Geographic variation and thermal adaptation in Bicyclus anynana

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Geographic variation and thermal adaptation in *Bicyclus anynana*

de Jong, Maria Adriana

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Geographic variation and thermal adaptation in *Bicyclus anynana*

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Chapter 1

Introduction

Maaike A. de Jong

Introduction

Understanding the mechanisms that enable organisms to cope with and adapt to environmental variation lies at the heart of ecological and evolutionary research. In this thesis, I investigate different aspects of adaptation to climate in the butterfly *Bicyclus anynana*, in particular by examining geographical variation among wild populations. I take an integrated approach to understanding how this species is adapted to spatially and temporally varying climatic conditions by combining studies at different levels of biological organization: the phenotypic, physiological and molecular genetic level. This introduction will provide the reader with the necessary background information to facilitate a better a fuller appreciation of the presented work. The first and main part of this chapter serves to place the research in a broader scientific perspective, as well as to highlight the relevance of the study and to elucidate the theoretical concepts that are central to the work. Subsequently, I will introduce the study species and give the rationale for using it as the study organism in this research. Finally, I will present the main objectives of the study and an overview of the content of the scientific chapters in the outline of the thesis.

1. SCIENTIFIC BACKGROUND AND RELEVANCE

Geographic variation within species

The concept of geographic variation in phenotypes has a long history in evolutionary biology, eventually leading Darwin and Wallace to simultaneously conceive the theory of evolution by natural selection (Darwin & Wallace 1858, Darwin 1859). Whereas both Darwin and Wallace mainly focused on geographical patterns of species distributions, the Modern Synthesis of evolutionary theory emphasized the importance of the genetic variation existing in wild populations as the basis for evolutionary processes (e.g. Huxley 1942). One of the main contributions leading to this theoretical development was Dobzhansky's work on geographical variation in wild populations of ladybird beetles and fruit flies (Dobzhansky 1937). Today, one of the central questions in biology remains how organisms adapt to divergent environmental conditions. Variation at the phenotypic level is commonly observed between populations of the same species from different geographical areas, in morphological, physiological, *life history* (see Box 1) and behavioural traits.

A powerful method to reveal patterns of local adaptation is to study variation in populations along *clines*, which can be defined as gradual phenotypic or genetic variation over environmental gradients across the geographical range of species (Endler 1977). This approach is especially useful in the study of thermal adaptation, since temperature generally correlates with latitude and altitude, and clinal variation along these environmental axes thus indicates temperature as the main selective agent. A well-known key example of latitudinal clinal variation is the widely observed increase in body size in animals towards the poles for nearly all major taxa (known as Bergmann's rule). The adaptive value of these clines in endotherms is generally

Box 1. Life history theory

Life history traits are directly involved in an organism's reproduction and survival, and are therefore closely linked to fitness. They include, for example, size and age at maturity, growth and development rate, number and size of offspring, and life span. Life history theory aims to understand the mechanisms that lead to variation in these traits within and among species. The combination of life history traits depends for a large part on the allocation of resources and genetic constraints, which both pose limits on the possible combinations between traits (*trade-offs*). For example, a classic case of a constraint resulting from resource allocation is the trade-off between egg size and egg number that is found for many organisms (Stearns 1992, Roff 1992).

ascribed to the decrease of body heat radiation in larger animals because of a lower surface area to volume ratio, enabling them to stay warmer in cold climates. However, this explanation does not apply to small-bodied ectotherms such as insects. Several adaptive theories have been put forward to explain these patterns in ectotherms, but so far have not led to a unifying theory (Angilletta & Dunham 2003).

Geographic differences in phenotypes can be caused by local adaptation, which is associated with genetic differentiation between populations, and *phenotypic plasticity*, which is a direct result of variation in environmental conditions. Both processes are important mechanisms by which organisms can adapt to environmental variation.

Interaction between genes and environment

Phenotypic plasticity, the ability of an organism with a given genotype to express different phenotypes in response to distinct environmental conditions, is a crucial mechanism in coping with environmental variation for many species. Although phenotypic plasticity can be a non-adaptive response resulting from physical or chemical sensitivity to environmental factors, there are many instances of adaptive phenotypic plasticity where the phenotypic response increases the fitness in the environment encountered. Classic examples include caste differentiation in social insects (e.g. Lüscher 1960) sun vs. shade leaves in sunflowers (Vogel 1968) and seasonal *polyphenism* in butterflies (Brakefield & Frankino 2009; see Box 2). Although the concept of phenotypic plasticity has been known for more than a century, only in the last few decades has it increasingly gained attention in the fields of evolutionary biology and ecology. Its significance as a major component of phenotypic variation is now being widely recognized (Pigliucci 2001, West-Eberhard 2003).

The *reaction norm* is an important analytical tool in the study of phenotypic plasticity, and can be defined as the response curve of a given phenotypic trait to an environmental gradient (Fig. 2). It thus links together the genotypic, environmental and phenotypic variables. The steeper the slope of a reaction norm is, the more plastic the phenotype

Box 2. Seasonal polyphenism in butterflies

One of the most striking and well-known examples of adaptive phenotypic plasticity is the seasonal polyphenism in many Lepidoptera species. Polyphenism is an extreme form of phenotypic plasticity, where multiple, discrete phenotypes can develop from a single genotype in response to differing environmental conditions (Shapiro 1976, Stearns 1989). There are many forms of polyphenism across a diversity of taxa, including sex determination in fish and reptiles (Crews et al. 1994) and wing-dispersal polyphenism in crickets (Zera & Denno 1997). In butterflies, polyphenism as an adaptation to contrasting seasonal conditions has been recorded for each life stage and can involve morphological, physiological and life history traits. Temperature and photoperiod are the main environmental cues determining seasonal polyphenism, but other factors, including larval diet, can also induce seasonal form. The best-documented component of seasonal polyphenism in butterflies involves differences among forms in the wing pattern, for which adaptive explanations include differences across the seasonal environment in crypsis, thermoregulation and sexual signaling (Kingsolver 1996, Brakefield & Frankino 2009). In the case of adaptive seasonal polyphenism each alternative form is expected to have its highest relative fitness in the environment in which it is usually found.

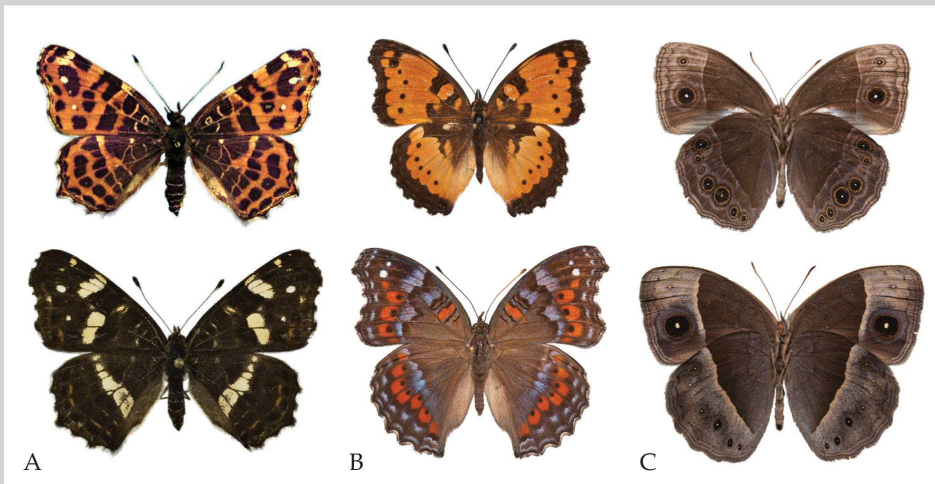

Figure 1. Examples of seasonal polyphenism in butterflies: (A) spring (top) and autumn (bottom) form of *Araschia levana*, (B) summer (top) and winter (bottom) form of *Precis octavia*, (C) wet season (top) and dry season (bottom) form of *Bicyclus safitza*. Photos courtesy of Pekka Malinen (A, bottom) and Oskar Brattström (B, C). Photo A (top) printed with permission of the Academy of Natural Sciences, Philadelphia, USA.

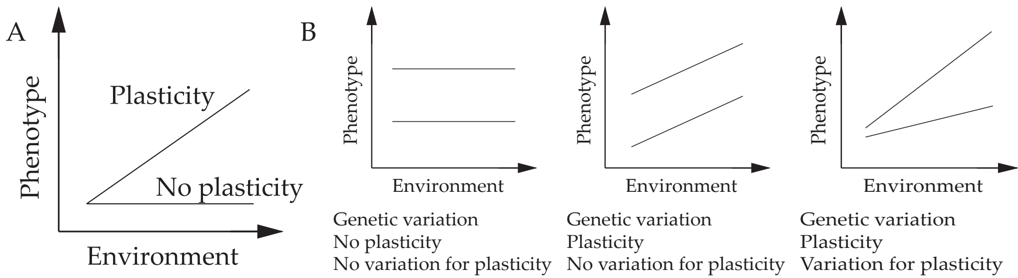

Figure 2. Illustration of the relationship between (linear) reaction norms and phenotypic plasticity, where reaction norms (lines) represent genotypes. (A) Reaction norms indicating a plastic vs canalized response. (B) Different scenarios involving the concepts of genetic variation, reaction norms and phenotypic plasticity. Redrawn after Pigliucci (2001).

is, while a flat reaction norm reveals an absence of phenotypic plasticity (a *canalized* phenotype; Fig. 2A). In order to partition phenotypic differences between populations into genetic and plastic components, individuals from the populations can be reared in a common environment (a *common garden* experiment) to remove phenotypic differences caused by plasticity. It is important to point out that the plasticity response has a genetic basis and can itself evolve as a trait when there is genetic variation and selection for the plasticity response in a population (Nussey et al. 2005). If a single trait of one genotype can be described by a single reaction norm, genotypes comprising populations can be described by bundles of reaction norms. The variation in the slopes of the reaction norms represents genetic variation for phenotypic plasticity within a population (Fig. 2B) and will determine the possibility of selection for the extent and shape of the plastic response. If populations differ in the bundles of reaction norms for their component genotypes, there can also be genetic geographic variation between populations for the plasticity response. In Chapter 2, geographic differentiation and genetic variation for phenotypic plasticity will be explored using a reaction norm approach.

Phenotypic plasticity is an exceedingly complex concept because it involves processes on all levels of biological organization, including gene expression, hormonal regulation of development, tissue-specific growth and physiological maintenance functions such as metabolism. It thus includes physiological, morphological and behavioural traits, and can be irreversible (which is often the case for *developmental plasticity* of morphological traits) or reversible (the form of plasticity known as *acclimation*). Another contributing factor to frequent debate and confounding use of terminology concerning phenotypic plasticity is undoubtedly our general lack of understanding how the genotype maps onto the expressed phenotypes through the interplay of developmental processes and environmental influence. One of today's major research challenges is to discover the genes and genetic processes involved in plasticity and the developmental pathways leading to the alternative phenotypes under environmental modulation. In my thesis, Chapter 3 investigates the underlying hormonal regulation of phenotypic plasticity during development.

Phylogeography

When interpreting patterns of geographic variation in the light of local adaptation, it is important to take into account neutral population genetic processes and the phylogeographic history of a species; both play a role in shaping the distribution of genetic variation within and across populations. *Phylogeography* is the study of historical events that have influenced the current geographic variation within a species. Studying the variation in neutral genetic markers within and across populations can reveal the footprints of historical demographic events including population expansion, population bottlenecks and migration. When these genetic signatures are linked to historical records on climatic and geographic conditions, a comprehensive picture of the demographic history of the populations may be reconstructed. For example, a considerable number of studies have revealed the effects of the quarternary ice ages on biodiversity. Periods of cold led to the retreat of many species into smaller refugia during the glacial maxima, with population expansions occurring during the interglacial periods (Hewitt 2000). Neutral marker information can also shed light on contemporary population genetic processes such as inbreeding, genetic drift and gene flow. Similar to past demographic events, these processes influence the distribution of genetic variation within and across populations, and hence the ability of populations to adapt to local conditions. Inbreeding (which may be the result of a population bottleneck) and genetic drift, in combination with restricted gene flow, lead to loss of genetic variation, decreasing the adaptive potential of populations. In contrast, high levels of gene flow can slow down or prevent local adaptation by reducing genetic differences between populations (Ehrlich & Raven 1969). Chapter 4 studies the population genetics and phylogeographic history of wild populations.

Ecological and evolutionary impacts of climate change

Climate affects all life on earth, and is one of the major environmental factors influencing the ecology and evolution of species. Especially ectothermic (cold blooded) organisms, which include the majority of animal biodiversity such as most fish, amphibians, reptiles and invertebrates, are sensitive in their basic physiological functions to variation in their thermal environment. It has been demonstrated that physiological trait limits can shape the distributions and ranges of species (Hoffmann & Blows 1994), which is further supported by the strong correlation between species richness and temperature (Allen et al. 2002).

Understanding the mechanisms by which organisms cope with and adapt to climate is particularly important in the face of current global climate change. As presented in the IPCC's (Intergovernmental Panel on Climate Change) most recent assessment report, global temperatures have increased at an accelerated rate over the past 50 years and will continue to rise over the following decades as a result of anthropogenic influence. Other long-term observations and predicted continuing changes include widespread changes in extreme temperatures and extreme weather events including droughts, heavy precipitation and heat waves. Especially in the tropics and subtropics, more intense and longer periods of drought have been recorded in the past decades, a

trend that is very likely to continue (IPCC 2007).

Ecological consequences of recent climate change have been documented for a broad array of taxa and on every continent (Parmesan 2006). The majority of these studies have reported on species range shifts (or *habitat tracking*), showing a shift towards the poles and higher altitudes as a general trend. However, with worldwide human-induced habitat reduction and fragmentation, for many species shifting range is not an option, especially when their dispersal capacity is limited. Recent studies have shown that especially tropical ectotherms face an extinction risk from global warming because their optimal performance temperature lies closer to the critical thermal maximum (Deutsch et al. 2008, Huey et al. 2009). For many species, their persistence will depend on the ability to cope with climate change by either phenotypic plasticity or genetic adaptation, two areas of research that are relatively underrepresented in the study of the impacts of climate change.

Phenotypic plasticity may increase an organism's ability to cope with climate change, depending on the magnitude and direction of the plasticity response, as well as the predictability of the environmental variation. Phenotypic plasticity responses to environmental change may also lead to a mismatch between phenotype and ecological conditions, as is illustrated by the change in timing of *phenology* (periodic events in the life cycle of plants and animals), which has been reported for various species (Root et al. 2003, Both et al. 2006, Parmesan 2006). Populations may also show genetic adaptation to climate change; several studies have shown a change in frequencies of genotypes associated with climate adaptation in response to recent global warming (Balanyá et al. 2006, Van Heerwaarden & Hoffmann 2007), although such studies are rare (for an overview see Gienapp et al. 2008). Studying candidate genes putatively involved in adaptation to climate can increase our understanding of the genetic mechanisms involved in adaptive differentiation. Furthermore, these genes can potentially be used as genetic markers to monitor species' molecular responses to climate change (Hoffmann & Willi 2008). By investigating intraspecific geographic variation and identifying patterns of past adaptation to local climate, we can gain insight into the evolutionary potential in response to future changes. Chapter 5 investigates geographical patterns of genetic variation in candidate genes associated with thermal adaptation using wild populations along a latitudinal cline.

2. STUDY SYSTEM

Bicyclus anynana, the Squinting Bush Brown, is a sub-Saharan butterfly species of the African genus *Bicyclus* (Satyrinae, Nymphalidae) which comprises more than 80 species. *B. anynana* is recognized as having three subspecies, the most widespread being *B. anynana anynana*, which occurs in Eastern and Southern Africa from Kenya to South Africa including the Comoros and the Tanzanian islands Zanzibar and Pemba. *B. anynana centralis* is morphologically very similar apart from subtle differences in wing pattern, and its range lies in western Uganda, Congo and northern Angola. *B. anynana socotrana*, which occurs only on the remote Yemeni island Socotra, has a more distinct wing pattern and smaller size (Condamin 1973).

The species' habitat predominantly consists of woodland and dry forest areas that

occur along the rivers, lakes and coast of savanna regions. *B. anynana* is a relatively common and a generalist species; the larvae feed on various grasses while the adults feed on fermenting fruit on the forest floor. The savanna ecosystem is characterized by a strong seasonality in rainfall, with intensity and frequency of the alternating wet and dry seasons depending on the latitude and geography of the region. As an adaptation to these contrasting environmental conditions, *B. anynana* displays seasonal polyphenism (see Box 2) for wing pattern, life history and physiological traits, expressing a distinct dry season and wet season form depending on developmental conditions (Brakefield & Reitsma 1991, Brakefield et al. 1996). In the well-studied laboratory population originating from Nkhata Bay in Malawi, where temperature is positively correlated with rainfall, temperature is the main environmental cue determining seasonal form during development. The wing pattern of dry season butterflies is cryptic with small or nearly absent eyespots, which allows them to blend in with a background of dead leaf litter and gives a fitness benefit in predator avoidance (Lyytinen et al. 2004, Brakefield & Frankino 2009). In the dry season form, the life history is adjusted to survive prolonged periods of food scarcity, and includes a larger body size and fat reserves, an altered metabolic rate and reproductive dormancy (Brakefield et al. 2007). In contrast, development and reproduction are rapid in the warmer wet season, when food conditions are highly favorable. Wet season form butterflies have large, conspicuous eyespots on the ventral wing surface, likely involved in sexual signaling (Oliver et al. 2009) and deflection of predatory attacks (Lyytinen et al. 2004). Fig. 3 shows a wet and dry season form of female *B. anynana* from South Africa.

B. anynana is a very suitable species for studying the mechanisms of adaptation to climate, because of its extensive phenotypic plasticity and genetic variation for a suite of life history and morphological traits as an adaptation to seasonal climatic variation. The species has a wide range and is usually abundant where it occurs. Moreover, the

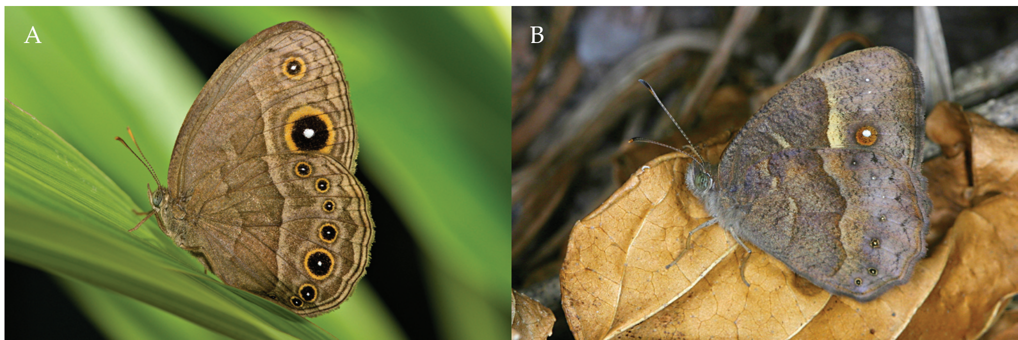

Figure 3. (A) Wet season form and (B) dry season form of *Bicyclus anynana*. Photos courtesy of Oskar Brattström (A) and Andre Coetzer (B).

biology of *B. anynana* is well known as a result of its role as a model species for research in ecological and evolutionary genetics. Its ecology has been studied in the field and laboratory (e.g. Brakefield & Reitsma 1991, Brakefield et al. 2007), and the genetic and developmental processes underlying phenotypic plasticity of wing pattern and life history traits are especially well studied. Consequently, abundant scientific knowledge and analytical means, including genetic and genomic tools, are available for this species (Brakefield et al. 2009). The majority of studies on *B. anynana* have used a laboratory-based population from Malawi that was founded in 1988. In this thesis, research on large-scale geographic variation in wild populations of *B. anynana* is presented for the first time.

3. THESIS OUTLINE

The work presented in this thesis combines studies of phenotypic plasticity and molecular genetics in relation to climate adaptation in *B. anynana*. Chapters 2 and 3 are focused on phenotypic plasticity of life history traits and wing pattern as an adaptation to seasonality. Chapters 4 and 5 investigate geographic patterns of genetic variation in wild populations.

In Chapter 2, I compare thermal reaction norms of several life history, physiological and wing pattern traits for a population from Malawi and one from South Africa. By using populations from different climatic regions, I investigate whether the seasonal plasticity response shows local adaptation, or whether the same response serves a broader range of climatic conditions. In this study, the trait reaction norms were measured over three temperatures, approximately covering the range of temperatures the butterflies experience in the field. In addition to reaction norm measurements at the population level, broad sense heritabilities and cross-environmental correlations were estimated for several traits in a family design to examine the adaptive potential of the plasticity response.

Chapter 3 follows up the results of Chapter 2, and is a detailed investigation of the hormonal dynamics underlying the development of the two distinct adult seasonal forms depending on temperature. Specifically, I assess whether the discrete developmental response of the phenotype is already present at the level of hormonal regulation, and to what extent different traits can respond independently to a shared underlying hormonal signal. The reaction norms resulting from Chapter 2 allowed for an estimation of a thermal 'switch-point' determining development into either a wet season or a dry season butterfly. Consequently, detailed reaction norms were measured for ecdysteroid and juvenile hormone levels during the critical phase of development over a range of 5 temperatures around this switch point. These measurements were coupled to reaction norms for life history, wing pattern and physiological traits over the same range of temperatures.

Chapters 4 and 5 take a molecular genetic approach to revealing geographical patterns of adaptation in *B. anynana*. Both chapters involve wild populations sampled along a latitudinal transect extending over most of the species range, including two subspecies and an island population. In order to make inferences about adaptive geographic variation, it is crucial to consider current and historical demographic processes that

influence the distribution of genetic variation within and between populations. With this objective, the phylogeography of the populations is investigated in Chapter 4. The genetic diversity, population structure and demographic history of the populations are analysed using a mitochondrial marker widely applied for this purpose.

Chapter 5 aims to reveal footprints of thermal selection in candidate genes by investigating geographic variation in amino acid polymorphisms. For this study, 19 genes associated with thermal adaptation were selected and tested for clinal variation of allele frequencies with latitude. The candidate genes include enzymes and other metabolites from the glycolytic pathway and the lipid pathway, and several genes involved in the biosynthesis of wing pattern pigmentation. Furthermore, six genes from the heat shock family and five genes involved in developmental pathways for which we did not expect clinal variation, we included as negative controls. In the interpretation of the findings, the phylogeographic structure resulting from Chapter 4 is taken into account. Finally, Chapter 6 summarizes and discusses the main conclusions of the scientific chapters and gives perspectives on future research in the light of the findings of this thesis.

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Chapter 2

Geographic variation in thermal plasticity of life history and wing pattern in *Bicyclus anynana*

Maaike A. de Jong, Fanja M.N.H. Kesbeke, Paul M. Brakefield and Bas J. Zwaan (2010), *Climate Research* **43** (1-2), 91-102

Geographic variation in thermal plasticity of life history and wing pattern in *Bicyclus anynana*

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ABSTRACT

Temperature is one of the main environmental cues regulating seasonal plasticity in insects. Global climate change may lead to a change in the predictive value of temperature for seasonal conditions, potentially resulting in a mismatch of phenotypic form and environment. The afrotropical butterfly *Bicyclus anynana* shows striking seasonal plasticity for wing patterns and life history traits. This polyphenism is an adaptation to contrasting patterns of rainfall over wet and dry seasons, and is mainly determined by temperature. To investigate the extent of local adaptation of the developmental plasticity response to regional climate, we compared the thermal reaction norms for several life history traits and wing pattern of two distant populations from regions with different temperature–rainfall associations. We found little to no population differentiation for the life history traits, while wing pattern showed substantially more geographic variation. Broad-sense heritabilities and cross-environment correlations for wing pattern and two life history traits indicated a potential for adaptation of the plasticity response of these traits. Our results indicate that thermal plasticity of wing pattern can be population-specific; thus climate change may lead to a mismatch of wing pattern to seasonal environment. Traits that can be further modified by acclimation during the butterfly's adult life span (starvation resistance, resting metabolic rate and egg size) showed no geographic differentiation for their developmental plasticity. This indicates that for these traits, adult acclimation plays an important role in coping with local climate.

Key words: acclimation, *Bicyclus anynana,* developmental plasticity, geographic variation, life history, seasonal polyphenism, temperature, wing pattern

INTRODUCTION

For many insects, developmental plasticity is a crucial mechanism to cope with seasonally varying conditions (Shapiro 1976, Gotthard & Nylin 1995). The ability to change the phenotype in response to environmental cues can be adaptive if the phenotypic variation increases fitness in the environments encountered (Stearns 1976). Adaptive seasonal plasticity has been observed for every stage of the insect life cycle,

and includes the timing of key life history events such as diapause (Tauber et al. 1986), plasticity of egg size (e.g. Fischer et al. 2003, Fischer & Karl 2010, this Special) and thermoregulation by wing melanisation (Shapiro 1976, Kingsolver 1996). An extreme form of developmental plasticity is polyphenism, which involves the development of discrete phenotypes rather than a continuous range of phenotypes in response to the environment (Shapiro 1976). Seasonally induced polyphenism allows insects with multiple generations per year to express the optimal phenotype in each season. Well-known examples are polymorphisms in dispersal (e.g. in aphids, Dixon & Kindlmann 1999) and seasonally varying wing morphs in Lepidoptera (Brakefield & Frankino 2009).

Current climate change is having far-reaching effects on biodiversity and species' distributions. In addition to well-documented shifts in species ranges, research is increasingly focusing on the roles of phenotypic plasticity and genetic adaptation in the responses of species to climate change (Chown et al. 2010, Van Doorslaer et al. 2010, Rezende et al. 2010, all this Special). Temperature is one of the main environmental cues involved in inducing seasonal plasticity in organisms. For seasonal polyphenisms, the inducing environment is often not the same as the selective environment (Nijhout 2003). The organism uses the cue (e.g. temperature) as a reliable predictor of other seasonal conditions (e.g. nutrient stress). However, global warming will have a major impact on average long-term temperature patterns, thereby potentially changing the predictive value of temperature for seasonal conditions. A profound and well-reported result of this is the change in timing of phenology observed for a diversity of species (Root et al. 2003, Parmesan 2006). For polyphenic species, a possible shift in development of phenotypic form relative to the seasonal transitions, resulting in a mismatch between phenotype and environment, could be a very relevant problem. Studying the extent and specificity of the plastic responses to temperature in polyphenic species can help us predict their ability to adapt to climate change.

In the afrotropical butterfly genus *Bicyclus* and especially in the species *B. anynana* (Nymphalidae), seasonal polyphenism has been well studied (Brakefield & Larsen 1984, Brakefield & Reitsma 1991, Brakefield et al. 2007). This species shows extensive developmental plasticity for wing pattern and life history traits as an adaptation to the strongly contrasting wet and dry seasons of its East African savannah habitat. In the warmer rainy season, food is abundant and conditions are highly favourable for both rapid larval growth and adult reproduction. In contrast, food is scarce or absent in the cooler dry season, and a survival-based strategy is better suited to this part of the year. The seasonal forms of *B. anynana* show striking differences in wing pattern: wet season butterflies typically have conspicuous eyespots and a white band on their ventral wing surfaces, while the cryptic dry season form shows almost no eyespots and is more or less uniformly brown. In addition to wing pattern, *B. anynana* shows developmental plasticity for development time, adult size, adult fat content, starvation resistance, life span, metabolic rate and egg size (Fischer et al. 2003, Brakefield et al. 2007, Pijpe et al. 2007).

Temperature is the single most important climatic variable determining seasonal phenotype in *Bicyclus anynana* from Malawi, where temperature is highly correlated with rainfall and, thus, provides a reliable indicator for the adaptive seasonal forms

(Kooi & Brakefield 1999). However, patterns of temperature–rainfall correlation vary strongly throughout the geographical distribution of *B. anynana*, and different regions are expected to have posed different selection pressures on the species' plastic responses to temperature (Roskam & Brakefield 1999). Studying genetic geographic differentiation between populations in relation to associated environmental differences is a common approach to assess the adaptive value of species responses to local conditions (Endler 1986). In the light of climate change, investigating local adaptation can also be used to predict potential adaptation to future conditions, i.e. temperature rises and changes in patterns of seasonality (e.g. Trotta et al. 2010, this Special).

In the present study, we compared two populations of *Bicyclus anynana* from different latitudes in their developmental plasticity response to temperature for wing pattern and several life history (related) traits. One population is from Malawi and lies in the tropics; the other population is located 1400 km to the south in subtropical South Africa. This large latitudinal distance results in substantial differences in local climate, and monthly average, minimum and maximum temperatures differ by as much as 5°C. However, for both localities, temperature and rainfall correlations are high. Thus temperature is a good indicator for rainfall in both regions, but specific temperatures correspond to different rainfall patterns between these populations; a temperature experienced in Malawi may be associated with the rainy season, while the same temperature is correlated with the dry season in South Africa. By comparing populations from regions that pose different selection pressures on the species' plastic responses to temperature, we aim to answer the following questions: (1) Is there evidence for geographic variation in the developmental plasticity response to temperature, or does the same plastic response cover a broader range of climates? (2) If the populations indeed show genetic adaptation of their plasticity response to local climate, is the extent of differentiation in the response to developmental temperature consistent for different traits?

To address these questions, we compared the populations in a common garden experiment and measured their reaction norms in response to three rearing temperatures for the following traits: developmental time, pupal mass (as a proxy for adult mass), adult fat content, starvation resistance, resting metabolic rate and wing pattern elements. In addition, to further investigate the adaptive potential of developmental plasticity to changing climates, we estimated broad-sense heritabilities and cross-environment correlations for developmental time, pupal mass, fat content and wing pattern in a split full-sibling family design for both populations.

MATERIALS AND METHODS

Stocks and rearing

Two populations of *Bicyclus anynana* were used, one from Malawi (MW) and one from South Africa (SA). The MW laboratory population was established from 80+ gravid females caught at Nkatha Bay (11° 45′ S, 34° 14′ E) in 1988. The SA laboratory population was established from 70+ gravid females caught in 2006 in False Bay Park of the St. Lucia Wetland Park, KwaZulu Natal (27° 58′ S, 32° 21′ E). Both populations were reared on

maize *Zea mays*, at sufficient numbers to maintain high levels of heterozygosity (at least 400 individuals per generation). Although the MW population has been maintained in the laboratory longer than the SA population, a recent report on 28 microsatellite markers showed overall high levels of heterozygosity and polymorphism for the MW population (Van 't Hof et al. 2005).

Experimental set-up

Prior to the experiment, both populations were reared under identical conditions (27°C; 12 h light:12 h dark cycle) for one generation to reduce maternal effects. We chose three treatment temperatures to measure reaction norms of the populations: 20, 23 and 27°C. The low (20°C) and high (27°C) temperatures are similar to those experienced by larvae of the dry and wet seasonal form, respectively, in the field in Malawi. The addition of an intermediate temperature (23°C) allowed us to assess potential non-linearity of reaction norms.

The populations were reared at the family and population levels in climate cells with high relative humidity (~70%) and a 12 h light:12 h dark cycle. For the full-sibling family design, offspring of single-mating pairs were divided over the 3 temperatures and reared on maize, each family in a sleeve-like gauze cage. The following traits were measured: larval and pupal development time, pupal weight (as a proxy for adult weight), relative fat content and wing pattern. At the population level, larvae were reared on maize in cages containing ~300 individuals each. The following traits were measured: larval and pupal development time, pupal weight, starvation resistance and resting metabolic rate. All measurements on live adults were conducted at the same temperature as the rearing temperature.

Measurements

Larval development time and pupal development time were recorded. Pupae were weighed to the nearest 0.1 mg at 1 d (23 and 27°C) or 2 d (20°C) after pupation and transferred to individual plastic pots to eclose. At the family level, emerged butterflies were frozen at –20°C within 1 d after eclosion. For assessment of wing pattern, three males and three females per temperature were measured from 20 families of the MW population and 18 families of the SA population. At the population level, the butterflies were either randomly selected for live measurements or frozen at –20°C one day after eclosion to determine fat content and wing pattern.

Relative fat content

Butterflies were stored at –20°C until measurement. After removal of wings, legs and antennae, the bodies were dried for 48 h at 40°C and weighed to the nearest 0.01 mg to determine dry mass. The fat mass (triglyceride and fatty acid) was then extracted by incubating and shaking (100 rpm) the bodies in a 2:1 dichloride-methane solution at room temperature for 48 h. This step was repeated once with fresh solution, and subsequently the bodies were dried for 24 h at 40°C. The fat content was calculated per

individual by subtracting the fat-free dry mass from the initial dry mass. Relative fat content was calculated as the percentage of fat mass of the initial dry mass.

Starvation resistance

One day after eclosion, butterflies were transferred to cylindrical hanging cages and separated according to sex to prevent mating. Butterflies were numbered individually and kept at a maximum of 15 individuals per cage to minimise density stress. Water-saturated cotton wool was provided ad libitum in the cages to prevent desiccation of the butterflies. Individual deaths were scored daily at a fixed time.

Resting metabolic rate

Adult CO_2 respirometry (ml CO_2 h^{-1}) was measured 1 d after eclosion, as an index for resting metabolic rate. A Li-Cor LI-6251 CO_2 analyzer in a Sable Systems push-through respirometry set-up was used to measure individual respiration. During measurement, butterflies were kept in cylindrical glass containers (4 × 9 cm, diameter × length) in a dark, temperature-controlled climate cabinet. Butterflies were measured in the nocturnal stage of their daily cycle to obtain inactive (resting) respiration rates (Pijpe et al. 2007). Data of two repeated measurements were analysed using Expedata software (Sable Systems) and averaged to obtain individual CO_2 respiration rates.

Wing pattern

The ventral surface of one hind wing of each individual was photographed and scanned using a Leica DC200 digital camera connected to a Leica MZ12 binocular microscope. The resulting images were analysed with ImagePro software to calculate the following wing pattern elements indicative of seasonal form: (1) surface area of the inner black disk of the fifth eyespot; (2) surface area of the white centre of the fifth eyespot; (3) width of the median band; (4,5,6) Red, green and blue (RGB) spectrum of the dark area in the fourth wing cell (located outside of the median band), respectively; (7,8,9) RGB spectrum of the light area in the fourth wing cell (located in the median band), respectively; and (10) contrast between light and dark in the fourth wing cell (Wijngaarden & Brakefield 2001). In addition, inter-pupil distance was measured as an estimate of wing size (Beldade & Brakefield 2002). Analysis showed that wing pattern elements (1–10) did not significantly co-vary with inter-pupil distance and thus were not corrected for size.

Statistical analysis

Population level

Data were analysed using three-way ANOVA with population, temperature and sex as fixed factors. Resting metabolic rate was analysed with pupal weight as a covariate. The wing pattern data (10 measurements) were reduced using a principal component

analysis (PCA), and the first two components were used for further analysis (see Results for details). *Post hoc* Tukey-Kramer Honestly Significant Difference (HSD) tests were used to assess population- and sex-specific responses to temperature where appropriate.

Family level

Between and within family variance components and their significance were estimated per population per sex and per temperature, using ANOVAs with family as a random factor. Broad-sense heritabilities were calculated as twice the intra-class correlation coefficient, which is the fraction of total variation due to differences between family groups (Falconer & Mackay 1996). Genetic correlations (r_G) across two temperatures were estimated using mixed-model ANOVA. In this method, r_G is estimated by dividing the covariance of family means across temperatures by the geometric mean of the between-family variance components (Fry 1992). The covariances of family means across temperatures and their significance were calculated per population per sex, using ANOVAs with temperature as a fixed factor and family as a random factor.

RESULTS

Life history traits

Reaction norms

Fig. 1 shows the reaction norms of pupal weight, relative fat content, starvation resistance and resting metabolic rate in response to temperature for life history and related traits, for each sex. The reaction norm for development time is not shown.

Development time. The factors population ($F_{1,872}$ = 162.61, $p < 0.0001$), temperature ($F_{2,872}$ = 6823.84, $p < 0.0001$) and sex ($F_{1,872}$ = 104.5123, $p < 0.0001$) had a significant effect on development time. The MW population had a shorter development time than the SA population for each temperature (average difference = 5.8%). For both populations, development time differed significantly between rearing temperatures for both sexes (20 > 23 > 27°C). For both populations and each temperature, males had a significantly shorter development time than females (average difference = 5.0%).

There was a significant interaction between population and temperature ($F_{2,872}$ = 31.16, $p < 0.0001$), caused by a relatively larger difference between populations at 20°C than at the higher temperatures. This might be caused by laboratory adaptation of MW: the stock population was typically reared at 20°C, which may have favoured a relatively faster development time at this temperature over time.

Pupal weight. There was a significant effect of population ($F_{1,869}$ = 185.92, $p < 0.0001$), temperature ($F_{2,869}$ = 4.42, $p = 0.0123$) and sex ($F_{1,869}$ = 964.43, $p < 0.0001$) on pupal weight (Fig. 1A). For both sexes, the SA population had a lower pupal weight than the MW population or all temperatures (average difference = 10.3%). There was no interaction between population and temperature, indicating a similar plasticity response of the populations to temperature. Per population and per sex, between-temperature differences

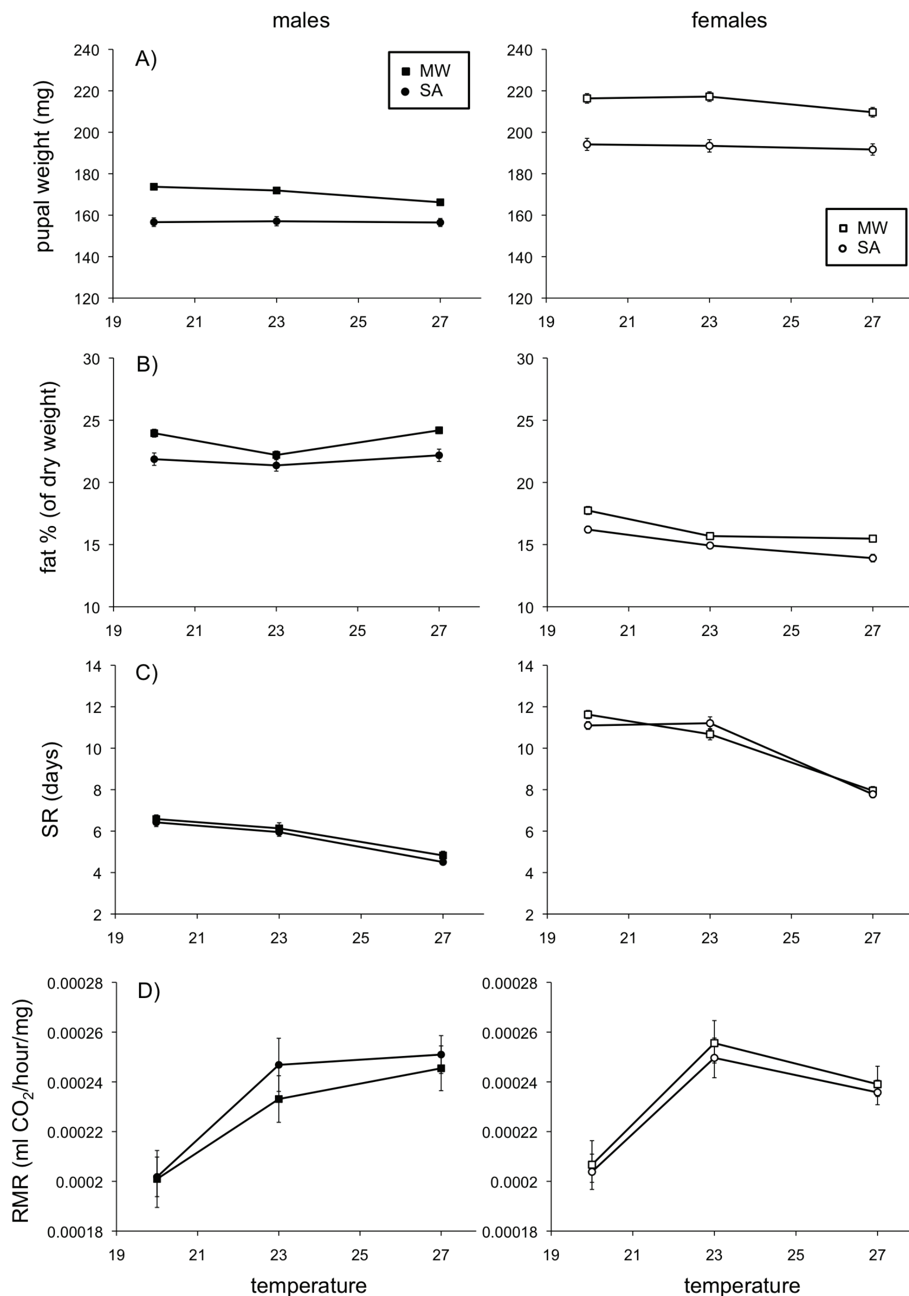

Figure 1. *Bicyclus anynana*. Thermal reaction norms of life history traits: (A) pupal weight; (B) relative fat content; (C) starvation resistance (SR); and (D) resting metabolic rate (RMR). MW: Malawi; SA: South Africa. Error bars: ±1 SE.

were not significant in *post hoc* tests (20 = 23 = 27°C), although there was a consistent trend for both sexes and populations (20 > 23 > 27°C). Pupal weight was consistently lower in males than in females for both populations (average difference = 24.7%).

Relative fat content. The factors population ($F_{1,654}$ = 112.87, p < 0.0001), temperature ($F_{2,654}$ = 14.8219, p < 0.0001) and sex ($F_{1,654}$ = 126.73, p < 0.0001) had a significant effect on fat content (Fig. 1B). Fat content was consistently lower in the SA population than in the MW population for both males and females. The variation explained by temperature was relatively small compared to population and sex. Significant interactions between temperature and population ($F_{2,654}$ = 3.37, p = 0.035), and temperature and sex ($F_{2,654}$ = 17.6235, p < 0.0001) indicated population- and sex-specific responses to temperature. *Post hoc* tests revealed differences in reaction norm shape between populations for both males (MW: 20 = 23 < 27°C; SA: 20 = 23 = 27°C) and females (MW: 20 > 23 = 27°C; SA: 20 = 23 = 27°C).

Starvation resistance. Temperature had a significant negative effect on starvation resistance ($F_{2,789}$ = 182.76, p < 0.0001). Populations showed no significant differences in their starvation resistance reaction norms, and there was no significant interaction between population and temperature (Fig. 1C). There was a significant effect of sex ($F_{1,789}$ = 1237.50, p < 0.0001), with females being on average 78.5% more starvation resistant than males. A significant interaction of sex and temperature ($F_{2,789}$ = 21.64, p < 0.0001) indicated sex-specific responses to temperature. *Post hoc* tests for separate sexes revealed a significant difference between 23 and 27°C but not between 20 and 23°C for females (20 = 23 > 27°C). For males, there were significant differences in starvation resistance between each temperature (20 > 23 > 27°C), although the difference between 20 and 23°C was much smaller than between 23 and 27°C.

Resting metabolic rate. There was a significant positive effect of temperature on resting metabolic rate ($F_{2,743}$ = 30.82, p > 0.0001), but not of population or sex (Fig. 1D). There were no significant interactions between factors. A *post hoc* test revealed a significant difference between 20 and 23°C, but not between 23°C and 27°C (20 < 23 = 27°C).

Heritabilities and cross-temperature correlations

Table 1 gives broad-sense heritabilities for development time and pupal weight for each population, sex and temperature, as well as cross-temperature correlations between 20 and 27°C, 20 and 23°C, and 23 and 27°C. All heritabilities were significant and ranged from moderate to high. Heritabilities were similar for the two traits, and showed no clear or consistent differences between populations, sexes or temperatures. All correlations were positive and significantly different from zero.

Wing pattern

Table 2 gives the first two principal components (PC1 and PC2) of 10 wing pattern measurements (size and colour of pattern elements) associated with seasonality. Combined, they explained about 81% of the variation in the data. The first eigenvector had the highest loadings for the bright colours, while the second eigenvector showed the highest loadings for the eyespot size elements. Both eigenvectors had similar

Table 1. *Bicyclus anynana*. Broad-sense heritabilities and cross-temperature correlations (r_G) for development time and pupal weight. MW: Malawi; SA: South Africa; F: female; M: male. *p < 0.05; **p < 0.01; ***p < 0.001.

		Heritability			Cross-temperature correlation		
Line	Sex	20°C	23°C	27°C	20-27°C	20-23°C	23-27°C
Development time							
MW	F	0.27***	0.49***	0.36***	0.61***	0.76***	0.85***
	M	0.41***	0.49***	0.24**	0.64***	0.77***	1.00***
SA	F	0.40***	0.49***	0.46***	0.72***	0.24***	0.68***
	M	0.22*	0.78***	0.75***	0.74***	0.49***	0.70***
Pupal weight							
MW	F	0.58***	0.73***	0.72***	0.73***	0.95***	0.81***
	M	0.44***	0.65***	0.28***	0.67***	0.89***	0.83***
SA	F	0.40***	0.41***	0.64***	0.67***	0.69***	0.95***
	M	0.53***	0.41***	0.78***	0.88***	0.74***	0.95***

Table 2. *Bicyclus anynana*. Loadings on PC1 and PC2 for 10 wing pattern measurements, and the percentage of variation explained by each principal component.

Trait	PC1	PC2
5th spot bright area	0.24	-0.39
5th spot dark area	0.14	-0.48
Distance node-band	-0.11	0.46
Dark colour R	0.34	0.31
Dark colour G	0.35	0.31
Dark colour B	0.30	0.36
Bright colour R	0.40	-0.02
Bright colour G	0.41	-0.01
Bright colour B	0.40	-0.02
Dark-bright contrast	0.30	-0.29
% explained	57.03	23.62

loadings for dark colour and contrast. Note that the sign of the band measurement is an artefact of the way the trait is measured: with increasing band size, the value of the variable decreases.

Reaction norms

Fig. 2 shows reaction norms of the two populations for PC1 and PC2 for each sex. There was a significant effect of the factors population ($F_{1,865}$ = 29.45, p < 0.0001), temperature ($F_{2,865}$ = 171.77, p < 0.0001) and sex ($F_{1,865}$ = 750.98, p < 0.0001) on PC1. Significant interactions between temperature and sex ($F_{2,865}$ = 18.75, p < 0.0001), and population and sex ($F_{1,865}$ = 4.51, p = 0.034) indicated a sex- and population-specific response to temperature. Subsequent *post hoc* testing per sex revealed no significant differences between the populations in the response of females (20 > 23 > 27°C). Males differed significantly between populations for the temperatures 23 and 27°C, and showed a different pattern in response to temperature (MW: 20 = 23 < 27°C; SA: 20 < 23 = 27°C).

The factors population ($F_{1,865}$ = 219.94, p < 0.0001), temperature ($F_{2,865}$ = 588.82, p < 0.0001) and sex ($F_{1,865}$ = 116.18, p < 0.0001) had a significant effect on PC2. A significant interaction between temperature and population ($F_{2,865}$ = 3.94, p = 0.02) meant

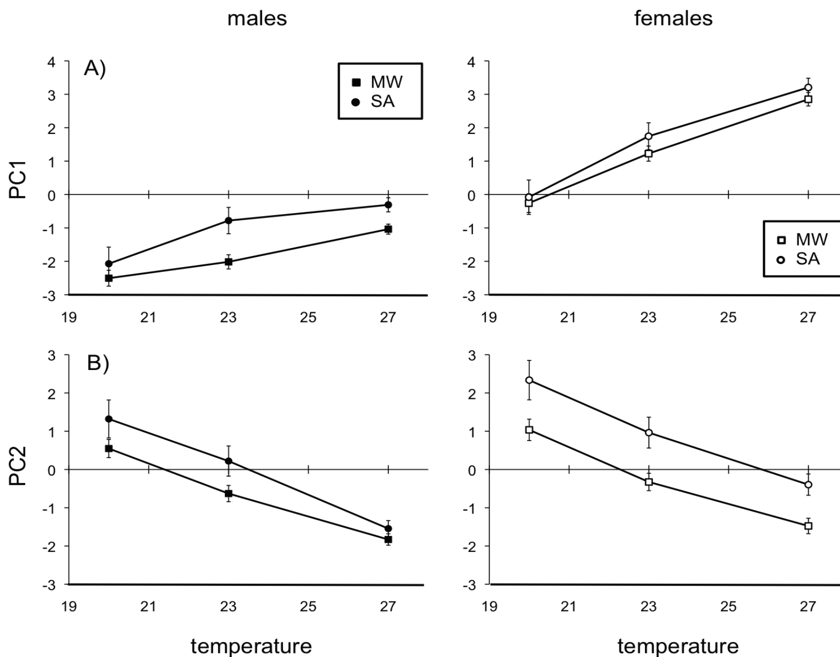

Figure 2. *Bicyclus anynana*. Thermal reaction norms of first and second principal component of 10 wing pattern elements. (A) PC1; (B) PC2. MW: Malawi; SA: South Africa. Error bars represent ±1 SE.

Table 3. *Bicyclus anynana*. Broad-sense heritabilities and cross-temperature correlations (r_G) for PC1 and PC2 of 10 wing pattern measurements. MW: Malawi; SA: South Africa; F: female; M: male. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

		Heritability			Cross-temperature correlation		
Line	Sex	20°C	23°C	27°C	20-27°C	20-23°C	23-27°C
PC1							
MW	F	0.92***	1.00***	0.82***	0.85***	0.91***	1.01***
	M	0.83***	0.88***	0.39	0.81***	0.83***	1.07***
SA	F	1.00***	1.00***	1.00***	0.92***	0.95***	0.93***
	M	0.92*	1.00***	1.00***	0.97***	0.98***	1.01***
PC2							
MW	F	0.88***	0.35	0.49*	0.65***	1.00***	0.71***
	M	0.72**	0.56*	0.14***	0.85***	0.76***	2.06***
SA	F	0.82***	0.77**	1.00***	0.77***	0.96***	0.82***
	M	0.40	0.46*	0.58**	0.91***	0.52***	0.90***

there was a difference in plasticity response to temperature. *Post hoc* testing showed a significant difference between populations for each temperature for both sexes, except for the males at 27°C. Both populations and both sexes responded in a similar pattern to temperature (20 > 23 > 27°C).

Heritabilities and cross-temperature correlations

Heritabilities and cross-temperature correlations between 20 and 27°C, 20 and 23°C, and 23 and 27°C for PC1 and PC2 for each population, sex and temperature are shown in Table 3. The range of heritabilities was moderate to high, mostly very high (> 0.8) and, with the exception of a few cases, significant. Heritabilities were slightly higher for PC1 than PC2, with no notable differences between populations, sexes or temperatures. All correlations were positive and differed significantly from zero.

DISCUSSION

Reaction norms

An important analytical concept in the study of phenotypic plasticity is the reaction norm, i.e. the phenotypic expression of a genotype across an environmental range (Schlichting & Pigliucci 1998). By measuring the reaction norms of geographically different populations under the same range of conditions (‘common garden’ approach), the level of genetic divergence between the populations for the plasticity response can be determined. A difference in the slope or shape of the reaction norm corresponds

to a genetic difference underlying the plastic response to environmental conditions (genotype–environment interaction).

Development time, weight and fat content

For both populations, development time decreased considerably with increasing temperature; this is widely observed for ectotherms and is generally viewed as non-adaptive plasticity (Nylin & Gotthard 1998). Both weight and fat content showed no clear directional pattern in developmental plasticity responses to temperature (Fig. 1A,B). It is commonly found that insects developing at lower temperatures become larger (Sibly & Atkinson 1994). While there was a trend showing increasing weight with decreasing temperature for both populations, these differences were not significant despite large sample sizes. Hence there does not seem to be a note-worthy effect of developmental temperature on size at maturity, at least not for the range of temperatures we used here. The shape of reaction norms for fat content differed between the populations. For SA, there was no effect of developmental temperature on fat content, while MW showed small but significant effects of temperature that differed between the sexes. Development time was consistently higher, and weight and fat content lower, for the SA population than the MW population for both sexes. We are cautious to account for these differences between populations in terms of adaptive differentiation because these traits might have been influenced by differences in food plant adaptation between the populations. Moreover, as the effect of temperature on development time may be entirely non-adaptive, and weight and fat content show little to no response to temperature, these traits may be of minor relevance in the context of the present study, i.e. concerning adaptive plasticity in response to temperature.

Starvation resistance and resting metabolic rate

Starvation resistance is a crucial trait in surviving periods of food shortage for many species. Previous work on *Bicyclus anynana* has shown that starvation resistance is a plastic trait highly dependent on both pre-adult and adult temperatures (Pijpe et al. 2007). Confirming prior reports, we found a strong effect of temperature, with increasing starvation resistance at lower temperatures, and females being more resistant to starvation than males (Fig. 1C). Interestingly, there was no population differentiation for the starvation resistance reaction norms between populations. Males and females of both the SA and MW populations showed a clear decrease in starvation resistance for the higher temperature. In other species, findings on population comparisons for starvation resistance vary. For *Drosophila* spp., for instance, there are reports of latitudinal variation for populations in India (Hoffmann & Harshman 1999) and North America (Schmidt et al. 2005), and altitudinal variation (Sørensen et al. 2005), while other studies found little or no level of differentiation at the geographic level (Robinson et al. 2000, Hoffmann et al. 2001).

Resting metabolic rate probably plays an important role in mediating survival under starvation in *Bicyclus anynana* (Pijpe et al. 2007) and other species such as *Drosophila* (e.g. Rion & Kawecki 2007). Similarly to starvation resistance, resting metabolic rate

shows developmental plasticity in response to temperature in *B. anynana* (Pijpe et al. 2007, V. Oostra et al. unpublished data). For both populations we observed a significant increase in resting metabolic rate for high temperature compared to the intermediate and low temperature (Fig. 1D). As with starvation resistance, we found no geographic differentiation in thermal reaction norm between the populations.

Wing pattern

Wing pattern is the most thoroughly studied seasonally plastic trait in *Bicyclus anynana*, and the adaptive benefit of the cryptic form in the dry season has been previously demonstrated (Lyytinen et al. 2004, Brakefield & Frankino 2009). Fig. 3 shows a wet season and a dry season form of a female *B. anynana* hind wing, with the measured areas indicated. Our results show a strong effect of developmental temperature on wing pattern for both PC1 and PC2 (Fig. 2), confirming the extensive plastic response of wing pattern to developmental temperature in this species. PC1 explained the larger part of the variation in the data (57%) and had the highest loadings of the colour measurements (Table 2). Wing colour in *B. anynana* differs among the sexes and seasonal forms. Males are darker than females, and the dry season form of both males and females is generally darker than the wet season form. This is evident in the reaction norm of PC1, which shows a strong response of temperature and clear sex differences (Fig. 2A). The two male populations differed significantly for the males, with SA males developing towards a more wet season form wing pattern at lower temperatures relative to MW

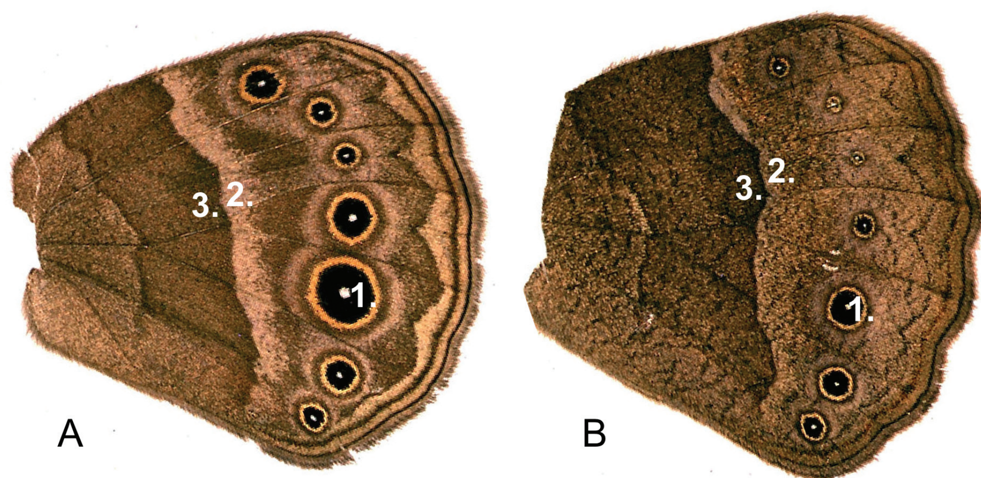

Figure 3. *Bicyclus anynana*. Hind wing of female *B. anynana* in the (A) wet season and (B) dry season. Numbers indicate wing pattern elements that were used to measure plasticity: (1) large eyespot of the hind wing; (2) light area of median band in fourth wing cell; and (3) area used to measure dark colour in fourth wing cell (located outside of the median band).

males. Although two populations are not sufficient to infer directional selection, the observed pattern is concordant with the overall lower temperatures in South Africa compared to Malawi. Females showed a similar but non-significant trend.

PC2 accounted for a smaller part of the variation (23.6%) and had the highest loadings of the size elements (e.g. eyespot size, Table 2). The size elements of the wings were strongly linked to seasonal form, with large eyespots and a clear white band in the wet season, as opposed to much smaller or absent eyespots and band in the dry season. There were also sex differences for the size elements, which were larger in females than in males, but these were not as pronounced as the colour differences. Both males and females showed inter-population differences for PC2, but in the opposite direction of PC1, resulting in overall higher values for the SA population than the MW population (Fig. 2B). This, in turn, indicates that the size elements are smaller (because the loadings of the size elements were negative on PC2) for the SA population. Eyespot size is expected to play a role in mate choice and to be under sexual selection (Breuker & Brakefield 2002, Robertson & Monteiro 2005), which could possibly account for the observed population differences.

Synthesis

To summarise, the populations showed very similar thermal reaction norms for the life history traits and resting metabolic rate, especially for those traits with a clear plastic response to temperature. In contrast, wing pattern showed more differentiation, in the intercept as well as the shape of the reaction norms. In order to identify the extent of population differentiation relative to thermal plasticity response, we performed PCAs on the life history traits, including resting metabolic rate (Fig. 4A), and on wing pattern (Fig. 4B). In the PCA of life history traits, the effects of temperature and sex were large, while the populations were grouped closely for each sex and temperature. The wing pattern PCA also showed a large effect of temperature and sex, but in comparison the separation between the populations along the PC axes was much larger for wing pattern than for the life history traits. Although studies on geographic variation using a range of temperatures are rare, we know of one such study that includes both life history traits and morphological wing traits (wing length and area) in *Drosophila melanogaster* (Van 't Land et al. 1999). In concordance with our findings, relatively small population differences were found between the reaction norms of life history traits compared to morphological wing traits (Van 't Land et al. 1999).

Finally, the overall non-linearity of the reaction norms corresponded well to the polyphenic nature of *Bicyclus anynana* in the wild (Brakefield & Reitsma 1991). Especially for starvation resistance and resting metabolic rate, the shape of the reaction norms suggests a discontinuous pattern. This was indeed confirmed for several traits by an experiment including more temperature treatments (V. Oostra et al. unpublished data), indicating that the transition between development into either dry- or wet-season form occurs in a relatively narrow temperature window, which is often the case for polyphenic species (Nijhout 2003).

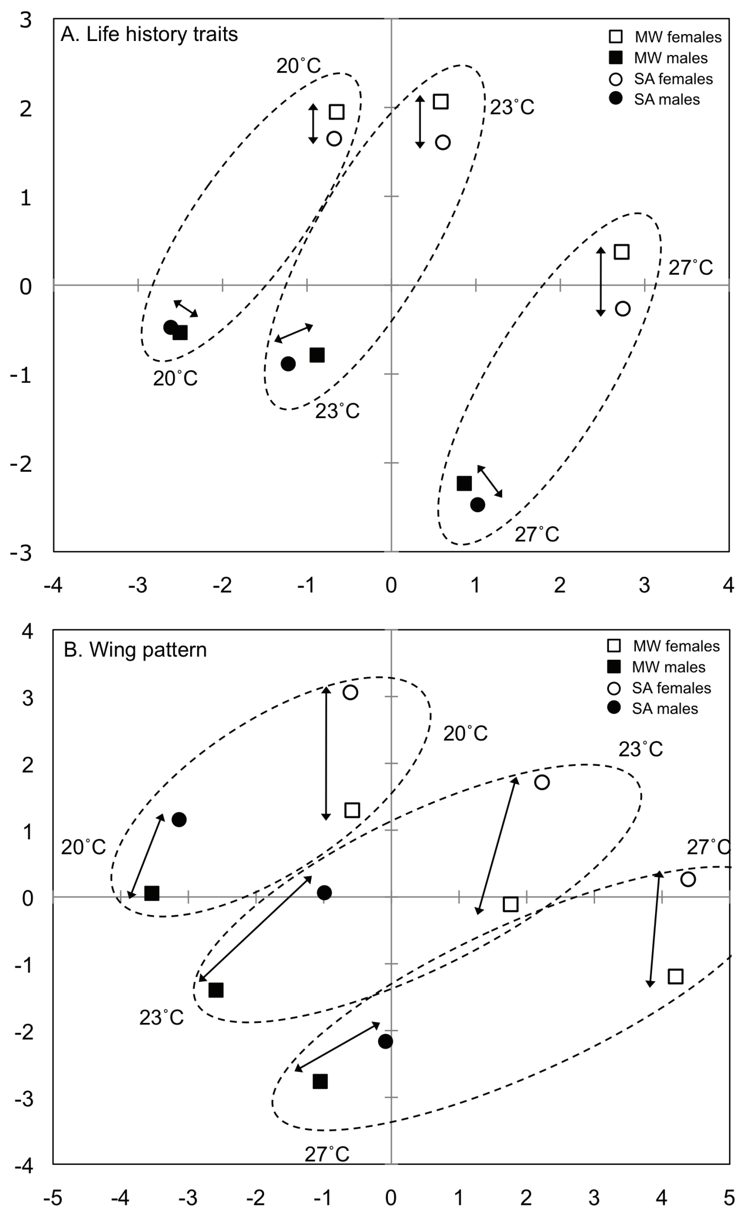

Figure 4. *Bicyclus anynana*. Principal component analyses on the averages of life history traits and wing pattern per temperature, sex and population. Dotted lines indicate temperature groups. Arrows indicate distances between populations. (A) Life history traits: larval development time, pupal development time, pupal weight, relative fat content and starvation resistance. PC1 and PC2 explained 91.3% of the variation. (B) Wing pattern: 10 wing measurements (see Materials and Methods). PC1 and PC2 explained 98.1% of the variation. MW: Malawi; SA: South Africa.

Heritabilities and cross-environment correlations

Genetic change of the plasticity response of a trait by selection requires that the trait is heritable and that there is genetic variation for plasticity (variation in slope of the reaction norms) (Via & Lande 1985, De Jong 1995). To gain insight into the potential of organisms to adapt to climate change, it is thus important to study the heritability of traits (Pertoldi & Bach 2007, Chown et al. 2010). In the present study, we estimated heritabilities using a full-sibling family design, resulting in broad-sense heritabilities that include both additive and non-additive genetic variance. Therefore, broad-sense heritabilities generally represent an overestimation of the heritable variation of traits (Roff 1997). Our results show considerably higher heritabilities for wing pattern (Table 3) than for development time and weight (Table 1) in both populations. This corresponds to the general finding that life history traits have lower heritabilities than morphological traits. Consequently, it is often assumed that life history traits have less genetic variation and evolve more slowly in response to directional selection because they are more closely linked to fitness (Roff & Mousseau 1987). This would be in agreement with our findings and could explain why we observed substantially more population differentiation for wing pattern than for the life history traits. However, the comparison between heritabilities of life history and morphological traits should be made with caution. When taking into account the different components comprising the trait variance (V_P), the low heritabilities of fitness-related traits can often be explained by their high residual variation (V_R: non-additive genetic and environmental factors). The additive genetic variance (V_A) would be a more precise measure to compare the selective response potential between traits (Houle 1992), but our experimental set-up did not allow for estimating narrow-sense heritability.

The trait values of a plastic trait for different environments can be considered as separate, genetically correlated characters (Falconer & Mackay 1996). These cross-environment correlations can be used as a measure of variation for the plastic trait, or the level of genotype–environment interaction. When the cross-environment correlation between genotypes is +1, the slopes of the reaction norms run parallel and there is no genotype–environment interaction. A correlation of $< +1$ indicates the presence of genetic variation for the plasticity response of the trait (Pigliucci 2005). Our data show high cross-temperature correlations for both life history traits (Table 1) and wing pattern (Table 3) for both populations. All correlations were lower than +1, indicating the presence of genetic variation for the plasticity response of the traits and the potential for adaptation by selection.

Developmental plasticity and adult acclimation

In order to unravel the complexity of phenotypic plasticity, it is important to distinguish between developmental plasticity (phenotypic change induced during development) and adult acclimation (reversible or flexible plasticity during the adult stage). A recent review concluded that many studies aimed at examining adult acclimation actually assessed the consequences of the developmental environment (Wilson & Franklin 2002), and so far only a few studies have examined the relative contributions of the two

forms of plasticity in a single experiment (e.g. Fischer et al. 2003, Terblanche & Chown 2006). For *Bicyclus anynana*, both developmental plasticity and adult acclimation are important in coping with seasonality (Fischer et al. 2003, Brakefield & Frankino 2009). Some traits, for example wing pattern and body size, are developmentally plastic, but fixed during the adult stage. Other traits, including resting metabolic rate and egg size, show both forms of plasticity. The initial state of these traits in early adult life depends on the developmental environment, but can be changed by acclimation depending on the adult environment. For example, egg size is initially dependent on developmental temperature, with females reared at cool temperatures producing fewer and larger eggs than females reared at warm temperatures. Cross-transferring females among temperatures can reverse this effect by acclimation over a period of ~10 d (Fischer et al. 2003). In the context of adaptive phenotypic plasticity, developmental plasticity serves as a prediction of the adult environment based on pre-adult conditions. Adult acclimation can thus be seen as the organism's ability to fine-tune its phenotype to the actual and potentially variable environmental conditions (beneficial acclimation hypothesis, Leroi et al. 1994).

Our results reveal an interesting pattern when taking both forms of plasticity into account. Of the traits that showed a clear developmental response to temperature, we found geographic variation in the thermal reaction norm of the irreversible trait wing pattern, but no visible differentiation in starvation resistance and resting metabolic rate, both of which are traits that can be influenced by acclimation during adult life. Moreover, in a different experiment we measured developmental plasticity of egg size using the same two populations and developmental temperatures (data not shown). We found no population differences between their thermal reaction norms for egg size. Phenotypic plasticity may buffer environmental variation, thus shielding the genetic response to selection (Falconer & Mackay 1996, West-Eberhard 2003). This, in turn, can slow down or prevent geographic differentiation. Our data suggest that, likewise, adult acclimation may shield developmental plasticity from evolutionary change.

CONCLUSIONS

Our study on geographic variation in the polyphenic response to temperature in *B. anynana* revealed a population specific response in wing pattern plasticity to developmental temperature. Therefore, when considering present-day climate change, a rise in temperature could consequently result in a phenotypic mismatch of wing pattern to season. High heritabilities and genetic variation for the plasticity response of wing pattern indicate a potential for adaptation. Despite the large difference in latitude between the populations and the differences in regional climate, we found little to no geographic variation for the life history traits that showed a clear plasticity response to developmental temperature. Furthermore, there was no geographic variation in the plasticity response of the traits that can be changed by acclimation in the adult stage. This indicates that adult acclimation plays a major role in coping with regional climate. Whether or not there is geographic variation in adult acclimation responses to temperature will make an interesting topic for future research.

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Chapter 3

Translating environmental gradients into discontinuous reaction norms via hormone signalling in a polyphenic butterfly

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Translating environmental gradients into discontinuous reaction norms via hormone signalling in a polyphenic butterfly

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ABSTRACT

Polyphenisms—the expression of discrete phenotypic morphs in response to environmental variation—are examples of phenotypic plasticity that may potentially be adaptive in the face of predictable environmental heterogeneity. In the butterfly *Bicyclus anynana*, we examine the hormonal regulation of phenotypic plasticity that involves divergent developmental trajectories into distinct adult morphs for a suite of traits as an adaptation to contrasting seasonal environments. This polyphenism is induced by temperature during development and mediated by ecdysteroid hormones. We reared larvae at separate temperatures spanning the natural range of seasonal environments and measured reaction norms for ecdysteroids, juvenile hormones (JHs) and adult fitness traits. Timing of peak ecdysteroid, but not JH titres, showed a binary response to the linear temperature gradient. Several adult traits (e.g. relative abdomen mass) responded in a similar, dimorphic manner, while others (e.g. wing pattern) showed a linear response. This study demonstrates that hormone dynamics can translate a linear environmental gradient into a discrete signal and, thus, that polyphenic differences between adult morphs can already be programmed at the stage of hormone signalling during development. The range of phenotypic responses observed within the suite of traits indicates both shared regulation and independent, trait-specific sensitivity to the hormone signal.

Key words: ecdysone, hormonal regulation, life history, phenotypic plasticity, reaction norm, seasonal polyphenism

INTRODUCTION

Phenotypic plasticity is the ability of individual genotypes to produce different phenotypes when exposed to environmental variation (Stearns 1989, Schlichting & Pigliucci 1998). Potentially, it allows organisms to persist in variable environments, and it is therefore of major evolutionary significance. Furthermore, phenotypic plasticity reveals how the developmental mechanisms that translate genotypes into phenotypes can be modulated by the environment and how sensitivity to the environment can be a source of phenotypic variation (Brakefield et al. 2003, West-Eberhard 2003, Gilbert & Epel 2008). The reaction norm concept describes phenotypic variation as a function of the environment and provides an experimental framework for studying developmental sensitivity to the environment (Debat & David 2001, Sultan 2007). A flat reaction norm represents a canalized phenotype, whereas a steep reaction norm represents a plastic phenotype. Polyphenisms can be seen as an extreme case of phenotypic plasticity, where alternative discrete phenotypes develop in response to environmental variation (Shapiro 1976, Stearns 1989, West-Eberhard 2003).

Hormones play crucial regulatory roles in coordinating the expression of physiological, behavioural and morphological traits into an integrated life history (Nijhout 1994, Zera et al. 2007, Ketterson 2009). The two major classes of insect hormones, ecdysteroids and juvenile hormones (JHs), have been implicated in many cases of insect polyphenisms, such as horned beetles, butterflies, social insects and sand crickets (Nijhout 2003, Zera 2007, Smith et al. 2008, Brakefield & Frankino 2009). While various studies have measured reaction norms across an environmental gradient for phenotypic traits (e.g. Trotta et al. 2006, Liefting et al. 2009), and others have measured differences in hormone dynamics between morphs at the extreme ends of a reaction norm (e.g. Brakefield et al. 1998), these approaches have rarely been combined (but see Anstey et al. 2009). It is therefore unknown whether discrete differences between adult morphs are already present at the endocrine level during development.

A polyphenism typically involves a suite of morphological, physiological and life-history traits that may respond to the same environmental signal (e.g. Pijpe et al. 2007, Brisson 2010). The central regulation of systemic hormone titres enables integration of traits at the organismal level, but can thereby potentially constrain their independent evolution (Ketterson & Nolan 1999, Zijlstra et al. 2004, McGlothlin & Ketterson 2008). On the other hand, sensitivity of the local tissue determines the response to the hormone, indicating scope for differentiated regulation of the traits comprising the polyphenism (Nijhout 1994). In contrast with theoretical advances (e.g. McGlothlin & Ketterson 2008), there is little empirical knowledge on the extent to which suites of traits regulated by the same hormone constitute integrated phenotypes across environmental gradients, or can respond independently.

With the present study, we aim to understand how hormonal mechanisms regulate a suite of fitness traits involved in the phenotypic plasticity in *Bicyclus anynana*. This afrotropical butterfly has evolved developmental plasticity as an adaptation to its seasonal environment (Brakefield & Reitsma 1991, Brakefield et al. 1996). In the warm wet season, butterflies have large, prominent eyespots on the ventral surface of their wings, which are probably involved in the deflection of predatory attacks (Lyytinen et

al. 2004). Butterflies of the cool dry season express a cryptic wing pattern with small to virtually absent eyespots. In the dry season in the field there is strong natural selection against conspicuous eyespots (Brakefield & Frankino 2009). Furthermore, these butterflies express an alternative physiology and life-history strategy that allows them to bridge the period of (nutritional) stress that the dry season represents (Brakefield et al. 2007). During the dry season, adults have altered metabolic rate, and accumulate more mass and higher fat content during the larval stage, important fitness traits associated with adult starvation resistance (Zwaan et al. 1991, Chippindale et al. 1996, Pijpe et al. 2007, De Jong et al. 2010). Finally, reproduction is delayed until the end of the dry season (Brakefield & Reitsma 1991, Fischer et al. 2003, Brakefield et al. 2007). The seasonal adult morphs are induced in response to temperature during a critical period of pre-adult development (Brakefield & Reitsma 1991, Brakefield et al. 1996). Analyses of the reaction norm for wing pattern have revealed a linear response to developmental temperature (Brakefield & Reitsma 1991, Wijngaarden et al. 2002), but it is unknown how the life-history traits respond to a gradient in environmental temperature.

Ecdysteroids have been found to be involved in regulating wing-pattern plasticity in *B. anynana* (Koch et al. 1996, Brakefield et al. 1998, Zijlstra et al. 2004), but it is unknown whether these hormones have a role in regulating the full suite of traits involved in the seasonal adaptation. Furthermore, it is unknown how ecdysteroid titres change along a continuous gradient in environmental temperature and how this response relates to those of the phenotypic traits. In this study, we apply the reaction norm concept to the hormone dynamics underlying the phenotypic response. The extension of the use of the reaction norm perspective to developmental and molecular processes regulating the phenotype promises to be a useful tool in the integrative study of phenotypic plasticity (Aubon-Horth & Renn 2009).

We manipulated the developmental environment by rearing cohorts of larvae under five different temperatures spanning the natural range of seasonal environments, with the lowest temperature corresponding to the dry-season environment and the highest to the wet-season environment. We measured the reaction norms for ecdysteroids and JHs during the critical pupal stage, as well as for size at maturity, relative abdomen to total body mass (as a measure of allocation of resources to early life reproduction versus flight ability), metabolic rate, fat reserves and ventral wing pattern—key fitness traits involved in the seasonal polyphenism.

MATERIALS AND METHODS

Experimental design

Cohorts of *B. anynana* used in this experiment were derived from an outbred wild-type population established in the laboratory in 1988. The experiment was carried out in two phases, one for the measurement of phenotypic traits and the other for measurement of hormone titres. In each phase, 2000 larvae were reared from egg to adult (n = 400 per temperature treatment). Eggs were collected from the wild-type population on a single day and kept at 23.5°C until hatching. Larvae were reared on maize (*Zea mays*) in climate-controlled chambers at 70 per cent relative humidity (RH) with a

12 L : 12 D light/dark cycle. After hatching, larvae were randomly divided over each of five climate-controlled chambers (19°C, 21°C, 23°C, 25°C and 27°C, ± 0.5°C) representing five temperature treatments, with a different allocation of temperature treatments to chambers in the two phases of the experiment. The lowest temperature corresponds to dry-season conditions in the field and the highest temperature to wet-season conditions Brakefield & Reitsma 1991. Temperature and RH were logged throughout the rearing process using data loggers (± 0.2°C) to ensure stability of environmental conditions.

Life-history traits

For each individual, we recorded development time as the number of days between hatching of the egg and eclosion of the butterfly. Pupae were weighed within 36 h after pupation to the nearest 0.1 mg. One day after eclosion, 100 males and 100 females per temperature treatment were haphazardly selected for resting metabolic rate (RMR) measurements. Fifty butterflies per rearing temperature per sex were measured at 19°C, and 50 at 27°C, in a climate-controlled chamber during the dark phase of the diurnal cycle. RMR was measured as the individual rate of CO_2 respiration (millilitre per hour) over a period of 20 min, following (Pijpe et al. 2007). Following RMR measurements, wings were cut off after which the butterflies were dried for 48 h at 55°C and weighed to the nearest 0.01 mg. Total fat (triglyceride and free fatty acids) was extracted by incubating the dried butterflies at room temperature in 2 : 1 (v/v) dichloro-methane : methanol for 96 h, followed by drying and weighing, yielding fat-free dry weight. Fat content was calculated by subtracting the fat-free dry weight from the initial dry mass. In order to estimate allocation of resources to different parts of the body, thorax and abdomen were dried and weighed separately.

Wing pattern

The ventral surface of one hindwing of each individual was photographed using a digital still camera connected to a binocular microscope (Leica). The images were analysed with ImagePro 6.0 software to measure the following wing pattern elements: (i) distance between the first and the fifth eyespot; (ii) radius of the inner black disc of the fifth eyespot; (iii) radius of the white centre of the fifth eyespot; and (iv) width of the median band (after Wijngaarden & Brakefield 2001).

Hormone titres

For female pupae of each temperature treatment, we measured ecdysone (Ecd), 20-hydroxyecdysone (20E), and JH-I, JH-II and JH-III titres at 11 time points throughout the earlier 55 per cent of the pupal stage, with five replicate pupae per time point. To correct for the direct effect of temperature on pupal development time, we scaled the time points for each temperature treatment separately to the total average duration of the pupal stage. For each temperature treatment, we chose 11 time points after pupation, corresponding to approximately 5 to 55 per cent of total pupal developmental time, spanning the relevant time window for ecdysteroid dynamics (Zijlstra et al. 2004). We

took 20 μl haemolymph samples from individual pupae, sampling each pupa only once. Hormone titres were measured from haemolymph by liquid chromatography–mass spectrometry (LC-MS), using the method developed by Westerlund & Hoffmann (2004) and Westerlund (2004), with minor modifications to the protocol (for details see Supplementary Material). This method allows for simultaneous quantification of all hormones from the same sample.

Statistical analyses

Life-history traits and wing pattern

Data were analysed using two-way analysis of variance (ANOVA) for each trait separately, with temperature treatment and sex as fixed effects. RMR and fat content were first analysed with dry weight as the only independent variable, of which the residuals were used as the dependent variables in the ANOVAs. Likewise, for relative abdomen mass we used the residuals of the model with abdomen dry weight as dependent, and total dry weight as independent variable. Finally, the four wing-pattern measurements were reduced using a principal component analysis (PCA; cf. Wijngaarden & Brakefield 2001), pooling data across the sexes. The first principal component (PC1) explained 50.5 per cent of the total variation and was associated with the traits that are indicative of seasonality (radius of the inner black disc of the fifth eyespot, radius of the white centre of the fifth eyespot and width of the median band). PC2 explained 30.3 per cent of the total variation and was associated with the distance between the first and the fifth eyespot, an index of size rather than seasonality. Thus, only PC1 was further analysed. *Post hoc* comparisons between specific levels of a factor were performed using Tukey's honest significant differences (HSD) tests.

Hormone titres

Previous work on *B. anynana* has shown that ecdysteroid titres peak at around 20 to 40 per cent of pupal development (hours after pupation as percentage of total pupal development time), with lower titres before and after. Titres of the two seasonal morphs have similarly shaped curves and similar absolute values, but show a difference in timing of peak titres (Koch et al. 1996, Brakefield et al. 1998, Zijlstra et al. 2004). To compare hormone dynamics across temperature treatments, we estimated the timing of the peaks for Ecd and 20E by fitting, for each hormone separately, the function

$$Y = e^{bt-at^2}$$

to the time series of each temperature, where Y is the hormone concentration (picograms per microlitre) at time t (relative time after pupation as fraction of total pupal time), and a and b are parameters determining the height and timing of the peak.

For each treatment, and for Ecd and 20E separately, we randomly drew one data point for each time point, using the five replicate pupae per time point, yielding five replicate time series per treatment per hormone. Through each time series, we fitted

the function with parameter values minimizing residual sum of squares. We thus obtained, per temperature treatment per hormone, five independent estimates of the two parameter values based on the five replicate pupae per time point. For this function, the timing of the peak t_{peak} is given by $b/2a$ (calculated by setting the first derivative of the function to 0), yielding five independent estimates of t_{peak}. This t_{peak} was subsequently used as dependent variable in a one-way ANOVA with temperature treatment as fixed factor. *Post hoc* comparisons between specific treatment levels were performed using Tukey's HSD tests. As we had no *a priori* expectations regarding JH-III concentration dynamics during the pupal stage, we used JH-III concentration as the dependent variable in a linear model with temperature treatment as fixed factor and relative time after pupation (as fraction of total pupal time) as covariate.

To estimate the potential effect of diurnal cycle on hormone concentrations (cf. Zhao & Zera 2004), we used, for each hormone separately, one-way ANOVA with hour of day at which a pupa was sampled as fixed factor and hormone concentration as dependent variable, followed by Tukey's HSD tests.

RESULTS

Reaction norms of phenotypic traits

All phenotypic traits involved in the seasonal variation showed a significant response to the gradient of developmental temperature. However, the precise shape of each reaction norm differed across traits. Some traits changed gradually and linearly along the temperature gradient, while other traits showed a discontinuous change at intermediate temperatures. Furthermore, for some traits there were marked differences between males and females in their response, while for other traits no such sex specificity was found.

In both sexes, development time decreased continuously with increasing developmental temperature; larvae developed faster under wet-season conditions. Though males developed faster than females ($p < 0.001$), the shape of the reaction norm was virtually identical between the sexes (Fig. 1a).

Across the temperature gradient, pupal mass was lower for males than for females ($p < 0.0001$), and in both sexes pupae were larger when reared at lower temperatures, corresponding to dry-season conditions. However, the shape of the reaction norm differed between the sexes. In males, pupal mass decreased in a continuous, linear manner with increasing developmental temperature, with intermediately sized pupae at intermediate temperatures. By contrast, in females, pupal mass did not change within the lower or higher ends of the reaction norm but showed a significant decrease between 21°C and 23°C (Fig. 1b).

Relative abdomen mass, as a measure of relative allocation to reproduction versus flight, was higher in adult females than in males, but only when they had developed at the three higher temperatures (i.e. wet-season conditions; $p < 0.01$). At lower temperatures, males did not differ from females. In females, the response to developmental temperature was discontinuous between the two lower and three higher temperatures, with a significant increase between 21°C and 23°C. For males,

the pattern was qualitatively similar (i.e. a relatively larger abdomen under wet-season compared with dry-season conditions), but the overall difference between the highest and the lowest temperature was smaller than for females (Fig. 1c).

At the three higher temperatures, females had lower adult relative fat content than males ($p < 0.01$), while at 19°C and 21°C males did not differ from females. Male relative fat content did not change along the temperature gradient, with the exception of 27°C, where it was lowest when compared with the other temperatures. In females, relative fat content decreased discontinuously with increasing developmental temperature (i.e. females developed highest fat content under dry-season conditions; Fig. 1d).

For both sexes and all developmental temperatures, adult RMR (the rate of CO_2 respiration at rest) was lower when measured at 19°C than when measured at 27°C ($p < 0.0001$; compare Fig. 2a with 2b). RMR at 19°C was higher for males when compared

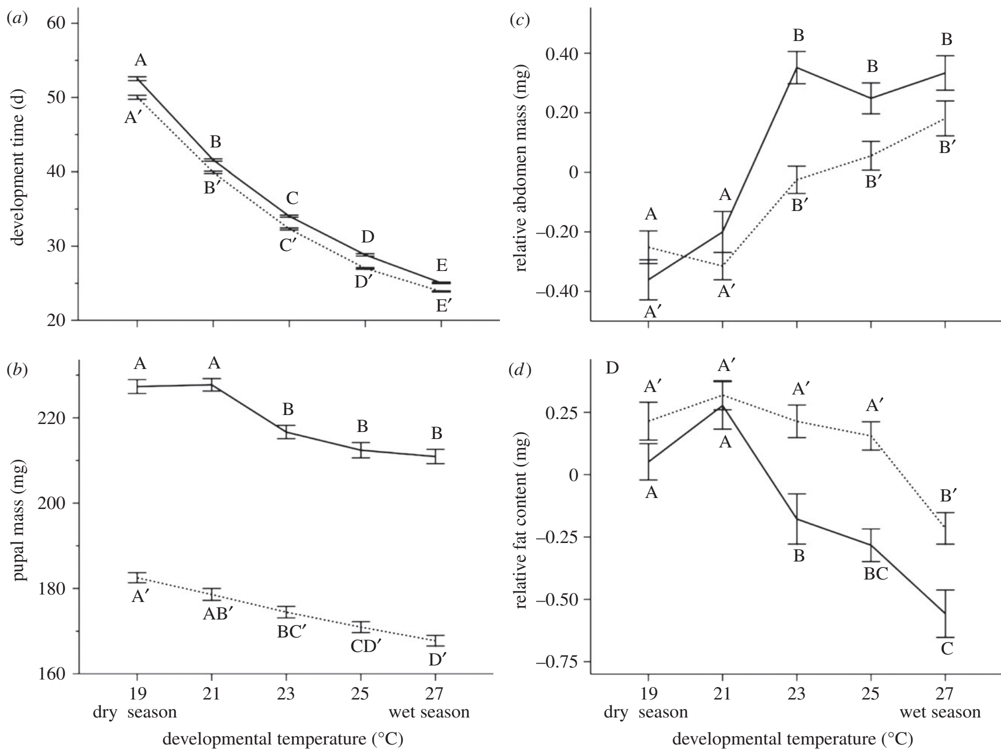

Figure 1. Effects of developmental temperature on (A) development time, (B) pupal mass, (C) relative abdomen mass (residuals from regression of abdomen dry mass on total dry mass) and (D) relative fat content (residuals from regression of fat content on dry mass). Females and males are represented by the solid and dotted lines, respectively. Error bars represent ± 1 s.e. with $50 < n < 150$. Significant differences across the temperature treatments (Tukey's HSD, $p < 0.05$) are indicated by different letters, coding for females and males separately.

with females across the developmental temperature gradient ($p < 0.0001$). In both sexes, RMR measured at 19°C showed a discontinuous shift along the developmental temperature gradient; adults developed at lower temperature (dry-season conditions) had higher RMR at 19°C than those developed at higher temperature (Fig. 2a). In both sexes, RMR measured at 27°C showed a similar decrease with increasing developmental temperature (i.e. butterflies developed highest RMR when reared under dry-season conditions). In males, the response was discontinuous while in females it was almost linear, with the exception of the highest developmental temperature. Furthermore, at the lowest developmental temperature, RMR measured at 27°C was lower in males than in females, while this was not the case at the higher temperatures (Fig. 2b).

Finally, PC1 of wing pattern changed linearly along the temperature axis in both males and females. Butterflies developed larger eyespots when reared under wet-season conditions, and the reaction norms differed in elevation between the sexes ($p < 0.001$; Fig. 3).

Dynamics and reaction norms of female hormone concentrations during pupal stage

Lack of diurnal cycle in hormone concentrations

Careful visual inspection of Ecd, 20E and JH-III concentrations plotted against time of day at which a sample was taken revealed no indication for a diurnal cycle for any of these hormones (cf. Zhao & Zera 2004). For Ecd and JH-III, no effect of time of day on concentration was found ($p > 0.1$). For 20E, there was a small but significant effect of time of day on concentration ($p = 0.05$), but *post hoc* comparisons between specific levels (i.e. hours) were not significant ($p > 0.1$).

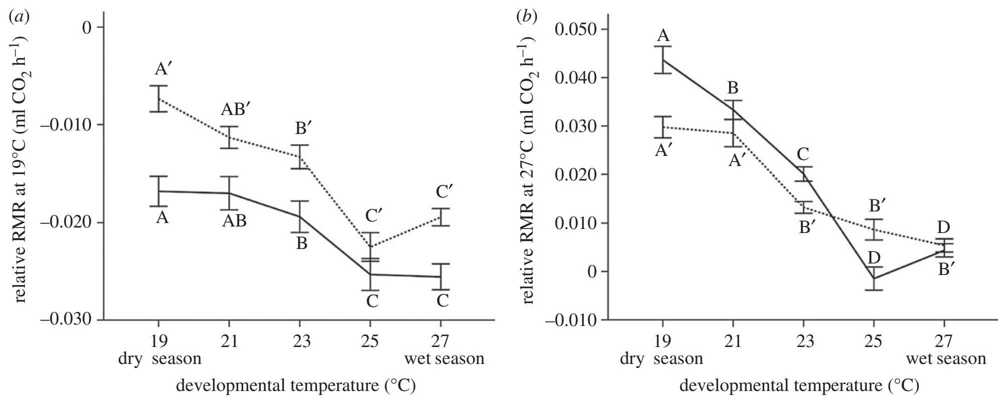

Figure 2. Effects of developmental temperature on adult relative RMR (residuals from regression of RMR on mass) measured at (A) 19°C and (B) 27°C. Note the difference in scale. Females and males are represented by the solid and dotted lines, respectively. Error bars represent ± 1 s.e. with n = 50. Significant differences across the temperature treatments (Tukey's HSD, $p < 0.05$) are indicated by different letters, coding for males and females separately.

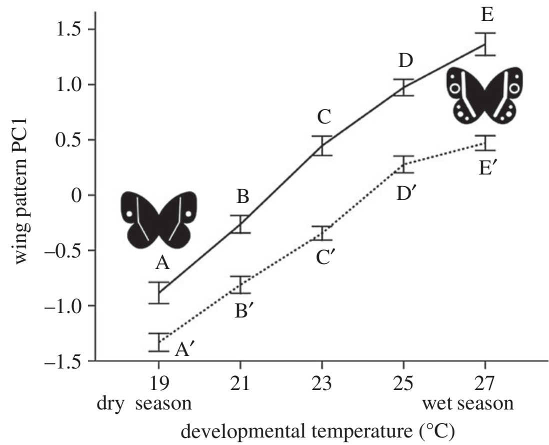

Figure 3. Effects of developmental temperature on the first principal component (PC1) of wing pattern, explaining 50.5% of variation in eyespot and band size. Females and males are represented by the solid and dotted lines, respectively. Error bars represent ± 1 s.e. with n = 50. Significant differences across the temperature treatments (Tukey's HSD, $p < 0.05$) are indicated by different letters, coding for females and males separately.

Ecdysone and 20-hydroxyecdysone

Both ecdysteroids showed qualitatively similar dynamics during the pupal stage, with low early concentrations, peak concentrations between 20 to 40 per cent of pupal development (hours after pupation as percentage of total pupal development time), and low late concentrations (Fig. 4). For both hormones, concentrations were in a similar range across all temperature treatments (Ecd: approx. 30–1800 pg μl^{-1}; 20E: approx. 100–2600 pg μl^{-1}), but varied with time after pupation. Ecd concentrations were below detection levels very early and very late in the pupal stage.

While the absolute concentrations were similar across temperature treatments, the timing of increase, peak and decrease of hormone concentration showed a marked shift between the temperature treatments. We formally compared hormone dynamics throughout the pupal stage across temperature treatments by constructing, for each ecdysteroid separately, nonlinear regression models with hormone concentration as dependent variable and relative time after pupation (as fraction of total pupal time) as independent variable (see Materials and Methods). All models were significant (95% confidence interval (CI) for p: 0.0002–0.0040) and captured most of the variation (95% CI for R^2: 0.79–0.86). Using the estimated parameters for each model, we calculated peak concentrations and their timings.

Peak Ecd concentrations did not differ across temperature treatments ($p > 0.7$). However, there was a significant shift in the timing of peak concentrations with increasing developmental temperature. Concentrations peaked late at lower temperatures (dry-season conditions) and early at higher temperatures (wet-season conditions; $p < 0.0001$), with a discontinuous shift between these two types of dynamics occurring between 21°C and 23°C, and no changes between the two lower or among the three higher temperatures (Fig. 4a,c).

Similarly, peak 20E concentrations did not differ across temperature treatments ($p > 0.1$), but there was a significant shift in the timing of peak concentrations with increasing developmental temperature. At lower temperatures (dry-season conditions),

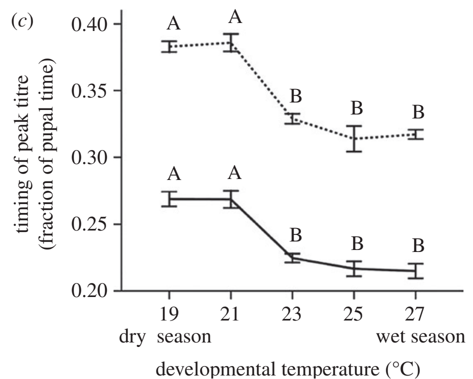

Figure 4. Effects of developmental temperature on ecdysone (Ecd) and 20-hydroxyecdysone (20E) dynamics during the pupal stage. Titres (± s.e.) throughout the pupal stage (fraction of total pupal development time) across the five temperature treatments (filled diamonds, 19°C; filled squares, 21°C; open triangles, 23°C; open squares, 25°C; open diamonds, 27°C) of (A) Ecd and (B) 20E. (C) Reaction norms of estimated time of peak (± s.e.) Ecd (solid line) and 20E (dashed line) titre for developmental temperature.

20E concentrations peaked late when compared with the higher temperatures (wet-season conditions; $p < 0.0001$), with a discontinuous shift occurring between 21°C and 23°C. Again, this shift was the only change in timing along the temperature gradient and no intermediate types of dynamics were observed at intermediate temperatures (Fig. 4b,c).

Ecd concentrations peaked earlier than those of 20E, with a time lag of approximately 10 per cent of pupal time (hours after pupation as percentage of total pupal development time), which was constant along the temperature gradient (Fig. 4c).

Juvenile hormones

JH-I was only detected in haemolymph of approximately 14 per cent of all pupae, with concentrations ranging from 1 to 35 pg μl^{-1} and no effect of developmental temperature. JH-II was below detection level in all analysed pupae. JH-III was detected in haemolymph of pupae of all developmental stages across all developmental temperatures, in a comparable range of concentrations (30–100 pg μl^{-1}; Fig. 5). There was no effect of relative time after pupation (as fraction of total pupal time) on JH-III titres ($p > 0.1$), but there was a small but significant effect of developmental temperature ($p < 0.0001$), with pupae developed at 19°C having highest, and pupae developed at 21°C lowest, JH-III concentrations.

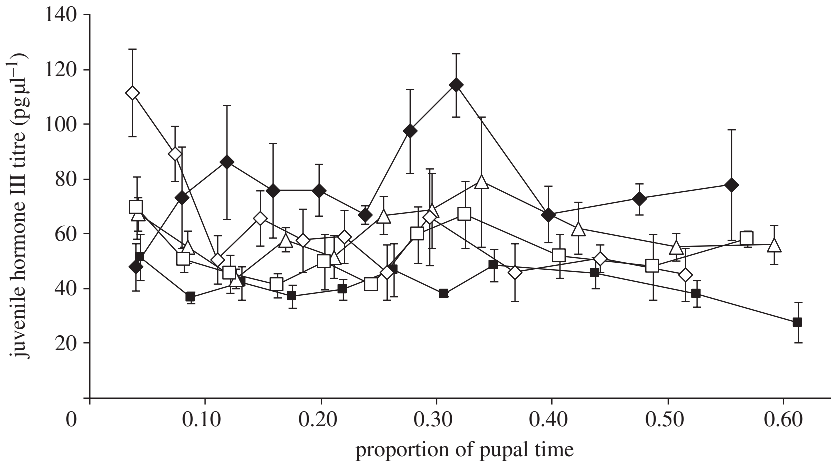

Figure 5. Dynamics during pupal stage of juvenile hormone (JH)-III titres (± s.e.) across the five temperature treatments. JH-I was only detected in approximately 14 per cent of all pupae, and JH-II was detected in none of the pupae. Filled diamonds, 19°C; filled squares, 21°C; open triangles, 23°C; open squares, 25°C; open diamonds, 27°C.

DISCUSSION

Hormone dynamics

The discontinuous expression of a polyphenic trait across an environmental gradient requires some form of a switch mechanism between alternative developmental trajectories. This developmental switch could arise by a change in: (i) the hormone titre; (ii) the sensitivity to the hormone; (iii) the hormone timing; and (iv) the window of sensitivity (Nijhout 2003). Experimental studies have linked each of these scenarios to polyphenisms, either by direct measurement of titres, titre regulators or sensitivity, or indirectly by hormone manipulation. Examples include Ecd titre changes linked to horn length in beetles (Emlen & Nijhout 1999); morph-associated differences in JH dynamics in a wing-polymorphic crickets (Zhao & Zera 2004); and differences in the timing of Ecd release between the two wing-pattern morphs of butterflies (Brakefield et al. 1998). However, these studies typically concern the hormonal dynamics under only two environmental conditions, which makes it impossible to discern whether these hormonal changes are continuous or discrete. Analysing the precise shape of the reaction norm at the hormone level reveals how a continuous environmental trajectory is translated into discrete alternative developmental trajectories (e.g. Anstey et al. 2009).

For the first time, we show that the dichotomy between adult phenotypic morphs can already be programmed at the stage of hormone signalling during development. Our results reveal a discontinuous shift in the timing of peak Ecd and 20E titres in response to a linear gradient of developmental temperatures (Fig. 4c). Both hormones show this shift in timing between 21°C and 23°C, while there are no significant differences in timing between the two lower temperatures (dry-season conditions), nor among the three higher temperatures (wet-season conditions). None of the measured JHs show a clear response to the temperature gradient (Fig. 5) and all are, therefore, unlikely to be involved in regulating the polyphenism. Our results indicate that a discontinuous response of ecdysteroid dynamics to the continuous environmental gradient underlies the polyphenism in *B. anynana*.

Phenotypic responses

In females, but not in males, the response of pupal mass to developmental temperature was discontinuous. Thus, despite large, continuous changes in development time in response to the temperature gradient (Fig. 1a), female larvae ultimately develop a discontinuous pattern in pupal mass (Fig. 1b). The correspondence between the responses of female pupal mass and ecdysteroid dynamics (Fig. 4) to the temperature gradient suggests that these traits share upstream regulators.

In adults, the relative contribution of the abdomen to total body mass showed a clear discontinuous response to developmental temperature (Fig. 1c), which was particularly pronounced in females. These results indicate a higher relative allocation to flight during the harsh dry season and to reproduction during the favourable wet season, especially in females. Adult fat content in males remained fairly constant across most

developmental temperatures, with the exception of the highest, whereas the response was more discontinuous in females (Fig. 1d). The response of RMR ranged from clearly discontinuous to more linear (Fig. 2). Overall, the responses of a number of adult traits to the temperature gradient are strikingly similar to the discontinuous response of pupal ecdysteroid dynamics (Fig. 4), indicating a regulatory role for ecdysteroid signalling during the pupal stage in shaping adult physiological and allocation traits.

In accordance with earlier results (Brakefield & Reitsma 1991, Wijngaarden et al. 2002), we found a linear response of ventral wing pattern to the temperature gradient (Fig. 3), contrasting with the discontinuous reaction norm for pupal ecdysteroid dynamics (Fig. 4). Previous studies, using hormone manipulation and artificial selection experiments, have demonstrated a functional role of pupal ecdysteroids in the regulation of wing pattern polyphenism, as well as genetic correlations between dynamics of ecdysteroid titres and wing pattern (Koch et al. 1996, Brakefield et al. 1998, Zijlstra et al. 2004). Combined, these findings strongly imply an additional level of regulation between the hormone signal and the response of the developmental pathways producing the wing pattern. This regulation is likely to involve changes in the window of hormone sensitivity (for example by altered timing of Ecd receptor expression (Nijhout 2003, Brakefield et al. 1998).

CONCLUSIONS

By applying a continuous temperature gradient to developing larvae and pupae, we showed that a discontinuous ecdysteroid signal during the pupal stage underlies the seasonal polyphenism in *B. anynana*. Furthermore, several fitness traits (such as relative abdomen mass, RMR, female pupal mass and fat content) displayed a similar, dimorphic response, indicating shared regulation of these traits. In contrast, ventral wing pattern, known to be regulated by ecdysteroids (Zijlstra et al. 2004), responded in a linear manner. Taken together, our findings suggest that the diversity in shapes of reaction norms of the traits involved in the phenotypic plasticity stems from variation in how each trait responds to the ecdysteroid dynamics.

In view of our results and of earlier findings that revealed a short window of sensitivity of the ventral wing pattern to 20E injections (Koch et al. 1996) and a complete lack of sensitivity of the dorsal wing pattern (Brakefield et al. 1998), we propose that variation across traits in windows of sensitivity to the systemic hormone signal can be a general mechanism underlying both linear and discrete responses to environmental gradients within suites of traits that share a hormonal regulator. This diversity in responses allows for both flexibility and integration of traits underlying adaptations to divergent environments.

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SUPPLEMENTARY MATERIAL

Hormone titre measurements by liquid chromatography - mass spectrometry (LC-MS)

Hormone quantification from haemolymph followed, with minor modifications, the LC-MS method developed by Westerlund & Hoffmann (2004) and Westerlund (2004), which allows for simultaneous quantification of all hormones from the same sample. Pupal haemolymph (20 µl) was extracted with a glass capillary and deposited into 300 µl 1 : 1 methanol : isooctane (v/v) solution. The mixture was vortexed for 20 s, allowed to stand at room temperature for 20 min and then centrifuged at 10,000 × g at 6°C for 20 min. With a glass Pasteur pipette both phases of the supernatant were transferred to a new glass vial and stored at -80°C until further analysis. Prior to hormone titre quantification by LC-MS, the isooctane phase of the sample was evaporated and sample volume was reduced to 20 µl using a vacuum centrifuge. The sample was then centrifuged at 10,000 × g for 10 min, after which the supernatant was transferred to a new glass vial and placed in the autosampler (Shimadzu SIL-10ADVP) connected to an Eldex Micro Pro HPLC system. Samples were separated on a 150 × 2 mm C18 reversed-phase column (ReproSil-Pur ODS-3, 5 µm, Dr. Maisch-GmbH, Ammerbuch, Germany) protected by a guard column (C18 cartridge, Phenomenex, Aschaffenburg, Germany) at a flow rate of 200 µl min^{-1} and column temperature of 37°C. The mobile phase consisted of a methanol/water gradient varying between 30% and 100% over 20 min. MS analysis was performed using electrospray ionisation in the positive mode on a Shimadzu LCMS-2010A operating under the following conditions: Probe high voltage was set at 4.50 kV, CDL voltage at -5.0 V and temperature at 250°C, the heat block at 200 °C. The nitrogen flow rate was 4 l min^{-1}. Post-run analysis was performed using the Shimadzu LCMSsolution Ver.3 software. To achieve absolute quantification of hormones, calibration curves were compiled by measuring a dilution series which consisted of samples made by spiking a *B. anynana* haemolymph mixture with a range of known concentrations of synthetic hormones: JH-I, JH-II (both purchased from SciTech, Prague, Czech Republic), JH-III, 20-hydroxyecdysone and ecdysone (all three purchased from Sigma Aldrich, USA). All solvents were HPLC grade.

Chapter 4

Phylogeography of *Bicyclus anynana* using the mtDNA COI gene

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Manuscript

Phylogeography of *Bicyclus anynana* using the mtDNA COI gene

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ABSTRACT

This study investigates the genetic diversity, population structure and demographic history of the afrotropical butterfly *Bicyclus anynana* using mitochondrial DNA (mtDNA). Samples from six wild populations covering most of the species range were compared for the cytochrome c oxidase subunit gene (COI). Molecular diversity indices show overall high mtDNA diversity for the populations, but low nucleotide divergence between haplotypes. Our results indicate relatively little geographic population structure, despite the extensive distributional range and expected limited gene flow between populations. We implemented neutrality tests to assess signatures of recent historical demographic events. Tajima's *D* test and Fu's F_S test both suggested recent population growth for the populations. The results were only significant for the southernmost populations when applying Tajima's *D*, but Fu's F_S indicated significant deviations from neutrality for all populations except the one closest to the equator. Based on our own findings and those from pollen and vegetation studies, we hypothesize that the species range of *B. anynana* was reduced to equatorial refugia during the last glacial period, and that the species expanded southwards during the past 10,000 years. These results provide crucial background information for studies of phenotypic and molecular adaptation in wild populations of *B. anynana*.

INTRODUCTION

The degree of isolation and the demographic history of populations are key factors influencing the potential of populations to adapt to divergent environmental conditions. Understanding the spatial genetic structure of populations is crucial for making inferences about adaptive geographic variation in species, and their adaptive potential to respond to future changes in the environment. Species-specific life history characteristics can influence geographic population structure, for example, an increased dispersal capacity leads to higher level of gene flow, which can slow down or limit geographic differentiation (Slatkin 1987). Historical biogeographical changes can

have an effect on the genetic variation within and between populations. For instance, past habitat reduction can cause a demographic bottleneck and a decrease in genetic variation, thereby decreasing the ability of a population to respond to selection. Variation in the genome, on the population and the geographic level, bears the footprints of past demographic events as well as ongoing and current population genetic processes such as gene flow. Studying the molecular phylogeny and population genetics of a species can provide insight into the historical biogeographic events and life history traits that shape the distribution of genetic variation over populations (Avise 2000).

The afrotropical butterfly *Bicyclus anynana* (Nymphalidea) is increasingly used as a model species in evolutionary genetics and life-history studies, but so far intraspecific phylogeographic information based on molecular data is lacking. The species inhabits the Eastern part of sub-Saharan Africa, where its range stretches from the equator to the subtropics, spanning an area of more than 3,000 kilometers (Condamin 1973). This region is largely dominated by savannah vegetation and characterized by a strong seasonality in rainfall, although the intensity and frequency of the alternating wet and dry seasons vary according to latitude. *B. anynana*'s preferred habitat is the edges of the dry forests that occur along the rivers, lakes and the coast of the savannah area, where the adults feed on fallen forest fruit, while the larvae develop on grasses. The species copes with the seasonal nature of its habitat by expressing alternate adult phenotypes as an adaptation to the contrasting seasonal environments (Brakefield & Reitsma 1991, Brakefield et al. 1996). This phenotypic plasticity enables survival of the harsh dry seasons without the need to migrate or diapause. The adult butterflies are weak flyers, generally resting in the shade during the warmest part of the day, and flying mainly during the morning and late afternoon. Although *B. anynana* is a relatively common species, its habitat is naturally very fragmented, and decreasing rapidly due to man-induced habitat fragmentation and degradation.

In this study, we use mitochondrial DNA (mtDNA) sequence data to examine the phylogeography of *B. anynana*. mtDNA is widely used as a tool in phylogeographic studies, due to its low or absent recombination, uniparental inheritance, conserved structure and relatively high evolutionary rate (Moritz et al. 1987, Harrison et al. 1989, Avise 2000). The analysis of intraspecific mtDNA variation can reveal information about the interconnectivity of populations and past demographic events such as population expansions (Avise 2000). The Cytochrome Oxidase I (COI) gene is one of the most frequently employed mtDNA genes to investigate phylogeographic patterns and histories at the inter- and intraspecies level, and has been extensively used for evolutionary studies in insects (Caterino et al. 2000). We investigate the range-wide phylogeography of *B. anynana* using six populations distributed throughout the species' range, and including a population of a different subspecies and an island population. We sequenced 1,500 bp of the COI gene for 25 individuals per population, and focused the analysis on: 1) the sequence diversity and variability, 2) population subdivision, and 3) the demographic history of the populations. The main aim of this study is to provide information on the isolation of the populations and their phylogeographic history, which will be especially interesting in the light of ongoing and future studies of adaptive phenotypic and genetic geographic variation in the species.

MATERIALS AND METHODS

Sample collection

We took a sample of 25 adults of *Bicyclus anynana* from each of six populations. The samples were collected in 2005 and 2006 from the following locations (followed by their abbreviations and geographic coordinates): Lake Mburo in Uganda (LM, 0° 38′ S, 30° 57′ E); Watamu in Kenya (WA, 3° 21′ S, 40° 1′ E); Ngezi forest on Pemba Island, Tanzania (PE, 4° 55′ S, 39° 42′ E); Zomba in Malawi (ZO, 15° 22′ S, 35° 19′ E); Mpaphuli Cycad Reserve in Limpopo, South Africa (LP, 22° 47′ S, 30° 37′ E); and False Bay Park of the Greater St. Lucia Wetland Area, KwaZulu Natal, South Africa (FB, 27° 58′ S, 32° 21′ E). A schematic map of Africa with the locations of the populations is given in Fig. 1. Butterflies were frozen alive at -80°C and stored at the same temperature until they were further used for DNA extraction.

DNA extraction, PCR, and sequencing

Genomic DNA was extracted from individual thoraces and legs using Qiagen's DNeasy tissue kit, following the manufacturer's instructions. The COI gene was amplified in two parts with a total length of ~1,500 bp, using two universal primer pairs as described in Wahlberg & Wheat (2008). The polymerase chain reaction (PCR) was conducted in a total volume of 20 µl, containing 1.0 µl of DNA template, 2.0 µl 10× buffer, 2.0 µl $MgCl_2$ (25 mM), 1.0 µl primer 1 (10 mM), 1.0 µl primer 2 (10 mM), 0.4 µl dNTPs (10 mM), 0.1 µl *Taq* polymerase (5 U/µl) and 12.5 µl Milli-Q water. PCR conditions consisted of an initial cycle at 95°C for 5 min, 40 cycles of 94°C for 30 s, 50°C for 30 s, 72˚C for 90 s, and a final extension at 72°C for 10 min. PCR products that yielded a clear band on agarose gel by electrophoresis were purified and sequenced using capillary electrophoresis

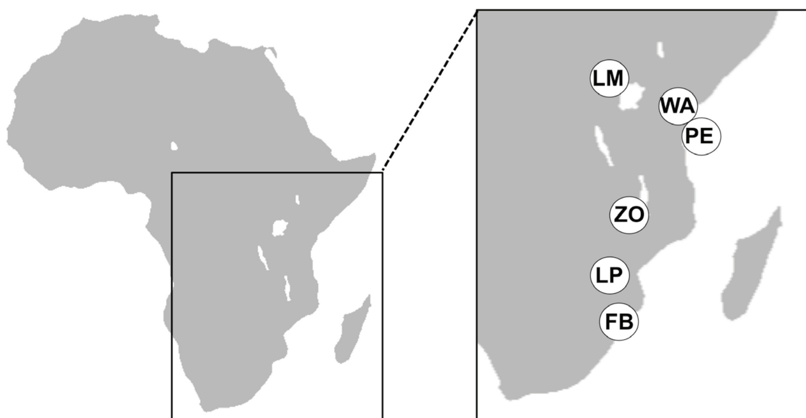

Figure 1. Overview of the locations of the analysed populations on the African continent. FB: False Bay; LM: Lake Mburo; LP: Limpopo; PE: Pemba; WA: Watamu; ZO: Zomba.

sequencing by Macrogen Europe.

Data analysis

Variation within and among populations

Individual sequences were aligned manually using the program BioEdit 7.0.9.0 (Hall 1999). All statistical parameters and tests were calculated using the program Arlequin 3.5 (Excoffier & Lischer 2010). Genetic diversity within populations was estimated by computing haplotype diversity (*H*; Nei, 1987), and nucleotide diversity (π; Nei, 1987). Haplotype diversity (also known as gene diversity) represents the probability that two randomly sampled alleles are different, while nucleotide diversity Is defined as the average number of nucleotide differences per site in pairwise comparisons among DNA sequences (Nei 1987). Relationships between haplotypes were estimated using the minimum spanning network method (also called molecular-variance parsimony technique). The haplotype network was computed under haplotype pairwise differences, giving the number of mutation steps between haplotypes. The network was subsequently drawn by hand (Fig. 2). Partitioning of genetic variation within and among populations was calculated using analysis of molecular variance (AMOVA) (Excoffier et al. 1992), by computation of conventional *F* statistics from haplotypes with 1,000 permutations.

Neutrality and demographic history

Statistical tests originally developed to assess the selective neutrality of mutations have been implemented to test for demographic expansion in recent years (Ramos-Onsins & Rozas 2002). These tests are designed to distinguish between neutrally evolving sequences under mutation-drift equilibrium, and sequences evolving under non-neutral processes including directional or balancing selection, and demographic expansion or contraction. In order to test for past population expansion, we used two statistical tests commonly used to analyze demographic events. Tajima's *D* uses the frequency of segregating nucleotide sites, while Fu's F_S (Fu 1997) uses the distribution of alleles or haplotypes. Both tests are based on the principle that a sudden population expansion that is associated with a non-neutral process will show a shift in the allele frequency spectrum compared to a neutral Wright-Fisher model consistent with population expansion under neutral evolution. The analyses were implemented in the program Arlequin 3.5 (Excoffier & Lischer 2010), p values were generated using 1,000 simulations under a model of selective neutrality.

RESULTS

Diversity indices

We analysed a combined length of 1,480 bp of the COI gene for a total of 150 individuals from six wild *B. anynana* populations. Over the whole data set, we identified

Table 1. Sample size (n), number of haplotypes (k), number of polymorphic sites (PS), haplotype diversity (*H*) ± SD and nucleotide diversity (π) ± SD per population.

Population	n	k	PS	*H*	π
False Bay	25	13	18	0.897 ± 0.043	0.0018 ± 0.0011
Limpopo	25	10	19	0.747 ± 0.082	0.0015 ± 0.0009
Zomba	25	12	14	0.883 ± 0.051	0.0019 ± 0.0012
Pemba	25	11	15	0.860 ± 0.050	0.0017 ± 0.0010
Watamu	25	12	12	0.917 ± 0.032	0.0018 ± 0.0011
Lake Mburo	25	12	14	0.893 ± 0.038	0.0023 ± 0.0014

54 polymorphic sites leading to the definition of 59 haplotypes. One haplotype was found in four populations (FB, LP, ZO and WA) and occurred frequently (21%) over the total data set. In addition to this common haplotype, six other haplotypes were shared by at least two populations. The shared haplotypes represented 47% of the total number of individuals. Finally, 52 were private (unique to a single population), most of them being singleton haplotypes. The number of haplotypes was comparable among populations, varying between 10 for LP to 13 for FB (Table 1). The genetic diversity was large in every population, ranging from $H = 0.75$ to $H = 0.92$ (Table 1), with a mean gene diversity per population of $H = 0.866$. In contrast, nucleotide diversity was relatively low for each population, ranging between $\pi = 0.0015$ for LP and $\pi = 0.0023$ for LM (Table 1). Although overall diversity was similar among populations, gene diversity and nucleotide diversity were both lowest for LP, followed by the island population PE. Gene diversity was highest for FB, while LM showed the highest nucleotide diversity (Table 1).

Geographical structure

Results from the AMOVA showed that the genetic variation within populations (65.16%) was much larger than the variation among populations (34.84%), indicating relatively little geographic population structure. Pairwise F_{ST} values between populations were all significant except for the comparison between the two southernmost populations FB and LP, which had a very low F_{ST} value of 0.008 (Table 2). The island population PE and the *centralis* subspecies population LM showed most differentiation in pairwise comparisons with the other populations. The highest pairwise F_{ST} value resulted from the comparison between these two populations ($F_{ST} = 0.576$). PE showed the least differentiation compared to WA, which is geographically the nearest population on the mainland. For the mainland populations FB, LP, ZO and WA, pairwise F_{ST} values generally increased with geographic distance, with the highest F_{ST}'s between the southern populations FB and LP compared with WA (Table 2).

Table 2. AMOVA haplotype F_{ST} results for pairwise population comparisons (lower diagonal) and associated significance indications (upper diagonal). FB: False Bay; LM: Lake Mburo; LP: Limpopo; PE: Pemba; WA: Watamu; ZO: Zomba; NS: not significant; ** p < 0.01; *** p < 0.001.

	FB	LP	ZO	PE	WA	LM
FB	-	NS	**	***	***	***
LP	0.008	-	**	***	***	***
ZO	0.066	0.080	-	***	**	***
PE	0.494	0.532	0.376	-	***	***
WA	0.326	0.369	0.168	0.212	-	***
LM	0.265	0.260	0.348	0.576	0.487	-

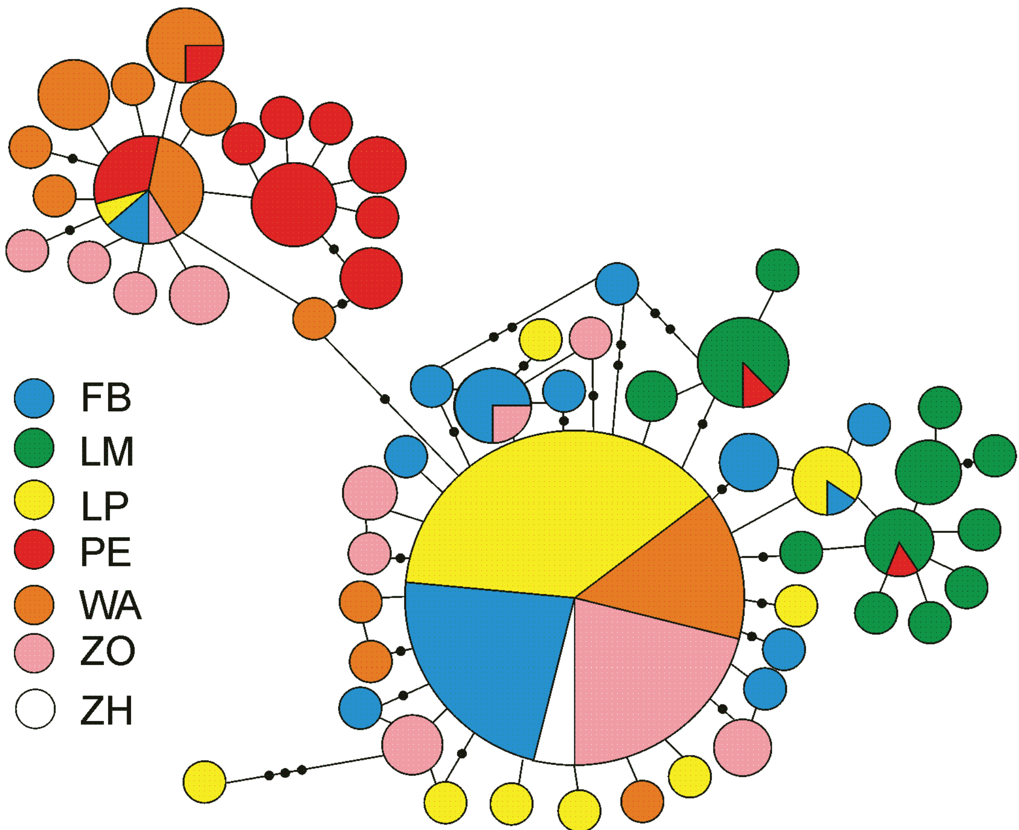

Figure 2. Haplotype network. Circle size is relative to number of haplotype copies present in dataset. A branch represents a single nucleotide change, black dots on branches represent inferred missing haplotypes (single nucleotide changes). ZH is a single haplotype from Harare, Zimbabwe. FB: False Bay; LM: Lake Mburo; LP: Limpopo; PE: Pemba; WA: Watamu; ZO: Zomba.

Table 3. Results of Tajima's *D* and Fu's F_S neutrality tests including associated p-values.

Population	Tajima's *D*	p	Fu's F_S	p
False Bay	-1.66	0.037	-6.22	0.002
Limpopo	-2.10	0.008	-3.50	0.026
Zomba	-0.87	0.176	-4.41	0.017
Pemba	-1.40	0.058	-4.04	0.014
Watamu	-0.68	0.291	-5.03	0.008
Lake Mburo	-0.31	0.450	-3.37	0.064

The haplotype network (Fig. 2) clearly reflects the results of the diversity indices and AMOVA presented above. It shows the common haplotype which was present in 4 of the populations, with many single haplotypes around it. The star-like shape of this part of the network indicates little geographical structure, in particular for the mainland populations FB, LP and ZO. For these populations the network shows very low levels of sequence divergence and a high frequency of unique mutations, which can be a signature of rapid population expansion. The haplotypes of the WA, and especially the PE and LM, form more distinct groups and hence show more geographic structure, although sequence divergence is generally low.

Demographic analysis

The results of Tajima's *D* test and Fu's F_S test are presented in Table 3, including associated simulated p values. Tajima's *D* values were negative for all populations, indicating an excess of rare nucleotide site variants compared to what would be expected under a neutral model of evolution. Only for the two southernmost populations, FB and LP, were these deviations from neutrality significant. The results of Fu's F_S test, which is based on the distribution of haplotypes, also show negative values for all populations, indicating an excess of rare haplotypes over what would be expected under neutrality. Following this test, the hypothesis of neutral evolution was significantly rejected for all populations except for the *centralis* subspecies population LM.

DISCUSSION

Sequence diversity and geographic population structure

Despite the wide distributional range, the fragmented nature of the habitat and low expected rate of long distance dispersal, our results show relatively little geographic differentiation among populations of *B. anynana*. The molecular diversity indices are similar between the populations, including the island population Pemba for which lower diversity indices might have been expected due to its isolation from the mainland (Table 1). Haplotype diversity lies in the range 0.75 to 0.92, which is high

when compared to many other species . Similarly high haplotype diversity values have been reported in other Lepidoptera species, such as the palearctic Small Tortoiseshell *Aglais urticae* (Vandewoestijne et al. 2004) and the invasive horse-chestnut leaf miner *Cameraria ohridella* in Europe (Valade et al. 2009), but there are also reports of lower haplotype diversity, e.g. in the Monarch butterfly *Danaus plexippus* (Brower & Boyce 1991).

Although haplotype diversity is high, low nucleotide diversity values indicate only small differences between haplotypes. This is also evident from the minimum spanning haplotype network, which shows mostly single nucleotide differences between haplotypes (Fig. 2). In terms of population differentiation, the haplotype network demonstrates that especially the populations FB, LP, ZO and WA (the 'mainland' populations) show relatively little divergence, and share the most common haplotype in the analysis. The southernmost populations, FB and LP, are especially closely related to each other as indicated by a very low and non-significant F_{ST} value (Table 2). For these two populations, the majority of single haplotypes are only one or two nucleotides removed from the shared, most common, haplotype. The island population PE does not share the latter haplotype, but still shares three haplotypes with the other populations. The haplotypes for this population are closest related to those of WA, which is also geographically the nearest sampled population with a distance of approximately 170 km (the shortest distance to the coast from Pemba Island is 50 km). The LM population shows most differentiation, with only two haplotypes shared with one other population (PE). With a distance of over a 1,000 km to the nearest sampled population, the LM population is geographically the most distant population in the analysis. Moreover, this population belongs to the subspecies *B. anynana centralis*, while the other populations are of the subspecies *B. anynana anynana*, a classification made on the basis of wing morphology (Condamin 1973).

Sufficient gene flow between populations can slow down or prevent the process of geographic differentiation, and leave a signature of little population structure over large areas. This is commonly observed in flying insect species, specifically in those species that migrate or are good dispersers. Examples include Monarch butterflies *D. plexippus* (Brower & Boyce 1991), bumble bees *Bombus terrestris* (Estoup et al. 1996), and dragonflies *Anax junius* (Freeland et al. 2003). In contrast, *B. anynana* are weak flyers and do not migrate, therefore it is unlikely that long distance dispersal occurs frequently in this species. Although differences between sequences are small, our data do indicate geographic structure, in particular for the island population (PE) and the *B. anynana centralis* population (LM). This suggests that, at least for these two populations, gene flow is limited. Gene flow could play a role in the similarity between the mainland populations, and especially between the southernmost populations that show no geographic differentiation. However, it is most likely that a shared recent demographic history accounts for the major part of the observed phylogeographic pattern.

Demographic history of the populations

The combination of high haplotype diversity and low nucleotide diversity, as observed in our data, can be a signature of a rapid demographic expansion from a small effective

population size (Avise 2000). In recent years, statistical tests that were originally developed to test selective neutrality of mutations, have been implemented to detect such population growth (Ramos-Onsins & Rozas 2002). These tests are generally based on the distribution of pair-wise differences between sequences within populations. Here, we chose to use two tests that are commonly used to detect population expansion and that differ somewhat in their approach. Tajima's *D* test (Tajima 1989) is based on the allele frequency distribution of segregating nucleotide sites. A positive value indicates a bias towards intermediate frequency alleles, while a negative value indicates a bias towards rare alleles, the latter being a signature of recent population expansion. Fu's F_S test (Fu 1997) is based on the distribution of alleles or haplotypes, and here too, negative values can indicate recent population growth. In the present study, Tajima's *D* test shows negative values for all populations, however, only the two southernmost populations FB and LP differ significantly from neutrality. Fu's F_S test resulted in significant values for all populations except LM which was negative but not significant (Table 3). It has been shown that Fu's F_S test is more powerful than Tajima's *D* (Ramos-Ousins & Rozas 2002), and this would explain the differences in significance for some populations. The overall negative values resulting from both tests indicate that there is an excess of rare mutations in the populations, which can imply recent population expansion. Alternatively, these values can result from balancing selection on a nearby locus, although studies demonstrating direct or indirect selection (through hitchhiking) on the mitochondrial genome in natural populations are rare (but see e.g. Ruiz-Pesini 2004).

The explanation of recent demographic expansion corresponds well to the widely observed patterns of population expansion in organisms across taxa following the last glacial period, which ended around 12,500 years ago. Cooler and dryer conditions during the glacial maximum led to worldwide shifts and contraction of forest areas into small refugia, thereby reducing the area of available habitat for many species (Prentice & Jolly 2000). An extensive number of studies have provided genetic evidence of the glacial effects of glacial periods on population histories for various species in Europe and North America, but evidence for the African continent is less abundant (Hewitt 2000). Pollen data have revealed that tropical rain- and seasonal forests and dry woodland in Africa were reduced, and replaced by savanna vegetation during the last glacial maximum (Flenley 1998; Prentice & Jolly 2000). It has also been shown that several taxa of tropical rain forest vegetation persisted in equatorial Africa during this period (Elenga et al. 2000), indicating the existence of forest refugia. Studies on butterflies, birds, reptiles, mammals and other animals support this theory (Hamilton 2001, Douglass & Miller 2003). Based on our results, which indicate increasingly recent population expansion towards the South, it is likely that the *B. anynana* species area expanded southwards during the Holocene from glacial equatorial habitat refugia.

In conclusion, our study reveals a general high genetic diversity within populations of *B. anynana*, but relatively little differentiation among populations, especially when taking into account the limited dispersal ability of the species and the fragmented nature of the habitat. The observed patterns of genetic variation within and between the populations are most likely caused by a recent shared demographic history in the form of a reduced species area in the last glacial period. Interestingly, despite the

indication that the populations underwent recent expansion from ice age refugia, the species shows population differentiation in wing pattern, not only for the relatively isolated populations of the subspecies *B. anynana centralis* (Condamin 1973) and Pemba Island (M.A. de Jong, personal observation), but also for more closely related populations on the mainland (De Jong et al. 2010). These findings suggest that despite recent population history, population differentiation in morphology and potentially in other traits may occur relatively rapidly in *B. anynana*. This study provides a much needed framework for investigation of adaptive functional variation at the phenotypic or molecular level in wild populations of *B. anynana*. In Chapter 5, these results will be discussed in relation to a study of adaptive molecular variation in candidate genes.

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Chapter 5

Footprints of selection in wild populations of *Bicyclus anynana* along a latitudinal cline

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Manuscript

Footprints of selection in wild populations of *Bicyclus anynana* along a latitudinal cline

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ABSTRACT

One of the major questions in ecology and evolutionary biology is how variation in the genome enables species to adapt to divergent environmental parameters. The use of latitudinal clines is a powerful approach in associating genetic variation with geographically varying thermal conditions. Here, we have taken a candidate gene approach to study footprints of thermal selection in six wild populations of the afrotropical butterfly *Bicyclus anynana*, sampled along a latitudinal cline covering a distance of ~3,000 km from the equator to the subtropics. We sequenced coding regions of 19 genes that are candidates for an association with thermal adaptation, including enzymes and other proteins from the glycolytic pathway and its branches, the lipid pathway, and genes involved in pigment biosynthesis. In addition, six genes from the heat shock family and five genes involved in developmental pathways for which we did not expect structural variation associated with a thermal gradient, were included as a type of negative control. We identified non-synonymous nucleotide polymorphisms in 11 candidate genes and tested these for significant clinal variation by correlation analysis of allele frequencies with latitude. As an additional analysis to infer evidence of selection we implemented the Beaumont-Nichols F_{ST} outlier method. Two metabolic enzymes of the glycolytic pathway, *Treh* and *UGPase*, showed significant clinal variation of which *UGPase* remained significant after multiple testing correction. In addition, the outlier analysis indicated a significantly higher F_{ST} value for the same amino acid polymorphism in *UGPase* than expected under a model of neutral evolution. In contrast, we found no evidence of clines with latitude in the heat shock proteins and developmental genes. The underlying phylogeographic structure of the populations based on the mtDNA COI gene and the silent SNPs of the candidate genes did not show a clinal pattern. Our results thus indicate that the observed clinal variation in *UGPase* and *Treh* may reflect adaptation to a geographic thermal gradient.

INTRODUCTION

A central goal in our quest to unravel the mechanisms of natural selection is to understand how variation in the genome enables species or populations to cope with, and adapt to divergent environmental conditions. Studying genes that are putatively under selection can shed light on the quantity and nature of genetic changes involved in adaptive differentiation, as well as on potential constraints on evolutionary responses. Identifying genetic variation involved in the local adaptation of wild populations is especially relevant in the context of present-day human-induced environmental change, including habitat fragmentation and climate change. As past evolutionary change is recorded in the genome, we can potentially use this information to make predictions about the evolutionary potential of wild populations in response to future environmental change. Moreover, genes involved in adaptive responses could be used as genetic markers in conservation efforts and to monitor species' molecular responses to selection imposed by environmental change (Hoffmann & Willi 2008).

An increasing number of studies indicate that genetic changes in particular loci can play an important role in the performance of organisms and fitness in relation to their environment (e.g. Mitton & Duran 2004, Hoekstra et al. 2006, Campbell 2010). Genetic changes leading to variation in responses to environmental conditions include changes in regulatory regions that induce the differential expression of genes. For example, variation in the expression of heat shock protein (Hsp) genes has been linked to the presence of transposable elements in the promoter regions in *Drosophila* species, with a likely role in thermal adaptation (Chen et al. 2007). Alternatively, changes in the coding regions of the genes can lead to amino acid variation, and potential structural differences, in the transcribed proteins. A well-studied example of this is amino acid polymorphism in the metabolic enzyme phosphoglucose isomerase (Pgi), which has been associated with fitness and performance differences particularly in relation to temperature in various Arthropod taxa, including butterflies (Hanski & Saccheri 2006), beetles (Rank et al. 2007) and amphipods (Patarnello & Battaglia 1992). In the Glanville fritillary butterfly, *Melitaea cinxia*, different Pgi alleles correlate with life history traits including dispersal, metabolic rate and population growth, and are linked to thermal performance (Hanski & Saccheri 2006, Saastamoinen & Hanski 2008).

One approach to infer evidence of selection at the molecular level is to associate genetic polymorphisms in populations with geographically varying environmental parameters. Temperature is generally recognized as one of the main environmental variables influencing and limiting organismal performance and fitness, and consequently determines the distribution and range of species (e.g. Fields 2001, Angilletta 2009). Latitudinal clines are a powerful tool in demonstrating patterns of past natural selection associated with temperature (Endler 1977), and have been described at the phenotypic and molecular level in a wide range of organisms, including flies (Hoffmann & Weeks 2007), fish (Schmidt et al. 2008), and plants (Hall et al. 2007). Although molecular clinal variation has been well studied in *Drosophila* (Sezgin et al. 2004, Hoffmann & Weeks 2007), there are few comparable studies in other insect groups with distinct biological properties. Butterflies provide model species in evolutionary and ecological studies (Brakefield & Frankino 2009), and are important bio-indicators because of their

sensitivity to environmental changes (Parmesan 2003). However, studies of molecular clinal variation in butterflies are very limited, apart from those on altitudinal variation in copper butterflies (Fischer & Karl 2010).

In the present study, we took a candidate gene approach to study footprints of selection in wild populations of the butterfly *Bicyclus anynana* along a latitudinal cline. *B. anynana* is an emerging model species for developmental and life history studies, with genetic information and tools becoming increasingly available (Brakefield et al. 2009). The species occurs in East Africa, where its range extends from equatorial Kenya to subtropical South Africa. Temperature values for means, minima and maxima increase towards the equator, while daily and annual mean temperature amplitudes decrease. We sampled six populations from the equator to the southernmost part of the range covering approximately 3,000 km, thereby extending over most of the species' latitudinal range (Condamin 1973). From the EST derived gene collection for *B. anynana* (Beldade et al. 2006, 2009), we selected a set of candidate genes potentially involved in temperature adaptation, mainly based on findings from research on *Drosophila*. The majority of candidate genes for which single-locus latitudinal clines are reported in the literature are metabolic enzymes (e.g. Sezgin et al. 2004). We selected genes coding for enzymes and other proteins involved in the glycolytic pathway and its important branches (*Gapdh2*, *Gdh*, *GlyP*, *Tpi*, *Treh*, and *UGPase*) and in the lipid pathway (*Apolphorin precursor*, *desat1*, *Lipase like*, *LpR*, *TAG Lipase*, and *Vg*), as well as an antioxidant gene (*Cat*).

In addition to the metabolic genes, we included several genes involved in the pigment biosynthesis of wing pattern pigmentation (*black*, *Catsup*, *ddc*, *light*, *ovo*, and *yellow*). Latitudinal and altitudinal clines for pigmentation are widely documented for a range of species, including mice (Hoekstra 2006), flies (Wittkopp et al. 2003), and butterflies (Ellers & Boggs 2002, Karl et al. 2009). The adaptive value of these patterns of variation along thermal gradients has been ascribed to the regulation of body temperature (Ellers & Boggs 2002, Karl et al. 2009), protection from UV radiation (Gunn 1998) and desiccation resistance (Rajpurohit et al. 2007). Studies have linked polymorphisms in coding regions of genes to phenotypic variation in pigmentation genes (e.g. Sturm et al. 2001, Hoekstra et al. 2006). Previously, in a two population comparison, we have shown population differentiation for thermal reaction norms of wing pattern elements (De Jong et al. 2010). In *B. anynana*, wing pattern is a fitness-related trait that plays a role in predator avoidance (Lyytinen et al. 2004) and sexual signalling (Robertson & Monteiro 2005). Although a relationship between wing pattern and thermal adaptation has not been described for this species thus far, the wing pattern pigmentation genes are an interesting group to screen for molecular clinal variation.

Lastly, we selected several genes from the heat shock protein family (*Hsp23*, *Hsp60*, *Hsp68*, *Hsp83*, *Hsc70-3*, *Hsc70-4*) and genes involved in developmental pathways (*Apc*, *dll*, *en*, *ovo*, and *wg*). Heat shock proteins and heat shock cognates have been shown to play an important role in temperature adaptation in a variety of organisms, but this is mainly through differential gene expression (e.g. Chen et al. 2007, Rinehart et al. 2007). Hence, these genes are generally considered less likely candidates to show clinal variation in the coding regions. Similarly, although the selected developmental genes play an important role in wing development and patterning, and may thus be

linked to geographic variation in pigmentation, research is revealing that phenotypic variation is mainly associated with these genes through gene regulation (Wittkopp & Beldade 2009). Therefore, we do not expect, *a priori*, a correlation between the structural sequences and latitude. This group can thus be seen as a type of negative control.

To detect patterns of selection related to temperature adaptation, we analysed clinal variation of amino acid polymorphisms in the studied genes by testing for significant correlations between population latitude and allele frequencies. To address the possibility of confounding the phylogenetic history of the species with patterns of natural selection, we compare our findings with the geographic structure of the populations based on the COI mitochondrial gene and putatively neutral silent SNPs of the nuclear genes. Finally, we implemented the Beaumont-Nichols F_{ST} outlier method (Beaumont & Nichols 1996) to identify loci under directional or balancing selection as an additional method to infer evidence of selection for the studied candidate genes.

MATERIALS AND METHODS

Populations and samples

In 2005 and 2006, six populations were sampled along a latitudinal cline from the following locations (from North to South, followed by their abbreviations, coordinates and sample sizes): Lake Mburo in Uganda (LM, 0° 38′ S 30° 57′ E, n = 50); Watamu in Kenya (WA, 3° 21′ S 40° 1′ E, n = 40); Ngezi forest on Pemba Island, Tanzania (PE, 4° 55′ S 39° 42′ E, n = 50); Zomba in Malawi (ZO, 15° 22′ S 35° 19′ E, n = 43); Mpaphuli Cycad Reserve in Limpopo, South Africa (LP, 22° 47′ S 30˚ 37′ E, n = 50); and False Bay Park of the Greater St. Lucia Wetland Area, KwaZulu Natal, South Africa (FB, 27° 58′ S 32° 21′ E, n = 50). Fig. 1 gives an overview of the locations of the populations on the African continent. For each population, the gender structure of the samples was

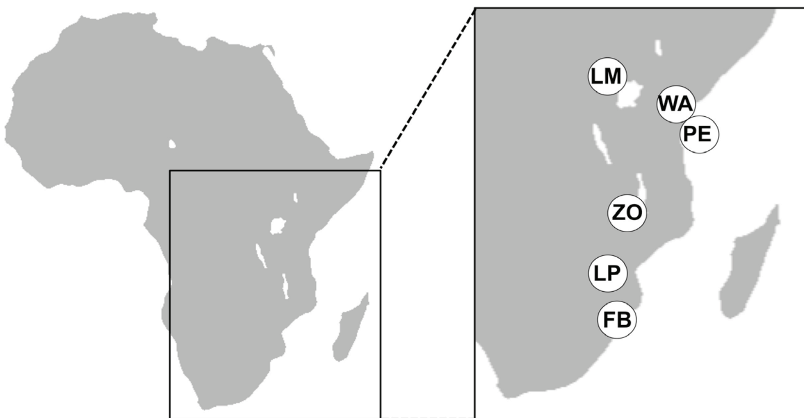


Figure 1. Overview of the locations of the analysed populations on the African continent. FB: False Bay; LM: Lake Mburo; LP: Limpopo; PE: Pemba; WA: Watamu; ZO: Zomba.

approximately evenly balanced between males and females. Samples were stored at -80°C until they were used for DNA extraction.

Candidate gene selection and primer design

We selected 19 candidate genes putatively involved in thermal adaptation from a published EST-derived gene collection of *B. anynana* (Beldade et al. 2006). This gene collection contains over 4,000 genes in the form of singletons and contigs assembled from expressed sequence tags, and was created using material from a *B. anynana* laboratory stock established from a wild population from Malawi in 1988. Although most candidate genes for this study were selected on the basis of *Drosophila* literature, we searched our *B. anynana* gene collection by tblastx analysis (e-score cut-off value of 1.0×10^{-5}) with *Bombyx mori* orthologs of the candidates to increase the chance of finding them in our gene collection. We obtained the *B. mori* orthologs from the Silkworm Genome Database (SilkDB, www.silkworm.genomics.org.cn).

The selected candidates include genes from the glycolytic pathway and its branches (*Gapdh2*, *Gdh*, *GlyP*, *Tpi*, *Treh*, and *UGPase*), the lipid pathway (*Apolphorin precursor*, *desat1*, *Lipase like*, *LpR*, *TAG Lipase*, and *Vg*), an antioxidant enzyme (*Cat*) and genes involved in pigmentation biosynthesis (*black*, *Catsup*, *ddc*, *light*, and *yellow*). In addition, we included six genes from the heat shock protein family (*Hsc70-3*, *Hsc70-4*, *Hsp23*, *Hsp60*, *Hsp68*, and *Hsp83*) and five genes involved in developmental pathways (*Apc*, *dll*, *en*, *ovo*, and *wg*) using the same method of selection from the EST-derived gene collection (Table 1 in Appendix).

For each gene, primers were designed to amplify lengths of 200 – 1,650 bp of the exonic regions, excluding the introns, resulting in one to three primer pairs per gene (Table 1 in Appendix). Based on the assumption of conserved intron positions between *Bicyclus anynana* and *Bombyx mori*, intron positions were identified by comparing coding sequence of the *B. mori* candidate orthologs with the genomic sequence from the SilkDB database. We designed the primers using the primer-BLAST tool with default settings on the website of the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). Primers were tested on five individuals per populations and accepted for further use when the PCR products yielded bands of the same size. Table 1 (Appendix) gives an overview of the genes included in the study, including their full names and abbreviations, contig numbers from the EST-derived gene collection for *Bicyclus anynana* (Beldade et al. 2006, 2009), functional assignment, sequences of the primers used for amplification of the genes, and sequence lengths of the amplicons.

DNA extraction, PCR and sequencing

Genomic DNA was extracted from individual thoraces and legs using Qiagen's DNeasy tissue kit and following the manufacturer's instructions. DNA concentrations were measured with Nanodrop spectrophotometry and Picogreen fluorometry. Various studies have shown that accurate estimates of allele frequencies based on PCR results can be obtained by precise pooling of the DNA of several individuals combined (reviewed in Sham et al. 2002). Thus, individual samples of identical concentrations

were combined in pools of three, resulting in 17 pools per population. PCRs were conducted in a 50 µl volume, with 3 µl DNA, 0.25 µl ExTaq (Takara), 5 µl 10× ExTaq buffer, 5 µl of each dNTP (2.5 mM), and 1 µl of each primer (10 pmol). PCR conditions consisted of an initial cycle at 94°C for 3 min, 33 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 5 min. Primer combinations for which the PCR product did not yield a band on agarose gel for one or more of the pooled groups were excluded from the analysis. After cleaning, PCR product concentrations were measured using Picogreen fluorometry and subsequently standardised, after which the PCR products for each population were pooled with equal molar concentration. Each population sample was prepared according to standard Illumina protocols for genomic DNA samples (more information can be found on the website www.illumina.com), loaded into a single lane of an eight-lane flow cell and sequenced by pair-end 75 bp reads on a Illumina Solexa Genome Analyzer. Measuring of DNA concentrations, pooling of samples, PCR reactions, and Illumina sequencing were performed by Macrogen Inc. (Seoul, Korea).

Analysis

SNP calling

Maq software (Li et al. 2008) was used for alignment of the 75 bp reads to the reference sequence, which was the contig sequence available for each candidate gene. For SNP discovery, SNPs were called when the minor allele frequency was 4% or higher in one or more of the populations. The alignment and SNP calling analyses were carried out by Macrogen Inc. (Seoul, Korea). To further reduce the chance of calling false positive SNPs and to reduce the size of the data set, we included only the SNPs with a minor allele frequency of at least 20% in one population, or a minor allele frequency of at least 5% in three populations for all further analyses. Reading frame positions for protein translation of candidate genes were determined by BLAST alignment to *Bombyx mori* orthologs from the SilkDB, which allowed for assessment of silent (synonymous) and substitution (non-synonymous) SNPs.

Population genetic parameters

SNP allele frequency data was converted to genotype files for each locus and population using WhichLoci software (Banks et al. 2003), assuming Hardy-Weinberg equilibrium for the loci, and given the initial sample sizes of the collected populations. Haplotype structure and linkage between SNPs could not be analysed because the reconstructed genotype data were based on allele frequencies from pooled samples. The population genetic parameters of allelic richness (AR, corrected for sample size) and unbiased gene diversity (or expected heterozygosity, *H*) were calculated per population per gene using the program FSTAT (ver. 2.9.3; Goudet 2002). Pairwise population differences were calculated using analysis of variance followed by a Tukey's honest significant differences (HSD) test in SPSS (ver. 14). Population pairwise F_{ST} values based on the silent SNPs and associated p-values were calculated per gene with Arlequin (ver. 3.5;

Excoffier & Lischer 2010) following Weir & Cockerham (1984) using 1,000 permutations, and averaged over the total set of genes.

Evidence of selection: clinal variation and F_{ST} *outlier analysis*

For the analysis of clinal variation, we used the amino acid polymorphisms that were polymorphic in at least three populations. We chose to focus on the SNPs that showed at least 20% difference in allele frequency between the most divergent populations to reduce loss of statistical power due to multiple testing. For each SNP, the association between allele frequency and geographic origin of the populations (latitude) was tested using Pearson's correlation analysis in SPSS (ver. 14) software. Multiple comparisons were corrected using Benjamini & Hochberg's (1995) False Discovery Rate procedure.

As an additional method to detect evidence of selection, we implemented the Beaumont & Nichols (1996) outlier method, which uses the distribution of loci based on the relationship between F_{ST} and H in an island model. As F_{ST} is a measure of population divergence, it can be used to reveal patterns of local adaptation. When a locus is under directional or balancing selection, it is expected to show respectively higher and lower F_{ST} values than neutral loci (Beaumont & Nichols 1996). When taking into account clinal variation, a steeper cline will generally be associated with a higher F_{ST} value, indicating stronger population divergence. Here, we were primarily interested in finding loci with outlier F_{ST} values among the replacement SNPs that were analysed for clinal variation. Outlier loci are defined as loci that show significantly more (directional selection) or less (balancing selection) differentiation among populations than predicted by a neutral model. We also included all synonymous SNPs in the outlier analysis because these SNPs are expected to be mostly under neutral evolution and thus provide a type of neutral baseline with which outlier loci can be compared. We calculated upper and lower outlier F_{ST} values including all SNPs, under an infinite alleles mutation model with 10,000 simulations and a confidence interval of 0.95, using the program LOSITAN (Antao et al. 2008).

RESULTS

Population genetic diversity indices and unique SNPs

Table 2 shows the average allelic richness (AR) and gene diversity (H) per candidate gene including the standard deviation for each population, and the total number of unique SNPs per population (SNPs occurring in only one population). Allelic richness measures genetic diversity as the average number of alleles in a sample, and was corrected for the differences in sample size between the populations. An analysis of variance showed that AR is significantly lower ($p < 0.0001$) for the LM population, which belongs to a different subspecies (*B. anynana centralis*), compared to the other populations. The WA populations and the island population PE have lower values than the FB, LP and ZO populations but these differences are not significant (Table 2). Gene diversity (or expected heterozygosity, H) represents the probability that two randomly sampled alleles are different, and the populations show a very similar pattern for this

Table 2. Allelic Richness (AR) and gene diversity (*H*) averaged over all genes, and number of unique single nucleotide polymorphisms (SNPs) per population. FB: False Bay; LM: Lake Mburo; LP: Limpopo; PE: Pemba; WA: Watamu; ZO: Zomba; SD: standard deviation

Population	AR ± SD	*H* ± SD	unique SNPs
FB	1.70 ± 0.18	0.21 ± 0.08	0
LP	1.70 ± 0.21	0.21 ± 0.08	0
ZO	1.70 ± 0.20	0.21 ± 0.08	0
PE	1.63 ± 0.21	0.18 ± 0.07	52
WA	1.64 ± 0.23	0.20 ± 0.09	0
LM	1.42 ± 0.19	0.12 ± 0.06	69

Table 3. Pairwise population F_{ST} values, averaged over all genes (lower diagonal), and associated p-values (upper diagonal). FB: False Bay; LM: Lake Mburo; LP: Limpopo; PE: Pemba; WA: Watamu; ZO: Zomba; *** p < 0.001

	FB	LP	ZO	PE	WA	LM
FB	-	***	***	***	***	***
LP	0.03	-	***	***	***	***
ZO	0.03	0.03	-	***	***	***
PE	0.30	0.29	0.30	-	***	***
WA	0.08	0.09	0.09	0.30	-	***
LM	0.50	0.49	0.50	0.56	0.49	-

measure as for AR. Again, LM shows the lowest diversity and is significantly different (p < 0.0001) from the other populations. WA and PE have slightly lower *H* values than FB, LP and ZO but these differences are very small and non-significant (Table 2). None of the populations FB, LP, ZO and WA have unique SNPs (SNPs that do not occur in other populations). In contrast, PE has 52 unique SNPs, while LM has the highest number with 69 unique SNPs (Table 2).

Pairwise population differentiation

We calculated average pairwise population F_{ST} values, from F_{ST} values for each gene based on the silent SNPs (Table 3). All pairwise F_{ST} values were significant (p < 0.001). Pairwise F_{ST} values are lower than 0.1 for any pairwise comparison within the populations FB, LP, ZO and WA. Comparisons between FB, LP and ZO give the smallest F_{ST} values (0.03), while WA shows slightly more differentiation from the first three populations (0.08-0.09). Pairwise F_{ST} values between PE and the other populations are relatively high. Differentiation between PE and the populations FB, LP, ZO and WA are very similar (0.29-0.30), while the pairwise F_{ST} value between PE and LM is the highest of

all comparisons (0.56). LM shows the most population differentiation with similar F_{ST} values for all pairwise comparisons (between 0.49 and 0.56).

Candidate genes

Table 4 (Appendix) gives the total sequenced length in base pairs (bp), the number of synonymous and non-synonymous SNPs and the ratio of synonymous and non-synonymous SNPs per 100 bp for each candidate gene. Sequenced lengths of genes varied between 200 and 1,645 bp, with an average of 774 bp. On average, there was 1 SNP for every 38 bp, corresponding to 2.6 bp per 100 bp. The majority of the identified SNPs were synonymous: we found nearly 20-fold as many synonymous SNPs as non-synonymous SNPs. Based on our SNP selection criteria (see Materials and Methods), further analyses of evidence of selection was focused on 14 replacement SNPs in 11 genes: *Treh*, *UGPase*, *TAGLipase*, *Vg*, *Hsp23*, *Hsp83*, *black*, *en*, *light*, *yellow*, and *wg* (Table 5).

Clinal variation

We present the results of a Pearson's correlation analysis on non-synonymous SNP allele frequencies against latitude. A summary is given in Table 5, which shows the F_{ST} value, Pearson's correlation coefficient (*r*) and associated p-value with significance indication per SNP for each gene. We found significant clinal variation for two out of three replacement SNPs in *Treh* (*Treh*^{335}, Fig. 2A and *Treh*^{550}, Fig. 2B), and for the only replacement SNP in *UGPase*

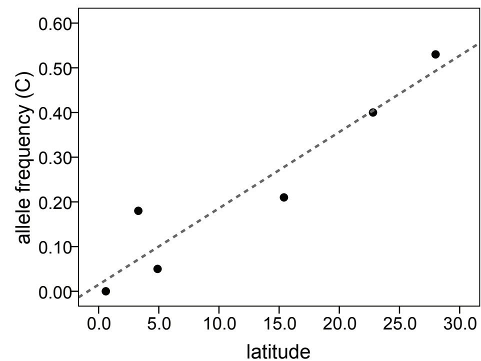
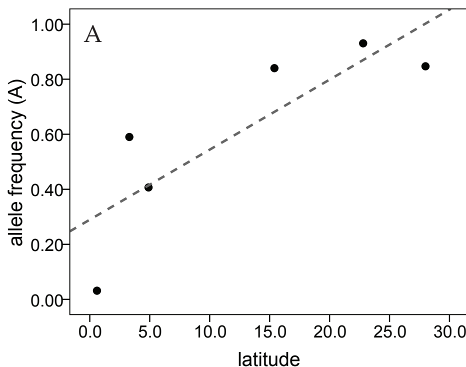


Figure 3. The latitudinal cline for SNP 408 of *UGPase*.



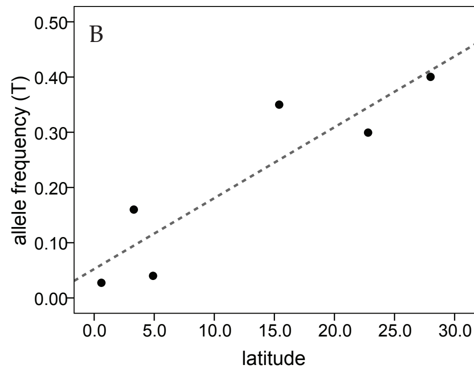


Figure 2. The latitudinal clines for (A) SNP 335 and (B) SNP 550 of *Treh*.

Table 5. Non-synonymous single nucleotide polymorphisms (SNPs) tested for significant clinal variation listed per gene, F_{ST} values, Pearson's correlation coefficient (*r*) and associated probabilities (p). Significant upper (+) or lower (-) F_{ST} outliers are indicated for the analysis including all populations (6) or populations FB, LP, ZO and WA (4). p-values that remained significant after False Discovery Rate (FDR) correction are indicated with *.

Gene name	SNP	F_{ST}	*r*	p	Outlier
Treh	335	0.451	0.840	0.036	
	549	0.536	0.388	0.116	
	550	0.137	0.905	0.013	
UGPase	248	0.306	0.966	0.002*	+ (4)
TAG Lipase I	424	0.133	0.078	0.884	
TAG Lipase II	32	0.152	0.044	0.935	
	296	0.534	0.451	0.369	
Vg	452	0.248	0.737	0.095	
	614	0.618	0.622	0.187	+ (6)
Hsp23	647	0.530	0.322	0.533	
Hsp83	551	0.006	0.098	0.854	- (6)
black	135	0.190	0.718	0.108	
light	782	0.802	0.707	0.116	+ (6)
yellow	585	0.248	0.764	0.077	
	591	0.565	0.507	0.305	+ (4)
wg	341	0.048	0.107	0.840	

(UGP^{248}, Fig. 3). After multiple testing correction using Benjamini & Hochberg's (1995) False Discovery Rate, only the cline for UGP^{248} remained significant.

Outlier analysis

To identify outlier F_{ST} values in the replacement SNPs, we first carried out the Beaumont & Nichols F_{ST} outlier analysis including all silent and replacement SNPs of the candidate genes for the six populations. This revealed one replacement SNP with a significant lower outlier F_{ST}: $Hsp83^{551}$ ($p < 0.001$), and two replacement loci with significant upper outlier F_{ST} values: Vg^{614} ($p < 0.01$) and $Light^{782}$ ($p < 0.001$; Table 5). These loci did not show significant clinal variation (Table 5), and the high F_{ST} values of the two upper outliers were caused by unique SNPs for the populations PE and LM. Overall, the upper F_{ST} values in the analysis (of both silent and replacement SNPs) were mainly determined by the large number of unique SNPs in PE and WA. Because we were more interested in outlier loci among polymorphisms shared between populations, we decided to also perform the analysis including only the four populations FB, LP, ZO and WA. These populations did not have any unique SNPs, and extend over most

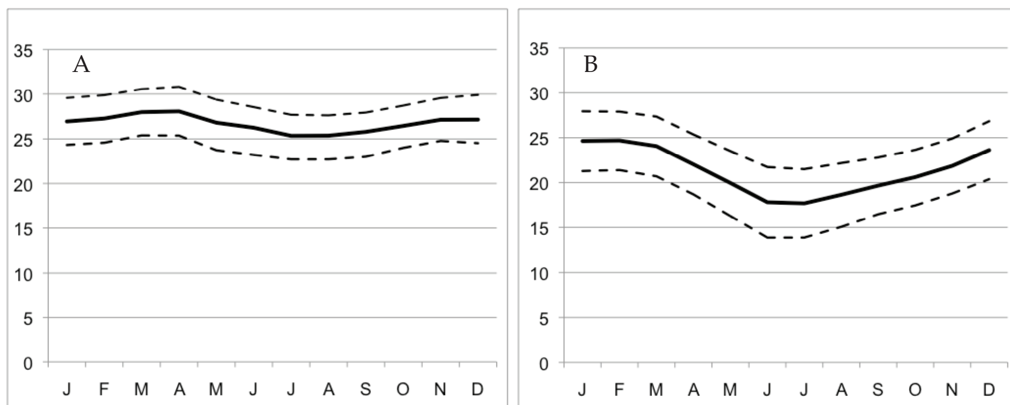


Figure 4. Average monthly temperatures for the localities of (A) the Kenyan WA population, and (B) the subtropical South African FB population. Solid line represents mean temperature in °C, and dashed lines represent average maximum and minimum temperatures. Climate data from the Global Historical Climate Network (GHCN).

of the sampled geographic area. This analysis resulted in two upper outlier F_{ST} values among the replacement SNPs: *Yellow*^{591} ($p < 0.05$) and *UGP*^{248} ($p < 0.05$), of which the latter also showed significant clinal variation (Table 5).

DISCUSSION

Climate

The distribution area of *B. anynana* spans a considerable temperature range with increasing overall temperatures and decreasing temperature amplitudes towards the equator. Fig. 4 shows climate charts for average minimum, mean and maximum temperatures for the Kenyan population near the equator (Fig. 4A) and the subtropical population from South Africa (Fig. 4B). Average monthly mean temperatures differ by as much as 8°C between these populations, and yearly temperature differences are considerably larger for South Africa than Kenya where temperatures remain fairly uniform throughout the year. Although there can be subtle deviations from a linear increase in temperature towards the equator, for example due to altitude differences, the regional scale shows a gradient in temperatures. The association of rainfall with latitude (not shown) is considerably more complex than temperature and does not show a clear gradient on a regional or local level.

Phylogeographic population structure

One potential problem with the interpretation of clinal variation is that the effects of spatially varying selection may be confounded with underlying phylogeographic patterns resulting from neutral evolution processes, such as drift and/or spatially

restricted gene flow (Gould & Johnston 1972, Endler 1977, Vasemägi 2006). To address this issue, we take into account the geographic population structure based on the mtDNA COI gene (De Jong et al. unpublished data; chapter 4 in this thesis), a marker widely used for inferring phylogenetic population structure (Avise 2000). This phylogeography indicated very little population differentiation for most populations (FB, LP, ZO and WA, further referred to as 'mainland' populations), while the island population PE and the population belonging to the different subspecies *B. anynana centralis* LM showed more differentiation. The study indicated that the low level of differentiation between the mainland populations is likely to be caused by recent population expansion from refugia during the last glacial maximum (De Jong et al. unpublished data; chapter 4 in this thesis). The most common haplotype, shared by the four mainland populations, did not show significant clinal variation in frequency with latitude.

Table 2 shows, for each population, gene diversity (expected heterozygosity) and allelic richness (average number of alleles), two statistics frequently used to measure genetic diversity. These indices (based on the nuclear genes) show a significantly lower genetic diversity for the LM population. PE also has a lower allelic richness and gene diversity than the mainland populations, but these differences are not significant. Both LM and PE have a substantial number of unique SNPs, as opposed to the mainland populations (Table 2), further indicating their relative isolation. The pairwise population differentiation based on the silent SNPs of the nuclear genes shows a similar pattern (Table 3). F_{ST} values among the four mainland populations are very small, indicating little differentiation, as opposed to the much higher values involving PE and particularly LM with these populations. The silent SNPs are likely to reflect the outcome of largely neutral evolution, although putative selection on these SNPs due to linkage/hitchhiking effects (Nielsen 2005) and other non-neutral processes (Chamary et al. 2006) cannot be excluded. An analysis with additional neutral markers could give a more conclusive perspective on the neutral population structure. To summarize, the mtDNA and silent SNPs indicate that the interpopulation structure does not show a clinal gradient. Furthermore, there is very little overall genetic divergence among the mainland populations, while the island population and especially the population of the *B. centralis* subspecies show much more differentiation.

Candidate genes

For this study, we sequenced coding sections of 20 candidate genes associated with temperature adaptation, and ten genes involved in wing pattern development. Despite conservative SNP calling, the genes were extremely polymorphic with on average 1 SNP per 38 bp. Previous studies on a single population of *B. anynana* also indicated a high level of polymorphism, with around 1 SNP per 50 bp (Beldade et al. 2006, 2009). This level of polymorphism is very high compared, for example, to humans with a reported SNP frequency in coding regions of 1 in 350bp (Cargill et al. 1999), and in the silkworm *Bombyx mori*, of 1 in 775bp (Cheng et al. 2004). On average, there were 15-fold more silent SNPs than replacement SNPs in *Bicyclus anynana*, indicating a generally strong balancing selection (Table 3). This is not surprising, considering the functional importance of these genes, which is also reflected in the relatively high level of

conservation at the protein level across species (e.g. in comparison with *Bombyx mori*). Because the silent SNPs are likely to mainly reflect the phylogeographic history of the populations, we only included the replacement SNPs for the analysis of footprints of selection.

Clinal variation in metabolic genes

The majority of evidence for adaptive clinal variation in coding polymorphisms results from studies on allozyme and candidate gene studies in metabolic genes in *Drosophila*. A well-known example is the parallel cline in the alcohol-dehydrogenase enzyme (Adh) in Australia, linked to latitudinal phenotypic variation in alcohol tolerance (Oakeshott et al. 1982). The recent advances in sequencing technology have sparked a renewed interest in studying clinal variation at the molecular level, resulting in an increase in the discovery of candidate genes displaying molecular clines. For example, Sezgin et al. (2004) reviewed and tested for clinal coding variation of metabolic enzymes in *D. melanogaster*, reporting on a total of nine genes displaying significant clines, and further reports have followed (Schmidt et al. 2008, Paaby et al. 2010).

Metabolic enzymes typically have limited thermal performance curves which are shaped by the ability to bind substrate (conformation) and the flexibility to change shape during catalysis (Hochachka & Somero 2002). The substitution of amino acids can alter the thermal properties of proteins (Fields 2001), and natural selection favours mutations that influence the conformational stability of enzymes (Marx et al. 2007). The more flexible an enzyme is, the faster it can change shape during catalysis, but this comes at the cost of a lower conformation stability. In general, higher temperatures favour greater conformational stability, while more flexible enzymes perform better at lower temperatures (Fields 2001, Hochachka & Somero 2002). Constraints on thermal performance of enzymes may be one of the most important factors determining the geographical distribution of ectotherms (Fields 2001).

In the present study, we identified significant clinal variation in replacement SNPs of two candidate genes involved in metabolic pathways: *UGPase* and *Treh*. These genes are both key enzymes in carbohydrate metabolism and widely found in plants, animals and microorganisms (Kleczkowski, 2004). Two of the three replacement SNPs in the approximately 1,000 bp sequence for *Treh* showed a significant correlation between allele frequency and latitude (Table 5; Fig. 2). The cline was very steep for $Treh^{335}$, for which the allele frequencies ranged from nearly zero to nearly one (Fig. 2A). The minor allele frequency of $Treh^{550}$ increased from nearly zero to 40% towards the south (Fig. 2B). In addition, there was also significant clinal variation for eight silent SNPs, distributed over the entire length of the fragment (data not shown). This is likely to be caused by linkage of the silent SNPs with the amino acid polymorphisms, and may reflect clinal variation in a common haplotype. *Treh* catalyzes the conversion of trehalose, an important storage carbohydrate, to glucose. In insects, *Treh* plays a crucial role in various physiological processes, including flight metabolism (Clegg & Evans 1961), and stress responses, including hypoxia (Chen & Haddad 2004), dessication (Worland et al. 1998, Timmermans et al. 2009), and thermal stress (Friedman 1978, Worland et al. 1998).

For *UGPase*, the single replacement SNP on the 500 bp sequence showed a significant cline (Fig. 3), as did two silent SNPs nearby (data not shown). *UGPase* catalyzes the reversible formation of UDP-glucose, an important step in the synthesis of glycogen, which is, like trehalose, an important storage carbohydrate (Alonso et al. 1995). The outlier analysis including the four mainland populations revealed the amino acid polymorphism in *UGPase* as an upper outlier locus. Relative to the overall low F_{ST} between these populations, *UGPase* had a much higher F_{ST} value, indicating directional selection on this locus. Crucially, for both *Treh* and *UGPase*, clines in the coding regions of the genes have also been reported for *D. melanogaster* in North America (Sezgin et al. 2004), suggesting a potential role for these enzymes in thermal adaptation across taxa. In the *Drosophila* study, an amino acid polymorphism in *Treh* and a silent SNP in *UGPase* show significant clinal variation (Sezgin 2004).

After correction for multiple testing, only the cline for the amino-acid replacement in *UGPase* remained significant. Here, we chose for an explorative design by including a large number of genes to screen for evidence of selection. This approach increases the chance of finding significant clines in individual genes, but comes at a cost of reduced statistical power due to multiple testing. Because only six populations were sampled the correlation coefficient needs to be very high in order to reach a high level of significance to withstand the stringent FDR approach. Although the clinal patterns found in *Treh* were not significant after multiple testing correction, we believe this remains an interesting candidate gene for follow-up studies, due to the clinal signal for multiple SNPs in the sequenced fragment and the reported clinal variation in *Drosophila* (Sezgin et al. 2004).

Linkage between the genes could be a potential cause of a shared pattern of clinal variation. Although we have no linkage information for the genes in this study, we do know the positions of their orthologs on the *B. mori* genome, where *UGPase* and *Treh* are each located on different chromosomes. A recently published gene-based linkage map for *Bicyclus anynana* revealed a generally strong conservation of gene assignments to chromosomes (Beldade et al. 2009).

Pigmentation genes

We included several genes involved in pigmentation biosynthesis in the analysis because of widely reported clinal variation in pigmentation across taxa (Ellers & Boggs 2002, Wittkopp et al. 2003, Hoekstra et al. 2006, Karl et al. 2009). However, we did not find significant clines for the replacement SNPs in the regions we sequenced. Interestingly, one SNP in the *yellow* pigmentation gene was an upper outlier among the mainland populations, and one SNP in the *light* pigmentation gene was an upper outlier among all six populations, indicating an increased population differentiation for these loci (Table 5). The *B. anynana centralis* subspecies has been described on the basis of differences in wing pattern from the *B. anynana anynana* subspecies (Condamin 1973). Moreover, De Jong et al. (2010) found significant population differentiation in reaction norms on rearing temperature for wing pattern between the South African FB population and a population from Malawi. Thus, it is possible that these pigmentation genes are involved in local adaptation for wing pattern in *B. anynana*.

In addition to thermal adaptation in the form of UV protection and regulation of body temperature by melanization, several adaptive explanations have been put forward to explain patterns of geographic variation in pigmentation. These include crypsis, deflection of predators, and mate choice and species recognition (Lyytinen et al. 2004, Oliver et al. 2009). In *B. anynana*, sexual selection is likely to be an important selective force shaping wing pattern, and hence may play an important role in driving population differentiation and eventually speciation (Oliver et al. 2009). Also, wing pattern is likely to be involved in crypsis and predatory deflection in this species (Lyytinen 2004). Ongoing and future research may reveal which selective forces are driving population differentiation for wing pattern in *B. anynana*.

Heat shock proteins and developmental genes

Conform our expectations, we did not find significant clinal variation in the developmental genes and the heat shock family genes. The selected developmental genes play a crucial role during embryogenesis and throughout development in *B. anynana* and other organisms (Beldade 2002), and these genes are not known to be involved in thermal adaptation. The heat shock proteins had the fewest amino-acid replacements compared to the other genes: only one in *Hsp23* and one in *Hsp83*. The one replacement SNP in *Hsp83* was a lower outlier locus (in all six populations), indicating balancing selection (Beaumont & Nichols 1996). These results are not surprising since heat shock proteins are generally highly conserved, even across taxa. In addition to their established function in (thermal) stress responses, heat shock proteins are important housekeeping genes in cellular regulation; they function as molecular chaperones and are involved in folding and transportation of other proteins. The majority of studies indicate an upregulation of the expression of heat shock proteins in response to thermal stress and other stress responses (e.g. Fangue et al. 2006, Chen et al. 2007, Rinehart et al. 2007), which has been linked to genetic variation in the regulatory regions (Chen et al. 2007). There are reports on clinal variation in the coding regions (e.g. Frydenberg et al. 2003, Hemmer-Hansen et al. 2007), although these are rare.

CONCLUSIONS

In this study, we found significant clinal variation in amino-acid polymorphisms for the metabolic enzymes *UGPase* and *Treh*. In addition, the amino-acid polymorphism in *UGPase* was an outlier loci compared to the overall F_{ST} in four populations, indicating that this locus is under selection. For these genes, our data strongly suggest adaptive population divergence along a latitudinal gradient and imply local adaptation against a background of generally low population divergence, as indicated by mtDNA and silent SNPs. Our results are paralleled by reports on clinal variation in *UGPase* and *Treh* in *D. melanogaster*. Moreover, as expected, we found no evidence of clines with latitude in the heat shock proteins and developmental genes. Taken together, our findings indicate a putative role in thermal adaptation for the genes *UGPase* and *Treh*, which are, therefore, interesting candidates for follow-up studies linking variation in phenotypic traits to molecular variation within and among populations.

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Table 1. List of genes included in the study and the primers used to amplify the sequenced fragments per gene. For each gene the full name, abbreviation, contig number in published EST-Derived gene collection and functional assignment is given. Per primer pair, the forward and reverse sequences are given, and the amplicon length.

Full name	Abbreviation	Contig	Functional assignment	Forward primer	Reverse primer	Amplicon length
glyceraldehyde 3 phosphatedehydrogenase 2	*Gapdh 2*	C326	glycolytic pathway	CCCGCTAACA TGTCGAAAAT	GATACCAGCA GCAGCATCAA	903
glutamatedehydrogenase	*Gdh*	C1411	glycolytic pathway	CCAACGGCC CTACAACAC	TGGTAATTGGA TTCACGTTCA	181
glutamatedehydrogenase	*Gdh*	C1412	glycolytic pathway	GAAATCCCAG ACAGGCTCAA	ACCTCCTTTT GTTGGGGTTC	300
glutamatedehydrogenase	*Gdh*	C1412	glycolytic pathway	CGATTCTCAAC TGATGTGACG	AGCCTTTCTT CGCCAGCTC	190
glycogenphosphorylase	*Glyp*	S7458	glycolytic pathway	GAACGATACC CATCCAGCTC	TTCACTTGCA CGTCGAACAT	733
glycogenphosphorylase	*Glyp*	S6487	glycolytic pathway	TGAGCGTCCA AACAGATTCA	CGCTTGGGGT CTTTCTCATA	241
riosephosphateisomerase	*Tpi*	C7836	glycolytic pathway	GAAGCTGGGC TCAAAGTGAT	TGGCAGTCTT TCCAGTACCA	181
riosephosphateisomerase	*Tpi*	C7836	glycolytic pathway	ACATCCACG CGTCCCTTC	ACTCCGGTTT AAGGCTTGCT	171
riosephosphateisomerase	*Treh*	C6804	glycolytic pathway	ATGAAACGGT GAAGGGAGTG	ACTTCACGAG CCCTTAGCAA	931
UTP-glucose-1-phosphate uridylyltransferase	*UGPase*	C6877	glycolytic pathway	TGCAATCAGG AGAGTTGTGG	AAAGCCACTT TTCTGCCAAA	408
apolphirinprecursor	*Apolphirin precursor*	C7601	lipid pathway	CTCCGTGGTC ACCTTCTTTT	GAGAATGAGAA AGGCAAAGACA	530
apolphirinprecursor	*Apolphirin precursor*	C7015	lipid pathway	GGGACAACTCTC CTAAATCTATCC	TCAGTGACCTC AACAGTGACAA	208

Table 1 (continued).

Full name	Abbreviation	Contig	Functional assignment	Forward primer	Reverse primer	Amplicon length
desaturase 1	*desat1*	C7463	lipid pathway	AGGGGTCGTG GGTTCTGTAT	CCGTAAATATTCG TGTAGTCCACAT	198
desaturase 1	*desat1*	C7463	lipid pathway	GTACTCGATTG GGCACGAGA	AATCGCAGCA CAGGATCAG	191
lipaselike	*Lipaselike*	C5275	lipid pathway	TGGACCTTCA GTTCAATTCG	GACGTGTTTC GTGCCAGTC	890
lipaselike	*Lipaselike*	S4049	lipid pathway	AAGCCTTGGA GCTCACGTT	ATCATCCGCA TTGATTTTCC	556
lipophorinreceptor	*LpR*	S5824	lipid pathway	ATGTCGTTCGG ACGAGTTCA	CACTTCCATCA GGGCAGTCT	212
lipophorinreceptor	*LpR*	S5824	lipid pathway	CTCCGCCGAA GGTTATATCG	GACTTCATCA CTGCCGTCTG	251
lipophorinreceptor	*LpR*	C7081	lipid pathway	GACCCTCGG GACAGAACC	TCTTCTATGACA TCGCTCCAAA	194
lipophorinreceptor	*LpR*	C7081	lipid pathway	ACCAATCGAT GAAGGCAGTC	ATTCAAGGCT ATCGCACGAG	205
triacylglycerollipase	*TAG lipase*	S5789	lipid pathway	GGTTGGACCT TCGAAATGAA	GTGCCACGAG AAGTTCCAAT	618
triacylglycerollipase	*TAG lipase*	C2122	lipid pathway	CGTACGGACC AGTGCAGATA	TCCCACCCTT TGTTATCCAT	780
vitellogenin	*Vg*	C7110	lipid pathway	CATGCTGAAAAT CAATTATGACG	AAACCGCCGT TAGAGAAACA	866
catalase	*Cat*	C1371	anti oxidant	TCACCACCAA ATATGGAGCA	GTGAGAACCA TAGCCGTTCA	575
black	*black*	C4285	pigment synthesis	AAGTCTCACGG AGAGATCGAA	CTGCCATAAA CGCCAGAAG	200

Table 1 (continued).

Full name	Abbreviation	Contig	Functional assignment	Forward primer	Reverse primer	Amplicon length
black	*black*	C4285	pigment synthesis	CGCACCCTTA CTTCGTCAAC	TGGATAAAAGT AGGACCGAGCA	260
black	*black*	C4284	pigment synthesis	CAAAAAGACAAAT TTTATGATACATCC	ATATCCGATC CGAGCCTTTC	470
catecholaminesup	*Catsup*	C235	pigment synthesis	ACGTCACGTC AAGGTGAATG	AGGAGGGCGA GAATTTCTTT	1158
dopadecarboxylase	*ddc*	C3202	pigment synthesis	GACAAGTGGT GCCATCAGTG	GCTCCGCTAA GCATATCAGC	211
dopadecarboxylase	*ddc*	C3202	pigment synthesis	CTAGTCCCGC TTGCACTGA	TCCCACAAGT TTGGAAAGAA	236
dopadecarboxylase	*ddc*	C3202	pigment synthesis	GGCGACACTA GGAACGACTT	GGAGCCACA TAGCGGAAC	230
light	*light*	C3144	pigment synthesis	CTGTGAATGC GAAGGAGATG	TTGGGGTCCA CCTTCAAGTA	927
yellow	*yellow*	C4163	pigment synthesis	CGAATGTTTGT CCTTACTCCA	GGGCTTGTCG GTATTATTGG	817
heat shock protein 23	*Hsp23*	C4926	heat shock protein	TACCACACGA GAGCAACCAC	ATTCACTCGT CGGTTTCGAT	786
heat shock protein 60	*Hsp60*	C3306	heat shock protein	TAACCTACAA CCGGCGCTAA	ATGAGTTCGA GCTCGTCGTT	757
heat shock protein 60	*Hsp60*	C3307	heat shock protein	GGTAGTGAAC CGGCTGAAGA	TGGGGTTAGG CTCCTTTTCT	815
heat shock protein 68	*Hsp68*	C4558	heat shock protein	GAGCGAGGTT CGAGGAATTA	CCCTACATTCATA CTAGCGAATAAA	1088
heat shock protein 68	*Hsp68*	C4559	heat shock protein	CAAAATGCCA GCTATTGGAA	GCTTCGGTACT CGACGAAAG	837

Table 1 (continued).

Full name	Abbreviation	Contig	Functional assignment	Forward primer	Reverse primer	Amplicon length
heat shock protein 83	*Hsp83*	C1093	heat shock protein	CTTCGAGGAA CTTGCAGAGG	CCAGCGCTAG TTTCGACTTC	870
heat shock protein 83	*Hsp83*	C2544	heat shock protein	AGACGCTCTG GACAAGATCC	ATGGGATAGC CGATGAACTG	495
heat shock protein 83	*Hsp83*	S6762	heat shock protein	ATGACATCAC CCAGGAGGAG	GGCAAGTCCT CGCTGTCTAC	280
heat shock cognate 70-3	*Hsc70-3*	C3217	heat shock cognate	GGGTGTATACA AGAATGGACGA	GACCATGGCT GAGACCTCTT	319
heat shock cognate 70-3	*Hsc70-3*	C3218	heat shock cognate	ACTTTCGACG TGTCGCTTTT	TGGACTCAAC TCGCTCCTTT	1000
heat shock cognate 70-4	*Hsc70-4*	C880	heat shock cognate	AAGCACCTGC GGTAGGTATC	TGTGTTGTTG GGGTTCATTG	191
heat shock cognate 70-4	*Hsc70-4*	C880	heat shock cognate	CGTGCAAGCT GACATGAAAC	GAACTCCTGC ACGAAGTGGT	490
adenomatouspolyposis coli tumor suppressor	*Apc*	S782	developmental pathway	ATTGGAAAGT GCAGCCAGAT	CCACACTCTC GCTTCTCTCC	432
distal-less	*dll*	C3	developmental pathway	ACGAATTCAG TCCCCAAACA	TTGGTGCATAG GAGGAAAGG	375
engrailed	*en*	not published	developmental pathway	GAGCGCATCG TGTTGTTAAA	GAAGGGTATG GTTGGTGGTG	463
ovo	*ovo*	C2907	developmental pathway	CTTAGGCCCC TCGACTTAAA	GTTCTGCTGGA AGACCCAAA	842
wingless	*wg*	S2	developmental pathway	GCCTGTAAAA CGCTCCATCT	CCGAAGTTGG AACCTGTAGC	638

Table 4. List of candidate genes, length of the sequenced exon parts in base pairs (bp), number of non-synonymous (NS) single nucleotide polymorphisms (SNPs) per gene, number of synonymous (S) SNPs per gene, ratio of non-synonymous (NS) SNPs per 100 base pairs, and ratio of synonymous (S) SNPs per 100 base pairs.

Gene name	Length (bp)	# NS SNPs	# S SNPs	NS SNP per 100 bp	S SNP per 100 bp
Gapdh2	903	0	12	0	1.3
Gdh	671	0	12	0	1.79
GlyP	974	0	32	0	3.29
Tpi	352	0	5	0	1.42
Treh	931	3	30	0.32	3.22
UGPase	408	1	25	0.25	6.12
Apolphirin precursor	738	3	23	0.41	3.12
desat1	389	1	8	0.26	2.06
Lipase like	890	0	27	0	3.03
Lipase like	556	0	17	0	3.06
LpR	862	0	12	0	1.39
TAG Lipase	618	1	17	0.16	2.75
TAG Lipase	780	5	36	0.64	4.62
Vg	866	4	28	0.46	3.23
Cat	575	0	7	0	1.22
Hsc70-3	1319	0	35	0	2.65
Hsc70-4	681	0	12	0	1.76
Hsp23	786	1	12	0.13	1.53
Hsp60	1572	0	39	0	2.48
Hsp68	837	0	38	0	4.54
Hsp83	1645	1	45	0.08	3.59
Apc	432	0	11	0	2.55
black	930	1	19	0.11	2.04
Catsup	1158	0	13	0	1.12
ddc	677	1	13	0.15	1.92
dll	375	0	7	0	1.87
en	290	3	14	1.03	4.83
light	927	3	6	0.32	0.65
ovo	842	0	4	0	0.48
yellow	817	2	34	0.25	4.16
wg	200	1	3	0.50	1.50

Chapter 6

Summary, discussion, and perspectives

Maaike A. de Jong

Summary, discussion, and perspectives

The aim of this thesis is to investigate mechanisms of adaptation to climate, and in particular temperature, in the African butterfly *Bicyclus anynana*. The thesis takes an integrated approach to thermal adaptation and brings together studies at the phenotypic, physiological and genetic level. By examining geographical variation among wild populations, the work investigates how *B. anynana* is adapted to geographically varying thermal conditions. Because of the major influence of temperature on the ecology and evolution of species, the way organisms adapt to thermal variation has long captivated the attention of biological research. In recent years, the field of thermal adaptation has seen a surge of interest as a consequence of the impacts of recent climate change on biodiversity. The results presented in this thesis contribute to our general knowledge about the mechanisms of adaptation to environmental variation. In a broader perspective, they may also add to our understanding of whether, and how species may adapt to climate change.

The purpose of this concluding chapter is to give a short summary of the scientific chapters, to discuss the main results in a broader perspective and give suggestions for future research. The thesis is roughly divided into two sections; the main subject of chapters 2 and 3 are phenotypic plasticity in response to temperature, while chapters 4 and 5 are focused on neutral and adaptive geographic variation at the molecular genetic level.

PHENOTYPIC PLASTICITY AND ADAPTATION TO CLIMATE

A major component of adaptation to climate in *B. anynana* is the extensive phenotypic plasticity the species shows by expressing two distinct phenotypes adapted to the alternating wet and dry seasons of its habitat. This seasonal polyphenism forms the central topic of Chapters 2 and 3, in which geographic variation and the underlying hormonal dynamics of the polyphenism are respectively explored.

Chapter 2: geographic variation in phenotypic plasticity

Chapter 2 investigates geographic variation for seasonal plasticity in *B. anynana* by comparing thermal reaction norms for wing pattern and several life history traits of two populations, one from Malawi and one from South Africa. The main question addressed in this study is whether there is evidence for local adaptation to the specific temperature-rainfall associations of the regional climates of the populations, or whether essentially the same plasticity response to developmental temperature covers a broader range of climates. In the light of climate change this question is relevant because, when predicting species' responses to climate change, it is important to understand to what extent phenotypic plasticity allows organisms to cope with changing temperatures. The more specialized an organism is in its phenotypic plasticity response to temperature, the less likely it will be able to successfully cope with changing climatic conditions.

To compare the seasonal plasticity response to developmental temperature between

the populations, we measured reaction norms for wing pattern, development time, and adult size, resting metabolic rate and starvation resistance at three different developmental temperatures. These temperatures spanned the range of average temperatures experienced by the butterflies in the field. Interestingly, we found very little differentiation between the populations for the life history traits. In particular, the traits that can be further regulated by acclimation during the butterfly's adult life span (labile traits), namely, starvation resistance, resting metabolic rate and egg size, showed no geographic differentiation for their developmental plasticity despite a strong effect of developmental temperature. Hence, we hypothesize that for these traits adult acclimation plays an important role in coping with local climate. Although there is an extensive body of literature dedicated to phenotypic plasticity, studies distinguishing between developmental plasticity and adult acclimation are relatively rare, with many studies being designed in such a way that these two forms of plasticity are confounded (Wilson & Franklin 2002, but see Fischer et al. 2003, Terblanche & Chown 2006). In a follow-up of our results, it would be interesting to investigate in more detail the importance of adult acclimation in coping with climatic variation for the labile life history traits, and to determine the extent to which this form of plasticity can buffer the variation predicted as a result of climate change.

In contrast to the life history traits, the reaction norms for wing pattern showed a population-specific response, differing in the intercept as well as in shape between the populations. These results thus reveal a potential mismatch between wing pattern and environment in a scenario of changing temperatures associated with climate change. As the adaptive benefit of the wing pattern polyphenism in *B. anynana* has been demonstrated (Lyytinen et al. 2004, Brakefield & Frankino 2009) this could have negative implications for the fitness of the butterflies under such scenario's. Our results showed high broad-sense heritabilities and cross-temperature correlations for wing pattern, indicating a potential for adaptation for the intercept of the reaction norm. The observed geographic variation for wing pattern also indicates the adaptive potential of this trait, especially when coupled with the results of the phylogeographic analysis in Chapter 4, which showed a relatively recent range expansion for *B. anynana* (discussed in more detail below). This raises the issue of whether this potential would be sufficient to allow for rapid adaptation to human-induced climate change, although this will not be an easy question to answer.

One method of testing the evolutionary potential of traits is to apply artificial selection in the laboratory on one or more traits. This approach has been applied in previous research on a laboratory-established stock of *B. anynana* from Malawi which has usually been found to harbour sufficient genetic variation to respond to artificial selection targeted on single traits over a comparatively small number of generations (Brakefield 2003). However, traits involved in the polyphenism of *B. anynana* often show correlated responses to selection because of central regulation, for example, via a shared hormonal control (Zijlstra et al. 2004, Chapter 3 in this thesis). These trait correlations could impose negative effects on the fitness of the selected traits caused by trade-offs. Previous experiments in *B. anynana* have shown that genetically correlated traits can in some cases be uncoupled using antagonistic artificial selection (Beldade et al. 2002, Zijlstra et al. 2003, but see Allen et al. 2008). Thus, whether genetic correlations impose

constraints on thermal adaptation will depend on how temperature variation imposes selection on the individual traits and the amount of selectable genetic variation and covariation. Alongside this approach involving artificial selection to exploring genetic constraints, the fitness consequences of observed phenotypic changes in response to the selection should be assessed under (semi-) natural conditions (cf. Joron & Brakefield 2003; Frankino et al. 2005). This may help to reveal any trade-offs especially given that these are generally more difficult to observe under laboratory conditions.

Chapter 3: hormonal regulation of phenotypic plasticity

Chapter 3 examines in more detail the response of a suite of polyphenic traits and the underlying regulatory hormone dynamics to developmental temperature in the Malawi population that was also used in Chapter 2. The alternative polyphenic phenotypes can be the result of a discretely varying environment acting on a developmental program which responds in a continuous manner, or of a continuously varying environment acting on a threshold-like switch between alternative developmental trajectories (Nijhout 2003). The shape of the reaction norms for several of the life history traits in Chapter 2 suggested a discontinuous response, indicating that the transition between development into either the dry- or wet-season form occurs in a relatively narrow temperature window. Mechanisms by which discrete phenotypic morphs are produced in response to a continuously varying environment are still largely a black box (Nijhout 2003; Zera et al. 2007). Previous studies on regulation of phenotypic plasticity in *B. anynana* (Brakefield et al. 1998, Zijlstra et al. 2004) and other species (e.g. Anstey et al. 2009) have demonstrated the importance of neuroendocrine pathways in mediating the phenotypic response to environmental variation, but whether these hormone dynamics themselves respond in a discontinuous manner to a linear environmental gradient remains untested.

We measured the response to a range of five developmental temperatures of several life history traits and wing pattern and coupled this with precise measurements of Ecdysteroid hormone dynamics during pupal development. Our study revealed that Ecdysteroid hormone titers during morph differentiation show two discrete groups of dynamics in response to a linear environmental gradient: 19 and 21°C reared pupae showed a late, while 23, 25, and 27°C reared pupae showed an early hormone peak. This indicates a developmental switch at the hormone level between alternative developmental trajectories occurring between 21 and 23°C. We thus showed that the dichotomy between the seasonal morphs of *B. anynana* is already present at the endocrine level during early metamorphosis and we found that some traits responded in a similar discrete manner suggesting that these fitness traits are co-regulated. In contrast, other traits showed a linear response to the environmental gradient, and are therefore likely to differ in their regulation downstream of the measured hormone signal.

These results are relevant in the broader context of understanding how organisms interpret environmental cues and process these into adaptive changes in their phenotype. The central hormonal regulation of traits enables an integrated response of the phenotype to the environment, but can also potentially constrain their independent evolution. While several of the life history traits responded in a clear discontinuous manner to developmental temperature, wing pattern showed a linear reaction norm,

despite the underlying discontinuous hormone signal and the established role of hormone dynamic in the determination of this trait. This suggests an additional level of regulation between the Ecdysteroid signal and the developmental pathway shaping the wing pattern that is sensitive to developmental temperature. It is possible that such a mechanism could allow for a more flexible evolution of wing pattern than the life history traits, which might explain the observed population differentiation for wing pattern but not for the life history traits in Chapter 2. An interesting next step would be to study the developmental pathways involved in wing pattern determination downstream of the Ecdysteroid signaling during pupal development, for example the timing of Ecdysone receptor expression in the wing tissue, and ultimately compare these dynamics between populations that show geographic differentiation for wing pattern.

GEOGRAPHIC VARIATION: A MOLECULAR GENETIC APPROACH

In the second half of the thesis, I take a molecular genetic approach to study geographic variation among six wild populations of *B. anynana* along a latitudinal transect extending over most of the species range, from the equator to the subtropics. Chapter 4 investigates the phylogeographic history of the populations, reflecting patterns of neutral evolution and providing necessary background information for the interpretation of geographic variation in candidate genes in the context of thermal adaptation as presented in Chapter 5.

Chapter 4: phylogeography

The distribution of genetic variation within and among populations of a species is determined by natural selection as well as neutral evolutionary processes such as drift and gene flow. Therefore, it is important to take into account signatures of neutral evolution when inferring patterns of adaptive differentiation. In Chapter 4, we analysed the genetic diversity, population structure and demographic history of the populations using the mitochondrial gene COI, a marker widely used for the purpose of reconstructing intra- and interspecific phylogenies. Considering the large distances between the sampled populations, the fragmented nature of the habitat and the weak dispersive nature of *B. anynana*, we expected to find considerable neutral population differentiation. We found high genetic diversity within the populations; however, our results indicated relatively little geographic structure among the populations. The distribution of the variation for the mtDNA gene within and among the populations showed an increasing signature of recent population expansion southwards, which was especially evident for the two southernmost populations.

The observed signal of recent population expansion southwards corresponds well with widely observed patterns of population expansion across taxa following the last ice age (Hewitt 2000). Pollen studies have indicated that the cooler and drier conditions during the last glacial maximum led to a contraction of seasonal forests and dry woodland, the natural habitat of *B. anynana*, into smaller refugia in the equatorial region (Flenley 1998; Prentice & Jolly 2000). This suggests that Southern populations of the *B. anynana* species range only expanded southwards during the past 10,000 years.

Interestingly, our study included one population of a different subspecies (*B. anynana centralis*) and one island population (Pemba Island, Tanzania). Both these populations were more divergent for neutral markers, and they also have clear phenotypic differences in wing pattern (Condamin 1973, personal observation), although reaction norms have yet to be quantified. These observations, coupled with the results from Chapter 2 showing differences in wing pattern between populations from Malawi and South Africa, suggest morphological (and potentially other) traits may show relatively rapid population divergence in *B. anynana* despite the recent population history as discussed here. Measurement of wing pattern and other traits of these populations under controlled conditions could shed more light on the rate of population differentiation. In addition, an extension of the phylogeographic analysis including several neutral nuclear DNA markers would allow for a more precise estimation of the divergence time between populations. From this perspective, another potentially very interesting population to investigate is the one inhabiting Socotra Island (Yemen), described as a distinct subspecies (*B. anynana socotrana*). The butterflies of this population have a markedly different wing pattern and colouration than the common subspecies and are notably smaller in size (Condamin 1973, Steve Collins, personal communication), the latter possibly being an adaptation to reduce involuntary dispersal off the island by wind, as is commonly observed in island species (Whittaker & Fernández-Palacios 2006). A planned collecting expedition to Socotra in 2011 will provide an opportunity to study this population more closely.

In summary, the work presented in Chapter 4 is the first study on the range-wide phylogeographic structure in *B. anynana*. These results provide a valuable framework for future investigations of adaptive variation in wild populations of the species.

Chapter 5: clinal variation in candidate genes

With the rapidly increasing amount of genomic sequence information becoming available, one of the major challenges in biology remains linking variation in the genome to the phenotypic variation that enables organisms to cope with and adapt to environmental variation. Studying genes that are putatively under thermal selection can give insight into the genetic properties that allow organisms to adapt to temperature variation, and into the possible constraints on their adaptive potential. Because temperature generally correlates with latitude, studying populations that occur along a latitudinal gradient can reveal phenotypic or genetic variation involved in thermal adaptation.

In Chapter 5, we investigated clinal variation in the coding regions of 19 candidate genes associated with thermal adaptation, using the same six wild populations that were studied in Chapter 4. The majority of these genes code for enzymes and other proteins involved in central metabolism, in particular the glycolytic and lipid pathways. In addition, considering the widely documented latitudinal and altitudinal clines in pigmentation in a variety of organisms, we included genes involved in the biosynthesis of wing pattern pigmentation. Lastly, as a type of negative control, we included genes from two groups that we consider less likely to show clinal variation in the coding regions: the heat shock proteins and key developmental genes.

We analysed clinal variation of amino-acid polymorphisms in the candidate genes by testing for significant correlation between latitude and allele frequencies. First, we took into account the neutral interpopulation structure, which could possibly convey a clinal signal and thus may confound our interpretations of adaptive clinal variation in the candidate genes (discussed above). We did not find evidence for neutral clinal variation in the most common haplotype of the mtDNA COI gene (Chapter 4), nor for the putatively neutral silent SNPs of the nuclear genes. Two candidate genes coding for enzymes of the glycolytic pathway, *Treh* and *UGPase*, showed significant clinal variation, of which only the latter remained significant after correction for multiple testing. In addition, the clinally varying amino-acid polymorphism in *UGPase* had a significantly higher F_{ST} value than expected under neutral evolution, which is another indication of selection on this locus. Interestingly, research on *Drosophila melanogaster* has also identified significant clinal variation with latitude in both *UGPase* and *Treh* (Sezgin et al. 2004). No clinal variation was observed for the wing pattern pigmentation genes, however, amino-acid polymorphisms in the pigmentation genes *yellow* and *black* showed significant upper outlier F_{ST} values, indicating increased population differentiation for these loci. As expected, no clinal variation was observed for the control group genes. The observed clinal variation in *Treh* and especially *UGPase* may reflect adaptation to a thermal gradient, thus our results put these genes forward as interesting candidates for follow-up research.

The logical next step for future research would be to associate the candidate gene polymorphisms under putative selection with experimental phenotypic data. Experiments measuring thermal performance and tolerance of genotyped individuals could confirm whether these genetic polymorphisms play a role in thermal adaptation. Were such a role to be established, these genes could then be used as genetic markers to monitor molecular responses of wild populations to selection imposed by climate change. Research on other species has shown a change in frequencies of genotypes associated with climate adaptation in response to recent global warming (Balanyá et al. 2006, Van Heerwaarden & Hoffmann 2007). The planned sequencing of the *B. anynana* genome in the next few years and the increase of genomic tools becoming available for this species sketch an exciting prospect for extending our knowledge of the genetic architecture underlying thermal adaptation.

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Nederlandse samenvatting

Een centrale vraag in de biologie is hoe organismen zich aanpassen aan variatie in omgevingsfactoren en welke onderliggende mechanismen daarbij betrokken zijn. Het hoofdthema van dit proefschrift is adaptatie aan klimaat, en in het bijzonder aan temperatuur, in de tropische vlinder *Bicyclus anynana*. Door populaties uit verschillende geografische gebieden met elkaar te vergelijken op het fenotypische en genetische niveau, onderzoek ik in hoeverre er sprake is van lokale adaptatie aan regionale klimaatsomstandigheden.

De mechanismen van adaptatie aan temperatuur worden al sinds lange tijd onderzocht door biologen omdat de temperatuur een belangrijke invloed heeft op de ecologie en evolutie van organismen. In afgelopen jaren is de interesse voor het onderzoek aan temperatuuradaptatie sterk toegenomen doordat de gevolgen van klimaatverandering voor de biodiversiteit duidelijk meetbaar worden. De resultaten die in dit proefschrift worden gepresenteerd behandelen op genetisch, fysiologisch en fenotypisch niveau de mechanismen en processen betrokken bij, en beinvloed door temperatuursvariatie. In een breder perspectief vergroten ze het inzicht in de mate waarin organismen zich kunnen aanpassen aan klimaatverandering.

De ecologische gevolgen van klimaatverandering zijn waargenomen voor een grote diversiteit van soorten en op elk continent. De meeste studies hebben zich tot nu toe gericht op het verschuiven van de verspreidingsgebieden van soorten, en laten een algemeen patroon zien van verschuivingen in de richting van de poolgebieden en hogere altitudes. Het verschuiven van het verspreidingsgebied is vaak echter geen optie in verband met door de mens veroorzaakte habitatversnippering. Het voortbestaan van veel soorten zal afhangen van de mate waarin zij kunnen omgaan met veranderende temperaturen, door enerzijds genetische adaptatie, en anderzijds fenotypische plasticiteit. Fenotypische plasticiteit is de capaciteit van een organisme om het fenotype aan te passen in reactie op omgevingsfactoren. Organismen kunnen dus door middel van fenotypische plasticiteit vanuit hetzelfde genotype verschillende fenotypen ontwikkelen. Dit proefschrift behandelt zowel fenotypische plasticiteit als genetische adaptatie aan temperatuur in *B. anynana.*

De tropische dagvlinder *B. anynana* komt voor in het oostelijk deel van Afrika, van de evenaar tot in subtropisch Zuid-Afrika. Dit gebied, dat meer dan 3.000 km beslaat van noord naar zuid, wordt gekenmerkt door de afwisseling van natte en droge seizoenen, een karakteristiek gegeven van het savanne ecosysteem. De soort heeft zich op een bijzondere manier aangepast aan de variatie in omgevingsfactoren van de afwisselende seizoenen. Afhankelijk van het seizoen waarin de rupsen zich ontwikkelen, worden de vlinders gekenmerkt door twee verschillende fenotypen: de droge seizoensvorm verschilt van de natte seizoensvorm in diverse morfologische, fysiologische, en levensgeschiedenis (*life history)* eigenschappen (dit zijn eigenschappen die een directe rol spelen in de levencyclus en dus verband houden met de reproductie en levensduur van organismen). De ontwikkeling tot twee of meer discrete fenotypen

in respons op omgevingsfactoren wordt polyfenisme genoemd, en is een extreme vorm van fenotypische plasticiteit. Onderzoek heeft uitgewezen dat temperatuur de belangrijkste omgevingsfactor is die de seizoensvorm van *B. anynana* bepaalt tijdens de ontwikkeling van de rups. In het warme regenseizoen is er voldoende voedsel beschikbaar voor de rupsen en vlinders. De natte seizoensvorm is aangepast aan deze gunstige omstandigheden met een snelle ontwikkeling en reproductie, en een korte levensduur. De vleugels van deze vlinders vertonen opvallende oogvlekken, die waarschijnlijk een rol spelen in het vinden van partners voor de voortplanting en het ontwijken van aanvallen door predatoren. De droge seizoensvorm is beter uitgerust om periodes van voedselschaarste in het koelere droge seizoen te overleven. Deze vlinders zijn veel minder actief, hebben meer lichaamsreserves, een langere levensduur en stellen de reproductie uit tot het volgende regenseizoen. Bovendien is het vleugelpatroon aangepast om niet op te vallen tegen een achtergrond van droge vegetatie, waardoor de vlinders minder zichtbaar zijn voor predatoren.

B. anynana is een uitermate geschikt organisme voor onderzoek aan adaptatiemechanismen door de hoge mate van fenotypische plasticiteit en genetische variatie voor diverse fenotypische eigenschappen die verband houden met klimaatadaptatie. Bovendien is er veel onderzoek gedaan aan de soort in het verband van ecologische en evolutionaire studies, en is er uitgebreide kennis beschikbaar over de levensgeschiedenis, fysiologie en genetica van de vlinder.

Mijn proefschrift bestaat uit twee delen. De eerste helft van het proefschrift is gericht op fenotypische plasticiteit in B. anynana, waarbij Hoofdstuk 2 populaties uit verschillende geografische regios vergelijkt. Hoofdstuk 3 gaat dieper in op de fysiologische mechanismen die de ontwikkeling van het fenotype aansturen in reactie op omgevingsfactoren.

In Hoofdstuk 2 onderzoek ik geografische variatie voor de seizoensplasticiteit in respons op ontwikkelingstemperatuur door twee populaties met elkaar te vergelijken, één uit Malawi en één uit Zuid-Afrika. Ik onderzoek of er sprake is van lokale adaptatie van de populaties aan de specifieke klimaatomstandigheden van hun geografische lokaties, die van elkaar verschillen met betrekking tot temperatuur en regenval. Deze vraag is relevant in bredere zin in het perspectief van klimaatverwarming omdat de mate van temperatuurrespons van populaties iets zegt over de kwetsbaarheid voor klimaatsverandering. Als populaties lokale adaptatie vertonen voor de plasticiteitrespons op temperatuur zullen zij kwetsbaarder zijn voor afwijkende temperaturen als gevolg van klimaatverandering dan wanneer populaties dezelfde respons vertonen over een groter gebied.

Voor deze studie heb ik de fenotypische respons gemeten van verscheidene eigenschappen die een rol spelen in het seizoenspolyfenisme in reactie op een reeks van drie ontwikkelingstemperaturen die ongeveer de temperatuurvariatie beslaan in de natuurlijke habitat door het jaar heen. Mijn resultaten wezen uit dat de populaties zeer weinig tot geen verschil vertoonden in de plasticiteit van de eigenschappen gerelateerd aan fysiologie en de *life history*, waaronder ontwikkelingstijd, hongerresistentie en rustmetabolisme. Met name voor de eigenschappen die tijdens het volwassen stadium

nog kunnen worden bijgesteld afhankelijk van de temperatuursomstandigheden lieten de populaties precies dezelfde respons zien. In tegenstelling hiermee vertoonde het vleugelpatroon, dat na de ontwikkeling vastligt en niet meer kan veranderen, wél geografische variatie tussen de populaties. Dit heeft als implicatie dat in het geval van klimaatsverandering de vlinders het gevaar lopen een vleugelpatroon te ontwikkelen dat niet optimaal past bij het seizoen waarin zij zich bevinden. De nog plastische volwassen kenmerken kunnen echter een belangrijke rol spelen in adaptatie aan releatief kleine, lokale, klimaatsomstandigheden.

Hoofdstuk 3 gaat dieper in op de onderliggende fysiologische mechanismen die de plasticiteitsrespons op temperatuur reguleren. Het polyfenisme in *B. anynana*, de ontwikkeling van twee discrete fenotypen, suggereert een soort schakelmechanisme tijdens de ontwikkeling als reactie op de ontwikkelingstemperatuur. De resultaten van Hoofdstuk 2 impliceren dat deze schakel rond een vrij nauwe temperatuursmarge ligt voor sommige van de gemeten eigenschappen. Dit bracht mij er toe om een experiment te ontwerpen met een reeks van vijf ontwikkelingstemperaturen, en de respons op deze temperaturen van verscheidene eigenschappen te meten. Deze waren wederom het vleugelpatroon en een aantal fysiologische en *life history* eigenschappen zoals rustmetabolisme en ontwikkelingstijd. Deze metingen koppelde ik aan nauwkeurige metingen van de hormoonconcentraties tijdens de ontwikkeling in het popstadium in dezelfde temperatuurreeks.

Uit voorgaand onderzoek is gebleken dat hormonen een belangrijke rol spelen in de regeling van fenotypische plasticiteit. De EcdysteroÏden en de Juveniel Hormonen zijn de twee belangrijkste hormoongroepen in insekten, en de betrokkenheid van deze hormonen in de regulatie van polyfenisme is onder meer aangetoond in vlinders, kevers, krekels en sociale insecten. Studies met *B. anynana* hebben aangetoond dat het hormoon Ecdyson invloed heeft op de ontwikkeling van het vleugelpatroon en *life history* eigenschappen gerelateerd aan het seizoenspolyfenisme. Het was tot nu toe echter onbekend of het hormoonsignaal al tijdens de kritieke fase van de ontwikkeling de tweeledige respons van het polyfenisme liet zien. De resultaten die zijn gepresenteerd in Hoofdstuk 3 geven aan dat de tweeledige respons op temperatuur al aanwezig is op het niveau van het hormoonsignaal van Ecdyson. Sommige *life history* eigenschappen lieten eenzelfde binaire respons zien, met name rustmetabolisme en de allocatie van lichaamsreserves, wat impliceert dat deze eigenschappen centraal worden gereguleerd. Daarentegen liet het vleugelpatroon een lineaire respons zien, ondanks de aangetoonde invloed van Ecdyson op de ontwikkeling van het vleugelpatroon. Dit geeft aan dat er waarschijnlijk nog een niveau van regulatie aanwezig is tussen het Ecdysonsignaal tijdens het vroege popstadium en de uiteindelijke ontwikkeling van het vleugelpatroon. Deze resultaten dragen op een interessante wijze bij aan onze kennis over de mechanismen die betrokken zijn bij het vertalen van omgevingssignalen naar het uiteindelijke fenotype.

De tweede helft van het proefschrift behandelt twee studies met betrekking tot moleculair genetische variatie tussen zes wilde populaties die verspreid zijn van noord naar zuid over min of meer het gehele soortsgebied van *B. anynana.* De verdeling van

genetische variatie binnen en tussen populaties van een soort wordt bepaald door zowel natuurlijke selectie als neutraal evolutionaire processen zoals genetische drift en *gene flow* (de uitwisseling van genetisch materiaal tussen populaties als gevolg van verspreiding). Voor de interpretatie van geografische variatie in het kader van adaptatie is het dus belangrijk om de effecten van de neutrale evolutie op de populatievariatie in acht te nemen. Hoofdstuk 4 onderzoekt de genetische structuur binnen en tussen de populaties als gevolg van historische en hedendaagse processen van neutrale evolutie. Dit hoofdstuk levert cruciale achtergrondkennis op voor Hoofdstuk 5, dat zich richt op geografische variatie in kandidaat-genen die betrokken zijn bij temperatuuradaptatie.

In Hoofdstuk 4 wordt gebruik gemaakt van genetische variatie in het mitochondriale gen COI om de populatiestructuur te bepalen. Doordat mitochondriaal DNA voornamelijk op een neutrale manier evolueert, is dit gen zeer geschikt als moleculair marker om neutrale evolutieprocessen te bestuderen en wordt daarom ook veelvuldig gebruikt in fylogenetische studies. Voor ik aan de studie begon verwachtte ik dat de genetische variatie tussen de populaties groter zou zijn dan binnen de populaties, door de grote afstand tussen de populaties, het versnipperde habitat en de relatief lage lange-afstand vliegcapaciteit van de vlinders. De resultaten lieten een ander beeld zien: er was veel variatie binnen de populaties voor het COI gen, maar relatief weinig differentiatie tussen de populaties. Omdat een hoge mate van *gene flow* tussen de populaties onwaarschijnlijk is door bovengenoemde factoren moest er een andere verklaring zijn voor de lage mate van populatiedifferentiatie. Populatiegenetische tests toonden vervolgens een signatuur aan van recente populatiegroei van de evenaar naar het zuiden toe. Dit patroon komt sterk overeen met wijdverspreide patronen van populatie-expansie in verscheidene taxa in navolging van de laatste ijstijd. Vegetatiestudies hebben aangetoond dat de koelere en drogere omstandigheden tijdens het laatste glaciale maximum gepaard gingen met een reductie tot kleine gebieden rond de evenaar van de droge bossen die de habitat vormen van *B. anynana*. De resultaten geven aan dat de soort zich waarschijnlijk pas na de laatste ijstijd, gedurende de afgelopen 10.000 jaar, naar het zuiden toe heeft verspreid. In het licht van deze resultaten is het interessant op te merken dat ondanks de recente verspreiding er significante populatieverschillen zijn gevonden voor het vleugelpatroon, bijvoorbeeld zoals gepresenteerd in Hoofdstuk 2 en in beschrijvingen van de populaties in andere bronnen. Dit geeft aan dat de populatieverschillen in vleugelpatroon in relatief korte evolutionaire tijd zijn ontstaan.

In Hoofdstuk 5 heb ik gekeken naar genetische (DNA sequentie) variatie in kandidaat-genen in *B. anynana* door gebruik te maken van het gegeven dat de onderzochte populaties langs een natuurlijke geografische temperatuurgradiënt liggen. Wereldwijd gezien neemt de temperatuur af met toenemende breedtegraad (latitude): het is warmer aan de evenaar dan aan beide polen. Genetische variatie voor een gen dat toe- of afneemt met latitude is een belangrijke indicatie voor adaptatie aan de lokale temperatuursomstandigheden. Voor deze studie heb ik 19 kandidaat-genen geselecteerd op basis van aangetoonde associatie met temperatuuradaptatie in voorgaande studies aan voornamelijk *Drosophila melanogaster* (de fruitvlieg). Het grootste deel van deze genen is betrokken bij het centrale metabolisme, in het bijzonder bij de opbouw en

afbraak van glucose en lipiden. Het overige deel van de selectie bestond uit genen die een rol spelen in de synthese van pigmenten in de vleugelpatronen. Het is namelijk aangetoond dat melanisering van de vleugels verband kan houden met adaptatie aan temperatuur. De studie is specifiek gericht op het type variatie in de genen dat leidt tot aminozuurverandering in de uiteindelijke eiwitten waar de genen voor coderen. Een dergelijke aminozuursubstitutie kan leiden tot een verandering in het functioneren van het eiwit door bijvoorbeeld een verandering in de reactie-efficiëntie en/of de (temperatuur)stabiliteit van het eiwit. Ik heb het verband tussen de kandidaat-genen en temperatuuradaptatie getoetst door de populatiespecifieke allelfrequenties van deze aminozuurpolymorfismen in de genen te correleren met de breedtegraden van de populaties. Naast het selecteren van kandidaat-genen voor temperatuuradaptatie heb ik ook een groep genen gekozen waarvoor ik geen verband verwachtte met temperatuur. Deze controlegroep bestond uit genen betrokken bij de (vroege) ontwikkeling en de reguliere celfuncties.

De resultaten van deze studie laten een significant verband zien tussen aminozuurpolymorfismen in twee genen die coderen voor enzymen betrokken bij glycolyse: *Trehalase* en *UGPase*. Zoals verwacht waren er geen correlaties met de geografische gradiënt voor de genen in de controlegroep. Ik heb ook aangetoond dat de waargenomen significante correlaties niet veroorzaakt zijn door de achterliggende neutrale populatiestructuur. Deze resultaten zijn extra interessant in verband met parallelle resultaten in *D. melanogaster*, waarvoor ook een significant verband is aangetoond tussen deze twee genen en populatievariatie langs een noord-zuid gradiënt. Mijn resultaten laten daarom een mogelijke rol zien voor deze kandidaat-genen in adaptatie aan temperatuur. Vervolgonderzoek dat variatie in de genen associeert met fenotypische variatie in temperatuurtolerantie kan deze rol verder testen. Genen die een aangetoond verband houden met temperatuuradaptatie zouden kunnen worden toegepast als genetische markers waarmee eventuele veranderingen in de genetische opmaak van populaties in respons op klimaatverandering kunnen worden gevolgd. De in de nabije toekomst geplande ontcijfering van het hele genoom van *B. anynana* belooft vele mogelijkheden tot uitbereiding van de kennis over de genetische architectuur van temperatuuradaptatie.

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Curriculum vitae

Maaike de Jong was born on June 16th 1976 in Rotterdam, the Netherlands. After obtaining her Athenaeum diploma at the Rotterdam Montessori Lyceum in 1995, she moved to Amsterdam to study Cultural Anthropology. She realized after two years that she was more interested in nature than in people and started to study Biology at the same university, where she specialized in Ecology. She did her first research project on plant-predator interactions in the Population Ecology Group of Prof. Maus Sabelis. Subsequently she did a research project on plant population genetics in the Experimental Plant Systematics Group of Prof. Peter van Tienderen, for which she spent six months climbing the treetops of Monteverde forest in Costa Rica. She graduated *cum laude* in 2004, after which she stayed at the University of Amsterdam to work as a technical assistant in a EU project on risk assessment of GMO crops.

In September 2005 Maaike started her PhD research on geographical variation in *Bicyclus anynana* at Leiden University under the supervision of Prof. Paul Brakefield and Prof. Bas Zwaan. Fieldwork for this project led her to the African continent where she collected butterflies in Uganda, Kenya, Tanzania, Malawi and South Africa. She presented her scientific results at various international meetings, including the ESEB conferences in Uppsala, Sweden (2007) and Turin, Italy (2009), the International Entomology Congress in Durban, South Africa (2008), and the EU ThermAdapt funded workshops in Bialowieza, Poland (2009) and Sandbjerg, Denmark (2010). During her PhD she supervised several BSc and MSc students and participated in teaching the BSc Evolutionary Biology course at Leiden University.

Currently, Maaike is working as a postdoctoral researcher in Prof. Ilkka Hanski's Metapopulation Research Group at the University of Helsinki, where she studies ecological genetics of the butterfly *Melitaea cinxia*.

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