0.05$). With the truly polymorphic enzymes, there was again no correlation of genetic with geographic distance (Mantel test: PGM: $r = -0.078$; PGI: $r = 0.253$; G6PDH: $r = -0.104$; GOT1: $r = -0.193$; GOT2: $r = 0.241$; all $P > 0.05$).

Only with the four populations sampled in the first generation were there significant relationships between latitude and some of the allele frequencies found (PGM allele d: $R^2 = 0.91$, $P = 0.044$; PGM allele o $R^2 = 0.98$, $P = 0.012$; Figure 5.2). There was no difference with the time of capture for the different common alleles of PGI (b, d, e), PGM (d, f, g, o) or G6PDH (a, b). Only females that are heterozygous in PGM are active earlier in the day ($F_{1, 110} = 4.60$, $P = 0.028$).

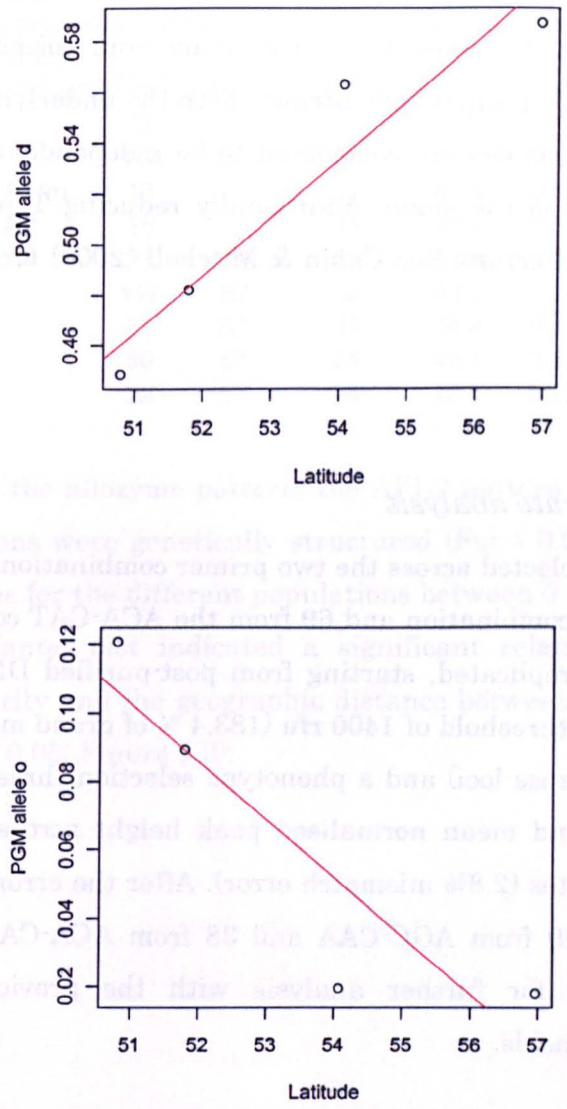

Figure 5.2 Relationship of allele frequency with latitude for two alleles of PGM. Populations are (from south to north): Frog Firle (*Lat: 50.79*), Bernwood (*Lat: 51.80*), Greenhow (*Lat: 54.07*), Mallaig (*Lat: 56.96*).

Within these four populations, individuals with PGM allele g are active only at high solar radiation (with allele g: 983.2 (± 156.1) W/m², without allele g: 543.7 (± 19.7) W/m²; $F_{1,190} = 7.804$; $P = 0.0057$). Individuals with PGM allele o can fly at higher windspeeds (with allele o: 3.2 (± 0.32) m/s², without allele o: 2.2 (± 0.2) m/s²; $F_{1,190} = 7.865$; $P = 0.0056$). G6PDH heterozygotes also can be active at higher windspeeds (heterozygotes: 2.8 (± 0.28) m/s², homozygotes: 2.18 (± 0.2) m/s²; $F_{1,191} = 4.7611$; $P = 0.0303$).

These relationships were investigated for each allele separately, resulting in 12 tests (3 for PGI; 4 for PGM; 2 for G6PDH; heterozygotes for the three loci). Adjustments (e.g. Bonferroni corrections) are not made for multiple comparisons because both the underlying enzyme sets and weather variables are considered to be independent, although the null hypothesis is the same. Additionally reducing Type I errors will increase Type II errors. See Cabin & Mitchell (2000) for full discussion of these issues.

5.3.2 AFLP

5.3.2.1 Error rate analysis

202 loci were selected across the two primer combinations (132 loci from the AGC-CAA combination and 69 from the ACA-CAT combination). 25 samples were replicated, starting from post-purified DNA material. A locus selection threshold of 1400 rfu (183.4 % of grand mean normalised peak height across loci) and a phenotype selection threshold of 400 rfu (52.4 % of grand mean normalised peak height across loci) gave the lowest error rates (2.8% mismatch error). After the error analysis, 57 of the 202 loci (19 from AGC-CAA and 38 from ACA-CAT combination) were retained for further analysis with the previously described selection thresholds.

5.3.2.2 Population genetic structure

The mean within population variation (analogous to Nei's gene diversity H_s) was 0.15 (± 0.007). The percentage polymorphic loci varied between sites from 40.4% to 66.7% (Table 5.9) and the expected heterozygosity (H_j) varied between 12.8% and 19.6% (Table 5.9).

Table 5.9 Population genetic data: n = number of individuals; #loc_P = number of polymorphic loci at the 5% level (loci with allelic frequencies lying within the range 0.05 – 0.95); PLP = percentage polymorphic loci at the 5% level; Hj = expected heterozygosity under Hardy-Weinberg proportions; S.E.(Hj) = standard error of Hj.

Population	n	#loc.	#loc_P	PLP	Hj	S.E.(Hj)
Friston	11	57	38	66.7	0.196	0.024
Frog Firle	105	57	24	42.1	0.135	0.020
Bedelands Farm	16	57	27	47.4	0.159	0.021
Aston Rowant	25	57	23	40.4	0.140	0.022
Grangelands	25	57	25	43.9	0.157	0.021
Bernwood	119	57	25	43.9	0.128	0.020
Otmoor	11	57	31	54.4	0.137	0.021
Greenhow	50	57	24	42.1	0.131	0.022
Mallaig	29	57	24	42.1	0.129	0.022

In contrast to the allozyme pattern, the AFLP pattern showed that the nine populations were genetically structured ($F_{ST} = 0.024$; $p < 0.0001$), with F_{ST} values for the different populations between 0 and 0.085 (Table 5.10). The Mantel test indicated a significant relationship between genetic similarity and the geographic distance between the populations ($r = 0.704$; $p < 0.05$; Figure 5.3).

Table 5.10 Pairwise F_{ST} values. FRIS = Friston, FROG = Frog Firle, BEDE = Bedelands Farm, ASTO = Aston Rowant, GRAN = Grangelands, BERN = Bernwood, OTMO = Otmoor, GREE = Greenhow, MALL = Mallaig

Population	FRIS	FROG	BEDE	ASTO	GRAN	BERN	OTMO	GREE
FROG	0.018							
BEDE	0.000	0.017						
ASTO	0.004	0.004	0.000					
GRAN	0.000	0.000	0.006	0.000				
BERN	0.012	0.003	0.003	0.002	0.000			
OTMO	0.013	0.003	0.000	0.000	0.006	0.000		
GREE	0.073	0.074	0.081	0.049	0.058	0.085	0.065	
MALL	0.036	0.048	0.035	0.037	0.045	0.046	0.021	0.073

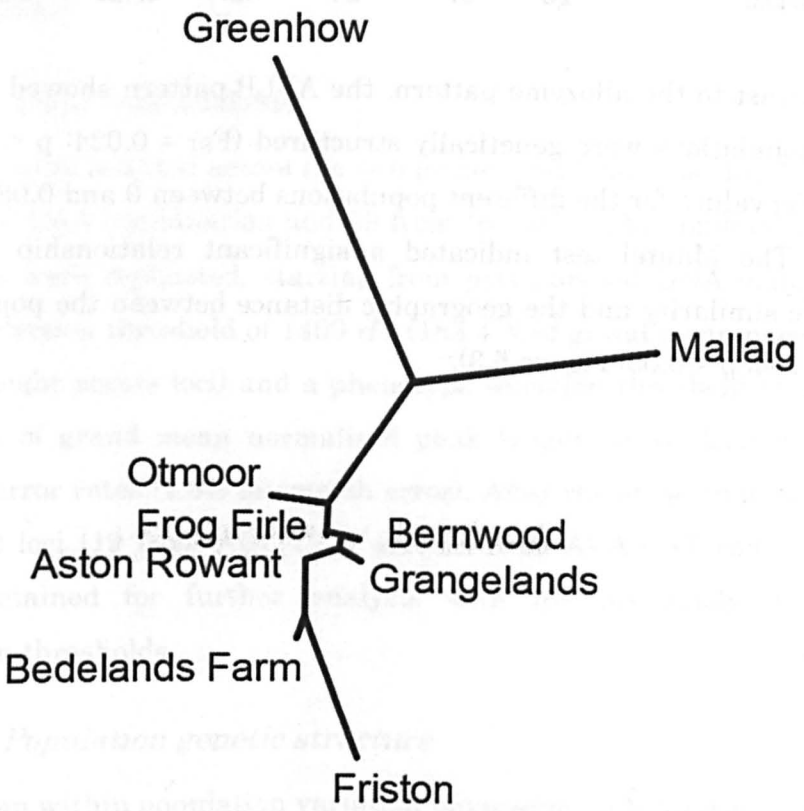

Figure 5.3 Isolation-by-distance for the nine populations. The length of the lines represents the distance between the populations.

5.4 DISCUSSION

The allozyme analysis revealed an overall genetic diversity for *P. icarus* which is lower than that known for related species (Schmitt *et al.*, 2002). It was lower (56.2% polymorphic loci) than found in previous studies on *P. icarus* in mainland Europe (77.9%; Schmitt *et al.*, 2003) and *P. coridon* (76.4%; Schmitt *et al.*, 2002). There are fewer alleles on the British Isles (3.41 alleles per locus) compared to the mainland European populations (7 alleles per locus; Schmitt *et al.*, 2003). This lower allelic diversity suggests that *P. icarus* has experienced a bottleneck, either during its colonisation of the British Isles or subsequent to colonization. This lower allelic diversity may have consequences in terms of the evolutionary potential of this species (Olivieri, 2009) as lower diversity can reduce the adaptability of species to changing environments (Hedrick, 2001). Generally a pattern of reduced genetic diversity with latitude is found as a consequence of post-glacial expansion and colonisation history (Hewitt, 2000). However, possibly because of the small spatial scale of the latitudinal cline in this study or because of the already quite impoverished genetic constitution of the populations this pattern was not found. Twelve of the seventeen studied loci were monomorphic or only had a few rare alleles. In comparison with Europe the genetic diversity is low, but it is not known whether the amount of variation found is approaching the minimum for persistence.

Despite the low allelic diversity, polymorphisms in the truly polymorphic loci were maintained. High levels of polymorphisms in PGI, PGM and G6PDH have been found in previous studies on butterflies (Watt, 1977; Goulson, 1993; Saccheri *et al.*, 1998; Schmitt *et al.*, 2002; Vandewoestijne & Van Dyck, 2010) and a selective advantage of heterozygotes has previously been given as an explanation for the maintenance of such polymorphisms (Dahlhoff & Rank, 2000; Haag *et al.*, 2005; Wheat *et al.*, 2005). In this study too, there was a reduction in the frequency of the most common homozygotes for PGI within populations, regardless of the generally high F_{IS} (heterozygote

deficiency). In most populations a different allele of PGM was predominant, possibly the result of different selective pressures in the different populations. This suggests that there is not one allele which is consistently superior in all populations, giving another explanation for the existence and maintenance of polymorphisms. One population, Greenhow, where *P. icarus* flies in one generation is located at a higher altitude (415m above sea level), with strong seasonal differences compared to the surrounding valleys at similar latitudes, where it is bivoltine. The frequency of the alleles of PGI, PGM and G6PDH in this population differs slightly from all the other populations and it seems likely that a lack of overlap in flight seasons of this population and the surrounding valley populations, allows the alleles in this location to drift a little further away from the other populations.

Only in the spring generation butterflies were there correlations of alleles of these three enzymes with latitude (Figure 5.2). Along a latitudinal cline, one of the most important variable environmental factors for invertebrates is temperature; the pattern that was found in the first generation in these alleles could be the result of varying thermal selection pressures. Possibly because of seasonal variation in allele frequencies (Watt, 1977) this pattern disappears when the summer generation butterflies are incorporated in the analysis. The overwintering generation in all latitudes within the UK goes through a cold winter period, the severity of which varies with latitude. Thus the latitudinal effect may be stronger in this than the summer generation. The lack of relationship between genetic distance and geographic distance for these three enzymes further suggests that the pattern of allelic frequencies is a not the result of an isolation-by-distance effect, but that selection possibly has shaped the pattern in these three extremely variable glycolytic enzymes. This is further confirmed by the presence of the isolation-by-distance pattern in the more neutral AFLPs. These results are a first step towards identifying candidate loci (PGI, PGM, G6PDH) which vary in relation to thermal gradients in *P. icarus*. Some of the alleles of these loci varied with solar radiation, windspeed or time of activity, but because of the lower frequency of

these less common alleles, a high sample size is not always guaranteed. Further functional studies are therefore needed to investigate the relationship between temperature and allelic variation.

The overall level of genetic diversity but low differentiation between populations (mean genetic distance (Nei, 1978) = 0.0236; F_{ST} = 0.121) is typical for a high density or highly mobile species (Hastings & Harrison, 1994; Hanski, 1998). However, *P. icarus* has previously been described as a species with a rather sedentary character (Emmet & Heath, 1989; Asher *et al.*, 2001). Low differentiation between populations might imply that *P. icarus* is a mobile species, but the lack of an isolation-by-distance pattern could also be explained by extinction and recolonisation dynamics with recolonisation events by means of long-distance dispersal (Vandewoestijne *et al.*, 2004). The reduction in allelic diversity (reduction in possible variability between populations) and the non-neutrality of the truly polymorphic enzymes (Watt, 1977; Watt, 1983; Haag *et al.*, 2005; Wheat *et al.*, 2005) could cause populations to be rather similar to each other, masking an isolation-by-distance pattern and reducing genetic differentiation. Careful interpretation of the results of phenotypic variation is therefore necessary as these characteristics may not be entirely neutral to selection (Watt, 1977; Eanes & Koehn, 1978; Watt *et al.*, 1983; Haag *et al.*, 2005), hence these results do not necessarily reflect population genetic structure (Goulson, 1993; Joyce *et al.*, 2009). This becomes clear when the results from the allozyme electrophoresis are compared with those from AFLPs, a more neutral marker. On the basis of this marker, the nine populations do show an isolation-by-distance effect (Figure 5.3). This means that populations that are geographically close are more similar in their DNA composition. Even with low F_{ST} values (F_{ST} = 0.024) AFLP can reveal significant population genetic structuring (Miller *et al.*, 2002). Allozymes therefore could fail to detect a population genetic structure in populations with high gene flow or low levels of differentiation.

On the basis of this pattern it seems most likely that the colonisation of the UK by *P. icarus* has occurred from one southern population. The

presence of founder effects in PGI and ME and the strong differences in allelic frequencies between the populations in PGM, PGI and G6PDH (Table 5.5) suggest that despite the low genetic distances and low F_{ST} values, genetic differentiation is possible and the potential for local adaptation in the different populations exists.

5.5 INTEGRATION

Polyommatus icarus in the UK is genetically impoverished compared to where it has been studied by Schmitt *et al.* (2003) in mainland Europe, most likely because of a bottleneck during its colonisation. Despite this impoverishment, certain important glycolytic enzymes, for which allelic variation has previously been related to fitness differences in other butterflies (Watt, 1977; Eanes & Koehn, 1978; Haag *et al.*, 2005), are polymorphic, adding to the evidence that selection and variation in environmental conditions is maintaining polymorphisms in these enzymes. This is further confirmed by the discrepancies between the geographic pattern in the enzymes and the AFLPs. Additionally, some alleles of these polymorphic enzymes vary along a thermal cline, indicating a potential for thermal adaptation. Specific lab studies to investigate a direct relationship between presence of certain alleles and thermal performance are now needed to improve our understanding of why polymorphisms in these enzymes have been maintained across so many species and taxa (Bijlsma, 1980; Crawford & Powers, 1989; Zamer & Hoffmann, 1989; Eanes *et al.*, 1993; Dahlhoff & Rank, 2000; Ward *et al.*, 2004; Huestis & Marshall, 2006; Cosmidis *et al.*, 2008).

Chapter 6 Developmental responses to temperature and photoperiod

6.1 INTRODUCTION

The size of an adult butterfly is dependent on larval growth (Atkinson *et al.*, 1994), which is strongly influenced by temperature and photoperiod (Nylin *et al.*, 1989; Leimar, 1996; Nylin & Gotthard, 1998; Gotthard, 2008). Thus, accumulation of resources by larvae is an important life history component as adult size has large consequences for fitness (Stearns, 1992). Higher temperatures during development generally allow for increased activity in ectotherms, resulting in faster growth and a shorter development time, but producing smaller adults. By converse, lower temperatures reduce growth rate and lengthen development time (Atkinson *et al.*, 1994; Atkinson & Sibly, 1997; Davidowitz & Nijhout, 2004; Kingsolver *et al.*, 2004). This explains the commonly observed pattern of increased adult size with latitude in arthropods (Blackenhorn & Demont, 2004), although the opposite pattern has also been found (Nylin & Svard, 1991; Blackenhorn & Demont, 2004). The latter pattern is usually explained as a seasonal effect as season length decreases with latitude (Mousseau & Roff, 1989; Nylin & Svard, 1991) with short seasons truncating the time available for development.

Developing larvae have to use reliable end-of-season cues to 'predict' the time left until periods of unsuitable weather to 'choose' whether to develop directly or to spend the unfavourable period in a resting state; diapause (Danilevskii, 1965). The main cue for butterflies in seasonal systems is photoperiod (Danilevskii, 1965; Leimar, 1996; Nylin & Gotthard, 1998; Burke *et al.*, 2005) and the daylength at which fifty percent of the population goes into diapause is termed the critical photoperiod (Tauber *et al.*, 1986). Larvae on the pathway of direct development have the capability to speed up their development to make sure they reach the desired stage before the start of the unfavourable period (Nylin, 1992; Leimar, 1996; Nylin *et al.*, 1996; Gotthard, 1998).

In many species there is geographical variation in diapause expression and the general pattern is a longer critical photoperiod with increasing latitude (Masaki, 1961; Tauber *et al.*, 1986; Mousseau & Roff, 1989a; Kurota & Shimada, 2003). *Polyommatus icarus* has a large latitudinal range and the response of individuals from different latitudes to photoperiodic and temperature regimes is expected to differ according to their location if development is an adaptive response to prevailing environmental conditions.

Thus, it is often the interaction between photoperiod and temperature cues that trigger the pathway of (speeded) direct development or diapause, whereas temperature directly affects development time through its effect on activity levels and growth rates. Adult butterflies of *P. icarus* in Britain are larger in the colder higher latitudes, i.e. Scotland and Northern Ireland, than in the south of England (Emmet & Heath, 1989; Asher *et al.*, 2001), suggesting a temperature-size effect unconstrained by the requirement to develop quickly to fit in two generations a year as in the south of England.

Besides these plastic responses to environmental cues, there is an additional genetic component that can influence development. For example, in *Lycaena tityrus,* variation in growth rate and ability to cope with food stress is dependent on PGI genotype (Karl *et al.*, 2010). This could allow for local adaptation to predictable environmental conditions, but whether such patterns of local adaptation in developmental traits are more widespread and reflect a geographical pattern is at present not known. The most likely allozyme candidates for selective loci in butterflies are PGI, PGM and G6PDH (Watt, 1977; Carter & Watt, 1988; Goulson, 1993; Haag *et al.*, 2005).

Understanding how individuals respond to reliable cues (photoperiod) and variable factors (temperature), and the variability of these responses within populations provides essential information in

predicting how species respond to environmental changes (e.g., climate change and land management change; Hill & Hodkinson, 1995; de Vries *et al.*, 2011) and the likely consequences for population stability and persistence. Furthermore, the possibility of allozyme adaptation adds a new dimension to the study of development and could shine a new light on the variation in developmental responses to environmental change.

Here, the plastic response of development time and final size to the two main factors (temperature and photoperiod) is investigated in an experimental study, whereby larvae from different latitudes were reared at temperatures and photoperiods representative of the extremes of the studied cline. Additionally candidate allozyme loci are studied to investigate possibilities for local adaptation. Specifically, this chapter will test the following predictions:

(1) Larvae reared at low temperatures will develop more slowly and have a larger final adult size than those reared at higher temperature. Butterflies from more northern populations are expected to better cope with cold temperatures (i.e. less prolonged development time).

(2) Butterflies reared at longer photoperiods are expected to:

a) develop more slowly or go into diapause because the photoperiod indicates they are at a high latitude allowing only one generation with no need to speed up development for a second generation or,

b) develop more slowly than those at a shorter photoperiod, because the photoperiod suggest an earlier calendar date, relaxing the time pressure and allowing more time for development.

(3) Both the responses to photoperiod and temperature cues and the degree of plasticity of the response are expected to differ between populations, suggesting that these are not completely plastic. This may have strong implications for adaptation to changing environments.

(4) Because variation in PGI has previously been related to differences in developmental traits, this study investigates variation in three candidate loci (PGI, PGM, G6PDH) in four populations and their relationship with development time.

6.2 MATERIAL & METHODS

Eggs collected in Frog Firle (south east England) in the field in spring in two years (from 7 females (16-17/06/2008) and 14 females (07-09/06/2010)) and eggs from two northern sites (Greenhow: 14 females (13-14/07/2010) and Mallaig: 10 females (22-23/07/2010)) were transferred to a constant temperature room (20°C) and stored in small Petri dishes lined with damp filter paper to prevent desiccation. The time between collecting of the eggs and hatching of the first instars was on average 10 days. Total development time was calculated from the day of hatching of the eggs till the eclosion of the adult butterfly. On the day of hatching, larvae were transferred to potted *Lotus corniculatus* host plants that were placed in an incubator, with a 16L8D cycle, in a split-temperature design (14°C and 20°C). These temperatures are the relevant air temperatures for Scotland and England respectively and the photoperiod is the late-spring daylength for southern England. In the second part of the experiment, which was executed in the following year, freshly hatched caterpillars were reared at a constant 20°C but at a longer daylength (19L5D) to be compared with the 16L8D treatment at 20°C. This photoperiod corresponds to the late-spring daylength for Scotland. Offspring of each female were reared individually resulting in 7 families in the first part (average n = 6.4 larvae per family) and 14 families in the second part (average n = 1.6 larvae per family) for Frog Firle, 14 families for Greenhow (average n = 3.1 larvae per family) and 10 families for Mallaig (average n = 4.1 larvae per family). Initially between 6 and 10 larvae per family were set up, but in order to maintain high family diversity and still guarantee sufficient food resources, the number of larvae was reduced during the experiment. On the day of eclosion, adult butterflies (16 females, 21 males at 14°C 16L8D; 17 females, 29 males at 20°C16L8D; 6 females, 22 males at 20°C 19L5D) were weighed to the nearest 0.001 g (HR-120-EC, A&D Instruments) and a subset was frozen for allozyme analysis (see Chapter 5). No two individuals from the same family were frozen for this analysis. The results of these three treatments could then be

analysed as a set of two contrasts (14°C v 20°C at 16L8D; 16L8D v 19L5D at 20°C).

6.2.1 Analyses

General Linear Mixed Models (GLMMs) were fitted to explain the variation in the development time and final adult weight for both fixed effects, temperature and photoperiod treatment. Sex was used as a fixed effect; both site and family nested within site were used as random effects. During the experiment however, northern populations all entered diapause, essentially yielding no data. All results are therefore coming from one (southern) site and only the fixed effect sex and random effect family were used in the model. Because of limitations of the design, the long-photoperiod treatment was done in a different season than the short-photoperiod treatment, possibly confounding photoperiod and year. This implies that families are not repeated across the photoperiod treatments. However, because of the random sampling in the same locations and a fixed rearing protocol, with care, data can be compared.

Because of the possible relationship between development time and final weight, time was tested as a covariate in the GLMM for final weight. Final model selection was done by backward elimination of the least significant variables starting from a full model containing all variables and interaction terms of interest.

Allozymes were labelled with either presence or absence of an allele for each common allele. GLMs were used to explain the variation in development time and final weight separately, with each allele (fixed effect) and rearing temperature and their interaction as factors. Weight was again incorporated as a covariate in the analysis of development time and vice versa.

All data were tested for normality using the Shapiro-Wilk's normality test in R 2.11.1 (R Development Core Team, 2010).

6.3 RESULTS

Irrespective of temperature or photoperiod conditions all northern larvae (from Mallaig and Greenhow) entered diapause in their second instar. Larvae stopped feeding and remained motionless on the underside of the *Lotus* plants and did not restart feeding till after 60+ days, when observations were ended. A subset of the caterpillars was subjected to a cold-shock (14 days in 4°C, 24h dark) after which they were transferred back to 20°C and 19L5D. No feeding was observed for 10 days and observations were ended after this period. Thus all results that follow are from one site (Frog Firle in the south).

6.3.1 Development at different temperatures

Southern (Frog Firle) butterflies reared at 14°C developed much slower than at 20°C (weighted means: 14°C: 147.2 (± 2.2) days for females; 139.4 (± 1.7) days for males; 20°C: 61.1 (± 0.8) days for females; 57.3 (± 0.67) days for males, Figure 6.1), and there was a family effect (Temperature: $F_{1,74} = 4376.78$, $P < 0.0001$; Sex: $F_{1,74} = 17.21$, $P < 0.0001$; Family: $F_{6,74} = 3.45$, $P = 0.0047$). However, larvae that spent a longer time developing had a lower final weight (Temperature: $F_{1,75} = 6.65$, $P = 0.012$; Figure 6.2) and there was a family effect ($F_{6,75} = 2.88$, $P = 0.014$). In the analysis of weight there was no effect of sex ($P > 0.05$). The individuals in the low temperature treatment also displayed considerable variation in development time. Males developed faster than females, but there was no difference in the response to development temperature between the sexes (no significant interaction effect). There was a weak negative relationship between development time and weight ($r = -0.217$, $P < 0.049$). Development time was tested as a covariate for weight but was not found to be significant ($P > 0.05$) and was not retained in the final model.

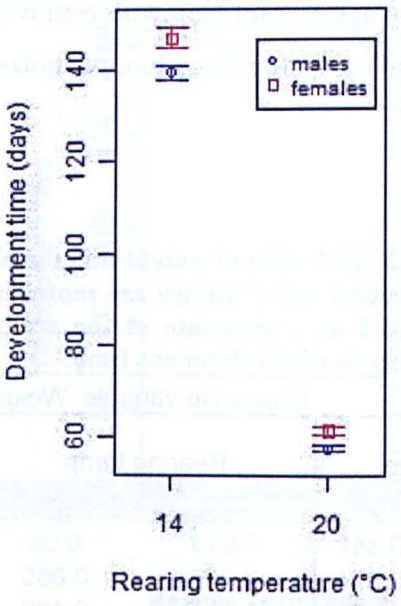

Figure 6.1 Development time (± standard error) of males and females in different rearing conditions. Depicted are the standard errors of the fitted full model.

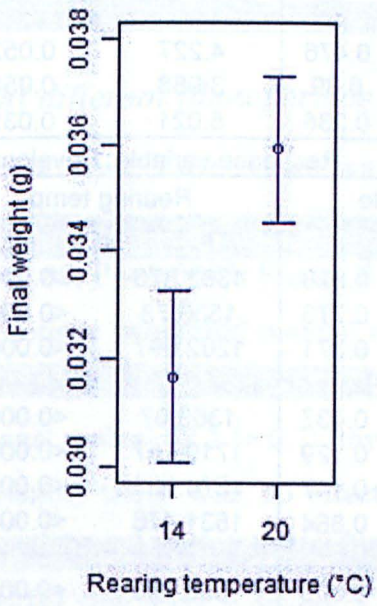

Figure 6.2 Final adult weight (± standard error) for butterflies reared under different temperatures.

In the subset that was frozen for allozyme analysis, no relationship was found between any of the alleles of G6PDH, PGI or PGM and development time or final weight (Table 6.1). However, in the analysis of development time there was a significant interaction between heterozygotes of PGM and rearing temperature (Table 6.2). There was

no effect of weight as covariate and this was removed from the analysis. PGM heterozygotes develop faster than homozygotes at 20°C but slower at 14°C (Figure 6.3).

Table 6.1 Results of the ANOVA of final weight and development time. F-ratios (with their degrees of freedom) and P-values are represented for each variable. Development time was used as a covariate in the analysis of weight; weight was a covariate in the analysis of development time.

	Response variable: Weight					
	Allele		Rearing temp		Development time	
G6PDH	$F_{1,23}$	P	$F_{1,23}$	P	$F_{1,23}$	P
allele a	0.036	0.852	3.913	0.06	5.48	0.028
allele b	0.823	0.374	3.746	0.065	5.166	0.033
heterozygote	0.663	0.424	2.904	0.102	4.454	0.046
PGI						
allele b	1.515	0.231	2.665	0.116	4.073	0.055
allele d	0.13	0.721	4.354	0.048	5.777	0.025
allele e	0.087	0.771	4.309	0.049	5.686	0.026
heterozygote	0.365	0.552	4.051	0.056	5.541	0.027
PGM						
allele f	0.526	0.476	4.227	0.052	5.598	0.027
allele o	0.02	0.89	3.988	0.058	5.283	0.031
heterozygote	0.97	0.335	5.021	0.035	6.606	0.017
	Response variable: Development time					
	Allele		Rearing temp		Weight	
G6PDH	$F_{1,23}$	P	$F_{1,23}$	P	$F_{1,23}$	P
allele a	0.411	0.528	1302.376	<0.0001	5.48	0.028
allele b	0.085	0.773	1536.73	<0.0001	5.166	0.033
heterozygote	0.832	0.371	1202.697	<0.0001	4.454	0.046
PGI						
allele b	0.626	0.437	1363.07	<0.0001	4.073	0.055
allele d	2.477	0.129	1710.157	<0.0001	5.777	0.025
allele e	2.251	0.147	1574.581	<0.0001	5.686	0.026
heterozygote	0.022	0.884	1531.476	<0.0001	5.541	0.027
PGM						
allele f	0.062	0.805	1398.043	<0.0001	5.598	0.027
allele o	1.629	0.215	1433.66	<0.0001	5.283	0.031
heterozygote	2.31	0.143	1568.785	<0.0001	6.606	0.017

Table 6.2 Results of the ANOVA of development time and PGM Heterozygotes.

	$F_{1,21}$	P
Rearing temp	1889.696	<0.0001
Heterozygote	4.765	0.041
Weight	5.320	0.031
Rearing temp*Heterozygote	11.162	0.003

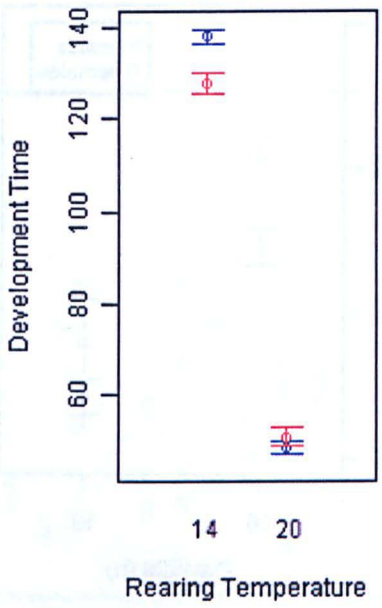

Figure 6.3 Relationship between rearing temperature and development time for PGM homozygotes (red) and heterozygotes (blue). Error bars indicate standard errors.

6.3.2 Development at different photoperiods

For the southern larvae (Frog Firle) there was a significant effect of photoperiod and sex on development time (Photoperiod: $F_{1,71} = 51.08$, $P < 0.0001$; Sex: $F_{1,71} = 14.83$, $P = 0.0003$; Figure 6.4). Butterflies reared in a 16L8D regime took longer (weighted means: males: 57.3 (± 0.6) days; females: 61.1 (± 0.8) days) than those reared under a 19-hour light regime (weighted means: males: 52.1 (± 0.7) days; females: 54.0 (± 1.3)). In the analysis of weight, there was no effect of sex. The short-day butterflies all developed into heavier butterflies (16L8D: 359 (± 0.12) mg; 19L5D: 202 (± 0.15) mg; $F_{1,72} = 67.39$, $P < 0.0001$; Figure 6.5). Because in this part of the experiment, butterflies were reared in two different years, family could not be used as a variable. Neither development time nor weight was significant as a covariate in the analyses. There was a positive relationship between development time and weight ($r = 0.461$, $P < 0.0001$; Figure 6.6).

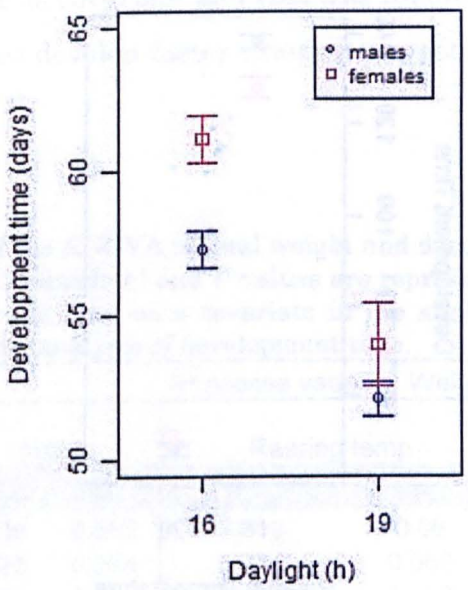

Figure 6.4 Development time (± standard error) of males and females in different photoperiods.

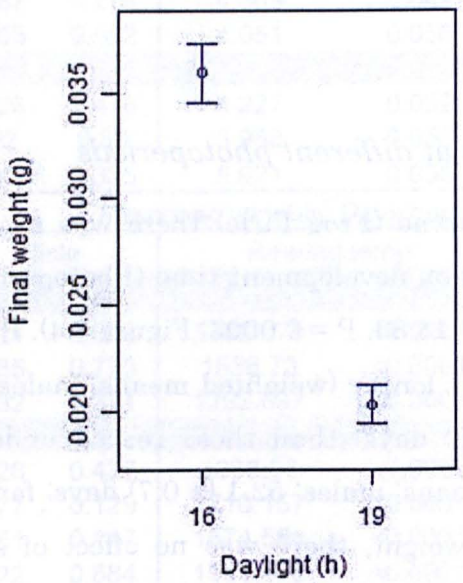

Figure 6.5 Final adult weight (± standard error) of butterflies reared in different photoperiods.

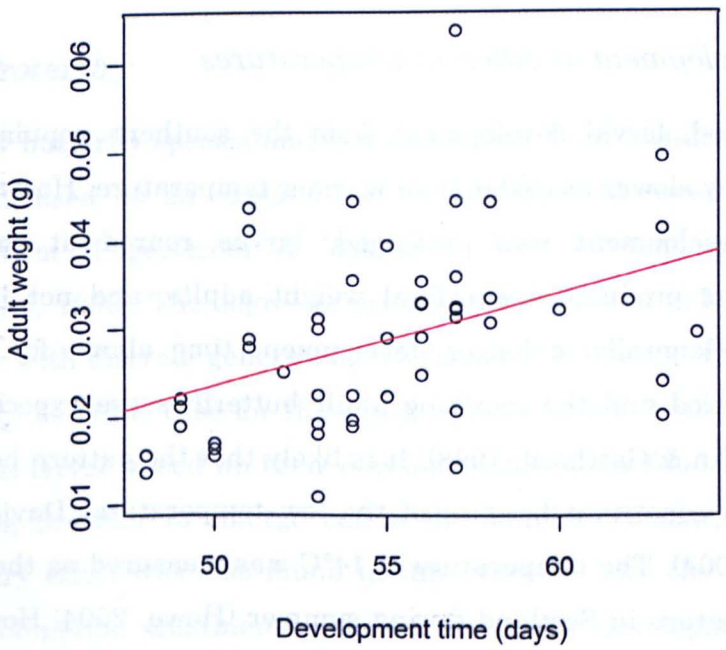

Figure 6.6 Relationship between development time and adult weight on the day of eclosion ($R^2 = 0.212$).

6.4 DISCUSSION

6.4.1 Development at different temperatures

As predicted, larval development from the southern population was significantly slower at colder than warmer temperature. However, even though development was prolonged, larvae reared at the colder temperature produced lower final weight adults, and not higher as expected. Generally, a longer development time allows for a longer feeding period and the resulting adult butterflies are expected to be larger (Nylin & Gotthard, 1998). It is likely that the pattern here is the result of a constraint because of the low temperature (Davidowitz & Nijhout, 2004). The temperature of 14°C was measured as the average air temperature in Scotland during summer (Howe, 2004; Howe *et al.*, 2007). This does not necessarily mean that *P. icarus* butterflies in Scotland or at even higher latitudes are suffering from strong environmental constraints on development, since solar radiation combined with appropriate microclimate choice could allow for larvae to experience higher temperatures than the average air temperature (Howe *et al.*, 2007). This could explain why there is a reduction in size in the lab and not in the field (see Chapter 3). Because larvae from the northern populations went into diapause, the latitudinal comparison could not be made.

The higher variability of development times of bivoltine populations at cold temperatures may reduce the chances of sibling mating in marginal conditions, where population density is likely to be small (Shreeve *et al.*, 1996). In more favourable conditions in southern locations, population densities are usually high enough so that variability in development times becomes less important and higher activity levels in these conditions will increase encounters with non-siblings. In other species this higher degree of variation in development times in colder conditions has previously been observed too (e.g. *Aricia agestis* (Burke *et al.*, 2005),

Pararge aegeria (Breuker, pers. comm.)). The reasons for this are currently unexplained but warrant future investigation.

6.4.2 Protandry

In many butterfly species males eclose earlier than females (protandry) which is used as an explanation for the often observed sexual size dimorphism (Fagerström & Wiklund, 1982; Wiklund *et al.*, 1991; Zonneveld, 1996). The degree of protandry is expected to be stronger in systems with discrete generations (Wiklund & Forsberg, 1991; Nylin *et al.*, 1993) as is the case for this study species in the British Isles. Male *P. icarus* larvae speed up their development in their final instar before pupating in order to emerge before the females (Leimar, 1996). This protandry effect was also found in this study in both the temperature and photoperiod treatments. The shorter male development did not however, result in differences between the sexes in final weight of the adults. In *P. icarus* the size dimorphism is variable, but occurs at all conditions (Leimar, 1996). This suggests that protandry is a trait that can be uncoupled from sexual size dimorphism (Nylin *et al.*, 1993), indicating that it is a trait that is actively selected for in *P. icarus* in seasonal environments.

6.4.3 Development at different photoperiods

The photoperiods the larvae were subjected to were those of a southern and a northern latitude. *Polyommatus icarus* occurs within the British Isles in two generations in the south (bivoltine) and only one in the north (univoltine) with a transition zone in North-Yorkshire (around 54° latitude) (Emmet & Heath, 1989; Asher *et al.*, 2001). The expected response was therefore that larvae from southern populations, subjected to the northern photoperiods, would behave like northern individuals and have a slower development or enter diapause (as the result of a plastic univoltine strategy). However, the larvae from the southern population when reared at longer daylengths responded with a shorter development time and a lower final weight. Because the experimental conditions of photoperiod and temperature are not naturally

encountered by these butterflies, they could have misinterpreted these cues. It is also possible that for *P. icarus* from Frog Firle temperature is a more important cue for diapause induction and colder temperatures are needed before these butterflies enter diapause. The longer, northern photoperiod to which they were subjected indicates a shorter growing season and this would then make it necessary to develop faster to produce the next generation. Larvae from more northern populations, Mallaig and Greenhow, subjected to southern photoperiods, were expected to develop directly as to give rise to a second generation. The response found however, was that all larvae went straight into diapause under both northern and southern photoperiod treatments. This is indicative of an obligate diapause strategy.

These findings suggest that the response to diapause cues is not entirely plastic as was also demonstrated for *Pieris brassicae* (Spieth *et al.*, 2011) and the flexibility in voltinism in this species is not as previously described (Emmet & Heath, 1989; Asher *et al.*, 2001). In a geographic comparison, southern populations appear to be more plastic in their voltinism strategies than northern ones. Further studies subjecting larvae from populations along a latitudinal cline to different thermal and photoperiod treatments will allow for a clearer understanding of the variation in voltinism.

6.4.4 Allozymes and development

Many studies have recently investigated the relationship of allozymes and performance (Watt, 1977; Goulson, 1993; Haag *et al.*, 2005; Hanski & Saccheri, 2006; Niitepõld *et al.*, 2009; Orsini *et al.*, 2009; Saastamoinen *et al.*, 2009; Vandewoestijne & Van Dyck, 2010). However, influence of allelic variation on development is hardly studied. One study has found that the PGI genotype of *Lycaena tityrus* can have an influence on growth rate, but not on total development time (Karl *et al.*, 2010). Previous studies on butterflies have found relationships between genotypes of PGI, PGM and G6PDH and different life history traits (Watt, 1977; Carter & Watt, 1988; Goulson, 1993; Haag *et al.*,

2005). However, for none of these three loci was there a direct effect on development time or final weight in this butterfly. *Polyommatus icarus* individuals that are heterozygote at the PGM locus could develop faster than homozygotes in warm conditions (20°C), but were slower in the cold treatment (14°C), but there was no effect on adult size, possibly as a result of canalisation (Waddington, 1942). As a result, in warm conditions, PGM heterozygotes can spend less time as a caterpillar without trade-offs between size and development time. In the cold treatment, homozygotes have a slight advantage over heterozygotes, suggesting that specific allozymes confer advantage in these conditions. A reduction in PGM heterozygote frequency with latitude would add power to the explanation of canalised development. However, across the studied populations, no trend of either increased or decreased heterozygosity in PGM was found. Because only a subset of the experimental individuals was used in the analysis and the larvae from the northern populations went into diapause in all conditions, sample sizes are quite low and the full design could not be executed. To distinguish between canalised development and heterozygote advantage in PGM, insight in the development patterns of univoltine populations and their heterozygosity are needed.

6.4.5 Implications for conservation

Generally, there is a negative relationship between temperature and final adult size (Roff, 1980; Atkinson *et al.*, 1994; Davidowitz & Nijhout, 2004; Nijhout *et al.*, 2010). In this study, southern butterflies reared under warm temperatures were larger than those reared under colder temperatures and the reverse pattern was found in the field: second generation individuals of *P. icarus* butterflies are generally smaller than the first (spring) generation (see Chapter 3).

The experimental procedures used here used a constant photoperiod, and the field and laboratory findings indicate an interaction between photoperiod and temperature cues on development. If this is a general pattern for insects, then responses of species and populations to

environmental change via developmental processes could be more complex than if they are governed by photoperiod alone. Photoperiod in any location is a very stable predictor, but environmental changes (global warming, habitat modification) can influence the thermal characteristics of habitats with possible important consequences on the development duration and size of this butterfly. Changes in phenology have previously been described for butterflies as a consequence of a modification of the physical characteristics of a habitat, with strong effects on species persistence and abundance (Hill *et al.*, 2002; de Vries *et al.*, 2011). The interaction between photoperiod and temperature on size warrants further investigation, in particular in the field as there are potential implications for egg production, activity and flight performance.

Chapter 7 Integration

The Common blue butterfly *Polyommatus icarus* has been described as a highly variable butterfly (Frohawk, 1934; Emmet & Heath, 1989; Asher *et al.*, 2001) and previous work has quantified within and between population variation in behaviour and morphology in two populations in the British Isles (Howe, 2004; Howe *et al.*, 2007). The main aims of this project were to build further on this work and explore the nature of the variability in several traits in *P. icarus* by studying morphological, physiological and behavioural traits along a latitudinal cline. With common-garden and split-brood experiments, the causes of the variability could be explored: local adaptation, plasticity or a combination of both. Information on the population genetic structure of *P. icarus* in the Britih Isles was collected to investigate the potential for local adaptation (i.e. genetic isolation).

There were overall differences between the populations in the studied morphological traits (wing size, shape and melanisation), but there was no strong relationship with latitude as was expected (Watt, 1968; Nylin & Svard, 1991; Chown & Klok, 2003; Blackenhorn & Demont, 2004; Ellers & Boggs, 2004; Nygren *et al.*, 2008). The absence of this relationship suggests that previous functional interpretations of the morphological characteristics (Howe, 2004) are possibly not entirely correct.

Only for females was there an increase in size with latitude, while males across all populations did not differ in forewing size (Chapter 3). Female *P. icarus* thus follow a Bergmann cline, which is in contrast with the saw-tooth pattern that was expected for ectotherms in a seasonal system (Mousseau, 1997; Chown & Klok, 2003; Blackenhorn & Demont, 2004) and on the basis of a previous Swedish study with this species (Nygren *et al.*, 2008). An increase in size with latitude could be the result of decreasing temperatures and thus slower development and larger final size at northern latitudes (Blackenhorn & Demont, 2004).

The opposite pattern in the Swedish study could be the result of combining butterflies from different generations in the analysis, which may have resulted in a saw-tooth pattern. The field study conducted in this work only used spring generation butterflies because these would all have experienced a cold overwintering stage. Directly developing summer generation butterflies experience a completely different set of environmental conditions compared to northern univoltine butterflies, making comparisons difficult. The choice to use only butterflies that have experienced larval overwintering thus would result in a more comparable approach.

In the experimental stage however, the cold (northern) treatment for southern larvae resulted in a slower development and smaller adult butterflies for both males and females (Chapter 6). Southern larvae reared at northern photoperiods were expected to behave as a univoltine population, but instead they sped up their development resulting in smaller adults. This suggests that latitudinal variation in size is the result of a complex interaction of factors and that there is a strong genetic component influencing development time, growth rate and adult size. This is further confirmed by diapause induction in all northern (univoltine) larvae under all conditions and a strategy that can allow multivoltinism in southern (normally bivoltine) larvae. This means that even with high levels of gene flow (low F_{ST} values; Chapter 5) local adaptation is possible for certain developmental traits. This may be a result of a temporal segregation: the obligate univoltines in the north are temporally separated from both bivoltine generations. In the transition zone, both voltinism patterns can occur without overlap in flight period. Increasing global temperatures could therefore allow bivoltine populations to develop two complete generations at increasingly higher latitudes, effectively causing the univoltine populations to disappear.

Polyommatus icarus is known for its plasticity in sexual size dimorphism (Leimar, 1996; Nygren *et al.*, 2008). In an experimental treatment, adult males increase in size when reared under long

photoperiods while females reach an intermediate size in all conditions (Leimar, 1996). In a common garden situation females reared from southern latitudes are smaller than males, but those from more northern latitudes are larger (Nygren *et al.*, 2008). This is the first study to describe field variation in sexual size dimorphism along a latitudinal cline. This pattern could be the result of male butterflies in southern latitudes that compensate for the shorter development time by higher growth rates. Because of the added risks of high growth rates (Gotthard, 2000), reaching a large size for males is likely to be a trait that is actively selected for. Large male size generally is associated with territoriality and female polygamy (Wiklund & Forsberg, 1991). However, in the lab experiment under all circumstances, males emerge before females (protandry), which is associated with female monogamy (Wiklund & Forsberg, 1991). As these butterflies use a combination of perching and patrolling (Howe, 2004), both a large size (territoriality) and emerging early (protandry) could be of importance in this butterfly. Large bodies also are more thermally stable and this could be an important trait in colder latitudes, as the wings hardly contribute towards thermoregulation (Chapter 4). Another explanation for the lower variability in male size across latitudes is that the males are constrained because they do not grow larger at more northern latitudes, suggesting that they reach their physical maximum size across the British Isles. Future studies should focus on rearing larvae under various combinations of temperatures and photoperiods in order to understand the variation in development and adult size in this butterfly.

Larvae from northern latitudes are genetically adapted to a univoltine life cycle and develop more slowly than those from more southern latitude, but in the lab the slower development results in smaller adults (Nygren *et al.*, 2008; this work). In order to explain the large size of *P. icarus* butterflies at northern latitudes, there are two possible explanations. Larvae in the field experience a combination of photoperiod cues and fluctuating temperatures. Furthermore, by appropriate microclimate choice the effective temperature experienced

by larvae can be much higher than the measured ambient temperature. This could result in higher growth rates in the field compared to the constant environment in the lab. Another explanation is that larvae in northern populations use two growing seasons (two-year life-cycle) to attain a large size (Kukal & Kevan, 1987; Nylin & Svard, 1991; Wipking & Mengelkoch, 1994; Morewood & Ring, 1998; Johansson, 2003; Nygren *et al.*, 2008). This idea needs further investigating as this could possibly explain the wide range of environmental conditions this species can thrive in.

In several butterfly species there is an increase in butterfly wing melanisation with latitude or altitude (Watt, 1968; Guppy, 1986; Ellers & Boggs, 2002; Ellers & Boggs, 2004; Roland, 2006) and the higher degree of melanisation in colder environments is assumed to aid in thermoregulation (Clusella-Trullas *et al.*, 2007). The lack of a strong relationship between basal wing melanisation of *P. icarus* and latitude suggests a rather weak relationship with temperature. This is further confirmed by a lack of strong effects of this butterfly's morphological characteristics and basking postures on heating and cooling rates (Chapter 4). Also, morphological variation has previously found not to influence thermoregulation-related behaviour in this species (Howe, 2004). Because of its small size, solar radiation is readily absorbed by the body, and the wings of *P. icarus* butterflies possibly do not contribute significantly to thermoregulation. This could facilitate variation in wing morphology in response to other factors, such as mate signalling or predation. It also means that for this small butterfly microhabitat temperature and solar radiation are the most important factors determining body temperature and that climate change and vegetation change will directly affect its thermoregulation and thus activity. In larger butterflies, whose wings have a thermal function (e.g. *Colias*, *Pieris*), wing variation may be more constrained because of thermal constraints. Further studies on other small-sized butterflies should clarify whether there is a general pattern of wing morphology being relatively unconstrained by thermoregulatory requirements in such species.

There was no strong effect of wing size and melanisation on heating rates as was expected. There was no increase in heating rates with higher melanisation, as would be expected for a dorsal basker. Higher temperatures and faster heating rates in a basking posture that cannot allow reflectance basking (wings fully spread) demonstrate that direct heating of the body is important in this small butterfly. In the field, with the wings fully spread, wind flow over the body may cause a convective cooling and elevating the wings into a reflectance basking posture could reduce convective cooling and have an advantage in windier conditions. In the laboratory setting used here there was minimal airflow and a warm ambient temperature and it will therefore be interesting to investigate whether the same results emerge in more marginal conditions, with lower temperatures. The radiation loads used in the experimental procedures are not representative of those of the brightest days, where radiation can reach 800-1000 Wm^{-2}. In the conditions used the microhabitat temperature (i.e. the temperature next to the body) was strongly correlated with the take-off temperature of the butterflies. The results from the morphological studies of field collected specimens (Chapter 3) and the experimental setting (Chapter 4) suggest that, at least in ideal conditions, the thermal characteristics of *P. icarus* are mainly determined by a combination of environmental conditions and microsite choice. The apparent absence of physical adaptations in terms of thermoregulation implies a strong association with warm microclimates and may be an important factor which restricts this butterfly to open low growing areas of vegetation with patches of sheltered short sward which provides warm microclimates (Howe, 2004). Current land use and natural processes make these sites widespread, and thus facilitates a widespread distribution.

The genetic diversity of *P. icarus* on the British Isles is considerably lower than on mainland Europe (Schmitt *et al.*, 2003; De Keyser *et al.* (submitted)). Often a reduction in genetic diversity with latitude is found and is interpreted as being the result of post-glacial range expansion and colonisation history (Hewitt, 2000). Within the UK no

isolation-by-distance or a relationship of genetic diversity with latitude was found in the allozyme marker. However, based on a more neutral DNA marker, AFLPs, there was an isolation-by-distance pattern, but no reduction in genetic diversity (Chapter 5). This suggests that there is population structuring that can not be detected with the allozyme marker. The high similarity between all the populations in allozyme frequency most likely is the result of a combination of a restricted genetic constitution (twelve of the seventeen studied loci were monomorphic) and a maintenance of polymorphisms making populations more similar. At least in one enzyme, PGI, the relative higher frequency of heterozygotes could assist in the maintenance of polymorphisms. Variation in alleles of this enzyme is associated with fitness related traits in other butterflies (Watt, 1977; Haag *et al.*, 2005; Wheat *et al.*, 2006; Karl *et al.*, 2008; Saastamoinen *et al.*, 2009) and heterozygote excess at this locus is not uncommon (Haag *et al.*, 2005; Wheat *et al.*, 2005). Maintaining high allelic diversity could assist this species in persisting in a wide variety of environments and could help buffer against environmental changes. Despite high levels of gene flow, differentiation in PGM appears to occur (Chapter 5), but in this work I have not demonstrated how selection acts on the different alleles. Well designed field studies to determine if there are differences of activities of individuals with particular alleles under specific environmental conditions could be undertaken to demonstrate this. This was one of the original aims of this project, but very low population densities during the first study years precluded this. Frequencies of the rare alleles are relatively low in the field, and even though significant relationships were found between PGM alleles and environmental variables (Chapter 5), they need further testing with adequate sample sizes. In the development experiment, PGM heterozygotes had shorter development times without size trade-offs (Chapter 6). This work shows potential non-neutrality for some allozyme loci (mainly PGM and PGI) in *P. icarus* but further studies with larger sample sizes are now needed to try identify how the allelic variation translates to functional properties.

This work has shown that the interpretation of morphological adaptations in *P. icarus* is not straightforward. Wing morphology and body size did not uniformly affect heating or cooling rates and it is suggested, from this work, that the wings of butterflies with small bodies may not play a large part in thermoregulation. In the face of increasing habitat fragmentation and higher global temperatures, it is important to discover how exactly these small grassland butterflies thermoregulate. *Polyommatus icarus* is a common butterfly species, but has shown large fluctuations in population sizes. As it is one of the more common and widespread grassland butterfly species, gaining a functional understanding of how exactly it deals with environmental variation, may enable us to improve conditions for the rarer, specialised grassland butterflies, most of which are relatively small. It is possible that body size is extremely important in maintaining a suitable body temperature in colder climates. I therefore suggest focussing attention in future thermoregulation related research on this butterfly on body size and the degree of hair cover, especially in respect to differing environmental development conditions.

It is obvious that *P. icarus* in the British Isles is genetically strongly impoverished compared to the mainland European populations. Usually genetically poor populations are characterised by a low capacity to cope with changing environmental conditions and are more prone to extinction (Frankham, 2005). *Polyommatus icarus* is widespread and is a known coloniser which is also able to recover in abundance after large seasonal declines. It can also maintain high levels of polymorphisms in some allozyme loci. Future studies should direct their attention to PGM and PGI as potential candidates for loci under selection and whether selection maintains polymorphisms in these enzymes. High sample sizes of all specific alleles, could possibly be generated using lab stocks of the different genotypes for use in large field based enclosures to compare the activities and behaviours of different genotypes. The genetic impoverishment and the year-to-year fluctuations in population size could cause serious bottlenecks and even though some polymorphisms are maintained, most loci are monomorphic in the UK. The loss of

advantageous alleles or allele combinations could have serious consequences if they confer an advantage for different life history traits (Hedrick, 2001; Olivieri, 2009). Whether the variation that does exist is maintained by movement of individuals between population units, potentially counteracting any effects of small population sizes during unfavourable years is not known. These ideas and hypotheses remain open to testing.

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